Metabolism and function of β-1,3-glucan in marine diatoms

by

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A thesis submitted in partial fulfillment of the requirements for the academic title

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SUMMARY

β-1,3-Glucan (chrysolaminaran) is the principal storage polysaccharide in diatoms (Bacillariophyceae), the major primary producers in the sea. The glucan generally contributes a substantial fraction of the algal biomass, but its level varies markedly in response to growth conditions. The scope of this work was to study the metabolism and function of the polysaccharide in marine diatoms. Axenic cultures of the marine planktonic diatom *Skeletonema costatum* (Grev.) Cleve were used in the experiments. Glucan metabolism was studied by growing the alga in batch culture, and measuring metabolite fluxes by chemical analyses as well as by ^{14}C tracer technique using labeled bicarbonate.

A photobioreactor was developed for strictly controlled growth of microalgae. Fine pH regulation was obtained by relay-activated titration with dilute acid (HCl) and base (NaOH). Irradiance and temperature were also carefully controlled. Batch cultures were grown with a 14:10 h light:dark cycle, and pH curves were recorded during different growth phases.

A new method was developed for the combined determination of β-1,3-glucan and cell wall polysaccharides in diatoms, representing total cellular carbohydrate. The glucan is rapidly extracted by hot dilute H_2SO_4, and the cell wall polysaccharides are subsequently hydrolyzed by cold 80% H_2SO_4 overnight. Each carbohydrate fraction is finally determined by the phenol-sulphuric acid method. This procedure is simple and rapid compared to previous methods, and applies well to laboratory cultures as well as natural phytoplankton populations dominated by diatoms.

Synthesis and mobilization of β-1,3-glucan in N-limited *S. costatum* were studied by combined ^{14}C tracer technique and chemical analyses. Radiolabeled bicarbonate was added to the cultures, and ^{14}C incorporation in different metabolites was determined using biochemical fractionation. In a pulse phase, ^{14}C label was mainly incorporated in the glucan fraction (85%) during photosynthesis under nitrogen limitation. Subsequently, a ^{14}C chase was carried out by adding NH_4^+ and incubating the cells under different light conditions. Radiolabeled glucan decreased significantly (by 26% in the dark, and by 19% in low light) whereas radiolabeled amino acids, proteins and other polysaccharides increased significantly during NH_4^+ assimilation. Chemical analyses of β-1,3-glucan and cellular free amino acids supported the ^{14}C measurements. Changes in amino acid composition strongly indicated that *de novo* biosynthesis took place, with a Gln/Glu ratio increasing from 0.4 to 10. This study provides

new evidence of β-1,3-glucan supplying carbon skeletons for synthesis of amino acids and protein in diatoms. Mobilization of glucan yields glucose, which is further metabolized by the respiratory pathways to provide precursors as well as energy. The results from the ^{14}C chase also indicated significant synthesis of other polysaccharides or possibly RNA from glucan.

In a different study, dark carbon fixation in N-limited *S. costatum* was measured using ^{14}C-bicarbonate. Addition of NH_4^+ resulted in 4-fold increase in carboxylation rate, and biochemical fractionation showed that mainly amino acids were radiolabeled. Chemical analyses confirmed that cellular free amino acids increased rapidly (with increasing Gln/Glu), and showed that cellular glucan decreased significantly (by 28%) during NH_4^+ assimilation. The results strongly indicate that β-carboxylation provides C_4 precursors for amino acid synthesis, and β-1,3-glucan is likely to be the ultimate substrate for β-carboxylation. Moreover, a C/N uptake ratio of 0.33 indicated that β-carboxylation was related to protein synthesis.

A detailed study was made of the production of carbohydrates and amino acids by *S. costatum* during different growth phases. During exponential growth under diel light conditions, the glucan level oscillated between 17% (end of scotophase) and 42% (end of photophase) of cellular organic carbon, and the corresponding protein/glucan ratio alternated between 2.3 and 0.7. Concurrently, the cellular free amino acid pool oscillated between 8% (end of scotophase) and 22% (end of photophase) of cellular organic nitrogen, and the corresponding Gln/Glu ratio alternated between 0.05 and 2. Depletion of nitrogen from the medium resulted in rapid accumulation of glucan, reaching 75-80% of cellular organic carbon, whereas the cellular nitrogenous components decreased significantly. Consequently, the protein/glucan ratio decreased to <0.1. This study indicates that β-1,3-glucan functions both as a short-term diurnal reserve and a long-term stockpile reserve.

Field investigations by other workers suggest that glucan plays a very active role in the dynamics of natural diatom populations, and the protein/glucan ratio has been used as a sensitive parameter for nutrient status. The glucan dynamics may be involved in physiological control of buoyancy. Glucan accumulation by nutrient-deplete cells causes increased cellular density and sinking below the nutricline. Upon nutrient replenishment and mobilization of glucan, the cells rise toward the surface of the water column, thereby transporting deep nutrients to the euphotic zone. β-1,3-Glucan also seems to play an important role in the development of resting stages in diatoms.

LIST OF PAPERS

1. Granum E & Myklestad SM (2001) A photobioreactor with pH control: demonstration by growth of the marine diatom *Skeletonema costatum*. *J Plankton Res* (Accepted)

2. Granum E & Myklestad SM (2001) A simple combined method for determination of β-1,3-glucan and cell wall polysaccharides in diatoms. *Hydrobiologia* (Submitted)

3. Granum E & Myklestad SM (1999) Effects of NH_4^+ assimilation on dark carbon fixation and β-1,3-glucan metabolism in the marine diatom *Skeletonema costatum* (Bacillariophyceae). *J Phycol* 35:1191-9

4. Granum E & Myklestad SM (2001) Mobilization of β-1,3-glucan and biosynthesis of amino acids induced by NH_4^+ addition to N-limited cells of the marine diatom *Skeletonema costatum* (Bacillariophyceae*). J Phycol* 37:772-82

5. Granum E, Kirkvold S & Myklestad SM (2001) Cellular and extracellular production of carbohydrates and amino acids by the marine diatom *Skeletonema costatum*: diel variations and effects of nitrogen depletion. *Mar Ecol Prog Ser* (Submitted)

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LIST OF ABBREVIATIONS AND SYMBOLS

α	quantum yield of absorbed light
A	radioactivity
ADP	adenosine diphosphate
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartate
ATP	adenosine triphosphate
Chl	chlorophyll
DIC	dissolved inorganic carbon
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
DP	degree of polymerization
E	irradiance
f	isotope discrimination factor
Fd	ferredoxin
Gln	glutamine
Glu	glutamate
Gly	glycine
His	histidine
HPLC	high-performance liquid chromatography
Ile	isoleucine
Leu	leucine
LICF	light-independent carbon fixation
Lys	lysine
Met	methionine
mRNA	messenger ribonucleic acid
MW	molecular weight
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
P	photosynthetic rate
P_i	inorganic orthophosphate
PEPC	phosphoenolpyruvate carboxylase
PEPCK	phosphoenolpyruvate carboxykinase
Phe	phenylalanine
PP_i	inorganic pyrophosphate
PSI	photosystem I
PSII	photosystem II
RNA	ribonucleic acid
RUBISCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
SEM	scanning electron microscopy
Ser	serine
t	time
T_m	membrane transition temperature
TCA	trichloroacetic acid
Thr	threonine
Tyr	tyrosine
UDP	uridine diphosphate
UTP	uridine triphosphate
Val	valine

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1. INTRODUCTION

1.1 Diatoms

Diatoms (Bacillariophyceae) constitute a class of eukaryotic microalgae characterized by siliceous cell walls and golden-brown colour. They are widespread in marine as well as fresh waters, and also occur on rocks or soil. There are over 250 genera of living diatoms, with ~100,000 species (Round & Crawford 1990). Marine algae produce $35\text{-}50 \cdot 10^{15}$ g biomass each year, accounting for ~40% of the total net primary production on Earth (Falkowski 1994). Planktonic diatoms play a dominant role in the most productive areas of the ocean such as the upwelling areas and the continental shelves. One estimate is that marine planktonic diatoms alone contribute 20-25% of the global net primary production (Werner 1977).

Diatoms are unicellular algae that range in diameter from 5 to 200 μm. The protoplast is encapsulated in a silica-impregnated cell wall, and the siliceous parts are referred to collectively as the frustule (Round et al. 1990, Hoek et al. 1995). Based on the structure of the frustule, diatoms are divided into two groups (orders): centric (radial symmetry) and pennate (bilateral symmetry) (Fig. 1.1).

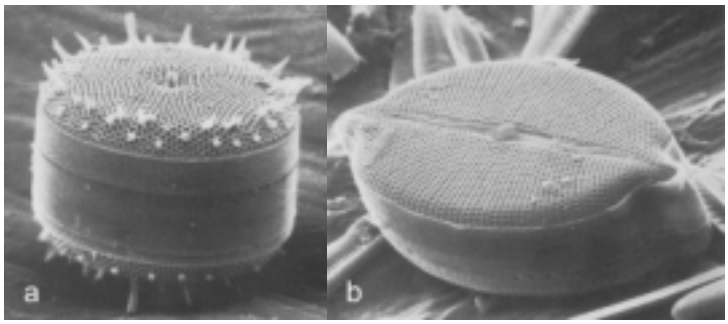

Fig. 1.1. Frustules of the centric diatom *Thalassiosira* (a) and the pennate diatom *Mastogloia* (b), SEM (Round et al. 1990).

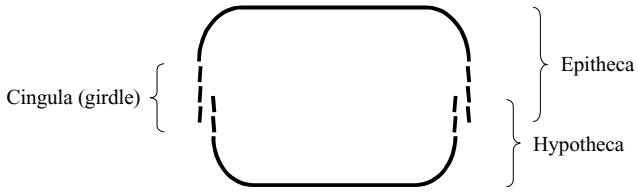

Fig. 1.2. The components of a diatom frustule. The epitheca overlaps the hypotheca, and each theca consists of a valve and a cingulum (girdle), which is composed of several bands (copulae).

The frustule consists of two halves: an epitheca and a hypotheca (Fig. 1.2). The epitheca overlaps the edge of the hypotheca, like the two halves of a petri dish. Each theca consists in turn of a large valve and a ring-like linking structure termed cingulum (girdle), which is composed of several bands (copulae). The valves are intricately ornamented, and the patterns are largely species-specific. Under vegetative growth, the epitheca and hypotheca slides apart, followed by open mitosis, division of the protoplast, and formation of new wall elements in each daughter cell. The newly synthesized half of the cell wall in each daughter cell is always a hypotheca, hence one of the daughter cells comes out smaller than the parent cell (except for some species with elastic girdle). Thus, average cell size in a diatom population decreases with each division. When the cells reach a minimal size, however, they can form auxospores, through which the diminution is reversed. The formation of auxospores is almost always linked to sexual reproduction. Some diatoms also develop resting spores, particularly when nitrogen is depleted. Many diatoms form chains in which the cells are juxtaposed by their valves and linked by silica or polysaccharide structures.

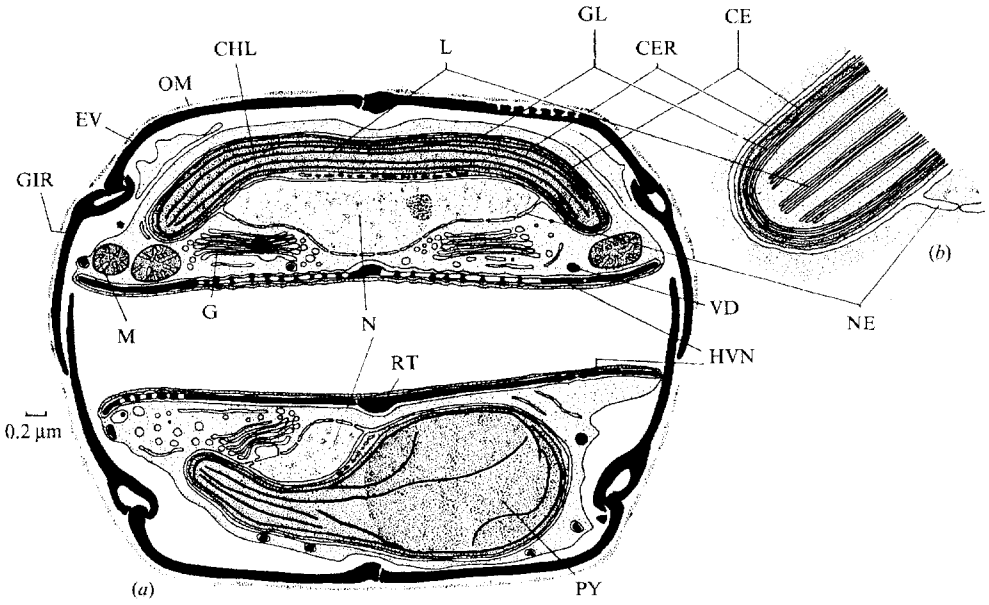

Fig. 1.3. Cross-section through the diatom *Amphipleura pellucida* directly after division (a) and part of its chloroplast (b) (Hoek et al. 1995). Abbreviations: CE, chloroplast envelope; CER, chloroplast endoplasmic reticulum; CHL, chloroplast; EV, epivalve of parent cell; G, Golgi body; GIR, girdle; HVN, hypovalve of daughter cell; L, lamella (stack of three thylakoids); M, mitochondrion; N, nucleus; NE, nuclear envelope; OM, organic material coating the silica frustule; PY, pyrenoid; VD, silica deposition vesicle.

Diatoms basically contain the usual plant cell organelles (Fig. 1.3). The nucleus is either suspended in the centre of the cell by a bridge of protoplasm or lies to one side of the cell, by the girdle or one of the valves. Chloroplast shape and number vary greatly, but the ultrastructure is similar. Thylakoids are arranged in bands of three (lamellae) with a girdle band around the whole. Each chloroplast is surrounded by four membranes, the outer two representing the chloroplast endoplasmic reticulum. The chloroplasts often lie close to the nucleus and in this case the endoplasmic reticulum is continuous with the nuclear envelope. Chloroplasts, mitochondria, Golgi bodies and other organelles are usually packed into a thin peripheral layer of cytoplasm sandwiched between the cell wall and one or two large central vacuoles.

1.2 Photosynthesis

Diatoms are predominantly photoautotrophic organisms, although some are able to live heterotrophically with a suitable source of organic carbon (facultative photoautotrophy). The process of oxygenic photosynthesis involves many intermediate steps, but the overall reaction can be written as:

$$CO_2 + 2H_2\mathbf{O} \xrightarrow[\text{Chl } a]{\text{Light energy}} [CH_2O] + H_2O + \mathbf{O}_2 \qquad (1)$$

where $[CH_2O]$ represents a general carbohydrate. Chlorophyll *a* (chl *a*) catalyzes the oxidation of H_2O to O_2 using light energy (indicated by boldface), while CO_2 is reduced to $[CH_2O]$ and H_2O in a different light-independent subprocess. Thus, the overall process is separated, both chemically and physically, into so-called light reactions and dark reactions, which are coupled by common intermediates and by enzyme regulation (Falkowski & Raven 1997). In the thylakoid membranes of the chloroplast, the light reactions are carried out by two photosystems linked in series (Fig. 1.4). In each photosystem, photons are absorbed by antenna pigments, and excitation energy is transferred to a reaction center chl *a*. Light-excited electrons are passed through an electron transport chain, which drives the pumping of protons across the thylakoid membrane. Electrons extracted from water in photosystem II (PSII) are sequentially transferred via quinones, the cytochrome *bf* complex, cytochrome c_6 and photosystem I (PSI) to ferredoxin (Fd). Excited electrons thus pass through a series of acceptors that convert electronic energy to chemical energy. Finally, NADPH is produced by ferredoxin-$NADP^+$ reductase using reduced Fd. The pH gradient across the thylakoid

membrane drives enzymatic ATP production (photophosphorylation). The phosphorylation of ADP to produce 1 mole of ATP is coupled to the translocation of 4 moles of H^+ from lumen to stroma. When $NADP^+$ levels are low, electrons from Fd can flow back to PSI through the cytochrome *bf* complex, thereby pumping extra protons across the membrane and generating ATP instead of NADPH (cyclic photophosphorylation). In the stroma of the chloroplast, CO_2 is fixed into carbohydrates through a cyclic series of reactions (Calvin cycle) using the NADPH and ATP generated in the light reactions (see chapter 1.3).

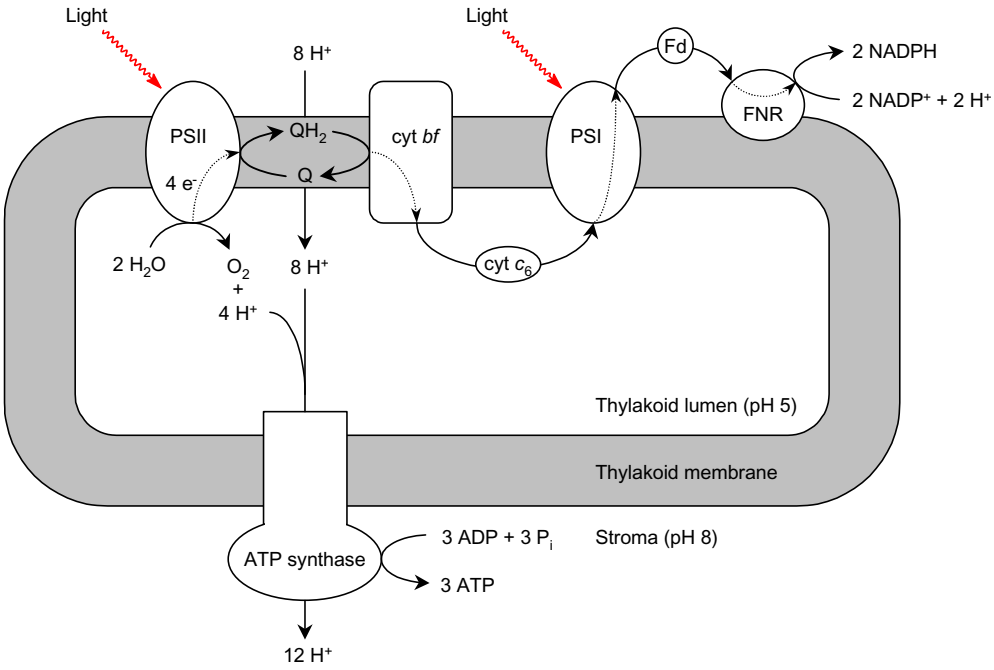

Fig. 1.4. Scheme of light reactions in the photosynthetic thylakoid membrane of chromophytes (dotted arrows indicate electron transport through protein complexes). Electrons extracted from water in photosystem II (PSII) are sequentially transferred through quinones (Q), the cytochrome *bf* complex (cyt *bf*), cytochrome c_6 (cyt c_6) and photosystem I (PSI) to ferredoxin (Fd). Finally, NADPH is produced by ferredoxin-$NADP^+$ reductase (FNR) using reduced Fd. Concurrent proton pumping drives ATP synthesis by ATP synthase.

The rate of photosynthetis is controlled by the efficiency of light utilization to drive the photosynthetic reactions. The light dependency is often described by a photosynthesis-irradiance curve (Fig. 1.5). In the light-limited region, gross photosynthetic rate (P) increases linearly with absorbed irradiance (E):

$$P = E\,\alpha \qquad (2)$$

where α (the slope of the curve) is the quantum yield of absorbed light. α is directly proportional to the functional absorption cross-section of PSII and the number of photosynthetic units (Falkowski 1992). As irradiance increases, the photosynthetic rate reaches a saturation level, P_{max}, which is related to the number of photosynthetic units and their maximum turnover rate. The saturation irradiance, E_k, is given as the intercept between α and P_{max}. Further increases in irradiance can lead to photoinhibition and reduction in the photosynthetic rate.

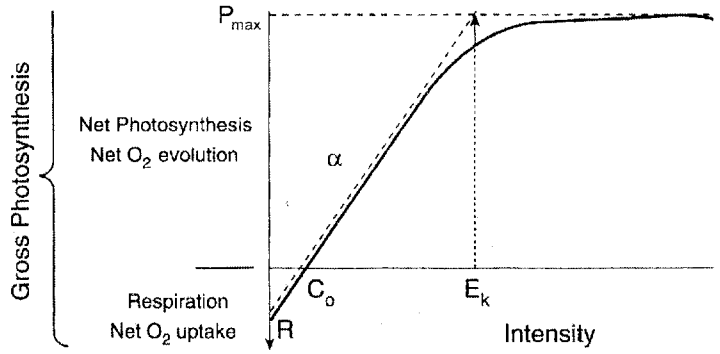

Fig. 1.5. Photosynthesis-irradiance curve derived from measurements of net O_2 exchange between the alga and the bulk fluid (Falkowski & Raven 1997). Abbreviations: α, quantum yield of absorbed light; C_o, compensation irradiance; E_k, saturation irradiance; P_{max}, maximum photosynthetic rate; R, respiration (assumed to be constant).

The principal products of the photosynthetic reactions (sugars, NADPH and ATP) are themselves substrates for biosynthetic reactions. Synthesis of many essential cellular components also requires nitrogen, phosphorus, sulphur and other elements, and their assimilation is tied to carbon fixation. Biosynthetic processes often involve oxidation reactions through respiratory pathways, which effectively operates as the inverse of photosynthetic reactions. Photosynthesis and respiratory pathways are coupled via fluxes of common substrates, and by the rate of production of reductant (NADPH) and energy (ATP) relative to demands for cell growth and maintenance.

1.3 Carbon fixation

Microalgae use ~95% of the photosynthetically generated NADPH and over 60% of the ATP to assimilate and reduce inorganic carbon through the Calvin cycle (Falkowski & Raven 1997). The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) catalyzes

the carboxylation and hydration of ribulose-1,5-bisphosphate to form two molecules of 3-phosphoglycerate:

$$\text{ribulose-1,5-bisphosphate} + CO_2 + H_2O \rightarrow 2\ (\text{3-phosphoglycerate}) \qquad (3)$$

RUBISCO constitutes ~15% of the chloroplast proteins and is presumably the single most abundant enzyme on Earth. The enzyme is present in high concentrations within the stroma in bodies called pyrenoids. In subsequent reactions, 3-phosphoglycerate is phosphorylated by ATP to form 1,3-bisphosphoglycerate, which is reduced by NADPH to glyceraldehyde-3-phosphate, with accompanying loss of one phosphate (Fig. 1.6). The remaining reactions of the Calvin cycle serve to regenerate ribulose-1,5-bisphosphate, and another ATP is invested in the phosphorylation of ribulose-5-phosphate. The overall synthesis of 1 molecule hexose from CO_2 requires 18 ATP and 12 NADPH molecules:

$$6\ CO_2 + 18\ ATP + 12\ NADPH + 12\ H_2O \rightarrow C_6H_{12}O_6 + 18\ ADP + 18\ P_i + 12\ NADP^+ + 6\ H^+ \qquad (4)$$

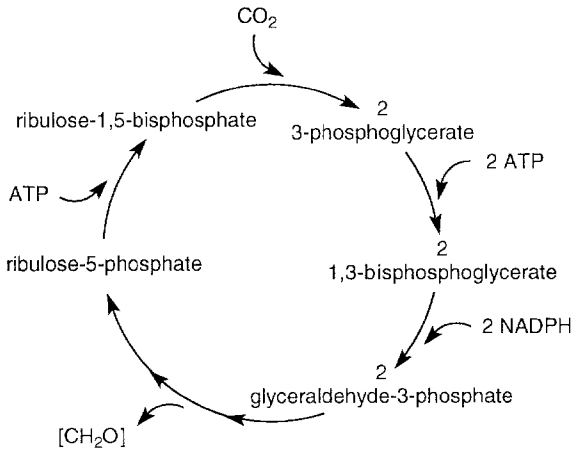

Fig. 1.6. Schematic view of the Calvin cycle showing CO_2 fixation and ATP- and NADPH-consuming steps.

RUBISCO can only use inorganic carbon in the form of CO_2 as a substrate. However, the hydrated forms of inorganic carbon are predominant in most aquatic systems. The chemical equilibria between dissolved inorganic carbon (DIC) species constitute a natural pH buffering system:

$$H_2O + CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2\ H^+ + CO_3^{2-} \qquad (5)$$

In seawater with an average pH of ~8.2 and ~2 mM DIC, over 95% of the inorganic carbon is present as HCO_3^-, and only 10 μM as CO_2 (Falkowski & Raven 1997). The inorganic carbon equilibria are easily imbalanced because uncatalyzed hydration-dehydration reactions (left-hand side of eq. 5) are very slow compared to biological interactions. However, the algal enzyme carbonic anhydrase catalyzes these reactions. In addition, many algae have carbon concentrating mechanisms that actively transport inorganic carbon from the medium into the cells when passive CO_2 diffusion is not adequate to support photosynthetic demands.

Photosynthesis is not the only form of carbon fixation. All organisms perform light-independent carbon fixation (dark carbon fixation), which is required to replenish intermediates in metabolic cycles that are withdrawn for anabolism. Biosynthesis of several essential amino acids, lipids, purines, pyrimidines and tetrapyrroles depends on such replacements, or *anaplerotic carboxylations*. The major anaplerotic carboxylation reactions are catalyzed by phosphoenolpyruvate carboxylase (eq. 6), phosphoenolpyruvate carboxykinase (eq. 7) and pyruvate carboxylase (eq. 8):

$$\text{phosphoenolpyruvate} + HCO_3^- \rightarrow \text{oxaloacetate} + P_i \quad (6)$$

$$\text{phosphoenolpyruvate} + CO_2 + ADP \rightarrow \text{oxaloacetate} + ATP \quad (7)$$

$$\text{pyruvate} + HCO_3^- + ATP \rightarrow \text{oxaloacetate} + ADP + P_i \quad (8)$$

These reactions are collectively termed β-carboxylation because the β-carbon of a C_3 substrate is carboxylated.

1.4 Nitrogen assimilation

Biosynthesis of amino acids and proteins requires the incorporation of nitrogen in the form of NH_4^+ into carbon skeletons. However, NO_3^- is the predominant form of assimilable nitrogen in most aquatic environments. Translocation of NO_3^- across the plasma membrane is carried out at the cost of ATP hydrolysis, and chemical reduction is catalyzed by two cellular enzymes. First nitrate reductase in the cytosol uses NADH or NADPH to reduce NO_3^- to NO_2^-, and then nitrite reductase in the chloroplast uses reduced ferredoxin to reduce NO_2^- to NH_4^+. Reduced ferredoxin is generally generated by photosynthetic electron flow (Raven 1976). However, algal cells can reduce NO_3^- in the dark provided that they have reserves of carbohydrates (Guerrero et al. 1981). Incorporation of NH_4^+ into amino acids is primarily

carried out by the sequential action of two enzymes in the chloroplast (Syrett 1981). First NH_4^+ is incorporated as amido group into glutamate (Glu) to form glutamine (Gln) by glutamine synthetase at the cost of ATP hydrolysis:

$$Glu + NH_4^+ + ATP \rightarrow Gln + ADP + P_i \quad (9)$$

The amido group of Gln is subsequently transferred to α-ketoglutarate and reduced by NADPH-dependent glutamate synthase, forming two Glu molecules:

$$Gln + \alpha\text{-ketoglutarate} + NADPH \rightarrow 2\ Glu + NADP^+ \quad (10)$$

One of the Glu molecules re-enters the assimilation pathway as a substrate for glutamine synthetase, thereby forming a cycle. The other Glu molecule is exported to the cytosol where transamination facilitates the syntheses of other amino acids.

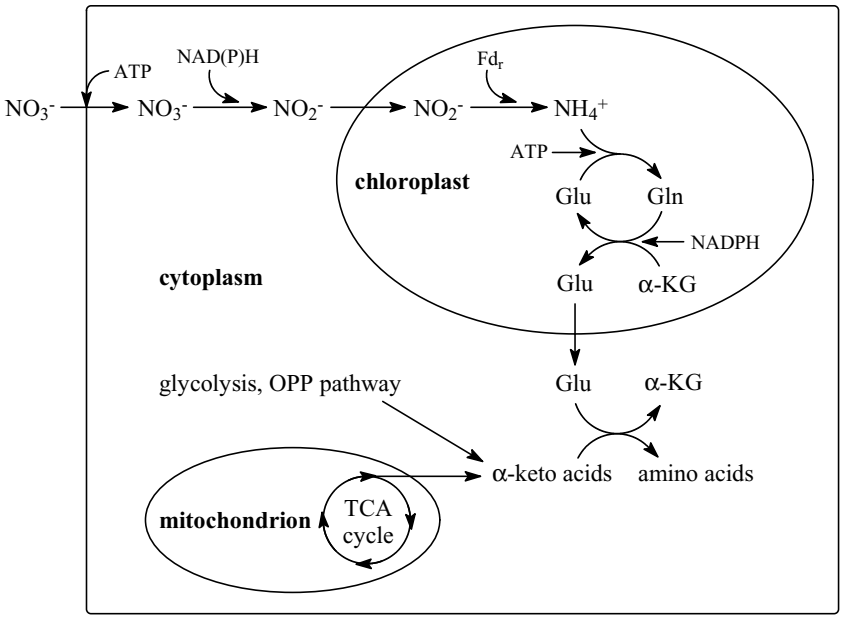

Fig. 1.7. General pathway of nitrogen assimilation in eukaryotic algae. NO_3^- is actively transported across the plasma membrane, and reduced to NO_2^- by nitrate reductase using NADH or NADPH. NO_2^- is imported to the chloroplast and reduced to NH_4^+ by nitrite reductase using reduced ferredoxin (Fd_r). NH_4^+ is first incorporated into glutamate (Glu) to form glutamine (Gln) by ATP-consuming glutamine synthetase, and the amido-N is subsequently transferred to α-ketoglutarate (α-KG) by NADPH-dependent glutamate synthase. The net production of Glu is exported to the cytoplasm where other amino acids are formed by transamination with α-keto acids provided by the respiratory pathways (glycolysis, oxidative pentose phosphate (OPP) pathway and tricarboxylic acid (TCA) cycle).

Organic carbon substrates for amino acids are provided by intermediates of the respiratory pathways (glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle). Intermediates from the tricarboxylic acid cycle used in amino acid synthesis must be replenished by β-carboxylation (see chapter 1.3). The pathway of nitrogen assimilation from NO_3^- to amino acids is illustrated in Fig. 1.7.

As demonstrated, the basic assimilatory pathways for inorganic nitrogen depend on organic carbon substrates, reductants and ATP that are supplied by both photosynthetic and respiratory pathways. Conversely, photosynthetic and respiratory processes are also dependent on nitrogen 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 level of free amino acids is further reflected in lower level of cellular protein, and usually by concurrent increase in carbohydrate or lipid content.

1.5 Biochemical composition

Apart from the frustule silica, the chemical composition of diatoms is quite similar to that of other microalgae (Parsons et al. 1961, Haug et al. 1973). Rapidly growing cells contain 30-60% protein, 10-50% carbohydrates, and 5-20% lipid (organic dry weight). Other important compunds are nucleic acids and nucleotides, pigments, vitamins, sugar alcohols, free amino acids and organic acids. However, chemical composition varies markedly as a function of growth conditions. Nutrient limitation generally leads to elevated levels of carbohydrate and lipid in relation to protein. Moreover, protein and carbohydrate pools oscillate in response to light:dark cycles.

Proteins play crucial roles in virtually all biological processes in diatoms as well as other cells. Nearly all biochemical reactions are catalyzed by specific protein enzymes. Proteins are also involved in transport and storage of metabolites and control of growth.

Lipids constitute a group of hydrophobic biomolecules that serve a variety of biological roles, such as energy reserves, signal molecules, and membrane components. Lipid or oil droplets often accumulate in the vacuoles or in cytoplasma under nutrient-limited conditions. The major class of membrane lipids is phospholipid, but the thylakoids contain galactolipid instead. The fatty acid composition of diatom lipids is characterized by high levels of C_{14}, C_{16} and C_{20} acids, particularly unsaturated 16:1 and 20:5 acids (Darley 1977).

Chlorophyll *a* is the central photosynthetic pigment, and constitute 1-6% of diatom cellular carbon (Parsons et al. 1961, Darley 1977). The main accessory pigments are the chlorophylls c_1 and c_2, and the carotenoids fucoxanthin, diadinoxanthin, diatoxanthin and β,β-carotene (Jeffrey & Vesk 1997). In a few diatom species, chlorophyll c_3 replaces chlorophyll c_1. The chloroplasts are usually golden-brown due to high levels of fucoxanthin.

Each cell contains a constant amount of the genetic molecule DNA (except during sexual reproduction), contributing 1-3% of diatom cellular carbon (Holm-Hansen 1969). In contrast, the RNA content generally increases with growth rate, and RNA/DNA ratios between <1 and 20 have been reported (Dortch et al. 1983).

Simple carbohydrates are the first products of photosynthetic carbon fixation (see chapter 1.3), and they provide precursors for all other biomolecules. Polysaccharides account for the bulk of diatom carbohydrates, and are often divided into three groups: (1) cell wall polysaccharides; (2) reserve glucan; and (3) extracellular polysaccharides.

In the diatom cell wall, an organic casing with structural polysaccharides coats the siliceous frustule. This casing consists of thin coating layers and the diatotepic layer on the inside of the frustule (Schmid et al. 1981, Stosch 1981, Volcani 1981). The diatotepum consists largely of acidic polysaccharides, often in the form of sulphated glucuronomannans (Ford & Percival 1965a, Percival et al. 1980). The casing also contains other heteropolysaccharides with widely varying proportions of galactose, glucose, mannose (and their corresponding uronic acids), fucose, rhamnose, xylose and other residues (Handa 1969, Allan et al. 1972, Hecky et al. 1973, Haug & Myklestad 1976). Strips of callose, a structural β-1,3-linked glucan, form a joint or gasket between the siliceous components (Waterkeyn & Bienfait 1987).

The principal storage product in diatoms is a β-1,3-glucan, which is often called chrysolaminaran. It is a water-soluble (1→3)-linked β-D-glucopyranan (DP 20-60) with occasional branching through C-2 and C-6 (Beattie et al. 1961, Ford & Percival 1965b, Paulsen & Myklestad 1978, McConville et al. 1986) (Fig. 1.8). Similar β-1,3-glucans presumably act as reserves in several other algal classes (Chrysophyceae, Euglenophyceae, Eustigmatophyceae, Haptophyceae, Phaeophyceae and Xanthophyceae). Due to the abundance of marine planktonic diatoms, β-1,3-glucan may rival cellulose as the polysaccharide with the highest annual production on Earth (Painter 1983). Despite its abundance, chrysolaminaran was rarely mentioned in early morphological investigations of

diatoms due to an absence of specific cytochemical reactions (Duke & Reimann 1977). However, some observations were made of hyaline bodies in the vacuole, which were assumed to be chrysolaminaran. Waterkeyn & Bienfait (1987) showed by fluorescence reaction with alkaline aniline blue that chrysolaminaran is localized in the vacuoles. The glucan accumulates during photosynthesis, particularly during nutrient limitation, and is consumed in darkness (Handa 1969, Myklestad & Haug 1972, Myklestad 1974). Such dynamics together with structural information and data on laminaran and paramylon metabolism indicate that chrysolaminaran is a photosynthetic reserve in diatoms (Craigie 1974, Darley 1977). Morris (1981) stated that pronounced synthesis of reserve carbohydrate and lipid depends on prolonged nutrient deficiency. However, more recent investigations have demonstrated substantial short-term variation in cellular β-1,3-glucan during both nutrient-sufficient and -deficient phases (Vårum & Myklestad 1984, Vårum et al. 1986, Myklestad 1988/1989).

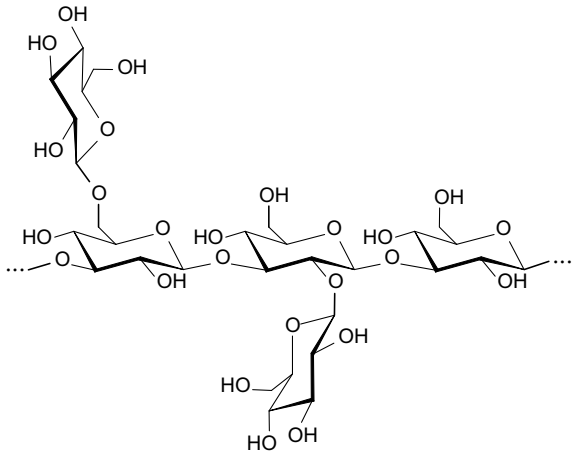

Fig. 1.8. Chemical structure of β-1,3-glucan with branches in C-2 and C-6.

Polysaccharides probably constitute the largest fraction of extracellularly produced components (Myklestad 2000). Most extracellular polysaccharides are complex heteroglycans, and their composition and structure can differ markedly from the intracellular ones (Smestad et al. 1974, 1975, Percival et al. 1980). They often form viscous or gelatinous mucilages that range from tight capsules to loose-slime matrices (Decho 1990). These secretions serve many functions in marine systems such as aggregation, ion exchange, protection from grazing, and attachment and locomotion of benthic species.

1.6 Scope

The scope of this contribution is to study the metabolism and function of β-1,3-glucan in marine diatoms. Previous investigations indicate that this polysaccharide has a very active metabolism, and the cellular glucan level varies markedly in response to growth conditions.

The marine planktonic diatom *Skeletonema costatum* (Grev.) Cleve was chosen as our experimental alga. This species is ubiquitous in coastal waters throughout the world, and has been subject for many ecological and physiological studies. The clone Skel-5 has been isolated from the Trondheimsfjord (Myklestad 1974) and maintained in axenic stock cultures at our laboratory.

We started out by constructing a strictly controlled cultivating system (photobioreactor), which was required to make accurate physiological studies of the alga. Moreover, a new method was developed for combined determination of β-1,3-glucan and cell wall polysaccharide in diatoms.

The metabolism of β-1,3-glucan was studied by growing the alga in batch culture, and measuring short-term fluxes of metabolites by chemical analyses as well as by ^{14}C tracer technique using labeled bicarbonate. The main strategy in the first part of the work was to accumulate glucan in the cells by photosynthesis under nitrogen limitation. Subsequently, the mobilization of glucan was triggered by replenishing nitrogen under various light conditions. Tracer technique was used to follow two separate fluxes of carbon during glucan mobilization, one is from glucan into amino acids and the other from β-carboxylation into amino acids (as well as other compounds).

In the second part of this work, algal production of carbohydrates and amino acids was investigated during different growth phases of *S. costatum*. Glucan dynamics were observed in response to diel light conditions and nitrogen depletion. An overall aim of these studies was to focus on the function of the storage polysaccharide β-1,3-glucan in the diatom.

2. METHODS

2.1 Organism and culture conditions

2.1.1 Organism and medium

The marine diatom *Skeletonema costatum* (Grev.) Cleve, clone Skel-5 was isolated from the Trondheimsfjord (Myklestad 1974), and is maintained in axenic stock cultures in the f/2 medium of Guillard & Ryther (1962) at 13°C. In the experiments, cultures were grown in different modifications of the f medium. The medium was based on filtered seawater of salinity ~32‰ from the Trondheimsfjord at 90 m depth, which was diluted to 3/4 with Milli-Q water (final salinity ~24‰).

2.1.2 Photobioreactor

An experimental reactor system was developed for strictly controlled growth of the alga (Fig. 2.1). The photobioreactor was designed as a closed CO_2 system with pH control. A detailed description of the reactor is provided in Paper 1.

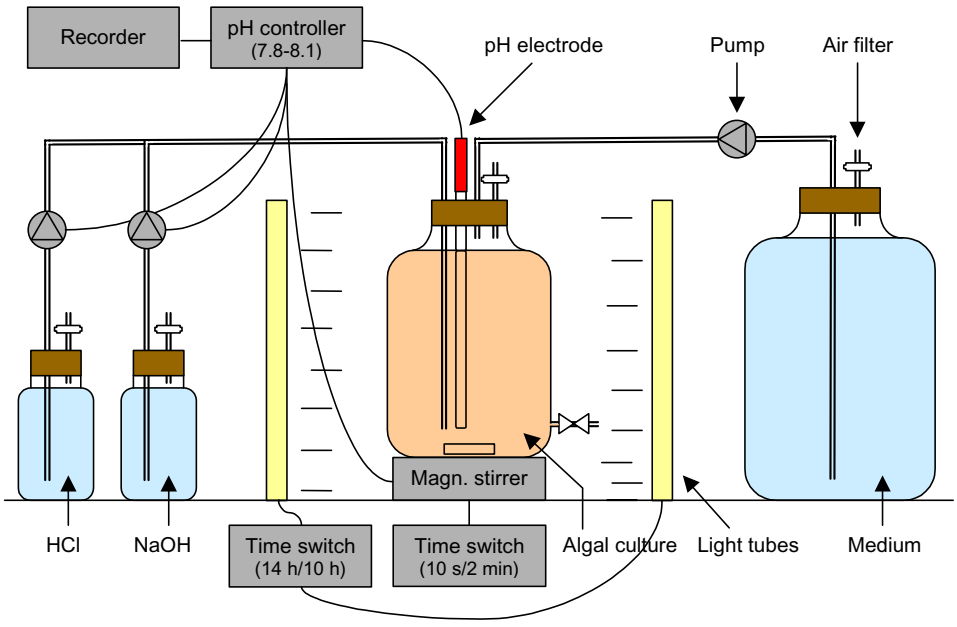

Fig. 2.1. Schematic drawing of experimental set up of the photobioreactor (Paper 1).

The reactor consisted essentially of a culture flask with magnetic stirring connected to a

medium reservoir and acid and base reservoirs (diluted HCl and NaOH, respectively). A pH electrode in the culture flask was coupled to a pH controller, which in turn was coupled to a recorder. By means of the pH controller, pH was measured continually, and kept within an interval of 7.8-8.1 by relay-activated titration with acid (in the light) and base (in the dark). Illumination was provided by racks of light tubes, and a light:dark cycle (usually 14:10 h) was set by a timer. The temperature of the culture cabinet was regulated to 13±1°C by a thermostat. The alga was inoculated aseptically in the reactor and grown as a batch culture. With slight modifications, the photobioreactor may also run as a continuous or semicontinuous system (Taraldsvik & Myklestad 2000).

The regulation of pH is illustrated by pH curves recorded during different growth phases of *S. costatum* (Fig. 2.2). During photosynthesis, pH increases mainly due to CO_2 fixation (see chapter 1.3), and to some extent increased alkalinity due to NO_3^- assimilation. Conversely, pH decreases in the dark due to CO_2 production by algal respiration. Case 1 shows pH as a function of time during 48 h of exponential growth ($2.5 \cdot 10^7$-$3.3 \cdot 10^8$ cells$\cdot l^{-1}$). The pH seemed to increase exponentially during the light period. In the dark, pH decreased approximately linearly, but faster each day due to increasing algal biomass.

Case 2 shows pH as a function of time during 48 h of the stationary phase following depletion of NO_3^- the preceding day (~$1.4 \cdot 10^9$ cells$\cdot l^{-1}$). The pH increased rapidly during the light periods, but progressively more slowly each day. This pH trend indicates active photosynthetic CO_2 fixation in the stationary phase, but gradually slower rates as the cells were loaded with reduced carbon reserves (glucan and lipid). In the dark, pH decreased slowly due to maintenance respiration.

Case 3 shows the effect of terminating pH control. The pH regulation was turned off in the middle of the photophase during exponential growth, resulting in progressively slower growth as pH increased freely ($4.0 \cdot 10^8$-$9.0 \cdot 10^8$ cells$\cdot l^{-1}$). Thus, high pH levels seem to inhibit growth of *S. costatum*.

This photobioreactor provides a convenient experimental apparatus for strictly controlled production of algal biomass and physiological studies. It is particularly effective for studying closed CO_2 systems with defined DIC concentration, for instance in ^{14}C tracer or DIC limitation experiments. Another possibility is to study the effects of pH on algal growth and metabolism (Taraldsvik & Myklestad 2000).

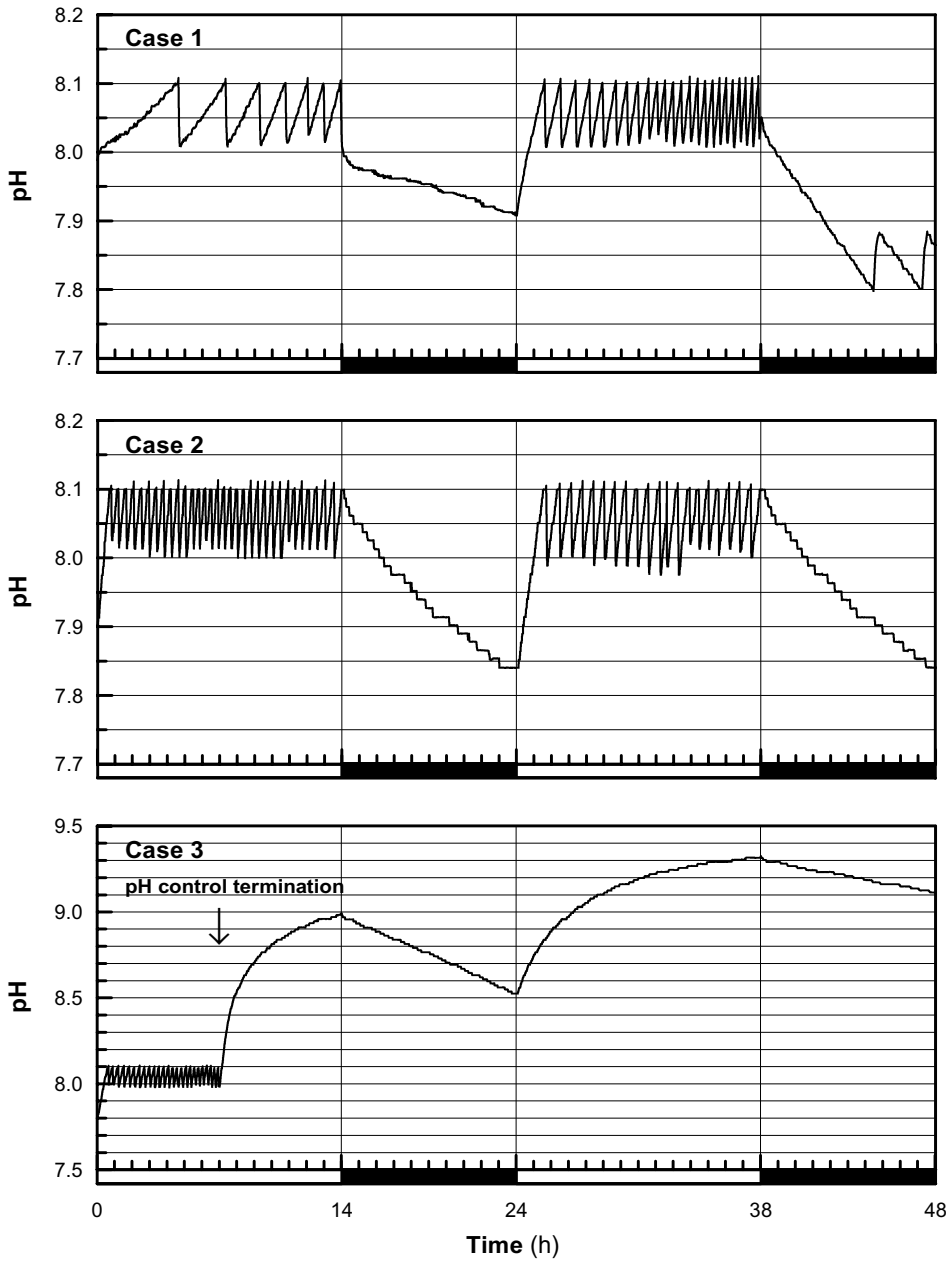

Fig. 2.2. pH as a function of time in *Skeletonema costatum* batch cultures with titration set points of 8.1 and 7.8, and a 14:10 h light:dark cycle (black bars indicate darkness) (Paper 1).

Case 1. Exponential growth ($2.5 \cdot 10^7$-$3.3 \cdot 10^8$ cells·l^{-1}).

Case 2. Stationary growth phase following NO_3^- depletion the preceding day ($1.4 \cdot 10^9$ cells·l^{-1}).

Case 3. Termination of pH control during exponential growth ($4.0 \cdot 10^8$-$9.0 \cdot 10^8$ cells·l^{-1}).

2.2 Polysaccharide analysis

A simple method was developed for combined determination of β-1,3-glucan and cell wall polysaccharides in diatoms. A detailed description of the method and demonstration for axenic cultures of *S. costatum* as well as natural phytoplankton are provided in Paper 2. The cellular glucan was first extracted by hot dilute H_2SO_4, and the cell wall polysaccharides were subsequently hydrolyzed by cold 80% H_2SO_4.

Cells from axenic cultures or field samples were harvested by filtration through 25-mm Whatman GF/C glass fiber filters under gentle vacuum (50 mm Hg). The filter was transferred directly to a glass vial, and stored at -20°C until analysis. Depending on cell size and the physiological condition 10^6-10^8 cells, or 0.5-2 mg dry matter, should be sufficient for analysis.

Cellular β-1,3-glucan was extracted by 5 ml 0.05 M H_2SO_4 at 60°C for 10 min using a water bath, and the extract was filtered (GF/C). The filter with cells was washed repeatedly with Milli-Q water, and dried overnight at 60°C. Cell wall polysaccharides were hydrolyzed by 0.5 ml 80% H_2SO_4 at 0-4°C for 20 h. The hydrolysate was diluted to 1 M H_2SO_4 by addition of 6.0 ml cold Milli-Q water, and filtered (GF/C).

The glucan extract and the cell wall hydrolysate were analyzed separately by the phenol-sulphuric acid method (Dubois et al. 1956). 0.5 ml 3% aqueous phenol and 5 ml concentrated H_2SO_4 were added to 2 ml sample (containing 5-100 μg carbohydrate) in a test tube, and the mixture was immediately stirred with a glass rod or vortexed. The tube was allowed to stand for 30 min, and then cooled in running water. Spectrophotometric extinction was measured in a 1-cm cell at 485 nm. The amount of reducing sugar was calculated using glucose as a standard with an extinction factor of ~0.1 $g \cdot l^{-1}$. A correction for the weight of non-hydrolyzed polysaccharide was made by a factor of 0.9. The determination was run in duplicate or triplicate with standard errors of 1-5%.

The method presented here provides a simple and rapid determination of β-1,3-glucan in diatoms. Myklestad & Haug (1972) and Myklestad (1978) have previously described methods for routine glucan determination where the cells were extracted twice with 0.05 M H_2SO_4 at 20°C for 1-2 h. Analyses of the marine diatom *Chaetoceros affinis* (Myklestad et al. 1972) and of natural phytoplankton populations (Haug et al. 1973) showed that glucose is the

predominant sugar in such acid extracts (>95%). Our new method is based on recent observations of phytoplankton membrane transition temperature, T_m (Myklestad & Swift 1998), and is much faster. T_m is defined as the temperature at which half the cellular soluble carbon is found extracellularly under standard conditions. This investigation revealed that the cells released virtually all cellular soluble carbon, including the reserve glucan, within 10 min incubation at a temperature >10°C above their T_m (28.5-41.5°C for diatoms). Membrane disorganization is the most probable mechanism for this release. On this basis we have chosen 60°C as the extraction temperature. The new method did not reveal significantly different results compared to the earlier method.

Hydrolysis of the cell wall polysaccharides was carried out using 80% H_2SO_4 in accordance with previous methods (Haug & Larsen 1962, Myklestad & Haug 1972). However, we have lowered the hydrolysis temperature from 20°C to only 0-4°C to slow down sugar decay reactions. Moreover, sequential extraction and hydrolysis of the same sample provide greater accuracy compared to previous methods. Myklestad & Haug (1972) took different samples for determination of glucan and total carbohydrate, and cell wall polysaccharide was estimated from the difference.

2.3 Radiocarbon fixation and biochemical fractionation

The radioisotope ^{14}C was used in tracer experiments by adding ^{14}C-labeled bicarbonate to the algal culture. This method is based on the ^{14}C technique introduced by Steemann-Nielsen (1952). At the end of the incubation, the organic ^{14}C is separated by acidification and purging of inorganic ^{14}C. Incorporation of organic ^{14}C in different fractions is determined by liquid scintillation counting. Photosynthetic partitioning of organic carbon into various classes of metabolites has previously been investigated by utilizing their different solubility in methanol/water, chloroform, and trichloroacetic acid (TCA) (Li et al. 1980, Hitchcock 1983, Lancelot 1984). The fractions in this scheme are generally assumed to be: methanol/water extract, low molecular weight (MW) metabolites (monosaccharides, amino/organic acids); chloroform extract, lipids; TCA extract, polysaccharides; and TCA residue, proteins. In diatoms, however, the reserve polysaccharide β-1,3-glucan is very soluble in water/methanol, and must also be included in the so-called low MW fraction (McConville et al. 1985, Myklestad 1988/1989). We have developed a modified fractionation method with further resolution of the 'low MW' fraction into amino acids (along with purines/pyrimidines) and β-1,3-glucan (along with monosaccharides and organic acids) by means of cation exchange

chromatography (Papers 3 and 4).

2.3.1 Cellular and extracellular carbon fixation

A specific amount of $NaH^{14}CO_3$ (usually 40 $\mu Ci \cdot l^{-1}$) was added to the culture, and samples (5 ml) were taken at different incubation times. Cells were harvested by filtration through 25-mm Whatman GF/C glass fiber filters under gentle vacuum (50 mm Hg) and washed immediately with filtered seawater (3×1 ml). The cells were purged of inorganic ^{14}C by exposing the filter to concentrated HCl vapor followed by CO_2 evaporation overnight. Cellular fixed ^{14}C (dpm) was measured by a Wallac 1410 scintillation counter using Wallac OptiPhase HiSafe 3 scintillation liquid (10 ml). Fixed ^{14}C was determined as the mean of two or three samples with standard errors of 3-5%. The rate of carbon fixation ($\partial C/\partial t$) was calculated from the rate of ^{14}C incorporation ($\partial A_{org}/\partial t$), the total activity of $NaH^{14}CO_3$ added to the culture (A_{tot}), the total DIC concentration, and a carbon isotope discrimination factor (f) according to Geider & Osborne (1992):

$$\frac{\partial C}{\partial t} = f \frac{\partial A_{org}}{\partial t} \frac{DIC}{A_{tot}} \qquad (11)$$

The medium filtrate was purged of inorganic ^{14}C by acidification to pH<2 with H_3PO_4 followed by CO_2 evaporation overnight. Extracellular fixed ^{14}C was measured by liquid scintillation counting as described above.

2.3.2 Biochemical fractionation of cellular fixed carbon

For biochemical fractionation, cells were harvested and purged of inorganic ^{14}C as described above. A 'low MW' fraction was isolated by extracting the filter with 3 ml Milli-Q water at 70°C for 30 min, and washing with 2 ml Milli-Q water. The extract was removed from the filter as supernatant after centrifugation (1400 g, 10 min), and the filter was dried at 60°C overnight. A lipid fraction was isolated by extracting the filter with 3 ml chloroform at 4°C for 10 min, and washing with 2 ml chloroform. A polysaccharide fraction (also containing nucleic acids) was isolated by extracting the filter with 3 ml 5% TCA at 95°C for 30 min, and washing with 2 ml 5% TCA. After TCA-extraction, an insoluble protein fraction remained on the filter. A scheme to illustrate the fractionation procedure is presented in Fig. 2.3.

The 'low MW' extract was separated by cation exchange chromatography into a basic fraction

(amino acids, purines, pyrimidines etc.) denoted 'amino acids' and an acid/neutral fraction (β-1,3-glucan, monosaccharides, organic acids etc.) denoted 'glucan'. A Pharmacia Resource S (methyl sulphonate) strong cation exchange column was equilibrated with 50 mM citrate, pH 2.0. The extract was filtered (0.2 μm), mixed 1:1 with 100 mM citrate, pH 2.0, and applied to the column. At first the acid/neutral fraction was eluted with 50 mM citrate, pH 2.0, and then the basic fraction with 20 mM $Na_2B_4O_7$, pH 9.0 with 0.3 M NaCl. The column was regenerated by flushing with 50 mM citrate, pH 2.0 with 0.5 M NaCl. Prior to chromatography, all buffers were filtered (0.2 μm) and degassed with helium.

Fixed ^{14}C in the five cellular fractions was measured by liquid scintillation counting as described above. A recovery of 85-99% was obtained by comparing the activity of all fractions with the total cellular activity (Paper 4).

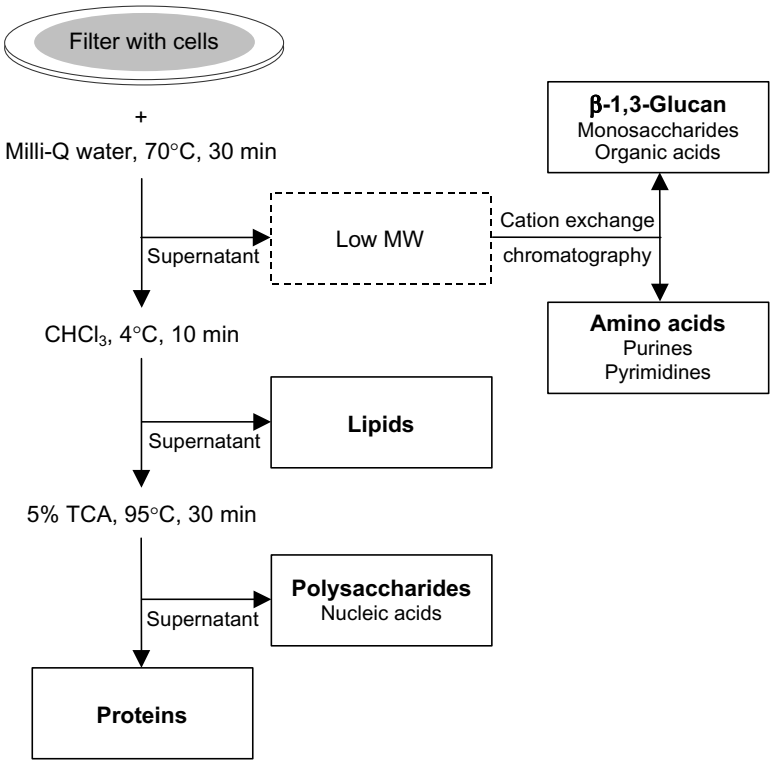

Fig. 2.3. Scheme of biochemical fractionation (Paper 4). The major component in each fraction is emphasized.

2.4 Additional analyses

Culture samples were directly used for microscopic cell counting (Bürker haemocytometer). Samples for analyses of cellular components were generally obtained by filtering the culture through Whatman GF/C glass fiber filters under gentle vacuum (50 mm Hg), and filtrates were used for analyses of dissolved components.

Cellular chlorophyll *a* (chl *a*) was extracted by cold acetone and measured by fluorometry (Holm-Hansen et al. 1965) or spectrophotometry (Jeffrey & Humphrey 1975). Cellular organic carbon and nitrogen were measured with an elemental analyzer (Carlo Erba NA 1500) (Kirsten 1979). Cellular free amino acids were extracted by hot Milli-Q water and analyzed by reverse phase HPLC (Waters Nova-Pak C_{18} column) with precolumn fluorescence derivatization with *o*-phthaldialdehyde (Lindroth & Mopper 1979) as modified by Flynn (1988).

Dissolved nutrients (NO_3^-, NH_4^+, P_i) were determined by standard seawater analyses (Strickland & Parsons 1972). Dissolved inorganic and organic carbon (DIC and DOC) were measured by a high-temperature catalytic oxidation analyzer (Dohrmann DC-190) (Sugimura & Suzuki 1988).

Further details and precision of methods are provided in the Papers.

3. METABOLISM OF β-1,3-GLUCAN

3.1 Biosynthesis

Biosynthetic investigations of β-1,3-glucans in algae are scarce. Goldemberg & Marechal (1963) prepared a paramylon synthase from *Euglena gracilis* (Euglenophyceae), which had highly specific UDP-glucose-β-1,3-glucan β-3-glucosyltransferase activity. Roessler (1987) measured activities of both UDP-glucose pyrophosphorylase and UDP-glucose-β-1,3-glucan β-3-glycosyltransferase (chrysolaminaran synthase) from the marine diatom *Cyclotella cryptica*. These studies suggest that glucan synthesis in diatoms follows the same general pathway as in most other organisms (Fig. 3.1). First UDP-glucose is formed from glucose-1-phosphate and UTP through the action of UDP-glucose pyrophosphorylase. The UDP-glucose then serves as the glucosyl donor for glucan polymerization at the non-reducing end by the glycosyltransferase. As far as we know, mechanisms of priming and branching have not been identified, but it seems likely that they are similar to those in the well characterized α-1,4-glucans. Synthesis of glycogen or starch requires a glycoprotein primer, and branching is carried out by rearrangement of the polysaccharide chain through the action of a transglycosylase (Stoddart 1984).

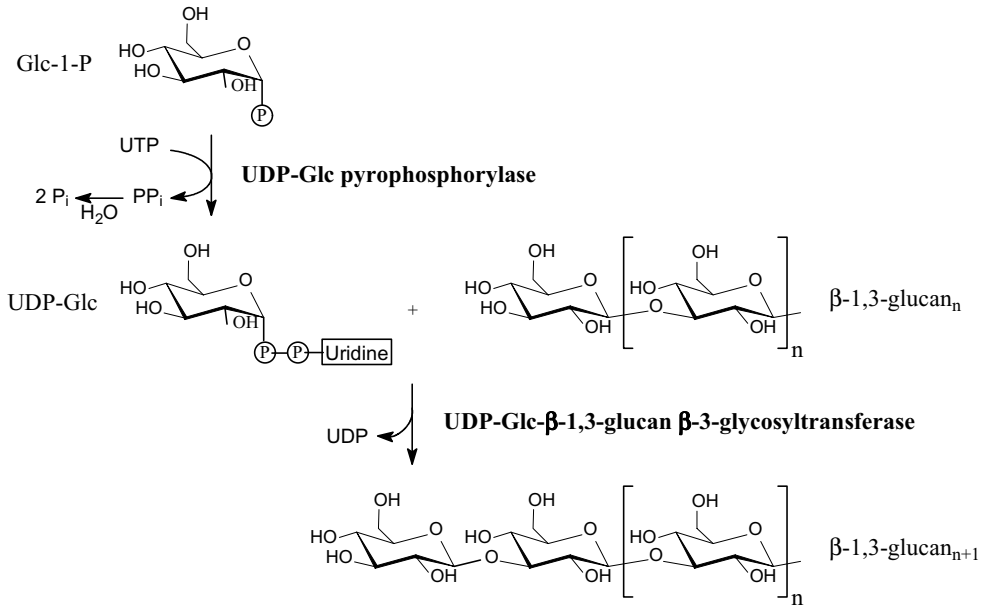

Fig. 3.1. Pathway of β-1,3-glucan biosynthesis. Abbreviations: Glc, glucose; Glc-1-P, glucose-1-phosphate.

In a study with the marine diatom *Skeletonema costatum*, the synthesis of β-1,3-glucan was investigated by ^{14}C pulse labeling (Paper 4). The diatom was grown in batch culture until NO_3^- was exhausted ($2.3 \cdot 10^9$ cells$\cdot l^{-1}$, 1.0 mg$\cdot l^{-1}$ chl *a*). The N-depleted cells were then incubated in high light (200 µmol photons$\cdot m^{-2} \cdot s^{-1}$) with ^{14}C-bicarbonate (4.5 h). Fixation of ^{14}C was measured, and biochemical fractionation was carried out according to our new method (see chapter 2.3.2). The C-specific rate of photosynthetic CO_2 fixation was calculated as 0.17 h^{-1} (114 fmol C$\cdot$cell$^{-1} \cdot h^{-1}$). The ^{14}C label was mainly incorporated into the glucan fraction (85%), and the rest was distributed between amino acids, proteins, other polysaccharides and lipids (3-5% each) (Fig. 3.2). Chemical analyses confirmed a high rate of glucan production whereas nitrogenous components were approximately constant (Fig. 3.3). In a similar study, Myklestad (1988/1989) reported 74% ^{14}C incorporation in the glucan fraction in the stationary growth phase, compared to 62% in the late exponential phase. The results show that synthesis of β-1,3-glucan is very active in diatoms during photosynthesis under both N-limited and N-sufficient conditions. Sugars produced by the Calvin cycle are rapidly converted to glucose-1-phosphate and polymerized into glucan by the UDP-glucose pyrophosphorylase and glycosyltransferase.

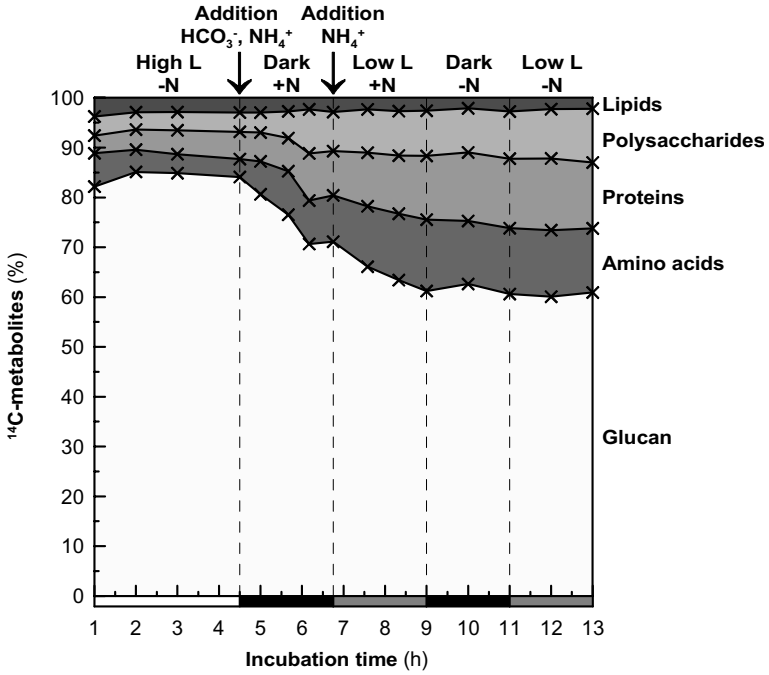

Fig. 3.2. Distribution of ^{14}C metabolites (%) in different biochemical fractions in *Skeletonema costatum* as a function of time (black bars indicate darkness; shaded bars indicate low light) (Paper 4).

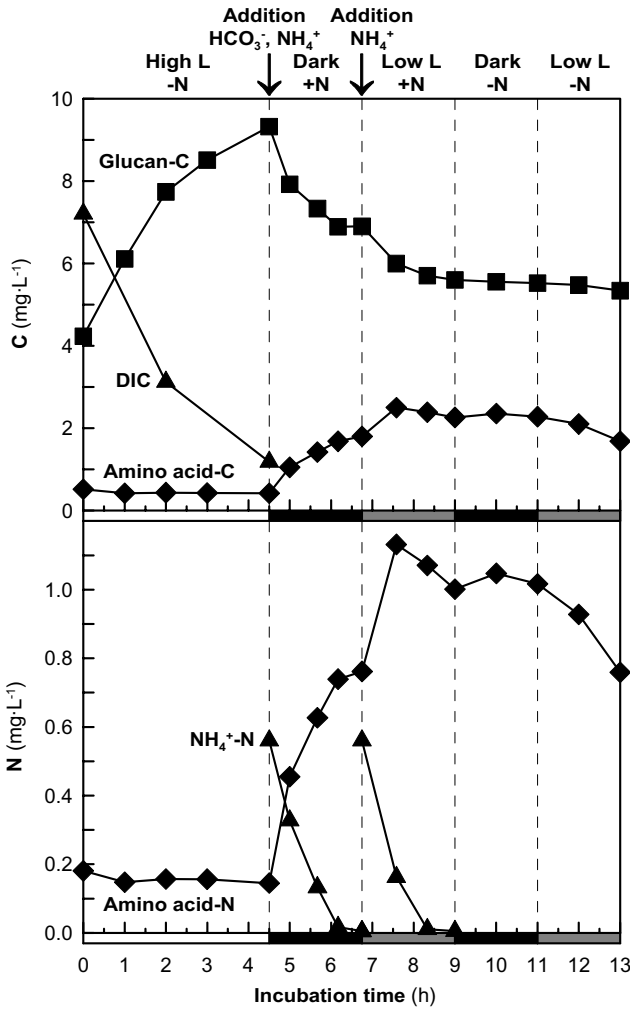

Fig. 3.3. Cellular β-1,3-glucan and free amino acids, DIC, and dissolved NH_4^+ in mass units of C and N per culture volume of *Skeletonema costatum* as functions of time (black bars indicate darkness; shaded bars indicate low light) (Paper 4).

Chrysolaminaran is localized in the vacuole of diatoms, and consequently, sugars produced by the Calvin cycle must be transported from the chloroplast via the cytosol into the vacuole. It is not clear how this transport is facilitated, and in what compartment the glucan synthesis takes place. However, polysaccharide synthesis in eukaryotes generally proceeds by the elaboration of endomembrane systems (Stoddart 1984). The growing saccharide chain is anchored to the primer in one membrane and its non-reducing terminal extends out to the glycosyltransferases in another parallel membrane. An ultrastructure study of the prymnesiophyte *Phaeocystis pouchetii* suggests that chrysolaminaran vesicles are formed in the Golgi body (Chang 1984).

3.2 Mobilization

Handa (1969) reported that β-1,3-glucan in *S. costatum* is used as a respiration substrate during incubation in darkness. Further investigations by Cuhel et al. (1984), Myklestad & Vårum (1984) and Myklestad (1988/1989) suggested that carbon skeletons from the glucan are used in protein synthesis during dark phases. Myklestad et al. (1982) found an exo-β-1,3-glucanase in several diatoms, which is probably active in glucan metabolism. Together these studies indicate that the storage polysaccharide can furnish both energy and precursors for cellular metabolism. However, the specific transformation of β-1,3-glucan into other metabolites has so far not been investigated.

Mobilization of β-1,3-glucan in *S. costatum* was investigated in the study described in chapter 3.1 (Paper 4). After a pulse labeling phase (4.5 h), the tracer was diluted with 1 mM unlabeled bicarbonate, and a ^{14}C chase was carried out by incubation in the dark and in low light (20 μmol photons·m^{-2}·s^{-1}) with additions of 40 μM NH_4^+ (2.3 h each). As a negative N control, the cells were finally incubated in the dark and in low light without a source of nitrogen (2 h each). Added NH_4^+ was assimilated at an initial N-specific rate of 0.11 h^{-1} (12 fmol N·$cell^{-1}$·h^{-1}) in both darkness and low light. In the dark period, labeled glucan decreased by 16%, while labeling of amino acids increased 2.6×, proteins 1.7×, and polysaccharides 2.0× (Fig. 3.2). Radiolabeled amino acids increased rapidly in the early stage of NH_4^+ assimilation, whereas proteins and polysaccharides were more rapidly labeled in the late stage. In the low light period, labeled glucan further decreased by 14%, while labeling of amino acids increased 1.5×, proteins 1.4×, and polysaccharides 1.2×. The lipid fraction remained constant at only 3% of radiolabeled metabolites. During incubation under similar conditions without a nitrogen source, the distribution of ^{14}C label was approximately constant. Throughout the experiment, radiolabeled extracellular metabolites ($DO^{14}C$) accounted for only 0.5% of cellular ^{14}C fixation.

Chemical analysis showed that cellular β-1,3-glucan decreased even more than indicated by the tracer results (26% in the dark, 19% in low light), which may be due to mobilization of a non-labeled glucan fraction (Fig. 3.3). The cells had already accumulated 4.2 mg glucan-C·l^{-1} before the ^{14}C pulse, compared to finally 9.3 mg glucan-C·l^{-1}. HPLC analysis showed that the level of cellular free amino acids was low during nitrogen depletion, but increased rapidly upon addition of NH_4^+ (4.3× within 2.3 h). The cellular pools of amino acids and glucan changed most rapidly in the early stage of NH_4^+ assimilation, and became constant, or slowly

decreased, after NH_4^+ was exhausted. Free amino acids only increased by 36% in the second NH_4^+ assimilation phase (low light), indicating that they are drawn off for protein synthesis at a higher rate when their cellular concentrations are higher.

Table 3.1. Composition of cellular free amino acids per culture volume of *Skeletonema costatum* (μM) as a function of time (Paper 4). The sum of all free amino acids was also calculated per cell.

	Phase										
	High L -N		Dark +N				Low L +N			Dark -N	Low L -N
Incubation time	0 h	4.5 h	0.5 h	1.2 h	1.7 h	2.3 h	0.8 h	1.6 h	2.3 h	2.0 h	2.0 h
Asp	1.16	0.72	1.20	1.56	2.03	2.37	2.41	2.25	2.32	3.03	1.42
Glu	2.22	1.66	1.45	1.66	2.82	4.67	2.85	3.85	4.63	4.47	3.18
Asn	0.44	0.26	1.09	1.53	1.77	1.84	3.49	3.59	3.81	3.90	3.07
His	-	-	-	0.02	0.04	0.05	0.08	0.12	0.12	0.14	0.02
Ser	0.57	0.60	0.36	0.28	0.27	0.33	0.27	0.27	0.30	0.34	0.19
Gln	1.09	0.70	11.0	16.1	20.2	16.4	28.4	25.2	21.4	21.3	17.7
Gly+Arg	0.89	0.82	1.03	1.27	1.76	2.55	2.86	3.26	3.65	4.00	2.30
Thr	0.42	0.36	0.28	0.31	0.34	0.56	0.45	0.51	0.59	0.59	0.30
Ala	1.10	0.92	1.10	1.23	1.57	2.15	2.05	1.92	2.13	1.77	0.91
Tyr	0.14	0.15	0.12	0.15	0.15	0.19	0.17	0.18	0.22	0.20	0.10
Val	0.32	0.28	0.20	0.19	0.22	0.32	0.21	0.23	0.26	0.23	0.13
Phe	0.08	0.10	0.04	0.04	0.05	0.08	0.07	0.14	0.12	0.06	0.01
Ile	0.25	0.25	0.13	0.13	0.14	0.19	0.16	0.15	0.16	0.16	0.07
Leu	0.27	0.26	0.15	0.13	0.15	0.23	0.16	0.15	0.18	0.17	0.08
Lys	0.55	0.54	0.34	0.31	0.34	0.47	0.38	0.39	0.39	0.43	0.22
Sum	9.5	7.6	18.5	24.9	31.8	32.4	44.0	42.2	40.2	40.7	29.7
Sum (fmol·cell^{-1})	3.6	2.9	7.1	9.6	12.2	12.5	16.9	16.2	15.5	15.7	11.4

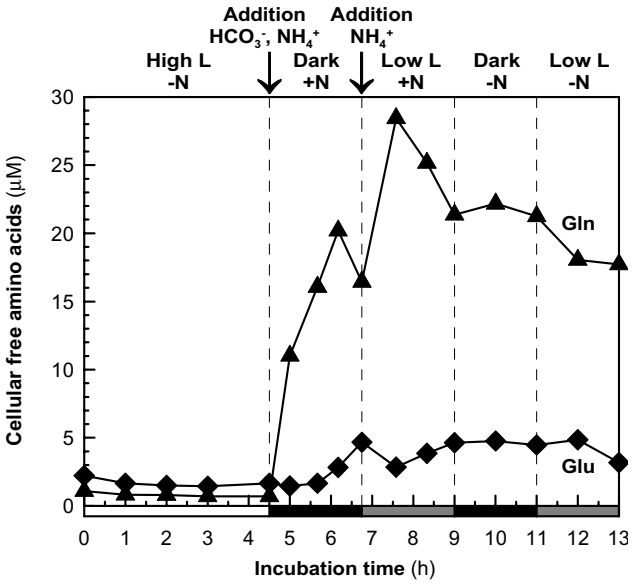

Fig. 3.4. Cellular free glutamate (Glu) and glutamine (Gln) per culture volume of *Skeletonema costatum* as functions of time (black bars indicate darkness; shaded bars indicate low light).

The composition of cellular free amino acids changed substantially at the onset of NH_4^+ assimilation (Table 3.1, Fig. 3.4). During NH_4^+ uptake, Gln accumulated very rapidly compared to the other amino acids, and the Gln/Glu ratio increased from 0.4 to 10. When NH_4^+ was exhausted, however, Gln quickly decreased while Glu still increased significantly. The variations in Glu and Gln probably reflect the main route of NH_4^+ assimilation: the glutamine synthetase-glutamate synthase cycle (see chapter 1.4). During NH_4^+ assimilation, the concentrations of Ala, Asn, Asp and Gly+Arg also increased considerably, while the other nine amino acids only slightly increased or decreased. Similar results were obtained in another study with dark NH_4^+ assimilation (Paper 3, chapter 3.3). The level of each amino acid reflects its rate of synthesis diminished by incorporation into proteins or utilization in synthesis of other components (purines, pyrimidines, etc.). Apart from the Gln/Glu ratio, variations in free amino acid composition are generally considered to be quite complex (Flynn 1990). However, the amino acids that increased significantly during NH_4^+ assimilation are all synthesized in short pathways from intermediates in the respiratory pathways. Ala and Asp are formed by transaminations of pyruvate and oxaloacetate, respectively, and Asn is formed from Asp by asparagine synthetase. Arg and Gly are formed in short pathways from Glu and 3-phosphoglycerate, respectively. In particular Asp, Glu and Gln have numerous metabolic roles, acting as nitrogen donors and precursors for other amino acids and nucleotides. Thus, the changes in amino acid composition upon nitrogen replenishment strongly indicate that *de novo* biosynthesis of amino acids took place and not protein degradation, which would provide quite a different distribution.

In a different experiment, glucan mobilization in saturating light was investigated under inorganic carbon limitation (Paper 4). As before, the diatom was grown in batch culture until NO_3^- was exhausted ($1.1 \cdot 10^9$ cells$\cdot l^{-1}$, 0.6 mg$\cdot l^{-1}$ chl *a*). The N-depleted cells were first incubated in high light (200 µmol photons$\cdot m^{-2} \cdot s^{-1}$) until DIC was exhausted (3 h), and then in high light with addition of 150 µM NH_4^+ under inorganic carbon limitation (6 h). In subsequent recovery phases, 1 mM bicarbonate was added, and the cells were incubated in the dark and in low light (20 µmol photons$\cdot m^{-2} \cdot s^{-1}$) (1.3 h each). Added NH_4^+ was assimilated at a N-specific rate of 0.023 h^{-1} (6.1 fmol N$\cdot cell^{-1} \cdot h^{-1}$) and cellular β-1,3-glucan decreased by 15% within 6 h (Fig. 3.5). The results indicate that glucan was mobilized in saturating light during NH_4^+ assimilation, even though inorganic carbon was modifying the metabolic rates. In this case, storage carhohydrate was probably the only source of easily available carbon for the alga since the net uptake of inorganic carbon was negligible.

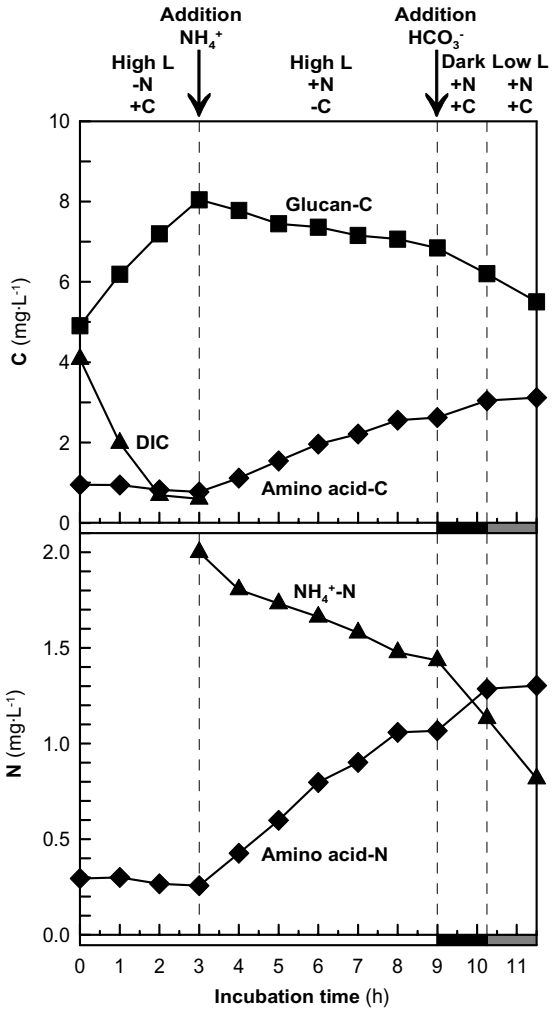

Fig. 3.5. Cellular β-1,3-glucan and free amino acids, DIC, and dissolved NH_4^+ in mass units of C and N per culture volume of *Skeletonema costatum* as functions of time (black bar indicates darkness; shaded bar indicates low light) (Paper 4).

HPLC analysis showed that the level of cellular free amino acids and the Gln/Glu ratio increased significantly upon addition of NH_4^+, but relatively slowly compared to DIC-replete cells in the dark (Table 3.2). In contrast with results under DIC-replete conditions, the levels of Asp and Gly+Arg dropped upon nitrogen replenishment, and Asn increased relatively slowly. These amino acids, however, increased rapidly upon addition of bicarbonate, suggesting that their rates of synthesis may be modified by inorganic carbon. The rates of NH_4^+ assimilation and glucan mobilization were also higher after bicarbonate was added. The results are consistent with the findings that anaplerotic reaction rates increase with inorganic

carbon (Holdsworth & Bruck 1977).

Table 3.2. Composition of cellular free amino acids per culture volume of *Skeletonema costatum* (μM) as a function of time (Paper 4). The sum of all free amino acids was also calculated per cell.

	Phase							
	High L -N +C		High L +N -C				Dark +N +C	Low L +N +C
Incubation time	0 h	3.0 h	1.0 h	2.0 h	4.0 h	6.0 h	1.3 h	1.3 h
Asp	2.53	1.03	0.24	0.24	0.36	0.45	3.18	4.36
Glu	2.16	1.13	0.99	1.83	2.62	3.49	1.92	3.75
Asn	0.20	0.17	0.44	0.50	0.56	0.64	1.40	2.07
His	-	-	-	-	0.13	0.20	0.22	0.26
Ser	0.34	0.40	0.26	0.23	0.44	0.55	0.45	0.43
Gln	0.27	0.29	8.1	13.4	24.9	28.1	34.9	32.4
Gly+Arg	2.20	3.85	1.94	1.16	1.85	1.61	1.86	2.93
Thr	0.60	0.37	0.43	0.40	0.42	0.55	0.49	0.72
Ala	2.54	1.88	1.75	3.19	3.03	3.99	4.31	5.29
Tyr	0.30	0.21	0.35	0.47	0.45	0.59	0.46	0.39
Val	1.72	1.51	1.33	1.33	1.37	1.47	1.32	1.02
Phe	0.17	0.15	0.15	0.18	0.24	0.30	0.19	0.22
Ile	0.86	0.75	0.61	0.63	0.68	0.72	0.60	0.38
Leu	0.85	0.86	0.73	0.76	0.89	0.95	0.78	0.44
Lys	2.93	2.66	2.30	2.18	1.95	1.69	1.49	1.71
Sum	17.7	15.3	19.6	26.8	38.7	45.3	53.6	56.4
Sum (fmol·cell^{-1})	16.1	13.9	17.8	24.4	35.2	41.2	48.7	51.3

This study strongly indicates that β-1,3-glucan provides precursors for amino acid synthesis, and eventually protein synthesis. Myklestad et al. (1982) demonstrated that the glucan in diatoms is hydrolyzed by an exo-β-1,3-glucanase, splitting off glucose at the non-reducing end. Glucose can be further metabolized by the respiratory pathways (glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle), providing precursors for amino acid synthesis (Fig. 3.6). Part of the ^{14}C-labeled 'amino acid'-fraction is probably accounted for by purines and pyrimidines, which, like the amino acids, are positively charged at low pH (see chapter 2.3.2). These bases are synthesized *de novo* from amino acids and other precursors. Results from the ^{14}C chase also indicated significant synthesis of other polysaccharides from mobilized β-1,3-glucan. Nucleic acids, however, also contribute to the ^{14}C-labeled 'polysaccharide' fraction. RNA probably accounts for some of the ^{14}C label, as active protein synthesis is strictly coupled to RNA synthesis. Most activated monosaccharide precursors that build up cell wall polysaccharides are synthesized in a few steps from glucose (Fig. 3.7). In the case of nucleic acids, ribose-5-phosphate is synthesized from glucose-6-phosphate through the pentose phosphate pathway, and forms nucleotides with purines or pyrimidines.

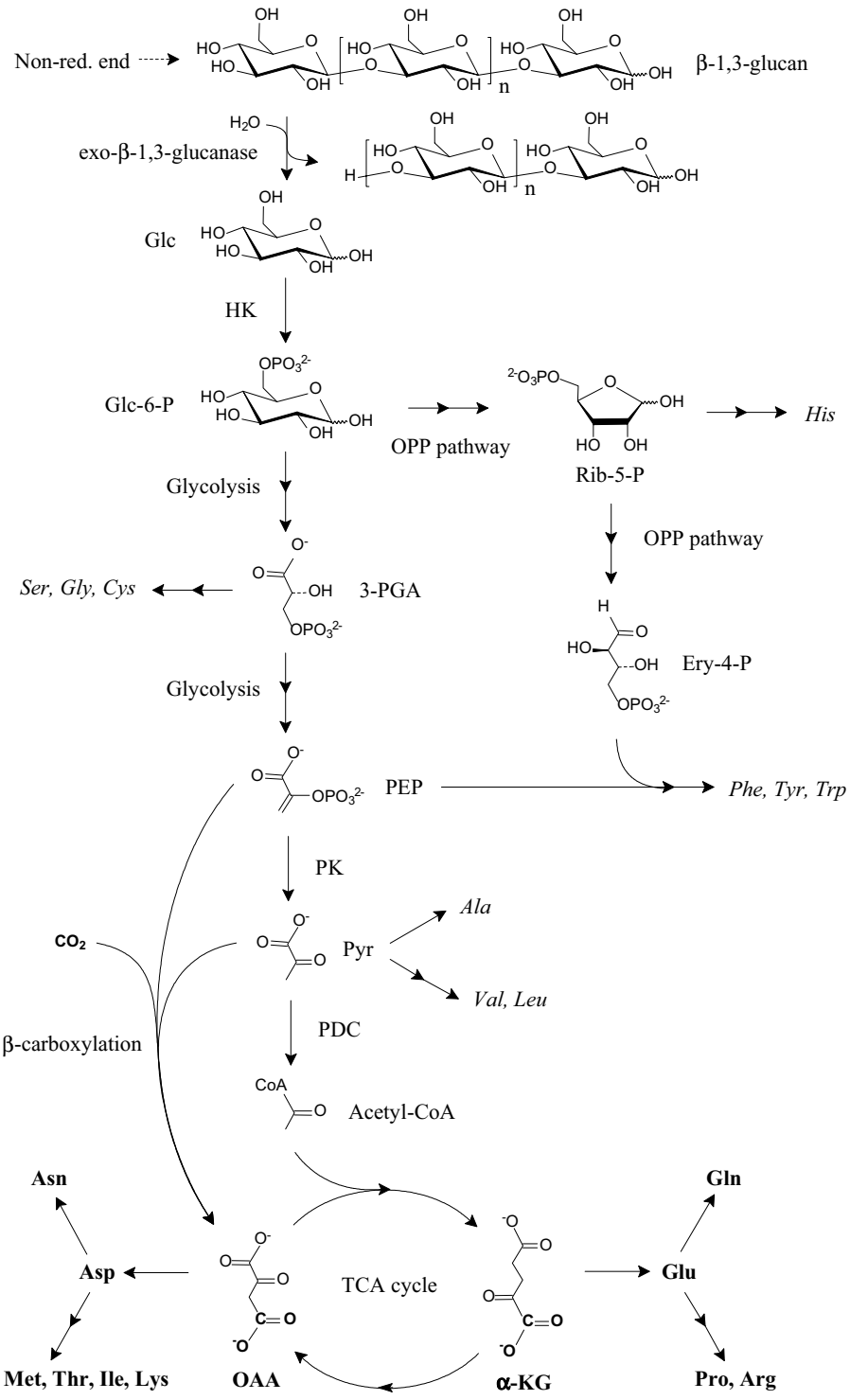

Fig. 3.6 (text on next page).

Fig. 3.6 (previous page). Scheme of metabolic pathways providing amino acid precursors from β-1,3-glucan (Paper 3). The glucan is hydrolyzed by exo-β-1,3-glucanase, splitting off glucose at the non-reducing end. Glucose is metabolized by the respiratory pathways (glycolysis, OPP pathway and TCA cycle), producing precursors for amino acid biosynthesis. Intermediates from the TCA cycle used in amino acid synthesis (oxaloacetate, α-ketoglutarate) must be replenished by β-carboxylation (indicated by bold type). Amino acids merely synthesized from intermediates in the glycolytic and OPP pathways, on the other hand, do not depend on β-carboxylation (italic type). Abbreviations: CoA, coenzyme A; Ery-4-P, erythrose-4-phosphate; Glc, glucose; Glc-6-P, glucose-6-phosphate; HK, hexokinase; OAA, oxaloacetate; α-KG, α-ketoglutarate; OPP, oxidative pentose phosphate; PDC, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; 3-PGA; 3-phosphoglycerate; PK, pyruvate kinase; Pyr, pyruvate; Rib-5-P, ribose-5-phosphate; TCA, tricarboxylic acid.

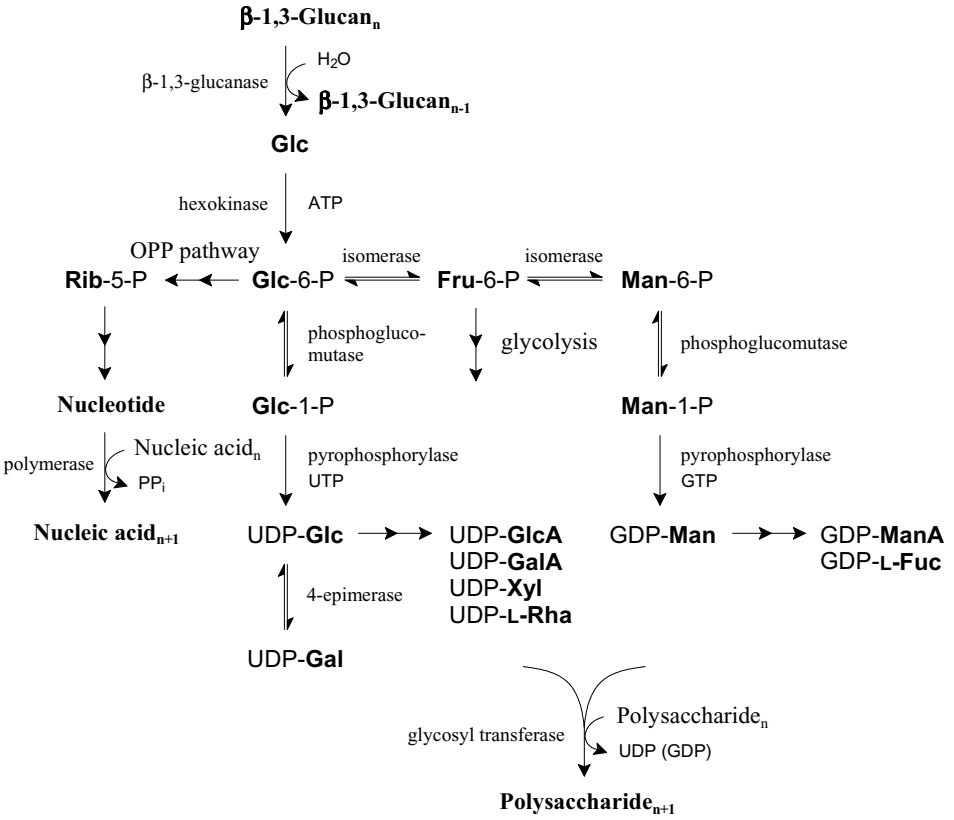

Fig. 3.7. Scheme of metabolic pathways providing precursors for polysaccharides and nucleic acids from β-1,3-glucan (Paper 4). The glucan is hydrolyzed by exo-β-1,3-glucanase, splitting off glucose at the non-reducing end. Different monosaccharide precursors are synthesized in few steps from glucose, and polymerized by glycosyl transferases. Alternatively, ribose-5-phosphate is synthesized from glucose-6-phosphate through the pentose phosphate pathway, and forms nucleotides with purines or pyrimidines, which can be polymerized. Abbreviations: Fru, fructose; Fuc, fucose; Gal, galactose; GalA, galacturonic acid; Glc, glucose; GlcA, glucuronic acid; Man, mannose; ManA, mannuronic acid; OPP, oxidative pentose phosphate; Rha, rhamnose; Rib, ribose; Xyl, xylose.

3.3 β-Carboxylation

Following the introduction of ^{14}C tracer technique, dark carbon fixation was observed in macrophytes (Craigie 1963) and in phytoplankton (Morris et al. 1971a). A practical problem in measuring photosynthesis is how to correct for such non-photosynthetic carbon fixation (Geider & Osborne 1992). In actively growing algae, the ratio of carboxylation in the dark compared to light saturation is typically <0.05, although it ranges from <0.01 to >0.4 (Mortain-Bertrand et al. 1988). Light-independent carbon fixation (LICF) is primarily accounted for by anaplerotic β-carboxylation, which is required to replenish intermediates in the tricarboxylic acid cycle that are withdrawn for synthesis of amino acids and other components (see chapter 1.3). The rate of β-carboxylation increases following addition of NH_4^+ to N-deficient but not N-replete cells (Morris et al. 1971b, Guy et al. 1989), and this has been used as a test for nitrogen limitation. Nitrogen assimilation by N-replete cells depends on active photosynthesis, whereas N-deplete cells can assimilate nitrogen in darkness at the expense of accumulated carbohydrate reserves (Syrett 1981). Studies with diatoms show that the substrate for β-carboxylation (phosphoenolpyruvate) originates from 3-phosphoglycerate (Calvin cycle intermediate) in light, and from polysaccharide in darkness (Kremer & Berks 1978, Appleby et al. 1980, Morris 1980, Mortain-Bertrand et al. 1987, 1988).

Effects of NH_4^+ assimilation on LICF in *S. costatum* were investigated by ^{14}C fixation in the dark (Paper 3). The diatom was grown in batch culture until NO_3^- was exhausted ($1.6 \cdot 10^9$ cells$\cdot l^{-1}$, 0.8 mg$\cdot l^{-1}$ chl *a*). The N-depleted cells were first incubated in the dark with ^{14}C-bicarbonate but without a source of nitrogen (2 h), and then in the dark with addition of 60 μM NH_4^+ (3 h). During nitrogen depletion, the C-specific carboxylation rate was only $0.88 \cdot 10^{-3}$ h^{-1} (1.0 fmol C$\cdot$cell$^{-1}\cdot h^{-1}$), and biochemical ^{14}C fractionation showed that mainly low MW metabolites were labeled (90%) (Fig. 3.8). Added NH_4^+ was assimilated at a N-specific rate of 0.10 h^{-1} (12 fmol N$\cdot$cell$^{-1}\cdot h^{-1}$), and the carboxylation rate increased 4-fold ($3.6 \cdot 10^{-3}$ h^{-1} or 4.0 fmol C$\cdot$cell$^{-1}\cdot h^{-1}$). The amino acid fraction accounted for virtually all net ^{14}C fixation during the first hour of NH_4^+ assimilation. As the carboxylation rate decreased after 1.5 h, the ^{14}C-labeled amino acid pool stabilized, while the macromolecular fraction (indicating protein) increased from 10% to 24% of the ^{14}C-metabolites. Radiolabeled extracellular metabolites ($DO^{14}C$) accounted for only 3.6% of cellular ^{14}C fixation. HPLC analysis showed that the level of cellular free amino acids was low during nitrogen depletion, but increased rapidly upon addition of NH_4^+ (3.2× within 3 h) (Fig. 3.9).

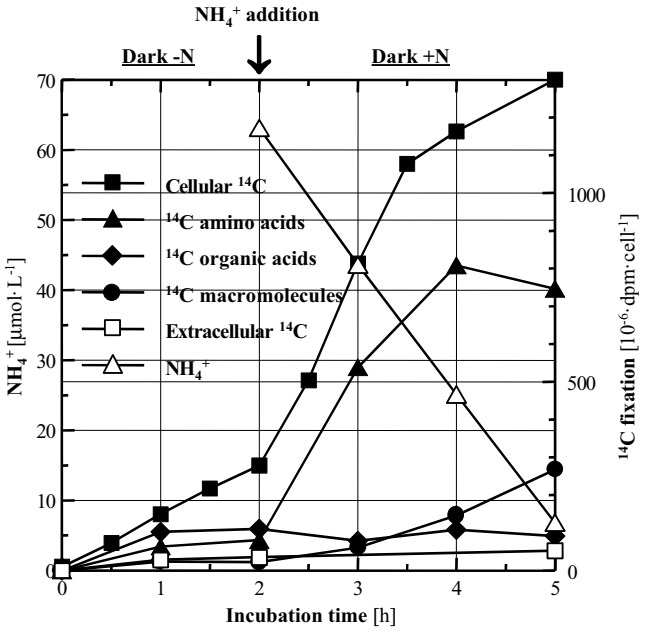

Fig. 3.8. Fixation of ^{14}C in different biochemical fractions and dissolved NH_4^+ in *Skeletonema costatum* culture as functions of time (Paper 3).

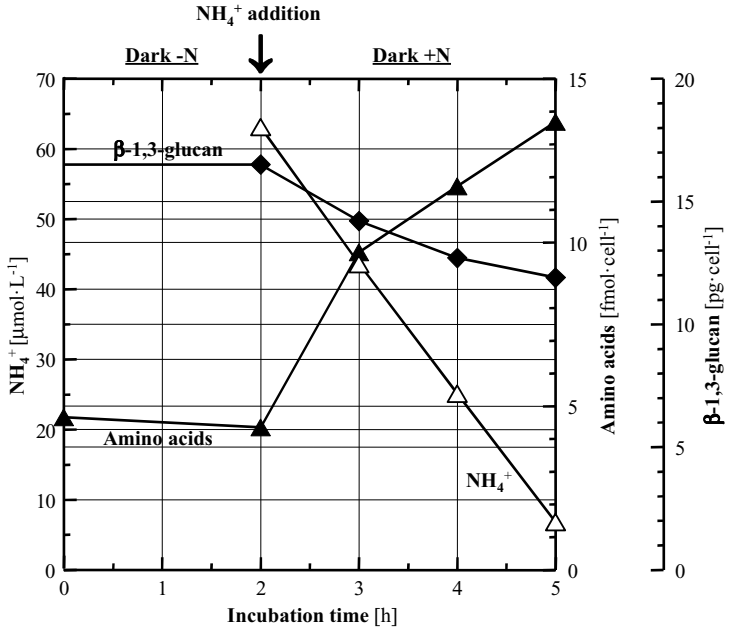

Fig. 3.9. Cellular concentrations of free amino acids and β-1,3-glucan and dissolved NH_4^+ in *Skeletonema costatum* culture as functions of time (Paper 3).

Cellular β-1,3-glucan was constant during nitrogen depletion, but decreased by 28% within 3 h of NH_4^+ assimilation. The results indicate mobilization of glucan for amino acid synthesis as described in chapter 3.2. In this study, however, amino acids were ^{14}C-labeled by LICF instead of ^{14}C-glucan mobilization. This investigation demonstrates that anaplerotic β-carboxylation provides C_4 precursors for amino acid synthesis, and β-1,3-glucan seems to be the ultimate substrate for β-carboxylation.

The molar C/N uptake ratio measured during dark NH_4^+ assimilation in this study was 0.33. A theoretical ratio of 0.35 was calculated based on the average amino acid composition of diatom protein as measured by Chuecas & Riley (1969). The requirement for nitrogen uptake was simply calculated as the number of N atoms per amino acid, whereas the requirement for LICF was calculated as one per amino acid that is derived from tricarboxylic acid cycle intermediates. Oxaloacetate is the primary product of β-carboxylation. Oxaloacetate can be reduced to malate by malate dehydrogenase or undergo a transamination reaction to form Asp. Alternatively, oxaloacetate or malate is transformed into α-ketoglutarate through the tricarboxylic acid cycle and forms Glu by means of glutamate synthase. Asp and Glu themselves are precursors for the amino acids Asn, Met, Thr, Ile, Lys, Gln, Pro and Arg (Fig. 3.6). The C/N uptake ratio thus indicates that β-carboxylation was related to protein synthesis. In a study with the chlorophyte *Selenastrum minutum*, Vanlerberghe et al. (1990) observed a C/N uptake ratio of 0.3, compared to a ratio of 0.34 calculated from amino acid composition of cell protein.

We have measured specific phosphoenolpyruvate carboxykinase (PEPCK) activity in cell-free extracts from *S. costatum* by the enzyme assay of Descolas-Gros & Fontugne (1985) (unpublished results). Enzymatic studies by many workers indicate that PEPCK is responsible for the LICF in chromophytes while phosphoenolpyruvate carboxylase (PEPC) plays this role in chlorophytes and vascular plants (Kremer & Küppers 1977, Johnston & Raven 1989, Descolas-Gros & Oriol 1992, Cabello-Pasini et al. 2000, 2001). Cabello-Pasini et al. (2001) immuno-localized PEPCK in the chloroplasts of *S. costatum* and the phaeophyte *Laminaria setchellii*, which is in contrast with the cytosolic localization of PEPC in chlorophytes and vascular plants. Thus, the flow of carbon might be different between these two groups of photoautotrophs. Moreover, the chloroplast-localization suggests that PEPCK is competing with RUBISCO for CO_2 during photosynthesis in chromophytes. Temporal partitioning of carboxylation may be controlled by pH fluctuations in the stroma, increasing from ~7.2 in the

dark to 8.0 during photosynthesis (Werdan et al. 1975, Flügge et al. 1980). PEPCK has a pH optimum at ~7.2 (Cabello-Pasini et al. 2000) and RUBISCO at >8.0 (Werdan et al. 1975), suggesting high RUBISCO activity in the light and high PEPCK activity in the dark. However, a study by Reinfelder et al. (2000) indicated C_4 photosynthesis in the marine diatom *Thalassiosira weissflogii*, where PEPC in the cytosol captures CO_2 in C_4 acids and PEPCK in the chloroplast releases CO_2 to RUBISCO by decarboxylation. It seems that further research is required to resolve the mechanisms of carbon fixation in diatoms.

4. β-1,3-GLUCAN DYNAMICS AND FUNCTION

4.1 Diel storage

In many photoautotrophs, carbon is stored as polysaccharide during the photoperiod, and then consumed at night to synthesize protein and other compounds. Such periodic synthesis and mobilization of storage products can be considered as *deterministic*, i.e. the result of an evolutionary response to predictable, periodic shortages of the product stored (Falkowski & Raven 1997). Hitchcock (1980) and Vårum et al. (1986) have observed significant diel oscillations in cellular carbohydrate in *Skeletonema costatum* with maximum at the end of the photophase and minimum at the end of the scotophase.

Diel variations in cellular carbohydrates and free amino acids were investigated in a study with *S. costatum* (Paper 5). Extracellular production was also measured, but is not discussed in this connection. The diatom was grown in batch culture with a 14:10 h light:dark cycle in N-limited media at two different strengths (150 or 50 μM NO_3^-). Figs. 4.1-4.2 show the growth and chemical composition of the cells when sampled every 24 h (4 h into the photophase). The exponential growth rate was 2.0 div. d^{-1}, and balanced growth apparently took place. However, additional sampling at the end of the light and dark periods revealed substantial diel changes in the chemical composition of the alga, although cell division was asynchronous (Figs. 4.3-4.4). During exponential growth, the glucan level oscillated between 2.7 (end of scotophase) and 13 pg C·$cell^{-1}$ (end of photophase), constituting 17-42% of cellular organic carbon. The corresponding protein/glucan ratio alternated between 2.3 and 0.7. The cell wall polysaccharides showed very low oscillations, and contributed 1.6-1.9 pg C·$cell^{-1}$ (6-10% of cellular organic carbon). Whereas total cellular organic carbon increased most rapidly during the early photophase, the glucan level increased most rapidly in the late part. These results are consistent with a study of diurnal rhythms in the same alga by Vårum et al. (1986). In the stationary phase, cellular glucan and organic carbon showed much lower diel variations (~10%), suggesting that glucan synthesis in the photophase was just sufficient to balance maintenance respiration in the scotophase.

During exponential growth, the rate of NO_3^- uptake was minimal in the scotophase compared to the photophase, which was reflected in diel variation in cellular free amino acids and organic nitrogen (Fig. 4.3). The amino acid pool oscillated between 0.2 (end of scotophase) and 0.8 pg N·$cell^{-1}$ (end of photophase), constituting 8-22% of cellular organic nitrogen.

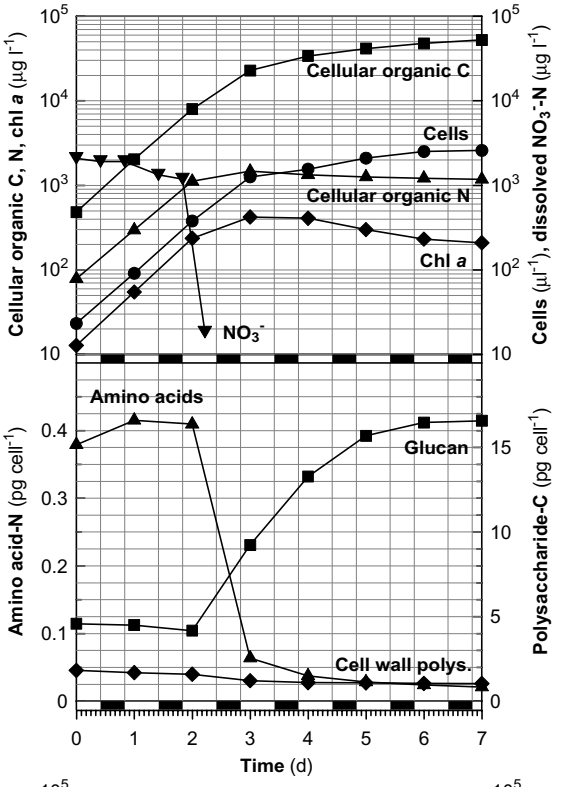

Fig. 4.1. Growth and chemical composition of *Skeletonema costatum* in 150 µM NO_3^- medium (Paper 5).

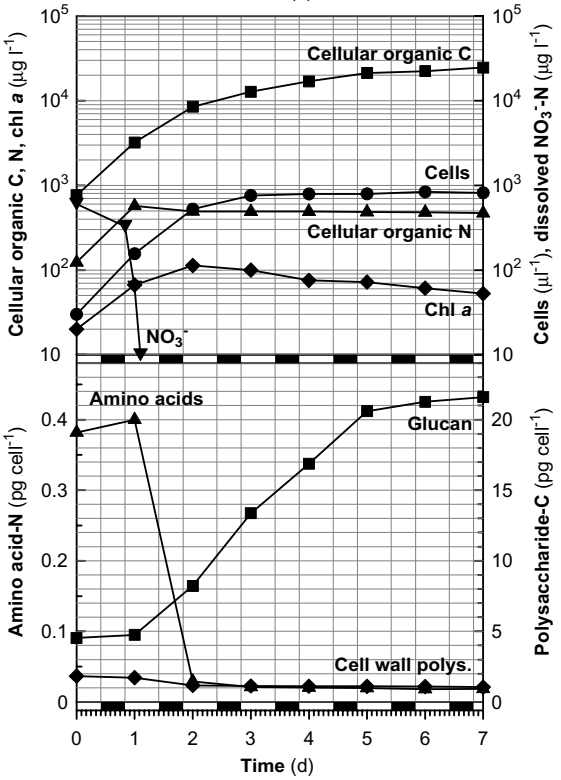

Fig. 4.2. Growth and chemical composition of *Skeletonema costatum* in 50 µM NO_3^- medium (Paper 5).

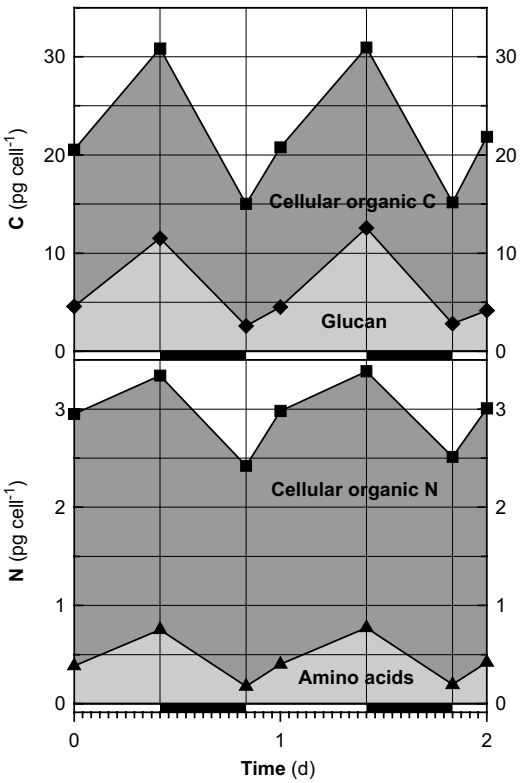

Fig. 4.3. Diel variations in cellular organic carbon and nitrogen and fractions of glucan and free amino acids during exponential growth phase of *Skeletonema costatum* (Paper 5).

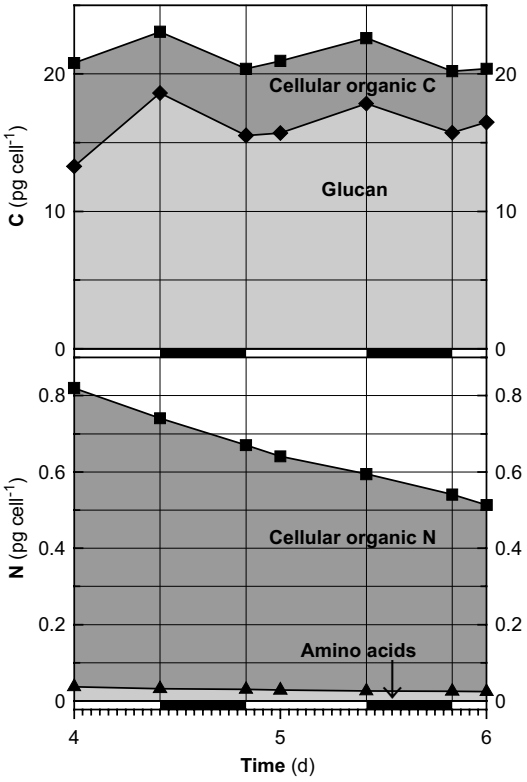

Fig. 4.4. Diel variations in cellular organic carbon and nitrogen and fractions of glucan and free amino acids during stationary growth phase of *Skeletonema costatum* (Paper 5).

In contrast with the glucan, the cellular pools of organic nitrogen and free amino acids accumulated more rapidly in the early photophase, indicating gradual saturation. In the scotophase, cellular organic nitrogen decreased per cell (due to cell division), but slightly increased per culture volume. Thus it seems some inorganic nitrogen in intracellular pools is assimilated into amino acids and proteins during the dark period. As NO_3^- uptake exceeds growth in the photophase, intracellular pools of NO_3^- and intermediate nitrogen assimilation products accumulate (Collos et al. 1992, Lomas & Glibert 2000).

The composition of cellular free amino acids also showed striking diel variation during exponential growth (Table 4.1). Gln oscillated between 2% (end of scotophase) and 34% of total amino acids (end of photophase), and the corresponding Gln/Glu ratio alternated between 0.05 and 2. A high rate of nitrogen assimilation probably induce increasing Gln/Glu through glutamine synthetase activity (see chapter 1.4). Even though all the other amino acids increased somewhat during the photophase (by factors of 1.3-10), they contributed much less than Gln and mostly decreased in percentage. In the stationary phase, the cellular quotas of nitrogenous components gradually decreased without significant diel variation.

Table 4.1. Cellular free amino acid composition (molar %) of *Skeletonema costatum* during exponential growth (day 0-2) at 'dawn' (end of scotophase) and 'dusk' (end of photophase) and stationary growth phase (day 5-7) (Paper 5).

	Growth phase		
	Exponential		Stationary
	'Dawn'	'Dusk'	
Asp	22	9	4
Glu	36	17	10
Asn	1	2	-
Ser	2	2	1
Gln	2	34	1
Gly+Arg	5	5	1
Thr	2	3	1
Ala	11	8	4
Tyr	1	3	4
Met	-	-	1
Val	8	7	18
Phe	-	2	4
Ile	3	3	22
Leu	3	1	25
Lys	5	4	4

The results clearly demonstrate uncoupling of photosynthetic CO_2 fixation, NO_3^- uptake and protein synthesis during exponential growth. By accumulating β-1,3-glucan as a photosynthetic reserve during the light period, the alga is able to perform biosynthetic

processes in the dark using the glucan as a carbon and energy source. Thus, glucan functions as a periodic short-term reserve. Moreover, the alga accumulates excessive nitrogen pools in the photophase to buffer the effect of low NO_3^- uptake and reduction in darkness. This is probably an important strategy for diatoms to optimize growth under diel light conditions.

4.2 Stockpile storage

Production and consumption of storage products can also be related to *stochastic* variations in the relative availability of different resources (Falkowski & Raven 1997). When a particular resource is in relative abundance, overplus stockpiling takes place 'just in case'. When a nutrient such as NO_3^- or P_i is exhausted from the medium, protein synthesis ceases whereas polysaccharide without nitrogen or phosphorus can still be synthesized. Glucan can be produced from photosynthetic CO_2 fixation as long as the enzymatic apparatus of the cell is still intact. This glucan can act as a long-term reserve that eventually is mobilized to furnish carbon skeletons and energy for cell maintenance and growth. Vårum & Myklestad (1984) observed that NO_3^- added to nutrient-limited *S. costatum* was assimilated in the dark while the glucan level was significantly reduced. In diatoms, β-1,3-glucan typically accounts for 10-30% of organic dry weight during exponential growth, but it may accumulate up to 80% under strong nutrient limitation (Myklestad 1974).

Effects of nitrogen depletion in *S. costatum* was investigated in the study described in chapter 4.1 (Paper 5). Upon NO_3^- exhaustion, cellular β-1,3-glucan increased rapidly for 3-4 d, and then stabilized at a level of 17-21 pg C·cell^{-1}, constituting 75-80% of cellular organic carbon (Figs. 4.1-4.2). The results indicate a high photosynthetic activity for several days until the cellular pool of glucan was saturated. During this period, biosynthesis of N-free carbohydrates was apparently not hampered. In contrast, the cellular nitrogen quota decreased by 80%, and consequently the protein/glucan ratio decreased to <0.1. The cell wall polysaccharide quota also decreased by 35% under nitrogen depletion. Previous studies have also shown that the glucan content of *S. costatum* may accumulate up to 80% of cellular organic carbon under strong nutrient limitation (Myklestad 1974, Vårum & Myklestad 1984). A protein/glucan ratio of <1 has been considered as nutrient deficiency. However, a slightly lower value than 1 was recorded for nutrient-sufficient *S. costatum* at the end of the photophase in the present study (see chapter 4.1).

The cellular free amino acid pool decreased by 90% within 24 h of nitrogen depletion, and

continued to decrease slowly throughout the stationary phase (Table 4.1). At the point of NO_3^- depletion, Gln decreased most rapidly, and the Gln/Glu ratio eventually decreased to 0.05. The proportions of small and acidic amino acids (Ala, Asp, Glu and Gly) decreased compared to large aliphatic amino acids (Ile, Leu and Val) in the stationary phase, resulting in a higher average C/N ratio for the cellular 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 nitrogen deprivation in diatoms. However, our results show that the effect of nitrogen depletion can easily be confounded with the effect of dark periods in N-sufficient cells, inducing equally low Gln/Glu ratios. The results indicate that the cells adapted to nitrogen limitation by reducing their cellular nitrogen contents to a minimum, thereby entering a low activity stage. Dortch (1982) found similar effects of nitrogen limitation on nitrogenous components in *S. costatum* and other diatoms including depletion of internal NO_3^- and NH_4^+. The concurrent reductions in growth rate and cell quota of nitrogen are in accordance with the Droop model, where specific growth rate is hyperbolically related to the cell quota of the limiting nutrient (Droop 1973).

β-1,3-Glucan seems to accumulate strongly in the stationary growth phase of diatoms, suggesting that it has a long-term stockpile storage function. This accumulation can also be connected to development of resting cells or resting spores (see chapter 5.3). Glucan synthesis appears to be a convenient carbon sink for active photosynthesis under nutrient depletion. This strategy may facilitate rapid growth as soon as nutrients are replenished using the glucan as an easily available source of precursors and energy.

5. ECOLOGICAL ASPECTS

5.1 β-1,3-Glucan dynamics

Field investigations of phytoplankton show that cellular glucan increases markedly during the day and decreases at night in accordance with laboratory cultures (Morris et al. 1981, Cuhel et al. 1984, Hama & Handa 1992). Combined accumulation of glucan and cessation of protein synthesis under nutrient limitation have also been demonstrated in natural phytoplankton populations dominated by diatoms (Haug et al. 1973, Hitchcock 1978, Sakshaug et al. 1983, Hama & Honjo 1987). Nutrient-sufficient conditions are characterized by protein/glucan ratios of >1. This ratio is easily determined and has been used as a sensitive parameter for characterizing physiological condition in natural diatom populations. However, species-specific variations occur, and diel variations must be taken into account. Thus, ecological studies suggest that the glucan dynamics in natural diatom populations are analogous to what we have observed in axenic cultures in the laboratory. Spring blooms dominated by diatoms may be comparable to the batch culture conditions we have used in our experiments.

5.2 Buoyancy control

Observations of sinking rate varying with growth stage suggest that buoyancy can be physiologically controlled by diatoms (Gross & Zeuthen 1948). The sinking rate of a diatom depend on its size, shape, and the difference between the density of the diatom and its medium. The density of the cell depends in part on its chemical composition, and major components are carbohydrate, protein, silica (all of which are more dense than seawater) and lipid (which is less dense than seawater) (Smayda 1970). Thus, accumulation of any of the denser components, such as glucan, could produce an increasing sinking rate, and vice versa. Another means of control is through the selective exchange of heavier ions for lighter ions in the vacuole (Anderson & Sweeney 1978). Waite et al. (1992) found a relationship between cell sinking rate and respiration rate in marine diatoms.

β-1,3-Glucan dynamics in diatoms give a wide scope for changes in carbohydrate content to affect cellular density. Increasing sinking rates with higher carbohydrate contents have been reported for marine planktonic diatoms such as *Coscinodiscus concinnus* (Granata 1991), *Rhizosolenia* spp. (Villareal et al. 1993) and *Thalassiosira weissflogii* (Richardson & Cullen 1995), although some observations are not consistent (Fisher & Harrison 1996). Nutrient-

deplete cells in the euphotic zone accumulate glucan, causing increased cellular density and sinking below the nutricline. Upon nutrient replenishment and reversion of the protein/carbohydrate ratio, the cells rise toward the surface again. Such vertical migration of algae with uncoupled carbon and nutrient assimilation enhances transport of deep nutrients to the euphotic zone, thereby increasing oceanic primary production. This strategy depends on the accumulation of carbon reserves in the form of glucan rather than triacylglycerols, which would have the opposite effect on cellular buoyancy. Compared to glucan, fat contains more potential metabolic energy per mass due to its highly reduced carbon and anhydrous character. However, fatty acids can not provide precursors for biosynthesis without the glyoxylate cycle, which has not been demonstrated in diatoms.

5.3 Resting stages

β-1,3-Glucan also seems to play an important role in the development of resting cells and resting spores in diatoms. Physiological distinctions in these life forms are higher C/N ratios, more condensed organelles, and more or larger vesicles containing storage product (Hargraves & French 1983, McQuoid & Hobson 1996). Photosynthetic and respiratory activities are depressed during resting stages. The marine planktonic diatom *Chaetoceros pseudocurvisetus* produces resting spores with large accumulation of glucan (70% of cellular organic carbon) as well as lipids (17%) under nitrogen depletion (Kuwata et al. 1993).

Resting spores occur frequently in marine centric species whereas freshwater diatoms and pennate species more often form resting cells (Round et al. 1990). It is commonly believed that these resting stages are a means of long- or short-term survival during periods of unfavorable conditions. Diatom blooms often culminate by formation and sinking of resting spores, suggesting that nutrient stress is the primary initiator of the resting phase (Sakshaug & Myklestad 1973, Garrison 1981, McQuoid & Hobson 1996). Formation and germination of resting cells and spores may be important for species succession, dispersal, and cycling of nutrients through the water column.

5.4 Microbial ecology

Due to the abundance of diatoms, glucan probably plays an important role as a carbon and energy source in the microbial ecology of the sea through grazing and bacterial activity. As far as we know, β-1,3-glucan is a unique product of algae in the sea. This polysaccharide is therefore particularly useful as an ecological indicator of phytoplankton growth.

6. CONCLUSION

6.1 Main findings

Synthesis and mobilization of β-1,3-glucan (chrysolaminaran) in the marine diatom *Skeletonema costatum* were studied by combined ^{14}C tracer technique and chemical analyses. Our results provide new evidence of glucan supplying essential carbon skeletons for synthesis of amino acids and other components in *S. costatum* in both darkness and subsaturating light, and even saturating light under inorganic carbon limitation. Moreover, anaplerotic β-carboxylation is active during amino acid synthesis to replenish C_4 intermediates withdrawn from the tricarboxylic acid cycle. Obviously, the glucan also furnishes energy and reducing power to the cell as it is metabolized by the respiratory pathways. Both energy (ATP) and electron donors (NADPH) are required to perform reductive biosynthesis of protein and other compounds.

During exponential growth of *S. costatum*, the glucan level oscillated markedly under diel light conditions. Depletion of nitrogen from the medium resulted in rapid accumulation of glucan, reaching 75-80% of cellular organic carbon, whereas the cellular organic nitrogen quota decreased significantly. These results indicate that glucan functions both as a short-term diurnal reserve and a long-term stockpile reserve. Glucan accumulation under nutrient depletion may also be connected to formation of resting cells or resting spores.

6.2 Future work

There are several interesting scopes for future work related to β-1,3-glucan metabolism in diatoms. Further research is required to establish the mechanisms, regulation and localization of glucan synthesizing and mobilizing enzymes as well as β-carboxylation enzymes. Moreover, tools have recently been developed for studies of the molecular biology of diatoms. Following isolation and sequencing of active genes, functional gene studies may provide a basic understanding of the cellular control of glucan metabolism. Another interesting field of study is the ecological aspects of β-1,3-glucan. Field investigations of natural phytoplankton are essential to reveal the physiological responses to various environmental conditions in the sea.

REFERENCES

Admiraal W, Peletier H & Laane RWPM (1986) Nitrogen metabolism of marine planktonic diatoms; excretion, assimilation and cellular pools of free amino acids in seven species with different cell size. *J Exp Mar Biol Ecol* 98:241-63

Allan GG, Lewin J & Johnson PG (1972) Marine polymers. IV. Diatom polysaccharides. *Bot Mar* 15:102-8

Anderson LWJ & Sweeney BM (1978) Role of inorganic ions in controlling sedimentation rate of a marine centric diatom *Ditylum brightwellii*. *J Phycol* 14:204-14

Appleby G, Colbeck J, Holdsworth ES & Wadman H (1980) β-Carboxylation enzymes in marine phytoplankton and isolation and purification of pyruvate carboxylase from *Amphidinium carterae* (Dinophyceae). *J Phycol* 16:290-5

Beattie A, Hirst EL & Percival E (1961) Comparative structural investigations on leucosin (chrysolaminarin) separated from diatoms and laminarin from the brown algae. *Biochem J* 79:531-7

Cabello-Pasini A, Smith GJ & Alberte RS (2000) Phosphoenolpyruvate carboxykinase from the marine diatom *Skeletonema costatum* and the phaeophyte *Laminaria setchellii*. I. Isolation and biochemical characterization. *Bot Mar* 43:559-68

Cabello-Pasini A, Swift H, Smith GJ & Alberte RS (2001) Phosphoenolpyruvate carboxykinase from the marine diatom *Skeletonema costatum* and the phaeophyte *Laminaria setchellii*. II. Immunological characterization and subcellular localization. *Bot Mar* 44:199-207

Chang FH (1984) The ultrastructure of *Phaeocystis pouchetii* (Prymnesiophyceae) vegetative colonies with special reference to the production of new mucilaginous envelope. *New Zeal J Mar Freshw Res* 18:303-8

Chuecas L & Riley JP (1969) The component combined amino acids of some marine diatoms. *J Mar Biol Ass UK* 49:117-20

Collos Y, Siddiqi MY, Wang MY, Glass ADM & Harrison PJ (1992) Nitrate uptake kinetics by two marine diatoms using the radioactive tracer ^{13}N. *J Exp Mar Biol Ecol* 163:251-60

Craigie JS (1963) Dark fixation of ^{14}C-bicarbonate by marine algae. *Can J Bot* 41:317-25

Craigie JS (1974) Storage products. In: Stewart WDP (ed) *Algal Physiology and Biochemistry*, Blackwell (Oxford) 206-35

Cuhel RL, Ortner PB & Lean DRS (1984) Night synthesis of protein by algae. *Limnol Oceanogr* 29:731-44

Darley WM (1977) Biochemical composition. In: Werner D (ed) *The Biology of Diatoms*, Blackwell (Oxford) 198-223

Decho AW (1990) Microbial exopolymer secretions in ocean environments: their role(s) in food webs and marine processes. *Oceanogr Mar Biol Annu Rev* 28:73-153

Descolas-Gros C & Fontugne MR (1985) Carbon fixation in marine phytoplankton: carboxylase activities and stable carbon-isotope ratios; physiological and paleoclimatological aspects. *Mar Biol* 87:1-6

Descolas-Gros C & Oriol L (1992) Variations in carboxylase activity in marine phytoplankton cultures. β-Carboxylation in carbon flux studies. *Mar Ecol Prog Ser* 85:163-9

Dortch Q (1982) Effect of growth conditions on accumulation of internal nitrate, ammonium, amino acids, and protein in three marine diatoms. *J Exp Mar Biol Ecol* 61:243-64

Dortch Q, Roberts TL, Clayton JR Jr & Ahmed SI (1983) RNA/DNA ratios and DNA concentrations as indicators of growth rate and biomass in planktonic marine organisms. *Mar Ecol Prog Ser* 13:61-71

Droop MR (1973) Some thoughts on nutrient limitation in algae. *J Phycol* 9:264-72

Dubois M, Gilles KA, Hamilton JK, Rebers PA & Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* 28:350-6

Duke EL & Reimann BEF (1977) The ultrastructure of the diatom cell. In: Werner D (ed) *The Biology of Diatoms*, Blackwell (Oxford) 65-109

Falkowski PG (1992) Molecular ecology of phytoplankton photosynthesis. In: Falkowski PG & Woodhead A (eds) *Primary Productivity and Biogeochemical Cycles in the Sea*, Plenum (New

York) 47-67
Falkowski PG (1994) The role of phytoplankton photosynthesis in global biogeochemical cycles. *Photosyn Res* 39:235-58
Falkowski PG & Raven JA (1997) *Aquatic Photosynthesis*, Blackwell (Malden, MA) 375 pp.
Falkowski PG, Sukenik A & Herzig R (1989) Nitrogen limitation in *Isochrysis galbana* (Haptophyceae). II. Relative abundance of chloroplast proteins. *J Phycol* 25:471-8
Fisher AE & Harrison PJ (1996) Does carbohydrate content affect the sinking rates of marine diatoms? *J Phycol* 32:360-5
Flügge UI, Freisl M, Heldt H & W (1980) The mechanism of the control of carbon fixation by the pH in the chloroplast stroma. *Planta* 149:48-51
Flynn KJ (1988) Some practical aspects of measurements of dissolved free amino acids in natural waters and within microalgae by the use of HPLC. *Chem Ecol* 3:269-93
Flynn KJ (1990) Composition of intracellular and extracellular pools of amino acids, and amino acid utilization of microalgae of different sizes. *J Exp Mar Biol Ecol* 139:151-66
Flynn KJ & Al-Amoudi OA (1988) Effects of N deprivation and darkness on composition of free amino acid pool in and on amino acid release from diatom *Phaeodactylum tricornutum* Bohlin. *J Exp Mar Biol Ecol* 119:131-44
Ford CW & Percival E (1965a) The carbohydrates of *Phaeodactylum tricornutum*. Part II. A sulphated glucuronomannan. *J Chem Soc* 1299:7042-6
Ford CW & Percival E (1965b) The carbohydrates of *Phaeodactylum tricornutum*. Part I. Preliminary examination of the organism, and characterisation of low molecular weight material and of a glucan. *J Chem Soc* 1298:7035-41
Garrison DL (1981) Monterey Bay phytoplankton II. Resting spore cycles in coastal diatom populations. *J Plankton Res* 3:137-56
Geider RJ & Osborne BA (1992) Using isotopes to measure gas exchange. In: Geider RJ & Osborne BA (eds) *Algal Photosynthesis: the Measurement of Algal Gas Exchange*, Chapman & Hall (New York) 32-70
Goldemberg SH & Marechal LR (1963) Biosynthesis of paramylon in *Euglena gracilis*. *Biochim Biophys Acta* 71:743-4
Granata TC (1991) Diel periodicity in growth and sinking rates of the centric diatom *Coscinodiscus concinnus*. *Limnol Oceanogr* 36:132-9
Gross F & Zeuthen E (1948) The buoyancy of plankton diatoms: a problem of cell physiology. *Proc R Soc Lond (Ser B)* 135:382-9
Guerrero MG, Vega JM & Losada M (1981) The assimilatory nitrate-reducing system and its regulation. *Ann Rev Plant Physiol* 32:169-204
Guillard RRL & Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. *Can J Microbiol* 8:229-39
Guy RD, Vanlerberghe GC & Turpin DH (1989) Significance of phosphoenolpyruvate carboxylase during ammonium assimilation: carbon isotope discrimination in photosynthesis and respiration by the N-limited green alga *Selenastrum minutum*. *Plant Physiol* 89:1150-7
Hama J & Handa N (1992) Diel variation of water-extractable carbohydrate composition of natural phytoplankton populations in Kinu-ura Bay. *J Exp Mar Biol Ecol* 162:159-76
Hama T & Honjo T (1987) Photosynthetic products and nutrient availability in phytoplankton population from Gokasho Bay, Japan. *J Exp Mar Biol Ecol* 112:251-66
Handa N (1969) Carbohydrate metabolism in the marine diatom *Skeletonema costatum*. *Mar Biol* 4:208-14
Hargraves PE & French F (1983) Diatom resting spores: significance and strategies. In: Fryxell GA (ed) *Survival Strategies of the Algae*, Cambridge University (New York) 49-68
Haug A & Larsen B (1962) Quantitative determination of the uronic acid composition of alginates. *Acta Chem Scand* 16:1908-18
Haug A & Myklestad S (1976) Polysaccharides of marine diatoms with special reference to *Chaetoceros* species. *Mar Biol* 34:217-22
Haug A, Myklestad S & Sakshaug E (1973) Studies on the phytoplankton ecology of the Trondheimsfjord. I. The chemical composition of phytoplankton populations. *J Exp Mar Biol Ecol* 11:15-26

Hecky RE, Mopper K, Kilham P & Degens ET (1973) The amino acid and sugar composition of diatom cell-walls. *Mar Biol* 19:323-31

Hitchcock GL (1978) Labelling patterns of carbon-14 in net plankton during a winter-spring bloom. *J Exp Mar Biol Ecol* 31:141-53

Hitchcock GL (1980) Diel variation in chlorophyll *a*, carbohydrate and protein content of the marine diatom *Skeletonema costatum*. *Mar Biol* 57:271-8

Hitchcock GL (1983) Photosynthate partitioning in cultured marine phytoplankton. I. Dinoflagellates. *J Exp Mar Biol Ecol* 69:21-36

Hoek C van den, Mann DG & Jahns HM (1995) *Algae: an Introduction to Phycology*, Cambridge University (New York) 623 pp.

Holdsworth ES & Bruck K (1977) Enzymes concerned with β-carboxylation in marine phytoplankter. Purification and properties of phosphoenolpyruvate carboxykinase. *Arch Biochem Biophys* 182:87-94

Holm-Hansen O (1969) Algae: amounts of DNA and organic carbon in single cells. *Science* 163:87-8

Holm-Hansen O, Lorenzen CJ, Holmes RW & Strickland JDH (1965) Fluorometric determination of chlorophyll. *J cons Cons int explor mer* 30:3-15

Jeffrey SW & Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1 and c_2 in higher plants, algae and natural phytoplankton. *Biochem Physiol Pflanzen* 167:191-4

Jeffrey SW & Vesk M (1997) Introduction to marine phytoplankton and their pigment signatures. In: Jeffrey SW, Mantoura RFC & Wright SW (eds) *Phytoplankton Pigments in Oceanography*, UNESCO (Paris) 37-84

Johnston AM & Raven JA (1989) Extraction, partial purification and characterization of phosphoenolpyruvate carboxykinase from *Ascophyllum nodosum* (Phaeophyceae). *J Phycol* 25:568-76

Kirsten WJ (1979) Automatic methods for the simultaneous determination of carbon, hydrogen, nitrogen, and sulfur, and for sulfur alone in organic and inorganic materials. *Anal Chem* 51:1173-9

Kremer BP & Berks R (1978) Photosynthesis and carbon metabolism in marine and freshwater diatoms. *Z Pflanzenphysiol* 87:149-65

Kremer BP & Küppers U (1977) Carboxylating enzymes and pathway of photosynthetic carbon assimilation in different marine algae - evidence for the C_4-pathway? *Planta* 133:191-6

Kuwata A, Hama T & Takahashi M (1993) Ecophysiological characterization of two life forms, resting spores and resting cells, of a marine planktonic diatom, *Chaetoceros pseudocurvisetus*, formed under nutrient depletion. *Mar Ecol Prog Ser* 102:245-55

Lancelot C (1984) Metabolic changes in *Phaeocystis poucheti* (Hariot) Lagerheim during the spring bloom in Belgian coastal waters. *Estuar Coast Shelf Sci* 18:593-600

Li WKW, Glover HE & Morris I (1980) Physiology of carbon photoassimilation by *Oscillatoria thiebautii* in the Caribbean Sea. *Limnol Oceanogr* 25:447-56

Lindroth P & Mopper K (1979) High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with *o*-phthaldialdehyde. *Anal Chem* 51:1667-74

Lomas MW & Glibert PM (2000) Comparisons of nitrate uptake, storage, and reduction in marine diatoms and flagellates. *J Phycol* 36:903-13

McConville MJ, Bacic A & Clarke AE (1986) Structural studies of chrysolaminaran from the ice diatom *Stauroneis amphioxys* (Gregory). *Carbohydr Res* 153:330-3

McConville MJ, Mitchell C & Wetherbee R (1985) Patterns of carbon assimilation in a microalgal community from annual sea ice, East Antarctica. *Polar Biol* 4:135-41

McQuoid MR & Hobson LA (1996) Diatom resting stages. *J Phycol* 32:889-902

Morris I (1980) Paths of carbon assimilation in marine phytoplankton. In: Falkowski PG (ed) *Primary Productivity in the Sea*, Plenum (New York) 139-59

Morris I (1981) Photosynthetic products, physiological state, and phytoplankton growth. In: Platt T (ed) *Physiological Bases of Phytoplankton Ecology*, Can Bull Fish Aquat Sci 210 (Ottawa) 83-102

Morris I, Smith AE & Glover HE (1981) Products of photosynthesis in phytoplankton off the Orinoco River and in the Caribbean Sea. *Limnol Oceanogr* 26:1034-44

Morris I, Yentsch CM & Yentsch CS (1971a) Relationship between light carbon dioxide fixation and

dark carbon dioxide fixation by marine algae. *Limnol Oceanogr* 16:854-8
Morris I, Yentsch CM & Yentsch CS (1971b) The physiological state with respect to nitrogen of phytoplankton from low-nutrient subtropical water as measured by the effect of ammonium ion on dark carbon dioxide fixation. *Limnol Oceanogr* 16:859-68
Mortain-Bertrand A, Descolas-Gros C & Jupin H (1987) Short-term ^{14}C incorporation in *Skeletonema costatum* (Greville) Cleve (Bacillariophyceae) as a function of light regime. *Phycologia* 26:262-9
Mortain-Bertrand A, Descolas-Gros C & Jupin H (1988) Pathway of dark inorganic carbon fixation in two species of diatoms: influence of light regime and regulator factors on diel variations. *J Plankton Res* 15:199-217
Myklestad S (1974) Production of carbohydrates by marine planktonic diatoms. I. Comparison of nine different species in culture. *J Exp Mar Biol Ecol* 15:261-74
Myklestad S (1978) β-1,3-Glucans in diatoms and brown seaweeds. In: Hellebust JA & Craigie JS (eds) *Handbook of Phycological Methods. Physiological and Biochemical Methods*, Cambridge University (New York) 133-41
Myklestad S, Djurhuus R & Mohus A (1982) Demonstration of *exo*-(β-1,3)-D-glucanase activity in some planktonic diatoms. *J Exp Mar Biol Ecol* 56:205-11
Myklestad S & Haug A (1972) Production of carbohydrates by the marine diatom *Chaetoceros affinis* var. *willei* (Gran) Hustedt. I. Effect of the concentration of nutrients in the culture medium. *J Exp Mar Biol Ecol* 9:125-36
Myklestad S, Haug A & Larsen B (1972) Production of carbohydrates by the marine diatom *Chaetoceros affinis* var. *willei* (Gran) Hustedt. II. Preliminary investigation of the extracellular polysaccharide. *J Exp Mar Biol Ecol* 9:137-44
Myklestad SM (1988/1989) Production, chemical structure, metabolism, and biological function of the (1→3)-linked, β-D-glucans in diatoms. *Biol Oceanogr* 6:313-26
Myklestad SM (2000) Dissolved organic carbon from phytoplankton. In: Wangersky P (ed) *The Handbook of Environmental Chemistry [D]. Marine Chemistry*, Springer (Berlin) 111-48
Myklestad SM & Swift E (1998) A new method for measuring soluble cellular organic content and a membrane property, T_m, of planktonic algae. *Eur J Phycol* 33:333-6
Painter TJ (1983) Algal polysaccharides. In: Aspinall GO (ed) *The Polysaccharides, Vol. 2*, Academic (New York) 195-285
Parsons TR, Stephens K & Strickland JDH (1961) On the chemical composition of eleven species of marine phytoplankters. *J Fish Res Bd Can* 18:1001-16
Paulsen BS & Myklestad S (1978) Structural studies of the reserve glucan produced by the marine diatom *Skeletonema costatum* (Grev.) Cleve. *Carbohydr Res* 62:386-8
Percival E, Rahman MA & Weigel H (1980) Chemistry of the polysaccharides of the diatom *Coscinodiscus nobilis*. *Phytochemistry* 19:809-11
Raven JA (1976) Division of labour between chloroplasts and cytoplasm. In: Barber J (ed) *The Intact Chloroplast*, Elsevier (Amsterdam) 403-43
Reinfelder JR, Kraepiel AML & Morel FMM (2000) Unicellular C_4 photosynthesis in a marine diatom. *Nature* 407:996-9
Richardson TL & Cullen JJ (1995) Changes in buoyancy and chemical composition during growth of a coastal marine diatom: ecological and biogeochemical consequences. *Mar Ecol Prog Ser* 128:77-90
Roessler PG (1987) UDPglucose pyrophosphorylase activity in the diatom *Cyclotella cryptica*. Pathway of chrysolaminarin biosynthesis. *J Phycol* 23:494-8
Round FE & Crawford RM (1990) Phylum Bacillariophyta. In: Margulis L, Corliss JO, Melkonian M & Chapman DJ (eds) *Handbook of Protoctista*, Jones & Bartlett (Boston) 574-96
Round FE, Crawford RM & Mann DG (1990) *The Diatoms. Biology and Morphology of the Genera*, Cambridge University (New York) 747 pp.
Sakshaug E, Andresen K, Myklestad S & Olsen Y (1983) Nutrient status of phytoplankton communities in Norwegian waters (marine, brackish, and fresh) as revealed by their chemical composition. *J Plankton Res* 5:175-96
Sakshaug E & Myklestad S (1973) Studies on the phytoplankton ecology of the Trondheimsfjord. III. Dynamics of phytoplankton blooms in relation to environmental factors, bioassay experiments and parameters for the physiological state of the populations. *J Exp Mar Biol Ecol* 11:157-88

Schmid A-MM, Borowitzka MA & Volcani BE (1981) Morphogenesis and biochemistry of diatom cell walls. In: Kiermayer O (ed) *Cytomorphogenesis in Plants*, Springer (Wien) 63-97

Smayda TJ (1970) The suspension and sinking of phytoplankton in the sea. *Oceanogr mar biol A Rev* 8:353-414

Smestad B, Haug A & Myklestad S (1974) Production of carbohydrates by the marine diatom *Chaetoceros affinis* var. *willei* (Gran) Hustedt. III. Structural studies of the extracellular polysaccharide. *Acta Chem Scand B* 28:662-6

Smestad B, Haug A & Myklestad S (1975) Structural studies of the extracellular polysaccharide produced by the diatom *Chaetoceros curvisetus* Cleve. *Acta Chem Scand B* 29:337-40

Steeman-Nielsen E (1952) The use of radio-active carbon (C^{14}) for measuring organic production in the sea. *J cons Cons int explor mer* 18:117-40

Stoddart RW (1984) *The Biosynthesis of Polysaccharides*, Macmillan (New York) 354 pp.

Stosch H-A von (1981) Structural and histochemical observations on the organic layers of the diatom cell wall. In: Ross R (ed) *Proceedings of the 6th Symposium on Recent and Fossil Diatoms*, Koeltz (Koenigstein) 231-52

Strickland JDH & Parsons TR (1972) *A Practical Handbook of Seawater Analysis, 2nd ed*, Bull Fish Res Bd Can 167 (Ottawa) 310 pp.

Sugimura Y & Suzuki Y (1988) A high-temperature catalytic oxidation method for the determination of non-volatile dissolved organic carbon in seawater by direct injection of a liquid sample. *Mar Chem* 24:105-31

Syrett PJ (1981) Nitrogen metabolism of microalgae. In: Platt T (ed) *Physiological Bases of Phytoplankton Ecology*, Can Bull Fish Aquat Sci 210 (Ottawa) 182-210

Taraldsvik M & Myklestad SM (2000) The effect of pH on growth rate, biochemical composition and extracellular carbohydrate production of the marine diatom *Skeletonema costatum*. *Eur J Phycol* 35:189-94

Vanlerberghe GC, Schuller KA, Smith RG, Feil R, Plaxton WC & Turpin DH (1990) Relationship between NH_4^+ assimilation rate and *in vivo* phosphoenolpyruvate carboxylase activity: regulation of anaplerotic carbon flow in the green alga *Selenastrum minutum*. *Plant Physiol* 94:284-90

Villareal TA, Altabet MA & Culver-Rymsza K (1993) Nitrogen transport by vertically-migrating diatom mats in the North Pacific Ocean. *Nature* 363:709-12

Volcani BE (1981) Cell wall formation in diatoms: morphogenesis and biochemistry. In: Simpson TL & Volcani BE (eds) *Silicon and Siliceous Structures in Biological Systems*, Springer (New York) 157-200

Vårum KM & Myklestad S (1984) Effects of light, salinity and nutrient limitation on the production of β-1,3-D-glucan and exo-D-glucanase activity in *Skeletonema costatum* (Grev.) Cleve. *J Exp Mar Biol Ecol* 83:13-26

Vårum KM, Østgaard K & Grimsrud K (1986) Diurnal rhythms in carbohydrate metabolism of the marine diatom *Skeletonema costatum* (Grev.) Cleve. *J Exp Mar Biol Ecol* 102:249-56

Waite A, Thompson PA & Harrison PJ (1992) Does energy control the sinking rates of marine diatoms? *Limnol Oceanogr* 37:468-77

Waterkeyn L & Bienfait A (1987) Localisation et rôle des β-1,3-glucanes (callose et chrysolaminarine) dans le genre *Pinnularia* (Diatomées). *La Cellule* 74:198-226

Werdan K, Heldt HW & Milovancev M (1975) The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on CO_2 fixation in the light and dark. *Biochim Biophys Acta* 396:276-92

Werner D (1977) Contributions of the study of diatoms to biology. In: Werner D (ed) *The Biology of Diatoms*, Blackwell (Oxford) 1-3

Paper 1

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A photobioreactor with pH control: demonstration by growth of the marine diatom *Skeletonema costatum*

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A simple combined method for determination of β-1,3-glucan and cell wall polysaccharides in diatoms

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Abstract

A new method is described for the combined determination of β-1,3-glucan and cell wall polysaccharides in diatoms, representing total cellular carbohydrate. The glucan is extracted by 0.05 mol l^{-1} H_2SO_4 at 60 °C for 10 min, and the cell wall polysaccharides are subsequently hydrolyzed by 80% H_2SO_4 at 0-4 °C for 20 h. Each carbohydrate fraction is determined by the phenol-sulphuric acid method. The method has been demonstrated for axenic cultures of the marine diatom *Skeletonema costatum* and natural marine phytoplankton populations dominated by diatoms. Cellular glucan and cell wall polysaccharides were determined with standard deviations of 1-3% and 2-5%, respectively.

Introduction

Diatoms (Bacillariophyceae) are the most abundant primary producers in the oceans. Carbohydrates occur as early products of photosynthesis and constitute one of the major classes of biomolecules, estimated to 10-70% of diatom organic matter (Romankevich, 1984). Polysaccharides account for the main parts of the carbohydrates and are often divided into three groups: (1) β-1,3-glucan, the food-reserve polysaccharide; (2) cell wall polysaccharides; and (3) extracellular mucilages. The present work applies only to the cellular groups (1-2).

The storage polysaccharide in diatoms is β-1,3-D-glucan, also called chrysolaminaran. It is a relatively short-chained (1→3)-linked β-D-glucopyranan (DP 20-60) with occasional branching through C-2 and C-6 (Beattie et al., 1961; Ford & Percival, 1965a; Paulsen & Myklestad, 1978; McConville et al., 1986). Fluorescence microscopy shows that chrysolaminaran in diatoms is localized in cellular vacuoles (Waterkeyn & Bienfait, 1987). Similar β-1,3-glucans presumably act as food-reserves in several other algal classes (Chrysophyceae, Euglenophyceae, Eustigmatophyceae, Haptophyceae (Prymnesiophyceae), Phaeophyceae, and Xanthophyceae). The glucan is rapidly produced during photosynthesis, but is mobilized during respiration in the dark (Handa, 1969; Vårum & Myklestad, 1984). In diatoms β-1,3-glucan typically accounts for 10-30% of organic dry weight in the exponential growth phase, but it may accumulate up to 80% in the stationary phase (Myklestad, 1974). When nutrients such as N or P are exhausted from the medium, protein synthesis ceases whereas glucan can still be produced from photosynthetic CO_2 fixation. Thus, the ratio of protein to glucan or carbohydrate has proved to be a sensitive and convenient parameter for characterizing the physiological condition of both laboratory cultures and natural phytoplankton populations (Haug et al., 1973; Myklestad, 1974; Hitchcock, 1978).

The β-1,3-glucan is water-soluble and can readily be extracted from the cells by hot water or dilute acid. Myklestad & Haug (1972) and Myklestad (1978) have described methods for routine determination of β-1,3-glucan in planktonic diatoms. Cells were extracted twice with

0.05 mol l^{-1} H_2SO_4 at 20 °C for 1-2 h, and the combined filtrates from the extractions were analyzed by the phenol-sulphuric acid method (Dubois et al., 1956). This method has also been used with epipelic diatoms from sediments (Smith & Underwood, 1998). The elevated temperature extraction reported here is based on the investigation by Myklestad & Swift (1998), and is much faster.

Structural polysaccharides are important as algal cell wall constituents. In diatoms the cell wall is composed of silica in an organic casing. This casing consists of thin coating layers and the diatotepic layer on the inside of the frustule (Schmid et al., 1981; Stosch, 1981; Volcani, 1981). The diatotepum consists largely of acidic polysaccharides, often in the form of sulphated glucuronomannans (Ford & Percival, 1965b; Percival et al., 1980). The casing also contains other heteropolysaccharides with widely varying proportions of galactose, glucose, mannose (and their corresponding uronic acids), xylose, fucose, rhamnose, and other residues (Handa, 1969; Allan et al., 1972; Hecky et al., 1973; Haug & Myklestad, 1976). Strips of callose, a structural β-1,3-linked glucan, form a joint or gasket between the siliceous valves (Waterkeyn & Bienfait, 1987).

Cell wall polysaccharides are generally insoluble in water, hence effective extraction or hydrolysis requires more harsh treatment. In commonly used quantitative methods the polysaccharides are hydrolyzed by concentrated acid or boiling dilute acid/alkali, and analyzed by the phenol-sulphuric acid method. In the method by Myklestad & Haug (1972) total cellular carbohydrate was analyzed by 80% H_2SO_4 hydrolysis at 20 °C for 20 h, and the cell wall polysaccharides were determined as the difference between total carbohydrate and glucan.

In this work we present a simple and convenient method for determination of β-1,3-glucan and cell wall polysaccharides in diatoms, which is partly a modification of previous methods. The reserve glucan and the cell wall polysaccharides are analyzed in combination by sequential extraction and hydrolysis. The present method is applicable to laboratory cultures as well as natural populations, and data is provided for both cases.

Materials and methods

Phytoplankton samples

The marine diatom *Skeletonema costatum* (Grev.) Cleve, clone Skel-5 isolated from the Trondheimsfjord (Myklestad, 1974) are maintained in axenic stock cultures in the f/2 medium of Guillard & Ryther (1962) at 13 °C. The culture medium is based on filtered seawater of salinity ~32 psu from the Trondheimsfjord at 90 m depth, which is diluted to 3/4 strength with Milli-Q water (final salinity ~24 psu). Data is provided for cells grown in a modified f/10 medium. All nutrients were at a concentration of f/10 except for $NaNO_3^-$ (50 µmol l^{-1}), NaH_2PO_4 (5 µmol l^{-1}), and Na_2SiO_3 (50 µmol l^{-1}), providing a slightly N-limited medium. The culture was grown in a 5-l batch photobioreactor (details to be published elsewhere) 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 (2×4) with an irradiance of 200 µmol m^{-2} s^{-1} as measured by a Biospherical Instruments (San Diego, CA) QSL-100 light meter. pH in the culture was continually monitored with a Consort (Turnhout, Belgium) R301 controller. The pH was kept within an interval of 7.8-8.1 by automatic titration with diluted HCl (in the light) and NaOH (in the dark).

Seawater samples were collected with 30-l Niskin bottles from ship R/V Harry Borthen I at Station Trollet in the Trondheimsfjord (central part of the fjord) at depths of 0, 3, and 10 m during the first spring bloom on March 29th 2001. The samples were stored in the dark and

cold and transferred to the laboratory within few hours.

Method

Cells from axenic cultures or field samples are harvested by filtration through 25-mm Whatman (Kent, UK) GF/C glass fiber filters under gentle vacuum (50 mm Hg). The filter is transferred directly to a glass vial, and stored at -20 °C until analysis. Depending on cell size and the physiological condition 10^6-10^8 cells, or 0.5-2 mg dry matter, should be sufficient for analysis.

Cellular β-1,3-glucan is extracted by 5 ml 0.05 mol l^{-1} H_2SO_4 at 60 °C for 10 min using a water bath, and the extract is filtered (GF/C). The filter with cells is washed repeatedly with Milli-Q water, and dried overnight at 60 °C. Cell wall polysaccharides are hydrolyzed by 0.5 ml 80% H_2SO_4 at 0-4 °C for 20 h. The hydrolysate is diluted to 1 mol l^{-1} H_2SO_4 by addition of 6.0 ml icecold Milli-Q water, and filtered (GF/C).

The glucan extract and the cell wall hydrolysate are analyzed separately by the phenol-sulphuric acid method (Dubois et al., 1956). 0.5 ml 3% aqueous phenol and 5 ml concentrated H_2SO_4 are added to 2 ml sample (containing 5-100 μg carbohydrate) in a test tube, and the mixture is immediately stirred with a glass rod or vortexed. The tube is allowed to stand for 30 min, and then cooled in running water. Spectrophotometric extinction is measured in a 1-cm cell at 485 nm. The amount of reducing sugar is calculated using glucose as a standard with an extinction factor of ~0.1 g l^{-1}. A correction for the weight of non-hydrolyzed polysaccharide (glucan or cell wall) is made by a factor of 0.9. The determination is run in duplicate or triplicate.

Additional analyses

The cells were counted with a haemocytometer (field samples were fixed and sedimented overnight). Dissolved NO_3^- and inorganic phosphate (P_i) were analyzed according to Strickland & Parsons (1972). Cellular chlorophyll *a* (chl *a*) was extracted by acetone and measured with a Turner Design (Sunnyvale, CA) 10 AU fluorometer according to Holm-Hansen et al. (1965). Cellular organic C and N were measured with a Carlo Erba (CE Instruments, Milan, Italy) NA 1500 elemental analyzer according to Kirsten (1979). Cellular protein was calculated as organic N×6.25. All variables were analyzed in triplicate with standard deviations of 2-10%.

Results

Axenic culture

The marine diatom *S. costatum* was grown in batch culture with a 14/10 h light/dark cycle for 5 days, and cellular β-1,3-glucan and cell wall polysaccharides were determined each day according to the method described. Figure 1 shows the time courses of cell density, cellular glucan, cell wall polysaccharides, and dissolved NO_3^-. On two occasions (indicated in Figure 1), separate samples were taken out in 10 replicates to investigate the precision of the polysaccharide method (Table 1). The cells grew exponentially (2.0 div. day^{-1}) until NO_3^- was depleted, and then gradually went into stationary phase. Cellular β-1,3-glucan accumulated heavily after NO_3^- was depleted, while cell wall polysaccharides only slightly increased. Levels of 3.0 mg l^{-1} glucan and 0.8 mg l^{-1} cell wall polysaccharides were measured in the late exponential phase at the point of NO_3^- depletion, while 31.4 mg l^{-1} glucan and 2.0 mg l^{-1} cell

wall polysaccharides were measured three days later in the stationary phase. Standard deviations for the glucan analyses were ~1%, and for cell wall polysaccharides 2-7%. The low precision level for cell wall polysaccharides in late exponential phase was mainly due to low sample carbohydrate concentrations.

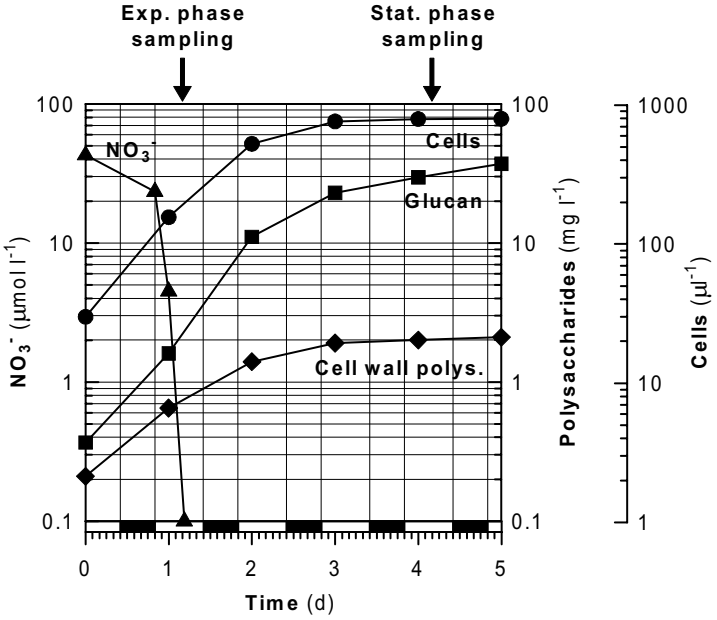

Figure 1. Time courses of cell density, cellular β-1,3-glucan, cell wall polysaccharides, and dissolved NO_3^- in a *S. costatum* batch culture (black bars indicate dark periods).

Table 1. Cellular β-1,3-glucan (G) and cell wall polysaccharides (CW) in a *S. costatum* batch culture at two instants during growth, n=10

	Growth phase			
	Late exponential		Stationary	
	G	CW	G	CW
Parallels (mg l^{-1})	3.00	0.88	31.9	2.02
	3.06	0.82	31.2	2.02
	3.10	0.74	31.5	2.06
	3.02	0.75	32.1	1.98
	3.04	0.69	31.4	2.12
	2.98	0.75	31.1	2.04
	3.01	0.80	31.6	1.96
	3.03	0.75	31.1	2.08
	2.98	0.82	31.3	2.00
	3.04	0.85	31.4	1.98
Mean (mg l^{-1})	3.03	0.79	31.4	2.03
s.d. (mg l^{-1})	0.04	0.06	0.3	0.05
Relative s.d. (%)	1.2	7.1	1.0	2.4

Natural phytoplankton

Seawater samples were collected from the Trondheimsfjord (Station Trollet) at depths of 0, 3, and 10 m on March 29th 2001, close to culmination of the first spring bloom. Table 2 shows the composition of phytoplankton species in the samples, and Table 3 shows the chemical composition (cellular chl *a*, cellular organic C and N, and dissolved NO_3^- and P_i). The phytoplankton populations were dominated by diatoms, especially *Chaetoceros* spp., and haptophytes, especially *Phaeocystis pouchetii* (predominantly as single cells). At depths of 0 and 3 m the total cell numbers and species composition were quite similar: 12-13 cells μl^{-1} with ~45% diatoms and ~55% haptophytes. At 10 m the total cell density was only 7 cells μl^{-1} with ~30% diatoms and ~70% haptophytes. The total algal biomass at 0-3 m contained about 13 $\mu g\ l^{-1}$ chl *a*, 0.60 mg l^{-1} C, and 0.10 mg l^{-1} N, while only half of this amount was measured at 10 m. The C/N ratios (6.6-6.9 mol mol^{-1}) suggest that the phytoplankton was nutrient sufficient, even though the levels of dissolved NO_3^- and P_i were quite low at 0-3 m (Table 3).

Table 2. Phytoplankton species composition (cells ml^{-1}) of seawater samples collected from the Trondheimsfjord on March 29th 2001

Class	Species	Depth (m)		
		0	3	10
Bacillariophyceae (diatoms)	*Chaetoceros* spp.	5,010	3,900	1,833
	Coscinodiscus sp.	3	1	-
	Navicula sp.	50	14	11
	Pseudonitzschia sp.	278	195	156
	Skeletonema costatum	885	930	168
	Thalassiosira spp.	41	30	42
	total diatoms	6,266	5,070	2,210
Cryptophyceae	indeterminate	75	45	45
Haptophyceae	*Phaeocystis pouchetii*	5,520	5,299	3,956
	indeterminate	1,473	1,472	828
	total haptophytes	6,993	6,771	4,784
total phytoplankton		13,334	11,886	7,039

The *Chaetoceros* spp. were predominantly *C. debilis* and *C. socialis*, the *Pseudonitzschia* sp. was probably *P. delicatissima* or *P. pungens*, and the *Thalassiosira* spp. were predominantly *T. polychorda* and other large spp.

Table 3. Chemical composition of seawater samples collected from the Trondheimsfjord on March 29th 2001

	Depth (m)		
	0	3	10
Chl *a* ($\mu g\ l^{-1}$)	12.4	13.3	7.2
Cellular organic C ($\mu g\ l^{-1}$)	602	565	291
Cellular organic N ($\mu g\ l^{-1}$)	107	99	49
Dissolved NO_3^- ($\mu mol\ l^{-1}$)	1.0	1.3	6.9
Dissolved P_i ($\mu mol\ l^{-1}$)	0.15	0.18	0.46

Cellular β-1,3-glucan and cell wall polysaccharides were measured at each depth in 8 replicates according to the method described (Table 4). The polysaccharide levels were also quite similar at the depths of 0 and 3 m: 0.24 mg l^{-1} glucan and 0.29 mg l^{-1} cell wall polysaccharides. The concentrations at 10 m were only 0.04 mg l^{-1} glucan and 0.13 mg l^{-1} cell wall polysaccharides. Standard deviations for the glucan analyses were 2-3%, and for cell wall polysaccharides ~5%. The protein/carbohydrate ratios were calculated as 1.2 at 0-3 m and 1.8 at 10 m, indicating nutrient sufficiency.

Table 4. Cellular β-1,3-glucan (G) and cell wall polysaccharides (CW) in seawater samples collected from the Trondheimsfjord on March 29th 2001, n=8

	Depth (m)					
	0		3		10	
	G	CW	G	CW	G	CW
Parallels (mg l^{-1})	0.242	0.287	0.251	0.305	0.043	0.119
	0.248	0.314	0.242	0.295	0.042	0.137
	0.233	0.285	0.232	0.306	0.044	0.123
	0.242	0.305	0.239	0.287	0.044	0.129
	0.239	0.300	0.239	0.324	0.043	0.129
	0.243	0.274	0.236	0.283	0.044	0.121
	0.242	0.285	0.242	0.297	0.042	0.121
	0.253	0.271	0.240	0.281	0.042	0.131
Mean (mg l^{-1})	0.243	0.290	0.240	0.297	0.043	0.126
s.d. (mg l^{-1})	0.006	0.015	0.005	0.014	0.001	0.006
Relative s.d. (%)	2.4	5.2	2.2	4.8	2.6	5.1

Discussion

The method presented here provides a simple and rapid determination of β-1,3-glucan in diatoms. Analyses of the marine diatom *Chaetoceros affinis* (Myklestad et al., 1972) and of natural phytoplankton populations (Haug et al., 1973) showed that glucose is the predominant sugar in acid extracts (>95%), even for field samples. In this contribution we present a new extraction procedure that is much faster than the previous (Myklestad & Haug, 1972). The new procedure is based on recent observations of phytoplankton membrane transition temperature, T_m (Myklestad & Swift, 1998). T_m is defined as the temperature at which half the cellular soluble carbon is found extracellularly under standard conditions. This investigation revealed that the cells released virtually all cellular soluble carbon, including the reserve glucan, within 10 min incubation at a temperature >10 °C above their T_m (28.5-41.5 °C for diatoms). Membrane disorganization is the most probable mechanism for this release. On this basis we have chosen 60 °C as the extraction temperature. The new method did not reveal significantly different results compared to the earlier method (Myklestad & Haug, 1972). We believe that this extraction procedure could also be applied to other classes of phytoplankton with similar β-1,3-glucans (e.g. Chrysophyceae and Haptophyceae).

Determination of the water-insoluble cell wall polysaccharides is more complex than the analysis of β-1,3-glucan. Complete hydrolysis of the polysaccharides to monomers can be difficult, especially with hexopyranuronosidic linkages, e.g. glucuronomannans (Painter, 1983). On the other hand, too harsh treatment can lead to decay of sugars due to side-reactions and dehydration reactions. The hydrolysis step in our procedure is carried out using 80% H_2SO_4 (Haug & Larsen, 1962; Myklestad & Haug, 1972). However, we have lowered

the hydrolysis temperature from 20 °C to only 0-4 °C to slow down any decay reactions. Moreover, the separate step with hydrolysis of cell wall polysaccharides after glucan extraction represents another improvement compared to previous methods. Myklestad & Haug (1972) took different samples for determination of glucan and total carbohydrate, and cell wall polysaccharides was estimated from the difference. Such estimates will be less accurate, as they depend on two different analyses. However, it is important that the remaining cell material after acid extraction is thoroughly washed and dried before hydrolysis.

Some workers have used a two step H_2SO_4 hydrolysis method for determination of particulate polysaccharides (Haug & Larsen, 1962; Pakulski & Benner, 1992). In the second step of this method the hydrolysate is diluted to 1 mol l^{-1} H_2SO_4 and boiled for a few hours. We have also carried out two step hydrolysis in one investigation with *S. costatum* cells and one with natural phytoplankton, but observed similar or insignificantly lower results. As a consequence we have omitted the boiling step with dilute acid in our method.

The results from the analyses of axenic *S. costatum* and natural phytoplankton populations are consistent with previous investigations (Haug et al., 1973; Myklestad, 1974). In contrast to the axenic diatom culture, the field samples contained more cell wall polysaccharides than β-1,3-glucan. However, the natural phytoplankton populations included higher numbers of haptophytes, predominantly *Phaeocystis pouchetii*, than of diatoms. On the other hand, it should be noted that the haptophytes are smaller and form a much lower percentage of the total biomass than of the cell numbers. Chrysolaminaran is also the most important storage product in haptophytes, but the cell wall composition is distinctly different from diatoms (Hoek et al., 1995). A haptophyte cell is covered by several layers of cellulose microfibrils (scales), which are glued together by a coating of complex acidic polysaccharides. Such haptophyte scales probably account for the high levels of cell wall polysaccharides measured in the field samples. The results are in agreement with previous investigations of natural phytoplankton populations with similar species composition from the Trondheimsfjord (Haug et al., 1973). In these investigations, however, the phytoplankton samples were collected by net hauls (mesh width 25 μm) compared to GF/C filters (1.2 μm) in the present investigation.

This new method provides a simple determination of the total cellular carbohydrate in diatoms, mainly represented by the food storage glucan and the cell wall polysaccharides. We have demonstrated that this procedure applies well to both laboratory cultures and natural phytoplankton populations dominated by diatoms. Cellular glucan and cell wall polysaccharides were determined with standard deviations of 1-3% and 2-5%, respectively. The lower precision levels coincided with the lower sample carbohydrate concentrations.

The sensitivity of the method may be a problem in field samples with very low biomass. The large filter volumes required for sampling during seasons of low phytoplankton growth or in open ocean areas are inconvenient. In such cases the much more sensitive TPTZ method (Myklestad et al., 1997) will be an alternative to the phenol-sulphuric acid for quantitative determination.

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References

Allan, G.G., J. Lewin & P.G. Johnson, 1972. Marine polymers. IV. Diatom polysaccharides. Bot. Mar. 15:102-8.

Beattie, A., E.L. Hirst & E. Percival, 1961. Comparative structural investigations on leucosin (chrysolaminarin) separated from diatoms and laminarin from the brown algae. Biochem. J. 79:531-7.

Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers & F. Smith, 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350-6.

Ford, C.W. & E. Percival, 1965a. The carbohydrates of *Phaeodactylum tricornutum*. Part I. Preliminary examination of the organism, and characterisation of low molecular weight material and of a glucan. J. Chem. Soc. 1298:7035-41.

Ford, C.W. & E. Percival, 1965b. The carbohydrates of *Phaeodactylum tricornutum*. Part II. A sulphated glucuronomannan. J. Chem. Soc. 1299:7042-6.

Guillard, R.R.L. & J.H. Ryther, 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. Can. J. Microbiol. 8:229-39.

Handa, N., 1969. Carbohydrate metabolism in the marine diatom *Skeletonema costatum*. Mar. Biol. 4:208-214.

Haug, A. & B. Larsen, 1962. Quantitative determination of the uronic acid composition of alginates. Acta Chem. Scand. 16:1908-18.

Haug, A. & S. Myklestad, 1976. Polysaccharides of marine diatoms with special reference to *Chaetoceros* species. Mar. Biol. 34:217-22.

Haug, A., S. Myklestad & E. Sakshaug, 1973. Studies on the phytoplankton ecology of the Trondheimsfjord. I. The chemical composition of phytoplankton populations. J. Exp. Mar. Biol. Ecol. 11:15-26.

Hecky, R.E., K. Mopper, P. Kilham & E.T. Degens, 1973. The amino acid and sugar composition of diatom cell-walls. Mar. Biol. 19:323-31.

Hitchcock, G.L., 1978. Labelling patterns of carbon-14 in net plankton during a winter-spring bloom. J. Exp. Mar. Biol. Ecol. 31:141-53.

Hoek, C. van den, D.G. Mann & H.M. Jahns, 1995. Algae: An Introduction to Phycology. University Press, Cambridge, 623 pp.

Holm-Hansen, O., C.J. Lorenzen, R.W. Holmes & J.D. Strickland, 1965. Fluorometric determination of chlorophyll. J. cons. Cons. int. explor. mer 30:3-15.

Kirsten, W.J., 1979. Automatic methods for the simultaneous determination of carbon, hydrogen, nitrogen, and sulfur, and for sulfur alone in organic and inorganic materials. Anal. Chem. 51:1173-9.

McConville, M.J., A. Bacic & A.E. Clarke, 1986. Structural studies of chrysolaminaran from the ice diatom *Stauroneis amphioxys* (Gregory). Carbohydr. Res. 153:330-3.

Myklestad, S., 1974. Production of carbohydrates by marine planktonic diatoms. I. Comparison of nine different species in culture. J. Exp. Mar. Biol. Ecol. 15:261-74.

Myklestad, S., 1978. β-1,3-Glucans in diatoms and brown seaweeds. In Hellebust, J.A. & J.S. Craigie (eds), Handbook of Phycological Methods. Physiological and Biochemical Methods. University Press, Cambridge: 133-41.

Myklestad, S. & A. Haug, 1972. Production of carbohydrates by the marine diatom *Chaetoceros affinis* var. *willei* (Gran) Hustedt. I. Effect of the concentration of nutrients in the culture medium. J. Exp. Mar. Biol. Ecol. 9:125-36.

Myklestad, S., A. Haug & B. Larsen, 1972. Production of carbohydrates by the marine diatom *Chaetoceros affinis* var. *willei* (Gran) Hustedt. II. Preliminary investigation of the extracellular polysaccharide. J. Exp. Mar. Biol. Ecol. 9:137-44.

Myklestad, S.M. & E. Swift, 1998. A new method for measuring soluble cellular organic content and a membrane property, T_m, of planktonic algae. Eur. J. Phycol. 33:333-6.

Myklestad, S.M., E. Skånøy & S. Hestmann, 1997. A sensitive and rapid method for analysis of dissolved mono- and polysaccharides in seawater. Mar. Chem. 56:279-86.

Painter, T.J., 1983. Algal polysaccharides. In Aspinall, G.O. (ed.), The Polysaccharides, Vol. 2. Academic Press, New York: 195-285.

Pakulski, J.D. & R. Benner, 1992. An improved method for the hydrolysis and MBTH analysis of dissolved and particulate carbohydrates in seawater. Mar. Chem. 40:143-60.
Paulsen, B.S. & S. Myklestad, 1978. Structural studies of the reserve glucan produced by the marine diatom *Skeletonema costatum* (Grev.) Cleve. Carbohydr. Res. 62:386-8.
Percival, E., M.A. Rahman & H. Weigel, 1980. Chemistry of the polysaccharides of the diatom *Coscinodiscus nobilis*. Phytochemistry 19:809-11.
Romankevich, E.A., 1984. Geochemistry of Organic Matter in the Ocean. Springer, Berlin, 334 pp.
Schmid, A.-M.M., M.A. Borowitzka & B.E. Volcani, 1981. Morphogenesis and biochemistry of diatom cell walls. In Kiermayer, O. (ed.), Cytomorphogenesis in Plants. Springer, Wien: 63-97.
Smith, D.J. & G.J.C. Underwood, 1998. Exopolymer production by intertidal epipelic diatoms. Limnol. Oceanogr. 43:1578-91.
Stosch, H.-A. von, 1981. Structural and histochemical observations on the organic layers of the diatom cell wall. In Ross, R. (ed.), Proceedings of the 6th symposium on recent and fossil diatoms. Koeltz, Koenigstein: 231-52.
Strickland, J.D.H. & T.R. Parsons, 1972. A Practical Handbook of Seawater Analysis, 2nd ed. Bull. Fish. Res. Bd Can. 167, Ottawa, 310 pp.
Volcani, B.E., 1981. Cell wall formation in diatoms: morphogenesis and biochemistry. In Simpson, T.L. & B.E. Volcani (eds), Silicon and Siliceous Structures in Biological Systems. Springer, New York: 157-200.
Vårum, K.M. & S. Myklestad, 1984. Effects of light, salinity and nutrient limitation on the production of β-1,3-D-glucan and exo-D-glucanase activity in *Skeletonema costatum* (Grev.) Cleve. J. Exp. Mar. Biol. Ecol. 83:13-26.
Waterkeyn, L. & A. Bienfait, 1987. Localisation et rôle des β-1,3-glucanes (callose et chrysolaminarine) dans le genre *Pinnularia* (Diatomées). La Cellule 74:198-226.

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Paper 3

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EFFECTS OF NH_4^+ ASSIMILATION ON DARK CARBON FIXATION AND β-1,3-GLUCAN METABOLISM IN THE MARINE DIATOM *SKELETONEMA COSTATUM* (BACILLARIOPHYCEAE)[1]

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The effects of NH_4^+ assimilation on dark carbon fixation and β-1,3-glucan metabolism in the N-limited marine diatom *Skeletonema costatum* (Grev.) Cleve (Bacillariophyceae) were investigated by chemical analysis of cell components and incorporation of ^{14}C-bicarbonate. The diatom was grown in pH-regulated batch cultures with a 14:10 h LD cycle until N depletion. The cells were then incubated in the dark with ^{14}C-bicarbonate, but without a source of N for 2 h, then in the dark with 63 μmol·L^{-1} NH_4^+ for 3 h. Without N, the cellular concentration of free amino acids was almost constant (~4.5 fmol·cell^{-1}). Added NH_4^+ was assimilated at a rate of 12 fmol·cell^{-1}·h^{-1}, and the cellular amino acid pool increased rapidly (doubled in <1 h, tripled in <3 h). The glutamine level increased steeply (45× within 3 h), and the Gln/Glu ratio increased from 0.1 to 2.4 within 3 h. The rate of dark C fixation during N depletion was only 1.0 fmol·cell^{-1}·h^{-1}. The addition of NH_4^+ strongly stimulated dark C fixation, leading to an assimilation rate of 4.0 fmol·cell^{-1}·h^{-1}, corresponding to a molar C/N uptake ratio of 0.33. Biochemical fractionation of organic ^{14}C showed no significant ^{14}C fixation into amino acids during N depletion, but during the first 1–2 h of NH_4^+ assimilation, amino acids were rapidly radiolabeled, accounting for virtually all net ^{14}C fixation. These results indicate that anaplerotic β-carboxylation is activated during NH_4^+ assimilation to provide C_4 intermediates for amino acid biosynthesis. The level of cellular β-1,3-D-glucan was constant (16.5 pg·cell^{-1}) during N depletion, but NH_4^+ assimilation activated a mobilization of 28% of the reserve glucan within 3 h. The results indicate that β-1,3-glucan in diatoms is the ultimate substrate for β-carboxylation, providing precursors for amino acid biosynthesis in addition to energy from respiration.

Key index words: **ammonium assimilation; anaplerotic β-carboxylation; carbon/nitrogen uptake ratio; dark carbon fixation; free amino acids; β-1,3-D-glucan; nitrogen limitation; *Skeletonema costatum***

Abbreviations: **DIC, dissolved inorganic carbon; DOC, dissolved organic carbon; Gln, glutamine; Glu, glutamate; MW, molecular weight; OPP, oxidative pentose phosphate; TCA, tricarboxylic acid**

Marine algae produce 35–50·10^{15} g biomass each year by means of photosynthetic CO_2 fixation (Falkowski and Raven 1997). This accounts for about 40% of the total net primary production of plants on earth. Diatoms have a dominant role in the most productive areas of the oceans and are estimated to contribute 20%–25% of the world's net primary production (Werner 1977). Their patterns of CO_2 fixation are therefore of great ecological interest. The introduction of ^{14}C tracer technique provided sensitive measurement of CO_2 fixation in phytoplankton (Steemann-Nielsen 1952).

In light, photoautotrophic cells perform photosynthetic CO_2 reduction by means of the Calvin–Benson cycle, effectively forming carbohydrate (Calvin and Bassham 1962). In addition, cells, including nonphotoautotrophic ones, fix significant amounts of CO_2 by nonreductive β-carboxylation. This anaplerotic process is essential to replenish intermediates in the tricarboxylic acid (TCA) cycle, when the intermediates are drawn off for biosynthesis of amino acids and other compounds. Early observations of dark carbon fixation in macrophytes (Craigie 1963) and in phytoplankton (Morris et al. 1971a) were accomplished by monitoring ^{14}C incorporation. In actively growing algae, the ratio of carboxylation in the dark compared to that under conditions of light saturation is typically <0.05, although it ranges from <0.01 to >0.4 (Mortain-Bertrand et al. 1988). The rate of β-carboxylation increases following the addition of NH_4^+ to N-deficient but not N-replete cells (Morris et al. 1971b, Guy et al. 1989), and this has been used as a test for N limitation. Assimilation of NH_4^+ by N-replete cells is dependent on CO_2 and light (photosynthesis), whereas N-deplete cells with accumulated carbohydrates can assimilate NH_4^+ in darkness (Thacker and Syrett 1972). In diatoms, the substrate for β-carboxylation (phosphoenolpyruvate) originates from 3-phosphoglycerate (an intermediate in the Calvin–Benson cycle) in light, and from a polysaccharide in darkness (Kremer and Berks 1978, Appleby et al. 1980, Morris 1980, Mortain-Bertrand et al. 1987, 1988). Most experiments on dark C fixation in marine algae have concentrated on long-term products and have shown that synthesis of proteins occurs at the expense of polysaccharides or lipids (Cuhel et al. 1984, Vårum and Myklestad 1984). Experiments relating to the first products (<1 min) of dark C fixation have shown mainly TCA cycle intermediates and

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amino acids (Glover et al. 1975, Mortain-Bertrand et al. 1988).

The main photosynthetic reserve polysaccharide in diatoms is a β-1,3-D-glucan, also called chrysolaminaran, which accumulates under nutrient limitation (Handa 1969, Myklestad and Haug 1972, Myklestad 1974). Hama and Honjo (1987) observed that the increased production of carbohydrate under low-nutrient conditions was due mainly to elevated glucose production, implying glucan. The glucan is consumed when diatoms are placed in darkness (Handa 1969, Vårum and Myklestad 1984), and apparently it serves as a respiratory substrate. Active exo-β-1,3-D-glucanase has been demonstrated in diatoms (Myklestad et al. 1982), indicating active glucan metabolism. The glucan is thought to be more than a substrate to furnish energy by respiration; it also is thought to provide essential carbon skeletons for amino acid biosynthesis (Myklestad 1988/1989).

The aim of this contribution is to study the effects of NH_4^+ assimilation on dark C fixation and β-1,3-glucan metabolism in the marine diatom *S. costatum* by analysis of changes in metabolite levels and ^{14}C labeling of different biochemical fractions.

MATERIALS AND METHODS

Organism and culture conditions. The 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 and Ryther (1962) at 13° C.

In the experiments, the cells were grown in a modified f/2 medium. The culture medium was based on filtered sea water of salinity ~32 psu from the Trondheimsfjord at 90 m depth, which was diluted to ¾ with Milli-Q water (final salinity ~24 psu). All nutrients were at a concentration of f/2 except for nitrate (f/6 = 294 μmol·L^{-1}) and phosphate (29 μmol·L^{-1}), providing a slightly N-limited medium (N/P = 10).

The diatom culture was grown in a photobioreactor (details to be published elsewhere), run as a stirred (at short intervals) 10-L batch reactor at 13° C with a 14:10 h LD cycle. Illumination was provided by racks of alternating Phillips TLD 18W/33 and TLD 18W/96 tubes (2 × 4) with an irradiance of 200 μE·m^{-2}·s^{-1} as measured by a QSL-100 light meter from Biospherical Instruments, San Diego, California. pH was measured continually and was kept within an interval of 7.8–8.1 by automatic titration with diluted HCl (in the light) and NaOH (in the dark).

Experiments with dark incubation and sampling were performed approximately 24 h after NO_3^- was depleted, and immediately after a 6-h light period. Dissolved inorganic carbon (DIC) was nearly exhausted, and extra bicarbonate was added to a final concentration of 0.7 mmol·L^{-1}. DIC was set at this low concentration to enhance ^{14}C-specific incorporation, but not any lower to avoid C limitation of dark C fixation. The cells were first incubated in the dark without a source of N (dark −N phase) for 2 h, then in the dark with about 70 μmol·L^{-1} NH_4Cl (dark +N phase) for 3 h. Before incubation in the dark, 1.5 L of culture were transferred to another photobioreactor, and 40 μCi·L^{-1} $NaH^{14}CO_3$ (specific activity 8.6 Ci·mol^{-1}) were added. Both cultures, however, were incubated simultaneously in the dark under similar conditions. Samples for measuring ^{14}C fixation were taken from the radiolabeled culture, and all other samples were taken from the unlabeled replicate.

Sampling and analysis. The cells were counted with a Bürker hemocytometer. Cell density was determined as the mean of six counts of about 160 cells each, with a sample standard deviation of 5%–7%.

The efficiency (quantum yield) of photochemistry in PSII (ϕ_p) was determined as the ratio of variable fluorescence (F_v) to maximum fluorescence (F_m) by measuring *in vivo* fluorescence before (F_0) and after (F_m) addition of 10 μmol·L^{-1} DCMU [3′-(3,4-dichlorophenyl)-1′,1′-dimethylurea] using a Turner Designs 10 AU fluorometer (Samuelsson and Øquist 1977):

$$\phi_p = \frac{F_v}{F_m} = \frac{F_m - F_0}{F_m}$$

The quantum yield was determined as the mean of two samples. In this method, estimates with <5% standard deviation are obtainable at low excitation intensities with sample chlorophyll concentrations of <1 mg·L^{-1} (Kulandaivelu and Daniell 1980).

The cells were harvested by filtration through 25-mm Whatman GF/C glass-fiber filters under gentle vacuum (50 mm Hg) and were stored at −20° C until analysis. Samples were filtered for analysis of cellular chl *a*, organic C and N, β-1,3-glucan, and free amino acids. The filtrates were used for analysis of medium NO_3^-, NH_4^+, DIC, and dissolved organic carbon (DOC).

Chlorophyll *a* was extracted with acetone and determined by spectrophotometric extinction (Jeffrey and Humphrey 1975) as the mean of two samples. Jeffrey and Humphrey reported a standard deviation of 2% for analysis of chl *a*.

Cellular organic C and N were measured in a Carlo Erba, Milan, Italy, elemental analyzer as the mean of two samples. Kirsten (1979) obtained errors of 1–2 μg in C and N measurements, corresponding to <1% error in our results. In our experiments, however, the precision seems to be lower (3%–8% error), probably due to problems with sample preparation.

Cellular β-1,3-D-glucan was determined by a modification (details to be published elsewhere) of the acid extraction method of Myklestad and Haug (1972). Cellular glucan was determined as the mean of three samples, with a standard deviation of 2%–5%.

Cellular free amino acids were extracted with hot water and determined by reversed-phase HPLC by precolumn fluorescence derivatization with *o*-phthaldialdehyde (Lindroth and Mopper 1979). Hot water extraction and modifications of the HPLC method were carried out as described by Flynn (1988), except that we used a Waters Nova-Pak, Milford, Massachusetts, C_{18} column and *o*-phthaldialdehyde reagent solution from Sigma, St. Louis, Missouri. A few amino acids were not chromatographically resolved because their peaks merged (His + Ser, Arg + Gly + Thr, and Tyr + Ala). Amino acid concentrations were determined as the mean of two samples. Repeatability of HPLC determination of amino acids obtained by Flynn (1988) was ±1.4%–8.8%, reported as 95% confidence limits. The concentrations of free amino acids were calculated per cell or per cell volume (200 μm^3·cell^{-1}). The diatom cell volume was calculated as that of a cylinder based on average diameter and length of 20 cells measured in a microscope.

Dissolved NO_3^- in the filtrates was determined by reduction to NO_2^-, diazotizing with sulfanilamide, and coupling with N-(1-naphthyl)-ethylenediamine. NH_4^+ was determined by the phenol-hypochlorite method (Strickland and Parsons 1972), both as a mean of two samples. The precision of both methods is ±5%, reported as 95% confidence limits. The N uptake rate was determined by linear regression analysis of dissolved NH_4^+ versus time. The N uptake rate was calculated both as moles of N taken up per unit cell per unit time (fmol·cell^{-1}·h^{-1}), and specific uptake rate as mass of N taken up per unit cellular mass of organic N per unit time (h^{-1}).

DIC was determined by alkalinity titration (Strickland and Parsons 1972) as the mean of two samples. The precision of the DIC measurement is ±3%, reported as 95% confidence limits.

DOC was measured by high-temperature catalytic oxidation (Sugimura and Suzuki 1988) as the mean of two samples using a Dohrmann, Cincinnati, Ohio, DC-190 analyzer with a standard deviation of 3%.

^{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). Samples were purged of inorganic ^{14}C by acidification (exposing the filters to concentrated HCl vapor) and evaporation of CO_2 overnight. Cellular fixed ^{14}C radioactivity (dpm)

TABLE 1. Chemical composition of an N-limited *S. costatum* batch culture as a function of time. The cells first were incubated in the dark without a source of N (dark −N phase) for 2 h, and then in the dark with 63 μmol·L^{-1} NH_4Cl (dark +N phase) for 3 h. The efficiency of photochemistry in PSII was measured as the ratio of variable fluorescence to maximum fluorescence (obtained by adding DCMU).

	Phase		
	Dark −N		Dark +N
Incubation time (h)	0	2	3
Cell density (10^9 cells·L^{-1})	1.6	—	1.6
Chl *a* (mg·L^{-1})	0.77	0.78	0.79
PSII photochemistry efficiency	0.61	0.63	0.65
Cellular organic C (mg·L^{-1})	22.0	21.2	20.3
Cellular organic N (mg·L^{-1})	2.9	2.8	3.1
DOC (mg·L^{-1})	4.7	4.5	4.7

was measured with a Wallac, Turku, Finland, 1410 scintillation counter using OptiPhase HiSafe 3 scintillation liquid (10 mL). Fixed ^{14}C was determined as the mean of two samples. In previous experiments (unpubl.), sample standard deviations for this method were estimated to be 3%–4% by using sets of four samples. The rate of dark ^{14}C fixation was determined by linear regression analysis of fixed ^{14}C versus time during the first 45–60 min of each phase. The C uptake rate ($\partial C/\partial t$) was calculated from the rate of dark ^{14}C fixation ($\partial A_{org}/\partial t$), the total activity of $NaH^{14}CO_3$ added to the culture (A_{tot} = 40 μCi·L^{-1}), and the total DIC concentration (0.7 mmol·L^{-1}) without any isotope discrimination factor (Geider and Osborne 1992):

$$\frac{\partial C}{\partial t} = \frac{\partial A_{org}}{\partial t}\frac{DIC}{A_{tot}}$$

The C uptake rate was calculated both as moles of C taken up per unit cell per unit time (fmol·cell^{-1}·h^{-1}) and specific uptake rate as mass of C taken up per unit cellular mass of organic C per unit time (h^{-1}).

Medium filtrate (5 mL) was purged of inorganic ^{14}C by acidification (pH < 2) with H_3PO_4 and evaporation of CO_2 overnight. Extracellular fixed ^{14}C was measured by liquid scintillation counting, as described above, and determined as the mean of two samples.

For biochemical fractionation, cells were harvested and purged of inorganic ^{14}C as described above. A low molecular weight (MW) fraction was extracted from the cells by hot water (2 mL Milli-Q water at 70° C). The extract was separated into a basic fraction (amino acids, purines, pyrimidines, etc.) and an acid/neutral fraction (organic acids, sugars, etc.) by cation exchange chromatography. A Pharmacia, Uppsala, Sweden, Resource S (methyl sulfonate) strong cation exchange column was equilibrated with start buffer, 50 mmol·L^{-1} citrate, pH 2.0. The low-MW extract was filtered (0.2 μm), mixed 1:1 with 100 mmol·L^{-1} citrate, pH 2.0, and applied to the column. At first the acid/neutral fraction was eluted with start buffer, then the basic fraction with 20 mmol·L^{-1} $Na_2B_4O_7$, pH 9.0, with 300 mmol·L^{-1} NaCl. The column was regenerated by flushing with 50 mmol·L^{-1} citrate, pH 2.0, with 500 mmol·L^{-1} NaCl. All buffers were filtered (0.2 μm) and degassed with He prior to chromatography. Left on the filter support was an insoluble macromolecular fraction (proteins, nucleic acids, etc.). Fixed ^{14}C in the three different fractions was measured by liquid scintillation counting, as described above, and determined as the mean of two samples.

RESULTS

Skeletonema costatum was grown in pH-regulated batch cultures with a 14:10 h LD cycle until N depletion ($1.6 \cdot 10^9$ cells·L^{-1}, 0.8 mg·L^{-1} chl *a*). The cells were then incubated in the dark with ^{14}C-bicarbonate, but without a source of N for 2 h (dark −N phase), then in the dark with 63 μmol·L^{-1} NH_4^+ for 3 h (dark +N phase).

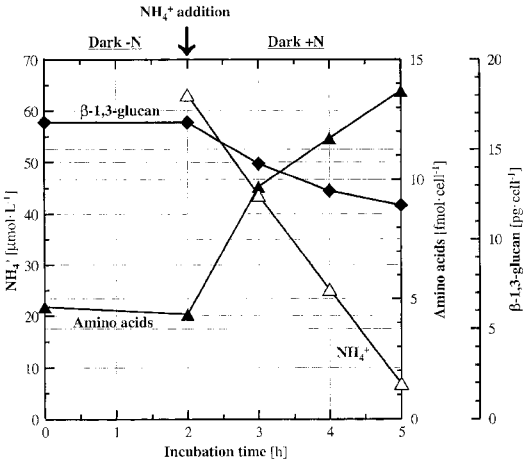

FIG. 1. Cellular concentrations of free amino acids and β-1,3-glucan and dissolved NH_4^+ in a N-limited *S. costatum* batch culture as functions of time. The cells first were incubated in the dark without a source of N (dark −N phase) for 2 h, then in the dark with 63 μmol·L^{-1} NH_4Cl (dark +N phase) for 3 h.

Table 1 shows the variation in chemical composition of the culture with time, and Figure 1 shows cellular concentrations of free amino acids and β-1,3-glucan and dissolved NH_4^+ as functions of time. Cell density, chl *a*, the efficiency (quantum yield) of photochemistry in PSII, cellular organic C, and DOC were approximately constant (no statistically significant variation) throughout the dark incubation period. During N depletion (dark −N phase), cellular free amino acids (~4.5 fmol·cell^{-1}), β-1,3-glucan (16.5 pg·cell^{-1}, 26.4 mg·L^{-1}), and organic N (2.8 mg·L^{-1}) were also constant. At the addition of NH_4^+ (dark +N phase), however, the free amino acid pool increased by 214% and cellular glucan decreased by 28% within 3 h, changing most rapidly in the early stage. NH_4^+ was assimilated at a rate of 12 fmol·cell^{-1}·h^{-1} (specific N uptake rate 0.098 h^{-1}), and cellular organic N increased by 11% within 3 h.

Table 2 shows the variation in composition of cellular free amino acids with time. During N depletion (dark −N phase), the composition of the free amino acid pool was approximately constant. At the addition of NH_4^+ (dark +N phase), however, the glutamine level increased steeply (45×), and accounted for 47% of the total increase in amino acids. There were also significant increases in glutamate (1.7×), aspartate (4.0×), and asparagine (16.5×). Figure 2 shows the glutamine (Gln) and glutamate (Glu) levels as functions of time, the Gln/Glu ratio being a sensitive indicator of N limitation. During NH_4^+ assimilation, the Gln/Glu ratio increased from 0.1 to 2.4 within 3 h.

Dark ^{14}C fixation and dissolved NH_4^+ as functions

TABLE 2. Cellular concentrations of free amino acids (fmol·cell $^{-1}$) in a N-limited *S. costatum* batch culture as a function of time. The cells first were incubated in the dark without a source of N (dark −N phase) for 2 h, then in the dark with 63 μmol·L^{-1} NH_4Cl (dark +N phase) for 3 h. The total concentration of amino acids was also calculated per cell volume (mmol·L $^{-1}$).

	Phase				
	Dark −N		Dark +N		
Incubation time (h)	0	2	1	2	3
Asp	0.71	0.67	1.71	2.26	2.74
Glu	0.93	1.08	1.68	1.81	1.89
Asn	0.04	0.04	0.31	0.47	0.66
His + Ser	0.35	0.16	0.22	0.28	0.25
Gln	0.13	0.10	2.66	3.60	4.50
Arg + Gly + Thr	0.52	0.39	0.85	1.10	1.45
Tyr + Ala	0.64	0.66	1.06	1.25	1.35
Met	0.02	0.04	0.06	0.03	0.06
Val	0.17	0.15	0.18	0.18	0.15
Phe	0.06	0.04	0.08	0.09	0.06
Ile	0.45	0.41	0.40	0.29	0.23
Leu	0.11	0.10	0.13	0.08	0.07
Lys	0.53	0.52	0.37	0.27	0.28
Total amino acids	4.66	4.35	9.70	11.71	13.68
Total amino acids (mmol·L^{-1})	23.3	21.8	48.5	58.6	68.4

of time are presented in Figure 3. During N depletion (dark −N phase), the C assimilation rate was only 1.0 fmol·cell $^{-1}$·h $^{-1}$ (specific C uptake rate $0.88 \cdot 10^{-3} \cdot h^{-1}$). Mainly low-MW metabolites (extracted by hot water) were labeled (90%), and of these, organic acids (acid/neutral fraction) accounted for 60% and amino acids (basic fraction) for 40%. A macromolecular (insoluble) fraction accounted for the rest of ^{14}C labeling (10%). The addition of NH_4^+ (dark +N phase) markedly activated ^{14}C fixation, and during the first hour, the C assimilation rate was 4.0 fmol·cell^{-1}·h^{-1} (specific C uptake rate $3.6 \cdot 10^{-3} \cdot h^{-1}$). During this period, the amino acid pool accounted for virtually all net ^{14}C fixation. As the cellular ^{14}C fixation rate decreased after 1.5 h, the rate of ^{14}C fixation in amino acids decreased even more, and after 2 h, the ^{14}C-labeled amino acid pool declined slightly. In contrast, ^{14}C labeling of the macromolecular fraction (indicating protein)

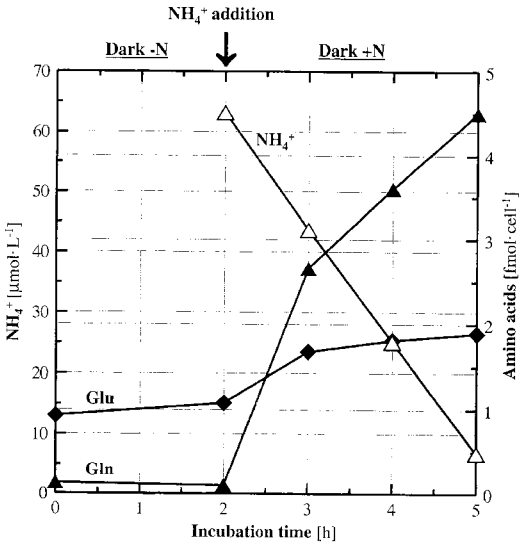

FIG. 2. Cellular concentrations of glutamate (Glu) and glutamine (Gln) and dissolved NH_4^+ in a N-limited *S. costatum* batch culture as functions of time. The cells first were incubated in the dark without a source of N (dark −N phase) for 2 h, then in the dark with 63 μmol·L^{-1} NH_4Cl (dark +N phase) for 3 h.

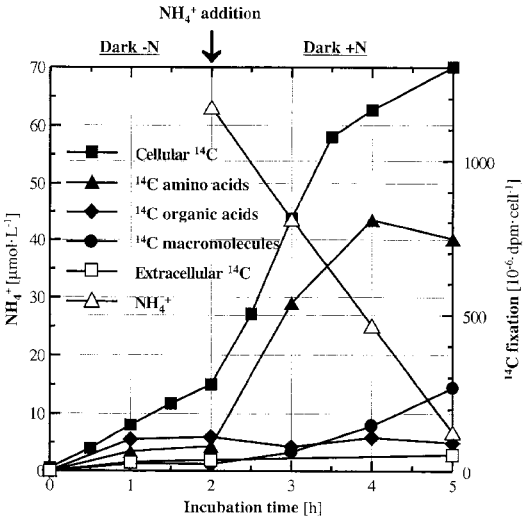

FIG. 3. ^{14}C fixation in different biochemical fractions and dissolved NH_4^+ in a N-limited *S. costatum* batch culture as functions of time. The cells first were incubated in the dark without a source of N (dark −N phase) for 2 h, then in the dark with 63 μmol·L^{-1} NH_4Cl (dark +N phase) for 3 h. Biochemical fractionation of cellular fixed ^{14}C into amino acids, organic acids and macromolecules was carried out by hot water extraction and cation exchange chromatography.

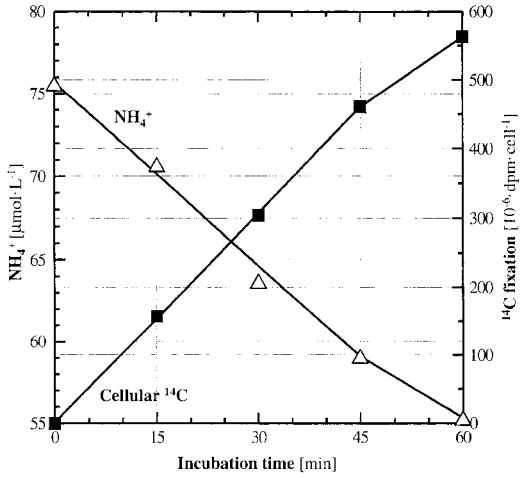

FIG. 4. Cellular ^{14}C fixation and dissolved NH_4^+ in a N-limited *S. costatum* batch culture as functions of time. The cells were incubated in the dark with 76 μmol·L^{-1} NH_4^+ for 1 h.

increased from 10% to 24% of cellular fixed ^{14}C during this period (2–3 h). There was no significant increase in ^{14}C labeling of organic acids during dark NH_4^+ assimilation. ^{14}C labeling of extracellular organic components (DOC) accounted for only 3.6% of cellular ^{14}C fixation.

An additional experiment was performed to examine the kinetics of C and N assimilation more closely during the early phase of N repletion in the dark. As before, *S. costatum* was grown in batch culture until N depletion (1.5·10^9 cells·L^{-1}, 1.1 mg·L^{-1} chl *a*), then incubated in the dark with 76 μmol·L^{-1} NH_4^+ (dark +N) and ^{14}C-bicarbonate for 1 h. Cellular ^{14}C fixation and dissolved NH_4^+ were sampled every 15 min, and the results are presented in Figure 4. Because the linearity of ^{14}C fixation and NH_4^+ assimilation with time seemed to decrease slightly after 45 min, uptake rates were calculated during the first 45 min, yielding 4.4 fmol C·cell^{-1}·h^{-1} and 14 fmol N·cell^{-1}·h^{-1}. Hence, the C/N uptake ratio was 0.31 in this, compared to 0.33 in the previous, experiment. After 1 h in the dark, the cells were incubated in light (200 μE·m^{-2}·s^{-1}) for 20 min, obtaining a photosynthetic C assimilation rate of 133 fmol·cell^{-1}·h^{-1}. Consequently, the ratio of dark- to light-saturated carboxylation was 0.033.

The C/N uptake ratio was correlated to amino acid biosynthesis by calculating the theoretical ratio of anaplerotic β-carboxylation to N assimilation required to produce cell protein (Table 3). A ratio of 0.35 was calculated, based on the average amino acid composition of the total cellular protein from six diatom species measured by Chuecas and Riley (1969). Due to acid hydrolysis, the amino acids Asn, Gln, and Trp were omitted in the calculations. The requirement for N assimilation was simply calculated as the number of N per amino acid, whereas the requirement for β-carboxylation was calculated as one per amino acid of the aspartate and glutamate families (derived from TCA cycle intermediates).

DISCUSSION

In light, photoautotrophic cells assimilate inorganic carbon mainly through the photosynthetic carbon reduction cycle (Calvin–Benson cycle) via Rubisco, reducing CO_2 and forming carbohydrate (Calvin and Bassham 1962). In the dark, however, cells fix inorganic carbon without any reduction of CO_2 taking place. Dark carbon fixation is required to replenish intermediates in metabolic cycles (e.g. the TCA cycle) when the intermediates are drawn off for anabolism (Falkowski and Raven 1997). An-

TABLE 3. Calculation of theoretical ratio of anaplerotic β-carboxylation to N assimilation as deduced from the average amino acid composition of the total cellular protein from six diatom species (Chuecas and Riley 1969).

Amino acid	Amino acid protein composition	N assimilation		β-carboxylation		
		Per amino acid	Per protein	Per amino acid	Per protein	Per N assimilation
Ala	0.095	1	0.095	0	0	0
Arg	0.085	4	0.340	1	0.085	0.25
Asp	0.072	1	0.072	1	0.072	1
Cys	0.014	1	0.014	0	0	0
Glu	0.072	1	0.072	1	0.072	1
Gly	0.096	1	0.096	0	0	0
His	0.015	3	0.045	0	0	0
Ile	0.044	1	0.044	1	0.044	1
Leu	0.086	1	0.086	0	0	0
Lys	0.029	2	0.058	1	0.029	0.5
Met	0.013	1	0.013	1	0.013	1
Phe	0.041	1	0.041	0	0	0
Pro	0.040	1	0.040	1	0.040	1
Ser	0.104	1	0.104	0	0	0
Thr	0.068	1	0.068	1	0.068	1
Tyr	0.009	1	0.009	0	0	0
Val	0.023	1	0.023	0	0	0
Total protein	0.906		1.220		0.423	0.347

aplerotic carboxylations thus provide essential components for biosynthesis and growth that cannot be produced from the Calvin–Benson cycle (e.g. amino acids, tetrapyrroles, pyrimidines, purines, and lipids). The major anaplerotic carboxylation enzymes are phosphoenolpyruvate carboxykinase, phosphoenolpyruvate carboxylase, and pyruvate carboxylase, of which the first shows a high activity in diatom extracts (Kremer and Berks 1978, Descolas-Gros and Oriol 1992). Because these enzymes catalyze the carboxylation of the β-carbon of 3C substrates, their activity is called β-carboxylation. The rate of β-carboxylation increases following the addition of NH_4^+ to N-deficient, but not N-replete, cells (Morris et al. 1971b, Guy et al. 1989). The stimulation of β-carboxylation induced by the added NH_4^+ probably reflects the anaplerotic formation of amino acids.

During the dark N-depletion period (dark −N) in our experiment, the level of cellular free amino acids did not change significantly (Fig. 1, Table 2). As expected, no net biosynthesis of amino acids took place, because no N source was available to the cells. When N was replete (dark +N), however, the amino acid pool increased rapidly (doubled in <1 h, tripled in <3 h). The levels of glutamine, glutamate, aspartate, and asparagine increased significantly, all of which are dependent on β-carboxylation for biosynthesis from TCA cycle intermediates (α-ketoglutarate and oxaloacetate). Glutamine alone accounted for 47% of the total increase in free amino acids, the Gln/Glu ratio increasing from 0.1 to 2.4 within 3 h (Fig. 2). This ratio provides a convenient estimate of N status in diatoms (Flynn and Al-Amoudi 1988). The rapid increase in glutamine at N repletion probably reflects the main biosynthetic route of NH_4^+ incorporation, which is the sequential action of glutamine synthetase and glutamate synthase (also called glutamine 2-oxoglutarate aminotransferase, or GOGAT) (Falkowski and Rivkin 1976, Syrett 1981), as illustrated in Figure 5. NH_4^+ is first incorporated as an amido group into glutamate to form glutamine by glutamine synthetase at the cost of ATP hydrolysis. The amido group of glutamine is subsequently transferred to α-ketoglutarate (also called 2-oxoglutarate) and reduced by NADPH-dependent glutamate synthase, forming two glutamate molecules. One of the glutamate molecules reenters the assimilation pathway as a substrate for glutamine synthetase, thereby forming a cycle. The α-amino group of the second glutamate molecule is transferred to α-keto acids by aminotransferases when other amino acids are formed. Vanlerberghe et al. (1990) inferred from *in vitro* activity and *in vivo* metabolite levels that glutamate (inhibitor) and glutamine (activator) were the main effectors of β-carboxylation in the green alga *Selenastrum minutum*. The results from our experiment suggest similar roles of glutamate and glutamine as effectors of β-carboxylation in diatoms.

During the N-deplete period (dark −N), dark ^{14}C fixation was only 1.0 fmol·cell^{-1}·h^{-1} (Fig. 3). A low degree of β-carboxylation is in accordance with low or no biosynthesis of amino acids and, hence, no need for TCA cycle intermediates. The onset of NH_4^+ assimilation (dark +N), however, strongly stimulated dark ^{14}C fixation (4.0 fmol·cell^{-1}·h^{-1}). Biochemical fractionation showed no net ^{14}C fixation in amino acids during N depletion, whereas during the first 1–2 h of NH_4^+ assimilation, amino acids were rapidly labeled, accounting for virtually all net ^{14}C fixation. These results suggest that anaplerotic β-carboxylation is activated to provide carbon skeletons for amino acid biosynthesis, in accordance with the findings of Guy et al. (1989) for the green alga *S. minutum*. Other workers have identified the first products (within <1 min) synthesized by dark ^{14}C fixation as TCA cycle intermediates (malate, oxaloacetate, etc.) and amino acids (aspartate, glutamate, etc.) (Glover et al. 1975, Mortain-Bertrand et al. 1988). Oxaloacetate is the primary product of β-carboxylation. Oxaloacetate can be reduced rapidly to malate by malate dehydrogenase or undergo a transamination reaction to form aspartate. Alternatively, oxaloacetate or malate is transformed into α-ketoglutarate via the TCA cycle and forms glutamate by means of glutamate synthase. Aspartate and glutamate themselves are precursors for biosynthesis of other amino acids (Fig. 6).

In the later stage (2–3 h) of NH_4^+ assimilation, net ^{14}C labeling of amino acids apparently ceased and was superseded by significant ^{14}C labeling of the macromolecular fraction, presumably protein. This

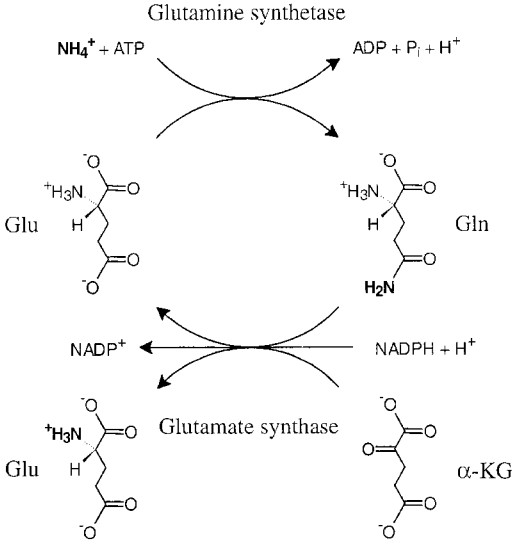

FIG. 5. Incorporation of NH_4^+ into amino acids by means of the glutamine synthetase–glutamate synthase cycle. The pathway of the incorporated NH_4^+ group is indicated by boldface. Abbreviations: α-KG, α-ketoglutarate; Gln, glutamine; Glu, glutamate.

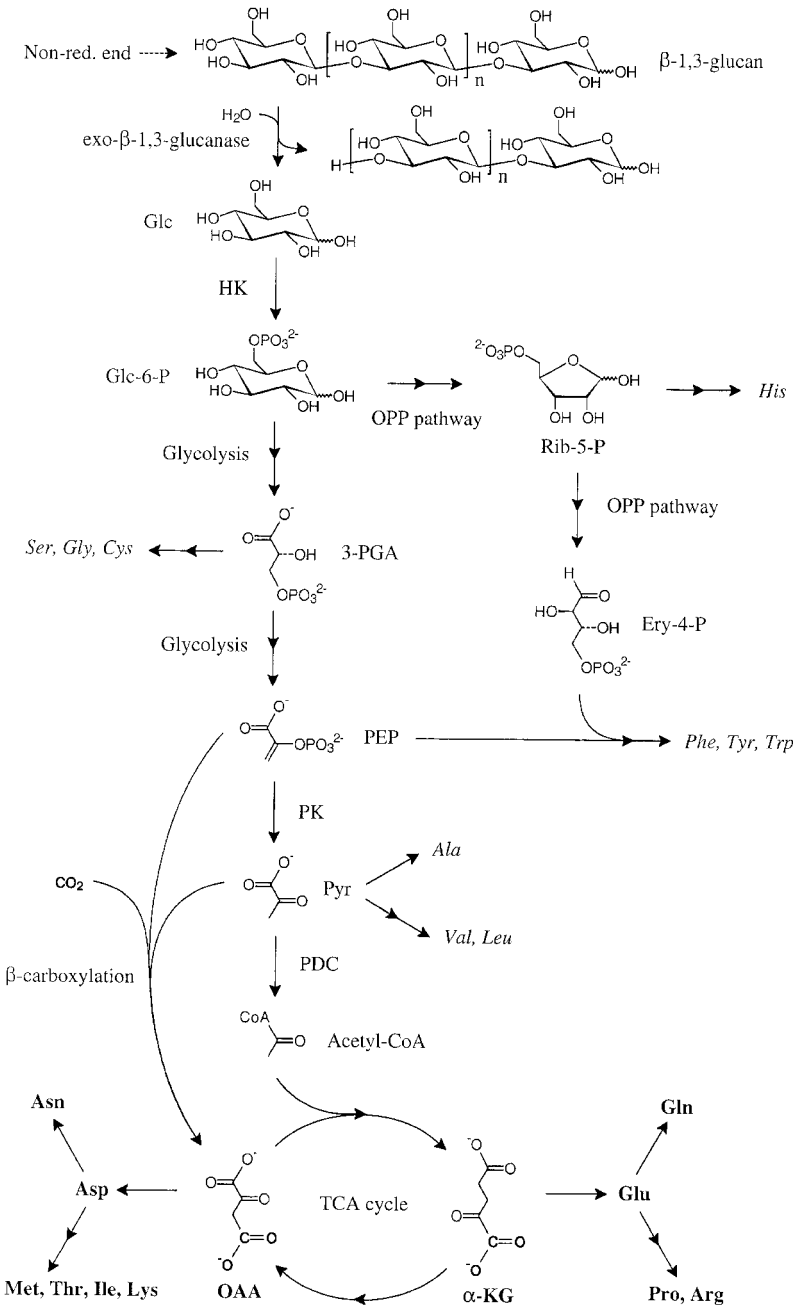

FIG. 6. Scheme of metabolic pathways providing precursors (C skeletons) for biosynthesis of amino acids, assuming β-1,3-D-glucan is the ultimate substrate. The glucan is hydrolyzed by exo-β-1,3-D-glucanase, splitting off D-glucose at the nonreducing end. Glucose is metabolized by the respiratory pathways (glycolysis, OPP pathway, and TCA cycle), producing precursors for amino acid biosynthesis. Intermediates from the TCA cycle used in amino acid synthesis (oxaloacetate, α-ketoglutarate) must be replenished by β-carboxylation; thus, amino acids formed from TCA intermediates are labeled during dark $^{14}CO_2$ fixation (indicated by boldface in this scheme). Amino acids merely synthesized from intermediates in the glycolytic and OPP pathways, on the other hand, are not labeled (italic type). Abbreviations: α-KG, α-ketoglutarate; CoA, coenzyme A; Ery-4-P, D-erythrose 4-phosphate; Glc, D-glucose; Glc-6-P, D-glucose 6-phosphate; HK, hexokinase; OAA, oxaloacetate; OPP, oxidative pentose phosphate; PDC, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; 3-PGA; 3-phosphoglycerate; PK, pyruvate kinase; Pyr, pyruvate; Rib-5-P, D-ribose 5-phosphate; TCA, tricarboxylic acid.

development suggests that the rate at which amino acids are drawn off for protein synthesis is rather similar to the rate of amino acid synthesis. These results corroborate previous reports of protein synthesis in darkness (Cuhel et al. 1984, Vårum and Myklestad 1984).

The assimilation rates measured in these experiments yielded an average molar C/N uptake ratio

of 0.32. This value agrees well with the calculated theoretical ratio of 0.35 for production of diatom protein (Table 3). This calculation, however, relies on the assumption that the cells produce only protein. A calculation that accounted for the synthesis of pyrimidines and purines would yield a lower ratio, closer to the experimental value of 0.32. Vanlerberghe et al. (1990) observed a C/N uptake ratio of 0.3 over a range of NH_4^+ assimilation rates in the green alga *S. selenastrum*, compared to 0.34 calculated from amino acid composition of cell protein.

During the dark N-deplete period (dark −N), the cellular content of reserve β-1,3-D-glucan was constant (Fig. 1). At the onset of dark NH_4^+ assimilation (dark +N), biosynthesis of amino acids coincided with a significant decrease in cellular glucan (28% within 3 h). These results suggest that glucan is the ultimate substrate for β-carboxylation and amino acid biosynthesis in the dark. In early experiments, Handa (1969) observed that the glucan in *S. costatum* is consumed when kept in the dark, and Vårum and Myklestad (1984) showed that nitrate assimilation in the dark led to pronounced reduction in the glucan content of *S. costatum* cells. Prior to the present experiments, the cells were loaded with glucan by growth under N limitation (Myklestad 1974). The enzyme exo-β-1,3-D-glucanase demonstrated in diatoms (Myklestad et al. 1982), readily splits off glucose from the nonreducing ends of β-1,3-D-glucan. Glucose is metabolized by the respiratory pathways (glycolysis, oxidative pentose phosphate (OPP) pathway, and TCA cycle) to form precursors for amino acid biosynthesis. Intermediates from the TCA cycle used in amino acid synthesis (oxaloacetate, α-ketoglutarate) must be replenished by β-carboxylation. A scheme that illustrates the metabolic pathways providing precursors for biosynthesis of amino acids, assuming β-1,3-glucan is the ultimate substrate, is presented in Figure 6.

Our results clearly indicate that β-1,3-glucan is more than a reservoir of energy: it also provides essential C skeletons for amino acid synthesis. Further work in our laboratory aims to confirm this and to study the regulation of the level of glucan in marine diatoms.

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Appleby, G., Colbeck, J., Holdsworth, E. S. & Wadman, H. 1980. β carboxylation enzymes in marine phytoplankton and isolation and purification of pyruvate carboxylase from *Amphidinium carterae* (Dinophyceae). *J. Phycol.* 16:290–5.

Calvin, M. & Bassham, J. A. 1962. *The Photosynthesis of Carbon Compounds.* Benjamin, New York, 126 pp.

Chuecas, L. & Riley, J. P. 1969. The component combined amino acids of some marine diatoms. *J. Mar. Biol. Ass. UK* 49:117–20.

Craigie, J. S. 1963. Dark fixation of ^{14}C-bicarbonate by marine algae. *Can. J. Bot.* 41:317–25.

Cuhel, R. L., Ortner, P. B. & Lean, D. R. S. 1984. Night synthesis of protein by algae. *Limnol. Oceanogr.* 29:731–44.

Descolas-Gros, C. & Oriol, L. 1992. Variations in carboxylase activity in marine phytoplankton cultures. β-carboxylation in carbon flux studies. *Mar. Ecol. Prog. Ser.* 85:163–9.

Falkowski, P. G. & Raven, J. A. 1997. *Aquatic Photosynthesis.* Blackwell, Malden, Massachusetts, 375 pp.

Falkowski, P. G. & Rivkin, R. B. 1976. The role of glutamine synthetase in the incorporation of ammonium in *Skeletonema costatum* (Bacillariophyceae). *J. Phycol.* 12:448–50.

Flynn, K. J. 1988. Some practical aspects of measurements of dissolved free amino acids in natural waters and within microalgae by the use of HPLC. *Chem. Ecol.* 3:269–93.

Flynn, K. J. & Al-Amoudi, O. A. 1988. Effects of N deprivation and darkness on composition of free amino acid pool in and on amino acid release from diatom *Phaeodactylum tricornutum* Bohlin. *J. Exp. Mar. Biol. Ecol.* 119:131–43.

Geider, R. J. & Osborne, B. A. 1992. *Algal Photosynthesis: The Measurement of Algal Gas Exchange.* Chapman and Hall, New York, 256 pp.

Glover, H., Beardall, J. & Morris, I. 1975. Effects of environmental factors on photosynthesis pattern in *Phaeodactylum tricornutum* (Bacillariophyceae). (I) Effect of nitrogen deficiency and light intensity. *J. Phycol.* 11:424–9.

Guillard, R. R. L. & Ryther, J. H. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.* 8:229–39.

Guy, R. D., Vanlerberghe, G. C. & Turpin, D. H. 1989. Significance of phosphoenolpyruvate carboxylase during ammonium assimilation. Carbon isotope discrimination in photosynthesis and respiration by the N-limited green alga *Selenastrum minutum*. *Plant Physiol.* 89:1150–7.

Hama, T. & Honjo, T. 1987. Photosynthetic products and nutrient availability in phytoplankton population from Gokasho Bay, Japan. *J. Exp. Mar. Biol. Ecol.* 112:251–66.

Handa, N. 1969. Carbohydrate metabolism in the marine diatom *Skeletonema costatum*. *Mar. Biol.* 4:208–14.

Jeffrey, S. W. & Humphrey, G. F. 1975. New spectrophotometric equations for determining chlorophylls a, b, c_1 and c_2 in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanzen* 167:191–4.

Kirsten, W. J. 1979. Automatic methods for the simultaneous determination of carbon, hydrogen, nitrogen, and sulfur, and for sulfur alone in organic and inorganic materials. *Anal. Chem.* 51:1173–9.

Kremer, B. P. & Berks, R. 1978. Photosynthesis and carbon metabolism in marine and freshwater diatoms. *Z. Pflanzenphysiol.* 87:149–65.

Kulandaivelu, G. & Daniell, H. 1980. Dichlorophenyl dimethylurea (DCMU) induced increase in chlorophyll a fluorescence intensity—an index of photosynthetic oxygen evolution in leaves, chloroplasts and algae. *Physiol. Plant* 48:385–8.

Lindroth, P. & Mopper, K. 1979. High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with *o*-phthaldialdehyde. *Anal. Chem.* 51:1667–74.

Morris, I. 1980. Paths of carbon assimilation in marine phytoplankton. *In* Falkowski, P. [Ed.] *Primary Productivity in the Sea.* Plenum Press, New York, pp. 139–59.

Morris, I., Yentsch, C. M. & Yentsch, C. S. 1971a. Relationship between light carbon dioxide fixation and dark carbon dioxide fixation by marine algae. *Limnol. Oceanogr.* 16:854–8.

——— 1971b. The physiological state with respect to nitrogen of phytoplankton from low-nutrient subtropical water as measured by the effect of ammonium ion on dark carbon fixation. *Limnol. Oceanogr.* 16:859–68.

Mortain-Bertrand, A., Descolas-Gros, C. & Jupin, H. 1987. Short-term ^{14}C incorporation in *Skeletonema costatum* (Greville)

Cleve (Bacillariophyceae) as a function of light regime. *Phycologia* 26:262–9.

Mortain-Bertrand, A., Descolas-Gros, C. & Jupin, H. 1988. Pathway of dark inorganic carbon fixation in two species of diatoms: influence of light regime and regulator factors on diel variations. *J. Plankton Res.* 15:199–217.

Myklestad, S. 1974. Production of carbohydrates by marine planktonic diatoms. I. Comparison of nine different species in culture. *J. Exp. Mar. Biol. Ecol.* 15:261–74.

Myklestad, S., Djurhuus, R. & Mohus, A. 1982. Demonstration of *exo*-(β-1,3)-D-glucanase activity in some planktonic diatoms. *J. Exp. Mar. Biol. Ecol.* 56:205–11.

Myklestad, S. & Haug, A. 1972. Production of carbohydrates by the marine diatom *Chaetoceros affinis* var. *willei* (Gran) Hustedt. I. Effect of the concentration of nutrients in the culture medium. *J. Exp. Mar. Biol. Ecol.* 9:125–36.

Myklestad, S. M. 1988/1989. Production, chemical structure, metabolism, and biological function of the (1→3)-linked β-D-glucans in diatoms. *Biol. Oceanogr.* 6:313–26.

Samuelsson, G & Öquist, G. 1977. A method for studying photosynthetic capacities of unicellular algae based on *in vivo* chlorophyll fluorescence. *Physiol. Plant* 40:315–9.

Steemann-Nielsen, E. 1952. The use of radio-active carbon (C^{14}) for measuring organic production in the sea. *J. Cons. Int. Explor. Mer.* 18:117–40.

Strickland, J. D. H. & Parsons, T. R. 1972. *A Practical Handbook of Seawater Analysis*, 2nd ed. Bull. Fish. Res. Bd. Can. 167, Ottawa, 310 pp.

Sugimura, Y. & Suzuki, Y. 1988. A high-temperature catalytic method for the determination of non-volatile dissolved organic carbon in seawater by direct injection of a liquid sample. *Mar. Chem.* 24:105–31.

Syrett, P. J. 1981. Nitrogen metabolism in microalgae. *In* Platt, T. [Ed.] *Physiological Bases of Phytoplankton Ecology*. Can. Bull. Fish. Aquat. Sci. 210, Ottawa, pp. 182–210.

Thacker, A. & Syrett, P. J. 1972. The assimilation of nitrate and ammonium by *Chlamydomonas reinhardi*. *New Phytol.* 71:423–33.

Vanlerberghe, G. C., Schuller, K. A., Smith, R. G., Feil, R., Plaxton, W. C. & Turpin, D. H. 1990. Relationship between NH_4^+ assimilation rate and *in vivo* phosphoenolpyruvate carboxylase activity. *Plant Physiol.* 94:284–90.

Vårum, K. M. & Myklestad, S. 1984. Effects of light, salinity and nutrient limitation on the production of β-1,3-D-glucan and exo-D-glucanase activity in *Skeletonema costatum* (Grev.) Cleve. *J. Exp. Mar. Biol. Ecol.* 83:13–25.

Werner, D. 1977. Contributions of the study of diatoms to biology. *In* Werner, D. [Ed.] *The Biology of Diatoms*. Blackwell, Oxford, pp. 1–3.

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Paper 4

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MOBILIZATION OF β-1,3-GLUCAN AND BIOSYNTHESIS OF AMINO ACIDS INDUCED BY NH_4^+ ADDITION TO N-LIMITED CELLS OF THE MARINE DIATOM *SKELETONEMA COSTATUM* (BACILLARIOPHYCEAE)[1]

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Mobilization of the reserve β-1,3-glucan (chrysolaminaran) in N-limited cells of the marine diatom *Skeletonema costatum* (Grev.) Cleve (Bacillariophyceae) was investigated. The diatom was grown in pH-regulated batch cultures with a 14:10-h light:dark cycle until N depletion. In a pulse-chase experiment, the cells were first incubated in high light (200 μmol photons·m^{-2}·s^{-1}) with ^{14}C-bicarbonate until dissolved inorganic carbon was exhausted. Unlabeled bicarbonate (1 mM) was then added, and the cells were incubated in the dark and subsequently in low light (20 μmol photons·m^{-2}·s^{-1}) with additions of 40 μM NH_4^+. In the ^{14}C pulse phase with high light and N depletion, β-1,3-glucan accumulated and accounted for 85% of incorporated ^{14}C. In the subsequent ^{14}C chase phases, added NH_4^+ was assimilated at an N-specific rate of 0.11 h^{-1} in both the dark and low light, and in both cases it caused a significant mobilization of β-1,3-glucan (dark, 26%; low light, 19%). Biochemical fractionation of organic ^{14}C showed that free amino acids were most rapidly labeled in the early stage of NH_4^+ assimilation, whereas proteins and polysaccharides were labeled more rapidly after 1.2 h. Analysis of the cellular free amino acids strongly indicated that *de novo* biosynthesis was occurring, with a Gln:Glu ratio increasing from 0.4 to 10 within 1.2 h. After the NH_4^+ was exhausted, the cellular pools of glucan and amino acids became constant or slowly decreased. In another experiment, N-limited cells were first incubated in high light until dissolved inorganic carbon was exhausted and were further incubated in high light with 150 μM NH_4^+ under inorganic carbon limitation. Added NH_4^+ was assimilated at an N-specific rate of 0.023 h^{-1}, and cellular β-1,3-glucan decreased by 15% within 6 h. Hence, β-1,3-glucan was mobilized during NH_4^+ assimilation, even though inorganic carbon was modifying the metabolic rates. The results provide new evidence of β-1,3-glucan supplying essential precursors for biosynthesis of amino acids and other components in *S. costatum* in both the dark and subsaturating light and even saturating light under inorganic carbon limitation.

Key index words: **ammonium assimilation; biochemical fractionation; free amino acids; β-1,3-D-glucan mobilization; nitrogen limitation; polysaccharides; *Skeletonema costatum***

Abbreviations: **DIC, dissolved inorganic carbon; DOC, dissolved organic carbon; Gln, glutamine; Glu, glutamate; L:D, light:dark; MW, molecular weight; TC, total carbon; TCA, trichloroacetic acid**

The principal storage polysaccharide in diatoms (Bacillariophyceae) is β-1,3-D-glucan, also called chrysolaminaran. It is a relatively short-chained water-soluble (1→3)-linked β-D-glucopyranan (DP 20-60) with occasional branching through C-2 and C-6 (Beattie et al. 1961, Paulsen and Myklestad 1978, McConville et al. 1986). Fluorescence microscopy shows that chrysolaminaran in diatoms is localized in cellular vacuoles (Waterkeyn and Bienfait 1987). Similar β-1,3-glucans are also found and named as chrysolaminaran in Chrysophyceae, Eustigmatophyceae, Haptophyceae (Prymnesiophyceae), and Xanthophyceae; as paramylon in Euglenophyceae; and as laminaran in Phaeophyceae. Because of the dominant role of diatoms in marine waters, β-1,3-glucan may rival cellulose as the polysaccharide with the highest annual production on earth (Painter 1983). The ratio of protein to glucan is a sensitive indicator of nutritional status in both laboratory cultures and natural phytoplankton populations (Myklestad and Haug 1972, Haug et al. 1973, Hitchcock 1978, Sakshaug et al. 1983). This is because glucan accumulates under nutrient limitation (see below), whereas protein synthesis decreases or stops.

Carbohydrates are the first products of photosynthesis in all algae (Calvin-Benson cycle) and provide precursors for all cell components. The main storage product of photosynthetic CO_2 fixation in diatoms is β-1,3-glucan. The two other main groups of polysaccharides in diatoms are cell wall heteroglycans and extracellular mucilages. These are predominantly complex polysaccharides containing residues of mannose, galactose, glucose (and their corresponding uronic acids), xylose, L-rhamnose, and L-fucose (Haug and Myklestad 1976, Painter 1983). Photosynthetic partitioning of organic carbon into various classes of metabolites has been investigated by using their different solubility in methanol/water, chloroform, and trichloroacetic acid (TCA) (Li et al. 1980, Hitchcock 1983, Lancelot 1984). The fractions in this scheme are generally assumed to be methanol/water extract, low molecular weight (MW) metabolites (monosaccharides, amino/organic acids); chloroform extract, lipids; TCA extract, polysaccharides; and TCA residue, proteins. In diatoms, however, the reserve polysaccharide β-1,3-glu-

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can is very soluble in water/methanol and must also be included in the low MW fraction (McConville et al. 1985, Myklestad 1988/1989). We recently developed a method for further resolution of the low MW fraction into amino acids (along with purines/pyrimidines) and β-1,3-glucan (along with monosaccharides and organic acids) by means of cation exchange chromatography (Granum and Myklestad 1999).

The cellular glucan content is relatively low in the logarithmic phase of growth, but a rapid accumulation occurs under nutrient limitation (Myklestad and Haug 1972, Myklestad 1974). The marine diatom *Skeletonema costatum* contains < 20% of total carbon as glucan in the log phase but accumulates up to 80% of its C as glucan in the stationary phase (Vårum and Myklestad 1984). Photosynthetic incorporation of ^{14}C-bicarbonate shows that the glucan fraction is quickly labeled in both log- and stationary-phase cells (McConville et al. 1985, Myklestad 1988/1989). Also, studies with ^{13}C-gas chromatography-mass spectroscopy show high glucose production in the stationary phase, implying the accumulation of glucan (Hama and Honjo 1987). Biosynthetic investigations of paramylon (Goldemberg and Marechal 1963) and chrysolaminaran (Roessler 1987) suggest that glucan synthesis follows the same general pathway as in most other organisms, through the action of the enzymes UDP-glucose pyrophosphorylase and glucosyl transferase.

The glucan is consumed when the alga is kept in the dark (Handa 1969, Vårum and Myklestad 1984), and apparently it serves as a respiratory substrate. In an investigation with *S. costatum* grown under different light:dark (L:D) regimes, the glucan content of log-phase cells decreased from ~50% of total C at the end of the day to 4%–25% at the end of the night (Vårum et al. 1986). Active exo-β-1,3-glucanase is produced in *S. costatum* and other diatoms, particularly under nutrient limitation, indicating glucan mobilization (Myklestad et al. 1982).

Algal cells are able to assimilate nitrogenous compounds in darkness provided that sufficient energy and carbon reserves are available (Syrett 1981, Cuhel et al. 1984). Previous investigations with *S. costatum* show that nutrient-limited cells readily assimilate both NO_3^- and NH_4^+ in the dark and that this is accompanied by a pronounced reduction in reserve glucan (Vårum and Myklestad 1984, Granum and Myklestad 1999). Also, addition of NH_4^+ activates rapid dark ^{14}C fixation into amino acids, indicating amino acid synthesis through anaplerotic β-carboxylation (Granum and Myklestad 1999).

The aim of this study was to further investigate the physiological function of β-1,3-glucan in *S. costatum* by combined ^{14}C-tracer technique and chemical analyses. Mobilization of glucan and biosynthesis of amino acids was induced by addition of NH_4^+ to N-limited cells under different light conditions (darkness, low light, and high light). A pulse-chase experiment with biochemical fractionation of organic ^{14}C was carried out to trace the incorporation of carbon skeletons from β-1,3-glucan into main metabolic products. We also investigated glucan mobilization during photosynthesis under severe dissolved inorganic carbon (DIC) limitation.

MATERIALS AND METHODS

Organism and culture conditions. The diatom *S. 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 and 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 three-fourths with Milli-Q water (Millipore, Bedford, MA) (final salinity ~ 24 psu). In the present experiments, the f/2 medium was modified by reducing nitrate to 300 μM (f/6) and phosphate to 30 μM, providing a slightly N-limited medium.

The cultures were grown in photobioreactors (unpublished data) and run as stirred (at short intervals) 5-L batch reactors at 13° C with a 14:10-h L:D cycle. Illumination was provided by racks of alternating Phillips TLD 18W/33 and TLD 18W/96 tubes (2 × 4) with an irradiance of 200 μmol photons·m^{-2}·s^{-1} as measured by a Biospherical Instruments (San Diego, CA) QSL-100 light meter. This irradiance is referred to as high light in this work, although it is in the low range of light saturation for the alga (Sakshaug and Andresen 1986). In some phases of the experiments, an irradiance of only 20 μmol photons·m^{-2}·s^{-1} was used and referred as low light (subsaturating light). pH in the culture was continually monitored with a pH electrode coupled to a Consort (Turnhout, Belgium) R301 controller and a TrendView (Dorset, UK) Multitrend recorder. pH was kept within a range of 7.8–8.1 by automatic titration with 0.1 M HCl (in the light) and 0.02 M NaOH (in the dark) effected by the pH controller using advanced relay regulation. pH in the culture is mainly determined by DIC and pH increases during photosynthetic CO_2 fixation due to shifting of the inorganic carbon equilibrium (pH decreases in the dark due to respiration). This experimental system is a closed CO_2 system (no aeration); hence, the cultures were eventually depleted in DIC. The point of DIC exhaustion (compensation point) can easily be derived from the pH curve, as the increase in pH levels off.

The present experiments were designed to achieve a high specific incorporation of CO_2 into cellular β-1,3-glucan by photosynthesis under N limitation, followed by glucan mobilization induced by addition of NH_4^+ under different light conditions (darkness, low light, or high light). The experiments were performed 1 day after NO_3^- was depleted and 1 h into the light period (after a 10-h dark period). In the first experiment, the N-limited cells were incubated in two parallel photobioreactors, one with 40 μCi·L^{-1} NaH^{14}CO$_3$ (specific activity 8.6 Ci·mol^{-1}) and one without. In a ^{14}C pulse phase the cells were incubated in high light until DIC was exhausted (3–5 h), and then 1 mM unlabeled $NaHCO_3$ was added. In subsequent ^{14}C chase phases, the cells were incubated in the dark and in low light with additions of 40 μM NH_4^+ (~2 h each). As a negative N control, the cells finally were incubated in the dark and in low light without a source of N (~2 h each). Different samples were taken at the beginning and end of each phase, and the most important parameters were sampled at intervals of 0.5–1 h within each phase. Samples for measuring ^{14}C fixation were taken from the radiolabeled culture, whereas all other samples were taken from the unlabeled replicate. The results from one representative experiment are reported.

In the second experiment, similar N-limited cells were incubated in a photobioreactor in high light until DIC was exhausted (3–5 h). Then 150 μM NH_4^+ was added, and incubation in high light was continued under inorganic carbon limitation with other components replete (~6 h). In subsequent recovery phases, 1 mM $NaHCO_3$ was added, and the cells were incubated in the dark and in low light (~1 h each). Samples for chemical analyses were taken during incubation, and the results from one representative experiment are reported.

Sampling and analyses. The cells were counted with a Bürker hemocytometer, and cell densities were determined as the mean

of six counts with SE 5%–7%. The efficiency (quantum yield) of photochemistry in PSII (ϕ_p) was determined as the ratio of variable fluorescence (F_v) to maximum fluorescence (F_m) (Samuelsson and Öquist 1977).

$$\phi_p = \frac{F_v}{F_m} = \frac{F_m - F_0}{F_m} \quad (1)$$

Culture samples were dark adapted for ~5 min, and *in vivo* fluorescence was measured before (F_0) and after (F_m) addition of 10 μM 3′-(3,4-dichlorophenyl)-1′,1′-dimethylurea using a Turner Designs (Sunnyvale, CA) 10 AU fluorometer. The quantum yield was determined as the mean of two samples. In this method, estimates with SE < 5% are obtainable at low excitation intensities with sample chl concentrations of <1 mg·L^{-1} (Kulandaivelu and Daniell 1980).

The cells were harvested by filtration through 25-mm Whatman (Kent, UK) GF/C glass fiber filters under gentle vacuum (50 mm Hg) and stored at −20° C until analyzed. Samples were filtered for analyses of cellular chl *a*, organic C and N, β-1,3-glucan, and free amino acids. The filtrates were used for analyses of dissolved NO_3^-, NH_4^+, total carbon (TC) and dissolved organic carbon (DOC).

Chl *a* was extracted by acetone and determined by spectrophotometric extinction (Jeffrey and Humphrey 1975) as the mean of two samples. Jeffrey and Humphrey reported SE 2% for analysis of chl *a*. Cellular organic C and N were measured with a Carlo Erba (CE Instruments, Milan, Italy) NA 1500 elemental analyzer (Kirsten 1979) as the mean of three samples with SE 3%–8%. Cellular β-1,3-glucan was determined by a modification (unpublished data) of the acid extraction method of Myklestad and Haug (1972). The glucan was extracted by 0.05 M H_2SO_4 at 60° C for 10 min and analyzed by the phenol-sulfuric acid method (Dubois et al. 1956). Cellular glucan was determined as the mean of three samples with SE 2%–5%.

Cellular free amino acids were extracted by hot water and determined by reverse-phase HPLC with precolumn fluorescence derivatization with *o*-phthaldialdehyde (Lindroth and Mopper 1979). Hot water extraction and modifications of the HPLC method were carried out as described by Flynn (1988), except that we used a Waters (Milford, MA) Nova-Pak C_{18} column and *o*-phthaldialdehyde reagent solution from Sigma (St. Louis, MO). Gly and Arg were not chromatographically resolved because their peaks merged and their combined concentration was based on a 1:1 ratio. Amino acid concentrations were determined as the means of two samples. The repeatability of the HPLC determination of amino acids obtained by Flynn (1988) was ±1.4%–8.8% ($P < 0.05$).

Dissolved NO_3^- in the filtrates was determined by reduction to NO_2^-, diazotizing with sulfanilamide, and coupling with *N*-(1-naphthyl)-ethylenediamine, and NH_4^+ was determined by the phenol-hypochlorite method (Strickland and Parsons 1972), both as a means of two samples. Strickland and Parsons reported a precision of ±5% ($P < 0.05$) for both methods. Dissolved TC and DOC were measured by high-temperature catalytic oxidation (Sugimura and Suzuki 1988) using a Dohrmann (Tekmar-Dohrmann, Cincinnati, OH) DC-190 analyzer with SE 3%, both as a means of two samples. Before DOC analysis, the samples were acidified to pH 2 and sparged with pure oxygen for 4 min to remove inorganic carbon. DIC was determined as the difference between TC and DOC.

^{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). Samples were purged of inorganic ^{14}C by acidification (exposing the filters to concentrated HCl vapor) and evaporation of CO_2 overnight. Cellular fixed ^{14}C radioactivity (dpm) was measured by a Wallac (PerkinElmer Life Sciences, Turku, Finland) 1410 scintillation counter using Wallac OptiPhase HiSafe 3 scintillation liquid (10 mL). Fixed ^{14}C was determined as the mean of two samples. In previous experiments (unpublished data), SE 3%–4% was estimated for this method by using sets of four samples. 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 and Osborne (1992):

$$\frac{\partial C}{\partial t} = f\frac{\partial A_{org}}{\partial t}\frac{DIC}{A_{tot}} \quad (2)$$

Medium filtrate (5 mL) was purged of inorganic ^{14}C by acidification (pH < 2) with H_3PO_4 and evaporation of CO_2 overnight. Extracellular fixed ^{14}C was measured by liquid scintillation counting as described above and determined as the mean of two samples.

For biochemical fractionation, cells were harvested and purged of inorganic ^{14}C as described above. Fractionation was performed by a modification of Li et al. (1980) with additional separation of the low MW fraction. A low MW fraction was isolated by extracting the filter with 3 mL Milli-Q water at 70° C for 30 min and washing with 2 mL Milli-Q water. The extract was removed from the filter as a supernatant after centrifugation (1400*g*, 10 min). The extract was acidified (pH 2.0) and separated by cation exchange chromatography into a basic fraction (amino acids, purines, pyrimidines, etc.) denoted "amino acids" and an acid/neutral fraction (β-1,3-glucan, monosaccharides, organic acids, etc.) denoted "glucan" (Granum and Myklestad 1999). After the low MW extraction, the filter was dried at 60° C overnight. A lipid fraction was isolated by extracting the filter with 3 mL $CHCl_3$ at 4° C for 10 min and washing with 2 mL $CHCl_3$. A polysaccharide fraction (also containing nucleic acids) was isolated by extracting the filter with 3 mL 5% TCA at 95° C for 30 min and washing with 2 mL 5% TCA. Left on the filter support was an insoluble protein fraction. A scheme that illustrates the fractionation procedure is presented in Figure 1. Fixed ^{14}C in the five different fractions was measured by liquid scintillation counting as described above and determined as the mean of three samples.

RESULTS

Glucan mobilization in the dark and in low light. In a ^{14}C pulse phase, N-limited *S. costatum* cells were incubated

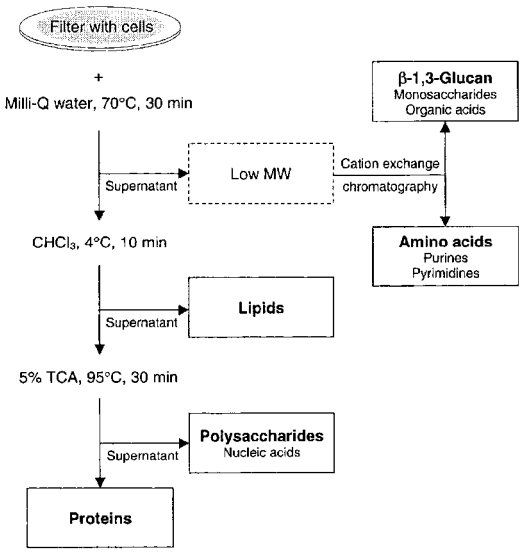

FIG. 1. Scheme of biochemical fractionation. The major components in each fraction are emphasized.

TABLE 1. Chemical composition per culture volume of *Skeletonema costatum* as a function of time.

	Phase					
	High L −N		Dark +N	Low L +N	Dark −N	Low L −N
	0 h[a]	4.5 h	2.3 h	2.3 h	2.0 h	2.0 h
Cell density, 10^9 cells·L^{-1}	2.3	2.6	—[d]	2.6	—	3.0
Chl *a*, mg·L^{-1}	1.0	1.0	1.2	1.3	—	1.8
PSII photochemistry efficiency[b]	0.59	0.53	0.59	0.61	—	0.61
Cellular organic C, mg·L^{-1}	21.2	25.1	24.2	24.0	—	24.0
Cellular organic N, mg·L^{-1}	3.8	3.8	4.1	4.3	—	4.3
Cellular organic C:N (molar)	6.5	7.7	6.9	6.5	—	6.5
DOC, mg·L^{-1}	2.3	2.3	2.4	2.4	2.4	2.4
DIC, mg·L^{-1}	7.2	1.2[c]	11.6	11.4	11.5	11.5

[a] Values in this row are incubation times.
[b] The efficiency of photochemistry in PSII was measured as the ratio of variable fluorescence to maximum fluorescence (obtained by adding dichlorophenyl dimethylurea [DCMU]).
[c] 1 mM $NaHCO_3$ was added to the culture at the end of the High L −N phase.
[d] — means no data.

in parallel photobioreactors with or without ^{14}C-bicarbonate in high light (200 μmol photons·m^{-2}·s^{-1}) until DIC was exhausted (High L −N). In subsequent ^{14}C chase phases, 1 mM unlabeled bicarbonate was added, and the cells were incubated in the dark and in low light (20 μmol photons·m^{-2}·s^{-1}) with additions of 40 μM NH_4^+ for 2.3 h each (Dark +N, Low L +N). As a negative N control, the cells finally were incubated in the dark and in low light without a source of N for 2 h each (Dark −N, Low L −N).

Table 1 shows the variation in chemical composition of unlabeled culture with time, and Figure 2 shows the time courses of cellular β-1,3-glucan and free amino acids, DIC, and dissolved NH_4^+ in mass units of C and N. During N depletion in high light, 6.0 mg·L^{-1} DIC was assimilated and 5.1 mg·L^{-1} cellular glucan-C was accumulated. The overall cellular organic C:N ratio (molar) increased from 6.5 to 7.7. Added NH_4^+ was assimilated at an initial N-specific rate of 0.11 h^{-1} (12 fmol·cell^{-1}·h^{-1}) in both the dark and low light. During dark N assimilation, cellular free amino acid increased 4.3 times, and glucan decreased by 26% within 2.3 h. The cellular pools of amino acids and glucan changed most rapidly in the early stage of NH_4^+ assimilation and became constant or slowly decreased after the added NH_4^+ was exhausted. During low light N assimilation, the amino acid pool further increased by 36% within 0.8 h and then slowly decreased, whereas cellular glucan further decreased by 19% within 2.3 h. As a result of the NH_4^+ pulses, the cellular organic C:N ratio (molar) decreased from 7.7 to 6.5. During incubation in the dark and low light without a source of N (−N control), the chemical composition of the culture was approximately constant, except for cellular free amino acids, which decreased by 27% during the final low light period.

Table 2 shows the variation in composition of cellular free amino acids with time. Upon addition of NH_4^+ in the dark, the Gln level increased steeply (29 times) and accounted for 80% of the total increase in amino acids within 1.7 h. Asn also increased somewhat steeply

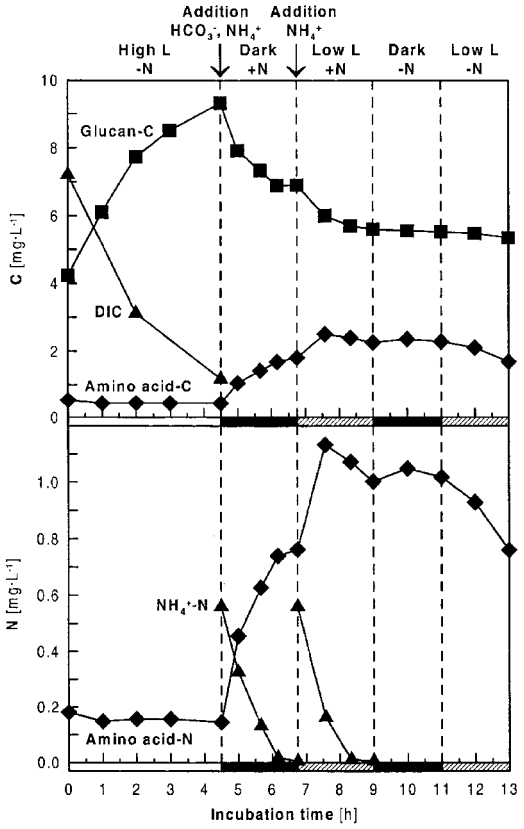

FIG. 2. Cellular β-1,3-glucan and free amino acids, DIC, and dissolved NH_4^+ in mass units of C and N per culture volume of *Skeletonema costatum* as functions of time (black bars indicate darkness; shaded bars indicate low light).

TABLE 2. Composition of cellular free amino acids per culture volume of *Skeletonema costatum* (μM) as a function of time.

	Phase										
	High L −N		Dark +N				Low L +N			Dark −N	Low L −N
	0 h[a]	4.5 h	0.5 h	1.2 h	1.7 h	2.3 h	0.8 h	1.6 h	2.3 h	2.0 h	2.0 h
Asp	1.16	0.72	1.20	1.56	2.03	2.37	2.41	2.25	2.32	3.03	1.42
Glu	2.22	1.66	1.45	1.66	2.82	4.67	2.85	3.85	4.63	4.47	3.18
Asn	0.44	0.26	1.09	1.53	1.77	1.84	3.49	3.59	3.81	3.90	3.07
His	—[b]	—	—	0.02	0.04	0.05	0.08	0.12	0.12	0.14	0.02
Ser	0.57	0.60	0.36	0.28	0.27	0.33	0.27	0.27	0.30	0.34	0.19
Gln	1.09	0.70	11.0	16.1	20.2	16.4	28.4	25.2	21.4	21.3	17.7
Gly + Arg	0.89	0.82	1.03	1.27	1.76	2.55	2.86	3.26	3.65	4.00	2.30
Thr	0.42	0.36	0.28	0.31	0.34	0.56	0.45	0.51	0.59	0.59	0.30
Ala	1.10	0.92	1.10	1.23	1.57	2.15	2.05	1.92	2.13	1.77	0.91
Tyr	0.14	0.15	0.12	0.15	0.15	0.19	0.17	0.18	0.22	0.20	0.10
Val	0.32	0.28	0.20	0.19	0.22	0.32	0.21	0.23	0.26	0.23	0.13
Phe	0.08	0.10	0.04	0.04	0.05	0.08	0.07	0.14	0.12	0.06	0.01
Ile	0.25	0.25	0.13	0.13	0.14	0.19	0.16	0.15	0.16	0.16	0.07
Leu	0.27	0.26	0.15	0.13	0.15	0.23	0.16	0.15	0.18	0.17	0.08
Lys	0.55	0.54	0.34	0.31	0.34	0.47	0.38	0.39	0.39	0.43	0.22
Sum	9.5	7.6	18.5	24.9	31.8	32.4	44.0	42.2	40.2	40.7	29.7
Sum, fmol·cell^{-1}	3.6	2.9	7.1	9.6	12.2	12.5	16.9	16.2	15.5	15.7	11.4

The sum of all free amino acids was also calculated per cell (fmol·cell^{-1}).
[a] Values in this row are incubation times.
[b] No data.

at the onset of NH_4^+ assimilation (4.2 times within 0.5 h) but increased only slightly after 0.5 h. Glu initially decreased slightly but then increased rapidly toward the end of the period (2.8 times within 2.3 h). Also, Ala, Asp, and Gly + Arg increased considerably (2.3–3.3 times within 2.3 h). With the subsequent addition of NH_4^+ in low light, Gln further increased 1.7 times within 0.8 h but then decreased by 25% toward the end of the 2.3-h period. Glu, on the other hand, decreased by 39% within 0.8 h and then increased to the initial level at the end of the period. Asn further increased 2.1 times within 2.3 h, whereas Asp remained constant. The Gln:Glu ratio increased from 0.4 to 9.7 within 1.2 h of the first N assimilation phase (dark) and further increased to a maximum of 10.0 within 0.8 h of the second N assimilation phase (low light). The Gln:Glu ratio decreased again after NH_4^+ was exhausted in each phase.

Table 3 shows ^{14}C fixation in different biochemical fractions, and Figure 3 shows the relative distribution (%) of cellular ^{14}C-metabolites as a function of time. In the ^{14}C pulse phase (N depletion in high light), the photosynthetic carbon assimilation rate was calculated as 0.17 h^{-1} (114 fmol·cell^{-1}·h^{-1}) during the first 2 h. The ^{14}C label was mainly incorporated in cellular β-1,3-glucan (85%), and the rest was distributed between amino acids, proteins, polysaccharides, and lipids (3%–5% each). In the following ^{14}C chase phases, significant redistribution of the label took place. Upon addition of NH_4^+ in the dark, labeled glucan decreased by 16%, whereas labeling of amino acids increased 2.6 times, proteins 1.7 times, and polysaccharides 2.0 times within 2.3 h. ^{14}C labeling of amino acids increased rapidly in the early stage of NH_4^+ assimilation, whereas proteins and polysaccharides were more rapidly labeled after 1.2 h. When NH_4^+ was added in low light, labeled glucan further decreased by 14%, whereas labeling of amino

TABLE 3. ^{14}C fixation per culture volume of *Skeletonema costatum* (dpm·μL^{-1}) in different biochemical fractions as functions of time.

	Phase										
	High L −N		Dark +N				Low L +N			Dark −N	Low L −N
	1.0 h[a]	4.5 h	0.5 h	1.2 h	1.7 h	2.3 h	0.8 h	1.6 h	2.3 h	2.0 h	2.0 h
Total cellular	38.8	72.7	70.0	70.6	70.1	71.9	71.8	71.6	70.7	71.4	74.1
Extracellular	—[b]	0.22	—	—	—	—	—	—	0.36	0.37	0.40
Glucan	30.5	59.1	56.1	45.2	45.9	46.8	44.1	41.6	38.9	38.9	38.8
Amino acids	2.5	2.5	4.6	5.2	5.7	6.1	8.1	8.7	9.1	8.5	8.2
Proteins	1.3	3.8	4.0	3.9	6.1	5.9	7.2	7.6	8.1	8.9	8.4
Polysaccharides	1.4	2.7	2.8	3.2	5.7	5.2	5.8	5.9	5.8	6.1	6.9
Lipids	1.4	2.1	2.1	1.6	1.5	1.9	1.6	1.8	1.6	1.8	1.4
Sum of fractions	37.1	70.3	69.6	59.1	64.9	65.9	66.7	65.6	63.5	64.2	63.7
Recovery, %	96	97	99	84	93	92	93	92	90	90	86

Recovery was calculated as the sum of ^{14}C-activity in all cellular fractions (glucan, amino acids, proteins, polysaccharides and lipids) divided by the ^{14}C-activity of nonfractionated cells (total cellular).
[a] Values in this row are incubation times.
[b] No data.

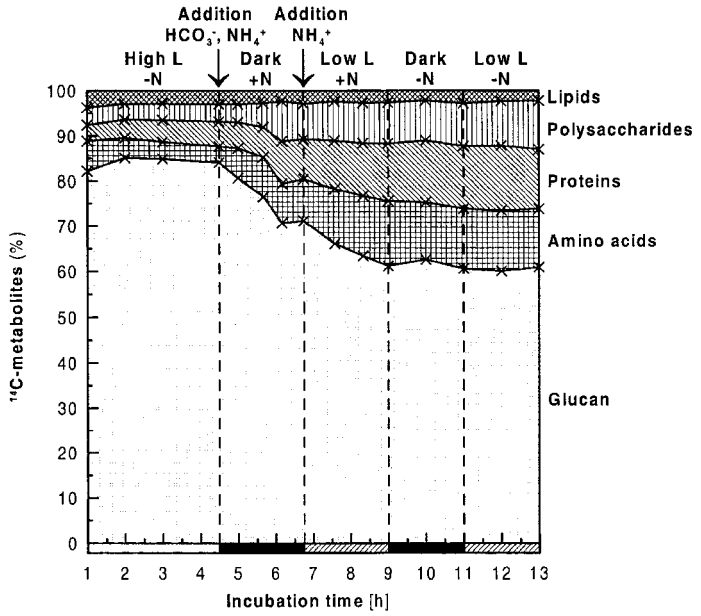

FIG. 3. Distribution of ^{14}C metabolites (%) in different biochemical fractions in *Skeletonema costatum* as a function of time (black bars indicate darkness; shaded bars indicate low light).

acids increased 1.5 times, proteins 1.4 times, and polysaccharides 1.2 times within 2.3 h. The lipid fraction remained constant at only 3% of ^{14}C-labeled metabolites. During incubation under similar conditions without an N source, the distribution of ^{14}C label was approximately constant. ^{14}C-labeled extracellular metabolites (DO^{14}C) accounted for only 0.5% of cellular ^{14}C fixation throughout the experiment.

Glucan mobilization under inorganic carbon limitation. N-limited *S. costatum* cells were incubated in a photobioreactor in high light (200 μmol photons·m^{-2}·s^{-1}) until DIC was exhausted (High L −N+C). Then, 150 μM NH_4^+ was added, and incubation in high light was continued for 6 h under inorganic carbon limitation with other components replete (High L +N−C). In subsequent recovery phases, 1 mM bicarbonate was added, and the cells were incubated in the dark and in low light (20 μmol photons·m^{-2}·s^{-1}) for 1.3 h each (Dark +N+C, Low L +N+C).

Table 4 shows the variation in chemical composition of the culture with time, and Figure 4 shows the time courses of cellular β-1,3-glucan and free amino acids, DIC, and dissolved NH_4^+ in mass units of C and N. During N depletion in high light, 3.5 mg·L^{-1} DIC was assimilated and 3.1 mg·L^{-1} cellular glucan-C was accumulated. In the following DIC limitation phase, 40 μM NH_4^+ was assimilated within 6 h, corresponding to a mean N-specific rate of 0.023 h^{-1}. Concomitantly,

TABLE 4. Chemical composition per culture volume of *Skeletonema costatum* as a function of time.

	Phase						
	High L −N +C		High L +N −C			Dark +N +C	Low L +N +C
	0 h[a]	3.0 h	2.0 h	4.0 h	6.0 h	1.3 h	1.3 h
Cell density, 10^9 cells·L^{-1}	1.1	1.1	—[d]	—	1.1	—	1.1
Chl *a*, mg·L^{-1}	0.58	0.56	—	—	0.54	0.55	0.62
PSII photochemistry efficiency[b]	0.55	0.52	0.54	—	0.51	—	0.58
Cellular organic C, mg·L^{-1}	21.3	25.0	24.4	24.0	24.0	—	24.1
Cellular organic N, mg·L^{-1}	3.7	3.7	4.0	4.2	4.4	—	4.6
Cellular organic C:N (molar)	6.7	7.9	7.1	6.7	6.4	—	6.1
DOC, mg·L^{-1}	3.7	3.8	4.6	4.9	4.9	—	4.9
DIC, mg·L^{-1}	4.0	<0.6	<0.6	<0.6	<0.6[c]	13.2	13.2

[a] Values in this row are incubation times.
[b] The efficiency of photochemistry in PSII was measured as the ratio of variable fluorescence to maximum fluorescence (obtained by adding DCMU).
[c] 1 mM $NaHCO_3$ was added to the culture at the end of the High L +N−C phase.
[d] No data.

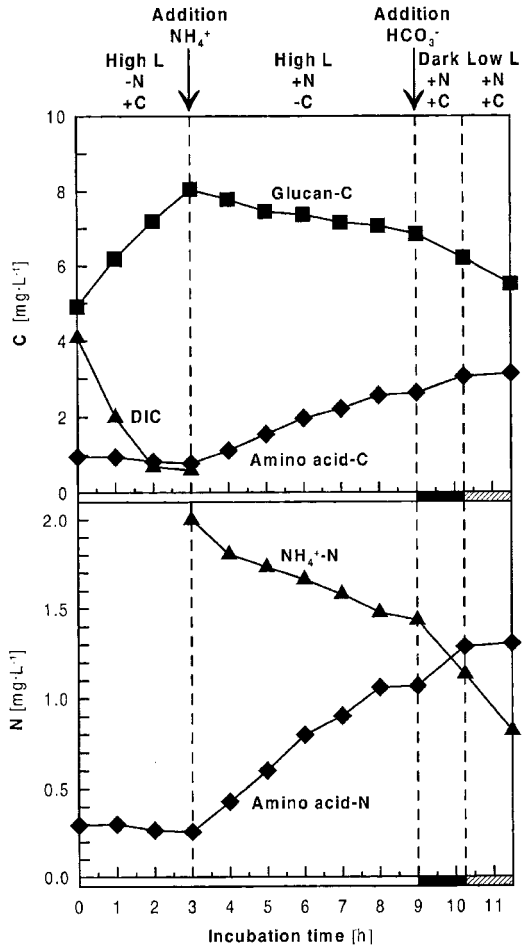

FIG. 4. Cellular β-1,3-glucan and free amino acids, DIC, and dissolved NH_4^+ in mass units of C and N per culture volume of *Skeletonema costatum* as functions of time (black bar indicates darkness; shaded bar indicates low light).

cellular free amino acids increased 3.0 times and glucan decreased by 15% (C specific mobilization rate $8.2 \cdot 10^{-3}$ h^{-1}). After addition of bicarbonate (recovery from DIC limitation), the NH_4^+ assimilation rate increased (0.053 h^{-1}) and reserve glucan decreased more rapidly (0.022 h^{-1}) in both darkness and low light. The amino acid pool increased by 18% in the dark but stabilized in low light.

Table 5 shows the variation in composition of cellular free amino acids with time, and Figure 5 shows the concentrations of Glu and Gln as functions of time. Upon addition of NH_4^+ during DIC limitation, the Gln level increased steeply within 1 h (28 times). After that, Gln increased more slowly and linearly for 3–4 h and stabilized at 28 μM, at a Gln:Glu ratio of 8.1. Asn increased 2.6 times, whereas Asp decreased by 77% within the first hour, and then both amino acids increased slowly throughout the DIC limitation phase. Also, Ala slightly decreased within the first hour (7%) and then increased significantly (2.1 times within 6 h), whereas Gly + Arg decreased throughout the −C phase (58%). After addition of bicarbonate (recovery from DIC limitation), the Gln:Glu ratio further increased to 18.2 within 1.3 h in the dark and then decreased to 8.6 within 1.3 h in low light. The levels of Asn, Asp, and Gly + Arg increased considerably (3.2, 9.7, and 1.8 times, respectively) during the recovery phases, whereas other amino acids only slightly decreased or increased.

DISCUSSION

Biochemical ^{14}C fractionation showed that N-limited *S. costatum* in high light incorporated 82%–85% of fixed carbon into cellular β-1,3-glucan (Fig. 3). In accordance with this, chemical analyses showed that accumulated cellular glucan-C accounted for 85% of the decrease in DIC (Fig. 2). In previous investigations with the same alga in stationary phase, 80% of ^{14}C-bicarbonate was incorporated into glucan (Myklestad 1988/1989) and glucan-C accounted for up to 80% of cellular carbon content (Vårum and Myklestad 1984). These investigations clearly demonstrate that β-1,3-glucan is the main storage product of photosynthetic CO_2 fixation in *S. costatum*. The high fraction of CO_2 incorporated into β-1,3-glucan is convenient for pulse ^{14}C labeling of glucan followed by a ^{14}C chase during glucan mobilization.

NH_4^+ was assimilated at a rate of 0.11 h^{-1} in both darkness and low light, whereas more reserve glucan was mobilized in the dark (26%) compared with low light (19%) (Fig. 2). A possible cause for this is that NH_4^+ assimilation was accommodated by some photosynthetic activity in low light. These results corroborate previous reports of glucan mobilization in the dark during NO_3^- and NH_4^+ assimilation in *S. costatum* (Vårum and Myklestad 1984, Granum and Myklestad 1999). The mobilization rate was highest in the beginning of each NH_4^+ assimilation phase, and the glucan content was constant or decreased very slowly after N depletion. Concomitantly, the pool of free amino acids increased most rapidly in the early stage of NH_4^+ assimilation and decreased slowly after N depletion. The total cellular pool of amino acids did not increase as much in the second NH_4^+ assimilation phase (low light). This development suggests that free amino acids are drawn off for protein synthesis at a higher rate when their cellular concentrations are higher.

At N repletion, the composition of the cellular free amino acids changed dramatically (Table 2). The level of each amino acid reflects its rate of biosynthesis diminished by incorporation into proteins or utilization in biosynthesis of other components (purines, pyrimidines, etc.). The concentrations of Ala, Asn, Asp, Gln, Glu, and Gly + Arg increased considerably, whereas the other nine amino acids only slightly increased or decreased. Ala, Asp, and Glu are easily formed by transamination of pyruvate, oxalacetate, and α-ketoglutarate,

TABLE 5. Composition of cellular free amino acids per culture volume of *Skeletonema costatum* (μM) as a function of time.

	Phase							
	High L −N +C		High L +N −C				Dark +N +C	Low L +N +C
	0 h[a]	3.0 h	1.0 h	2.0 h	4.0 h	6.0 h	1.3 h	1.3 h
Asp	2.53	1.03	0.24	0.24	0.36	0.45	3.18	4.36
Glu	2.16	1.13	0.99	1.83	2.62	3.49	1.92	3.75
Asn	0.20	0.17	0.44	0.50	0.56	0.64	1.40	2.07
His	—[b]	—	—	—	0.13	0.20	0.22	0.26
Ser	0.34	0.40	0.26	0.23	0.44	0.55	0.45	0.43
Gln	0.27	0.29	8.1	13.4	24.9	28.1	34.9	32.4
Gly + Arg	2.20	3.85	1.94	1.16	1.85	1.61	1.86	2.93
Thr	0.60	0.37	0.43	0.40	0.42	0.55	0.49	0.72
Ala	2.54	1.88	1.75	3.19	3.03	3.99	4.31	5.29
Tyr	0.30	0.21	0.35	0.47	0.45	0.59	0.46	0.39
Val	1.72	1.51	1.33	1.33	1.37	1.47	1.32	1.02
Phe	0.17	0.15	0.15	0.18	0.24	0.30	0.19	0.22
Ile	0.86	0.75	0.61	0.63	0.68	0.72	0.60	0.38
Leu	0.85	0.86	0.73	0.76	0.89	0.95	0.78	0.44
Lys	2.93	2.66	2.30	2.18	1.95	1.69	1.49	1.71
Sum	17.7	15.3	19.6	26.8	38.7	45.3	53.6	56.4
Sum, fmol·cell^{-1}	16.1	13.9	17.8	24.4	35.2	41.2	48.7	51.3

The sum of all free amino acids was also calculated per cell (fmol·cell^{-1}).
[a] Values in this row are incubation times.
[b] No data.

respectively. Asn and Gln are formed directly from Asp and Glu, respectively, whereas Arg and Gly are formed in short pathways from Glu and 3-phosphoglycerate, respectively. Especially Asp, Glu, and Gln have numerous metabolic roles (N donors, precursors for other amino acids, and nucleotides). Thus, the changes in amino acid composition upon N repletion strongly indicate

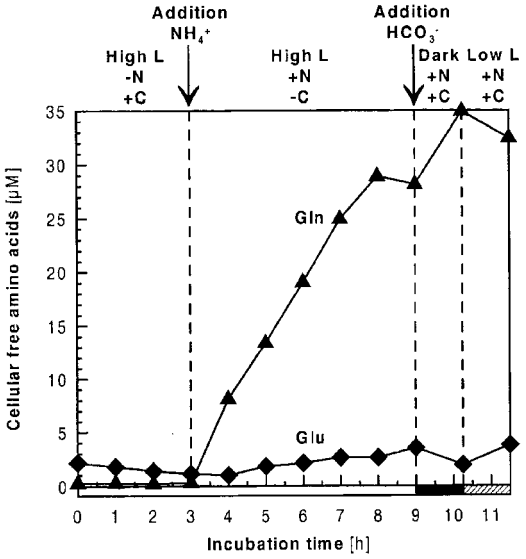

FIG. 5. Cellular free Glu and Gln per culture volume of *Skeletonema costatum* as functions of time (black bar indicates darkness; shaded bar indicates low light).

that *de novo* biosynthesis of amino acids took place and not protein degradation, which would provide quite a different distribution. Further evidence could be provided by ^{15}N labeling of "new" amino acids. However, ^{15}N is a stable isotope and thus difficult to combine with a radiolabel such as ^{14}C. Flynn (1990) considered that although the cellular free amino acid composition undoubtedly reflects a combination of both current C:N status and recent-past C:N metabolism, it is generally too complex for use as a metabolic fingerprint except for the Gln:Glu ratio.

The rapid increase in Gln upon N repletion probably reflects the main biosynthetic route of NH_4^+ assimilation: the glutamine synthetase-glutamate synthase cycle (Falkowski and Rivkin 1976, Syrett 1981). During rapid N assimilation, Gln seems to accumulate rapidly compared with Glu and other amino acids. When NH_4^+ is exhausted, however, Gln quickly decreases, whereas Glu still increases significantly. Thus, the Gln:Glu ratio evidently serves as a convenient indicator of N status in diatoms, as suggested by Flynn and Al-Amoudi (1988). Vanlerberghe et al. (1990) also claimed that Gln and Glu were the main effectors of β-carboxylation in the green alga *Selenastrum minutum*, and we have indications of similar effects in diatoms (Granum and Myklestad 1999). Anaplerotic β-carboxylation is required to replenish intermediates in the tricarboxylic acid cycle drawn off for biosynthesis of amino acids such as Arg, Asn, Asp, Gln, and Glu.

Fractionation of cellular organic ^{14}C was performed with 84%–99% recovery (Table 3). At the end of the ^{14}C pulse phase (N depletion in high light), the small remaining label was diluted with unlabeled bicarbonate and the total cellular ^{14}C was approximately constant in the chase phases (±3%). The apparently

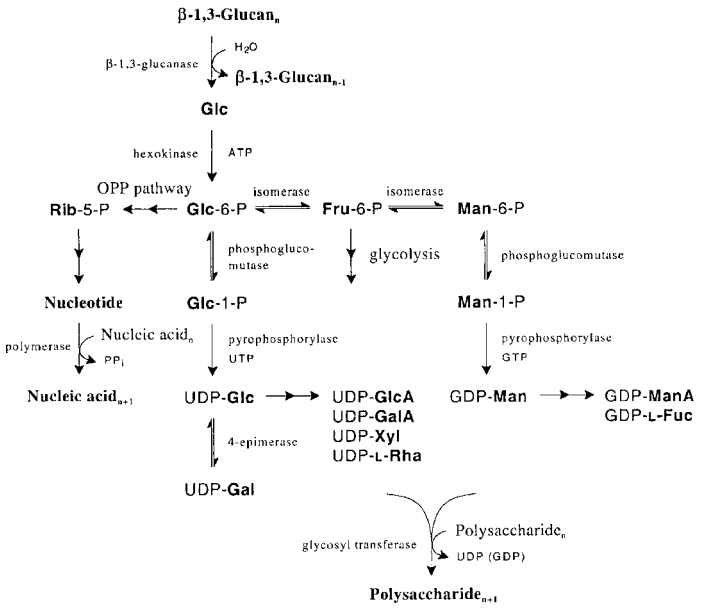

FIG. 6. Scheme of metabolic pathways providing precursors for polysaccharides and nucleic acids from β-1,3-glucan. The glucan is hydrolyzed by exo-β-1,3-glucanase, splitting off glucose at the nonreducing end. Different monosaccharide precursors are synthesized in few steps from glucose and polymerized by glycosyl transferases. Alternatively, ribose 5-phosphate is synthesized from glucose 6-phosphate through the oxidative pentose phosphate pathway and forms nucleotides with purines or pyrimidines, which can be polymerized. Fru, D-fructose; L-Fuc, L-fucose; Gal, D-galactose; GalA, D-galacturonic acid; Glc, D-glucose; GlcA, D-glucuronic acid; Man, D-mannose; ManA, D-mannuronic acid; OPP, oxidative pentose phosphate; L-Rha, L-rhamnose; Rib, D-ribose; Xyl, D-xylose.

low rate of respiration may indicate that respired $^{14}CO_2$ was refixed. The ^{14}C results show exactly the same main development of glucan mobilization as revealed from the chemical analyses. The only difference is that the redistribution of ^{14}C suggests a somewhat lower glucan mobilization (dark, 16%; low light, 14%) than the chemical analyses (dark, 26%; low light, 19%). This difference, however, may be accounted for by an unlabeled fraction of glucan in the ^{14}C culture. The cells had already accumulated 4.2 mg·L^{-1} glucan-C (45% of total) before ^{14}C addition. The chase of ^{14}C-metabolites shows that free amino acids were most rapidly labeled in the early stage of the first NH_4^+ assimilation phase, compared with proteins and polysaccharides, which were more rapidly labeled after 1.2 h. These results strongly indicate that β-1,3-glucan provides precursors for amino acid biosynthesis and eventually for incorporation into proteins. The glucan can readily be hydrolyzed by exo-β-1,3-glucanase, splitting off glucose at the nonreducing end (Myklestad et al. 1982). Glucose can be metabolized by the respiratory pathways, providing precursors for amino acid biosynthesis. Likely metabolic pathways have been proposed (Granum and Myklestad 1999). Part of the "amino acid" fraction is probably accounted for by purines and pyrimidines, which, like the amino acids, are positively charged at low pH (Fig. 1). These bases are synthesized *de novo* from amino acids and other precursors.

The results from the experiment carried out under inorganic carbon limitation indicated that NH_4^+ was taken up and assimilated in high light (Fig. 4). Concurrently, β-1,3-glucan was mobilized. In this case, reserve glucan was probably the only source of easily available carbon for the alga because the net uptake of inorganic carbon was negligible. Hence, the glucan furnished precursors for amino acid biosynthesis in the absence of photosynthetic carbon fixation in the light. As before, the Gln level and the Gln:Glu ratio increased rapidly upon N repletion but slowly compared with DIC-replete cells in the dark (Fig. 5). In contrast with the results of previous experiments, the levels of Asp and Gly + Arg dropped upon N repletion and Asn increased relatively slowly (Table 5). These amino acids, however, increased rapidly upon addition of bicarbonate, suggesting that their rates of biosynthesis may be modified by inorganic carbon. The higher rate of NH_4^+ assimilation and glucan metabolism after addition of bicarbonate is consistent with the findings that inorganic carbon is modifying the rate of anaplerotic reactions (Holdsworth and Bruck 1977). Even after bicarbonate addition, the metabolic rates are low compared with those in other experiments (Granum and Myklestad 1999, present study), suggesting that the cells did not recover completely from inorganic carbon limitation.

The results from the ^{14}C chase also indicated a significant biosynthesis of other polysaccharides from mobilized β-1,3-glucan (Fig. 3). Nucleic acids, however, also contribute to this "polysaccharide" fraction. RNA probably accounts for some of the ^{14}C label, because active protein synthesis is strictly coupled to RNA synthesis. A scheme of metabolic pathways providing precursors for polysaccharides and nucleic acids from β-1,3-glucan is presented in Figure 6. Most activated monosaccharide precursors that build up cell wall polysaccharides in diatoms are synthesized in a few steps from glucose. In the case of nucleic acids, ribose

5-phosphate is synthesized from glucose 6-phosphate through the oxidative pentose phosphate pathway and forms nucleotides with purines or pyrimidines.

Our results provide new evidence of β-1,3-glucan supplying essential carbon skeletons for amino acid biosynthesis in *S. costatum* in both the dark and subsaturating light and even saturating light under inorganic carbon limitation. Obviously, the glucan also furnishes energy and reducing power to the cell (most easily recognized in the dark) as it is metabolized by the respiratory pathways. Both energy (ATP) and electron donor (NADPH) are required to perform reductive biosyntheses, for instance incorporation of NH_4^+ into Glu through the glutamine synthetase-glutamate synthase pathway. The present contribution also indicates that β-1,3-glucan provides precursors for the synthesis of other polysaccharides and/or nucleic acids. Previous investigations with *S. costatum* showed that NH_4^+ assimilation activated rapid dark carbon fixation into amino acids, indicating amino acid synthesis through anaplerotic β-carboxylation (Granum and Myklestad 1999). The present contribution confirms that β-1,3-glucan is the ultimate substrate for β-carboxylation and amino acid biosynthesis. Work is in progress in our laboratory to further investigate the regulatory mechanisms of the glucan level in marine diatoms.

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Beattie, A., Hirst, E. L. & Percival, E. 1961. Comparative structural investigations on leucosin (chrysolaminarin) separated from diatoms and laminarin from the brown algae. *Biochem. J.* 79:531–7.

Cuhel, R. L., Ortner, P. B. & Lean, D. R. S. 1984. Night synthesis of protein by algae. *Limnol. Oceanogr.* 29:731–44.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. 1956. Colorimetric determination of sugars and related substances. *Anal. Chem.* 28:350–6.

Falkowski, P. G. & Rivkin, R. B. 1976. The role of glutamine synthetase in the incorporation of NH_4^+ in *Skeletonema costatum* (Bacillariophyceae). *J. Phycol.* 12:448–50.

Flynn, K. J. 1988. Some practical aspects of measurements of dissolved free amino acids in natural waters and within microalgae by the use of HPLC. *Chem. Ecol.* 3:269–93.

Flynn, K. J. 1990. Composition of intracellular and extracellular pools of amino acids, and amino acid utilization of microalgae of different sizes. *J. Exp. Mar. Biol. Ecol.* 139:151–66.

Flynn, K. J. & Al-Amoudi, O. A. 1988. Effects of N deprivation and darkness on composition of free amino acid pool in and on amino acid release from diatom *Phaeodactylum tricornutum* Bohlin. *J. Exp. Mar. Biol. Ecol.* 119:131–44.

Geider, R. J. & Osborne, B. A. 1992. Using isotopes to measure gas exchange. *In* Geider, R. J. & Osborne, B. A. [Eds.] *Algal Photosynthesis: The Measurement of Algal Gas Exchange.* Chapman and Hall, New York, pp. 32–70.

Goldemberg, S. H. & Marechal, L. R. 1963. Biosynthesis of paramylon in *Euglena gracilis. Biochim. Biophys. Acta* 71:743–4.

Granum, E. & Myklestad, S. M. 1999. Effects of NH_4^+ assimilation on dark carbon fixation and β-1,3-glucan metabolism in the marine diatom *Skeletonema costatum* (Bacillariophyceae). *J. Phycol.* 35:1191–9.

Guillard, R. R. L. & Ryther, J. H. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.* 8:229–39.

Hama, T. & Honjo, T. 1987. Photosynthetic products and nutrient availability in phytoplankton population from Gokasho Bay, Japan. *J. Exp. Mar. Biol. Ecol.* 112:251–66.

Handa, N. 1969. Carbohydrate metabolism in the marine diatom *Skeletonema costatum. Mar. Biol.* 4:208–14.

Haug, A., Myklestad, S. & Sakshaug, E. 1973. Studies on the phytoplankton ecology of the Trondheimsfjord. I. The chemical composition of phytoplankton populations. *J. Exp. Mar. Biol. Ecol.* 11:15–26.

Haug, A. & Myklestad, S. 1976. Polysaccharides of marine diatoms with special reference to *Chaetoceros* species. *Mar. Biol.* 34:217–22.

Hitchcock, G. L. 1978. Labelling patterns of carbon-14 in net plankton during a winter-spring bloom. *J. Exp. Mar. Biol. Ecol.* 31:141–53.

Hitchcock, G. L. 1983. Photosynthate partitioning in cultured marine phytoplankton. I. Dinoflagellates. *J. Exp. Mar. Biol. Ecol.* 69:21–36.

Holdsworth, E. S. & Bruck, K. 1977. Enzymes concerned with β-carboxylation in marine phytoplankter. Purification and properties of phosphoenolpyruvate carboxykinase. *Arch. Biochem. Biophys.* 182:87–94.

Jeffrey, S. W. & Humphrey, G. F. 1975. New spectrophotometric equations for determining chlorophylls *a, b,* c_1 and c_2 in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanzen* 167:191–4.

Kirsten, W. J. 1979. Automatic methods for the simultaneous determination of carbon, hydrogen, nitrogen, and sulfur, and for sulfur alone in organic and inorganic materials. *Anal. Chem.* 51:1173–9.

Kulandaivelu, G. & Daniell, H. 1980. Dichlorophenyl dimethylurea (DCMU) induced increase in chlorophyll *a* fluorescence intensity—an index of photosynthetic oxygen evolution in leaves, chloroplasts and algae. *Physiol. Plant.* 48:385–8.

Lancelot, C. 1984. Metabolic changes in *Phaeocystis poucheti* (Hariot) Lagerheim during the spring bloom in Belgian coastal waters. *Estuar. Coast. Shelf Sci.* 18:593–600.

Li, W. K. W., Glover, H. E. & Morris, I. 1980. Physiology of carbon photoassimilation by *Oscillatoria thiebautii* in the Caribbean Sea. *Limnol. Oceanogr.* 25:447–56.

Lindroth, P. & Mopper, K. 1979. HPLC determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with *o*-phthaldialdehyde. *Anal. Chem.* 51:1667–74.

McConville, M. J., Bacic, A. & Clarke, A. E. 1986. Structural studies of chrysolaminaran from the ice diatom *Stauroneis amphioxys* (Gregory). *Carbohydr. Res.* 153:330–3.

McConville, M. J., Mitchell, C. & Wetherbee, R. 1985. Patterns of carbon assimilation in a microalgal community from annual sea ice, East Antarctica. *Polar Biol.* 4:135–41.

Myklestad, S. 1974. Production of carbohydrates by marine planktonic diatoms. I. Comparison of nine different species in culture. *J. Exp. Mar. Biol. Ecol.* 15:261–74.

Myklestad, S., Djurhuus, R. & Mohus, A. 1982. Demonstration of *exo*-(β–1,3)-D-glucanase activity in some planktonic diatoms. *J. Exp. Mar. Biol. Ecol.* 56:205–11.

Myklestad, S. & Haug, A. 1972. Production of carbohydrates by the marine diatom *Chaetoceros affinis* var. willei (Gran) Hustedt. I. Effect of the concentration of nutrients in the culture medium. *J. Exp. Mar. Biol. Ecol.* 9:125–36.

Myklestad, S. M. 1988/1989. Production, chemical structure, metabolism, and biological function of the (1,3)-linked, β-D-glucans in diatoms. *Biol. Oceanogr.* 6:313–26.

Painter, T. J. 1983. Algal polysaccharides. *In* Aspinall, G. O. [Ed.] *The Polysaccharides.* Academic Press, New York, pp. 195–285.

Paulsen, B. S. & Myklestad, S. 1978. Structural studies of the reserve glucan produced by the marine diatom *Skeletonema costatum* (Grev.) Cleve. *Carbohydr. Res.* 62:386–8.

Roessler, P. G. 1987. UDP glucose pyrophosphorylase activity in the diatom *Cyclotella cryptica.* Pathway of chrysolaminaran biosynthesis. *J. Phycol.* 23:494–8.

Sakshaug, E., Andresen, K., Myklestad, S. & Olsen, Y. 1983. Nutrient status of phytoplankton communities in Norwegian waters (marine, brackish, and fresh) as revealed by their chemical composition. *J. Plankton Res.* 5:175–96.

Sakshaug, E. & Andresen, K. 1986. Effect of light regime upon growth rate and chemical composition of a clone of *Skeletonema costatum* from the Trondheimsfjord, Norway. *J. Plankton Res.* 8:619–37.

Samuelsson, G. & Öquist, G. 1977. A method for studying photosynthetic capacities of unicellular algae based on *in vivo* chlorophyll fluorescence. *Physiol. Plant.* 40:315–9.

Strickland, J. D. H. & Parsons, T. R. 1972. *A Practical Handbook of Seawater Analysis*, 2nd ed. Bull. Fish. Res. Bd. Can. 167, Ottawa, 310 pp.

Sugimura, Y. & Suzuki, Y. 1988. A high-temperature catalytic oxidation method for the determination of non-volatile dissolved organic carbon in seawater by direct injection of a liquid sample. *Mar. Chem.* 24:105–31.

Syrett, P. J. 1981. Nitrogen metabolism of microalgae. *In* Platt, T. [Ed.] *Physiological Bases of Phytoplankton Ecology*. Can. Bull. Fish. Aquat. Sci. 210, Ottawa, pp. 182–210.

Vanlerberghe, G. C., Schuller, K. A., Smith, R. G., Feil, R., Plaxton, W. C. & Turpin, D. H. 1990. Relationship between NH_4^+ assimilation rate and *in vivo* phosphoenolpyruvate carboxylase activity: regulation of anaplerotic carbon flow in the green alga *Selenastrum minutum*. *Plant Physiol.* 94:284–90.

Vårum, K. M. & Myklestad, S. 1984. Effects of light, salinity and nutrient limitation on the production of β-1,3-D-glucan and *exo*-D-glucanase activity in *Skeletonema costatum*. *J. Exp. Mar. Biol. Ecol.* 83:13–26.

Vårum, K. M., Østgaard, K. & Grimsrud, K. 1986. Diurnal rhythms in carbohydrate metabolism of the marine diatom *Skeletonema costatum* (Grev.) Cleve. *J. Exp. Mar. Biol. Ecol.* 102: 249–56.

Waterkeyn, L. & Bienfait, A. 1987. Localisation et rôle des β-1,3-glucanes (callose et chrysolaminarine) dans le genre *Pinnularia* (Diatomées). *La Cellule* 74:198–226.

Paper 5

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Cellular and extracellular production of carbohydrates and amino acids by the marine diatom *Skeletonema costatum*: diel variations and effects of nitrogen 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 two different strength. The exponential growth rate was 2.0 div. d^{-1}, and balanced growth took place apart from substantial diel variations. Inorganic C and N were primarily assimilated during the photophase, hence the elemental cell quotas increased accordingly. The level of reserve 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 6-10% 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. Gln emerged as the principal amino acid during photosynthesis, increasing from 0.2 to 12 fmol $cell^{-1}$, and the corresponding Gln/Glu ratio increased from 0.05 to 2. Upon NO_3^- exhaustion, the glucan level increased rapidly for 3-4 d, and then stabilized at 75-80% 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. Gln decreased most rapidly, and constituted <1% of the free amino acids in the stationary phase. Extracellular production accounted for 4% of the total algal 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 one 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 shows the carbohydrate and amino acid dynamics at the cellular level.

KEY WORDS: carbohydrates · free amino acids · production rate · excretion · diel variation · N limitation · marine diatom

INTRODUCTION

The diatoms (Bacillariophyceae) comprise the most abundant marine phytoplankton species, and are estimated to contribute 20-25% of the world net primary production (Werner 1977). The biochemical composition of diatoms is rather similar to that of other phytoplankton, where the major classes of metabolites are protein (30-50% of organic dry weight), carbohydrate (30-60%) and lipid (2-10%) (Parsons et al. 1961, Haug et al. 1973). However, the biochemical composition of each alga varies markedly as a function of growth conditions. Nutrient limitation generally leads to elevated levels of carbohydrate and lipid in relation to protein. Moreover, protein and carbohydrate pools oscillate in response to light:dark cycles.

Polysaccharides account for the bulk of the algal carbohydrates, and the main cellular groups are reserve and cell wall polysaccharides. The principal storage product in diatoms is β-1,3-glucan, also called chrysolaminaran, a (1→3)-linked β-D-glucopyranan (DP 20-60) 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 limitation (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 C metabolism (respiratory pathways). 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, photosynthesis and respiratory processes are also dependent on N supply. N 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 concurrent increase in carbohydrate or lipid contents.

Extracellular production should also be considered as a significant part of the algal primary production (Myklestad 2000). In general, phytoplankton releases 2-10% (PER) during rapid growth, increasing to 10-60% in the stationary phase. However, the absolute rate of release (per cell) may be higher during exponential growth (Myklestad et al. 1989). The physiological mechanisms for excretion are still poorly understood, but passive 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 15-50% of cell C as soluble compounds composed of small molecules, soluble proteins and reserve carbohydrate. 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 heteroglycans with a composition entirely different from the intracellular ones, and often form viscous or gelatinous mucilages (Allan et al. 1972, Myklestad et al. 1972, Percival et al. 1980). 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 genera *Chaetoceros* appears to have a generally high extracellular production (20-50%), particularly polysaccharides, while *Thalassiosira* spp. and *Skeletonema costatum* produce far less (1-7%) (Myklestad 1974, 1977).

This contribution presents a detailed study of carbohydrate and amino acid production in the marine diatom *S. costatum* during exponential and N-limited growth. As far as we know this is the first study of the combined cellular and extracellular production of these components. The levels of reserve 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.

METHODS

Organism and culture conditions. The 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 3/4 with Milli-Q water (Millipore, Bedford, MA), i.e. final salinity ~24 psu. In the present experiments, the cells were grown in modified N-limited f media containing 150 µM (case 1) or 50 µM NO_3^- (case 2). Other components were added at f/2 (case 1) or f/10 (case 2) strength, except P_i, 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, 3-5 mM $NaHCO_3$ was added as supplement to the natural bicarbonate content of 3/4 seawater (~1.7 mM).

The cultures were grown in photobioreactors (Granum & Myklestad 2001a), run as stirred (at short intervals) 5-l 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 (2×4) with an irradiance of 200 µmol m^{-2} s^{-1} as measured by a Biospherical Instruments (San Diego, CA) QSL-100 light meter. pH in the culture was continually monitored with a pH electrode coupled to a Consort (Turnhout, Belgium) R301 controller and a TrendView (Dorset, UK) Multitrend recorder. pH was kept within a range of 7.8-8.1 by automatic titration with 0.1 M HCl (in the light) and 0.02 M NaOH (in the dark) effected by the pH controller using advanced relay regulation.

A photobioreactor was incubated with exponentially growing cells. When the cell density reached 20-30 μl^{-1}, the cells were further incubated in two 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. In case 1, extra bicarbonate (without ^{14}C) was added on day 5 and 6 to avoid DIC limitation (2 mM each day). As a consequence, the ^{14}C label was diluted, and ^{14}C fixation underestimated carbon assimilation after day 5.

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 5-10%. The growth medium and culture samples from 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 (Kent, UK) 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 chlorophyll *a* (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 (Sunnyvale, CA) 10 AU fluorometer according to Holm-Hansen et al. (1965) as the mean of three samples with SE 3-5%. Cellular organic C and N were measured with a Carlo Erba (Milan, Italy) NA 1500 elemental analyzer (Kirsten 1979) as the mean of three samples with SE 3-8%. 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 0.05 M H_2SO_4 at 60°C, and the cell wall polysaccharides were subsequently hydrolyzed by cold 80% H_2SO_4 (Granum & Myklestad 2001b). Each carbohydrate fraction was determined by the phenol-sulphuric acid method (Dubois et al. 1956) as the mean of three samples with SE 1-5%.

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 (Milford, MA) Nova-Pak C_{18} column and *o*-phthaldialdehyde reagent solution from Sigma (St. Louis, MO). 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 two samples. The precision of this analysis is ±1-9% ($P<0.05$) according to Flynn.

Dissolved NO_3^- was determined by reduction and diazotizing reaction (Strickland & Parsons 1972) as the mean of two 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 (Cincinnati, OH) DC-190 analyzer with SE 3%, both as means of two 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 (1.1-1.4 mg l^{-1}). Dissolved mono- and polysaccharides were measured by the TPTZ method (Myklestad et al. 1997) as the mean of three samples with SE 6-10%.

^{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). Samples were purged of inorganic ^{14}C by acidification with HCl vapor, and evaporation of CO_2 overnight. Cellular fixed ^{14}C radioactivity (dpm) was measured by a Wallac (Turku, Finland) 1410 scintillation counter using Wallac OptiPhase HiSafe 3 scintillation liquid (10 ml). Fixed ^{14}C was determined as the mean of three samples with SE 5-10%. 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):

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

Medium filtrate was purged of inorganic ^{14}C by acidification (pH<2) with H_3PO_4 and evaporation of CO_2 overnight, and extracellular fixed ^{14}C was measured as described above.

Biochemical fractionation of cellular fixed ^{14}C was performed according to Granum & Myklestad (2001c). A low MW fraction was extracted by Milli-Q water at 70°C, and separated by cation exchange chromatography into a basic fraction (amino acids, purines, pyrimidines etc.) denoted "amino acids" and an acid/neutral fraction (β-1,3-glucan, monosaccharides, organic acids etc.) denoted "glucan" (Granum & Myklestad 1999). A lipid fraction was extracted by cold $CHCl_3$, and a polysaccharide fraction (also containing nucleic acids) was extracted by 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 at two different strengths, and sampled every 24 h (4 h into the photophase). Fig. 1 shows the growth and chemical composition of cells grown in 150 µM NO_3^- (case 1), and Fig. 2 shows the results for cells grown in 50 µM NO_3^- (case 2). The upper graphs in Figs. 1-2 show the growth in cell numbers, cellular organic C and N and cellular chl *a*. The cells grew exponentially until NO_3^- was exhausted, and then went through a transition phase and into the stationary phase. The exponential growth constant was 1.4 d^{-1}, corresponding to 2.0 div. d^{-1}, in both cases. 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* per cell decreased by even 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, day 5-7 (Table 1).

The lower graphs in Figs. 1-2 show the cellular quotas of carbohydrates and amino acids during growth. Cellular β-1,3-glucan was only 4.5 pg C $cell^{-1}$ during exponential growth. Upon NO_3^- exhaustion, the glucan increased rapidly for 3-4 d, and then stabilized at a level of 17-21 pg C $cell^{-1}$. Consequently, the 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 summerized in Table 2.

Additional sampling at the end of the light and dark periods revealed substantial diel changes in the chemical composition of the alga, especially during exponential growth, although cell division was asynchronous. Fig. 3 shows the diel variations in cellular quotas of organic C and N and fractions of glucan and free amino acids during exponential growth (case 1, day 0-2), and Fig. 4 shows diel variations during N depletion (case 1, day 4-6). During exponential growth, the glucan level oscillated between 2.7 (end of scotophase) and 13 pg C $cell^{-1}$ (end of scotophase), constituting 17-42% of cellular organic C. 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 6-10% 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 8-22% of cellular organic N. 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 (~10%), and the cellular quotas of N components gradually decreased without significant diel variation.

The composition of the cellular free amino acids also showed striking diel variations during exponential growth. Fig. 5 shows the diel changes in Gln and Glu during exponential growth (case 1, day 0-2), and Table 3 shows the average composition of cellular free amino acids as molar percentages in the exponential and stationary growth phases. During exponential growth, Gln oscillated between 0.2 (end of scotophase) and 12 fmol cell^{-1} (end of photophase), contributing 2-34% of the total free amino acids. The corresponding Gln/Glu ratio alternated between 0.05 and 2. Even though the other amino acids also increased somewhat in the photophase (by factors of 1.3-10), 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 depletion. 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.

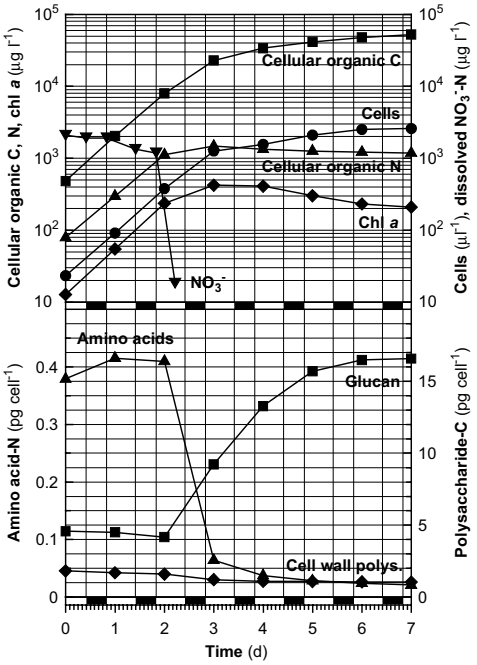

Fig. 1. Growth and chemical composition of *S. costatum* in 150 µM NO_3^- medium (case 1)

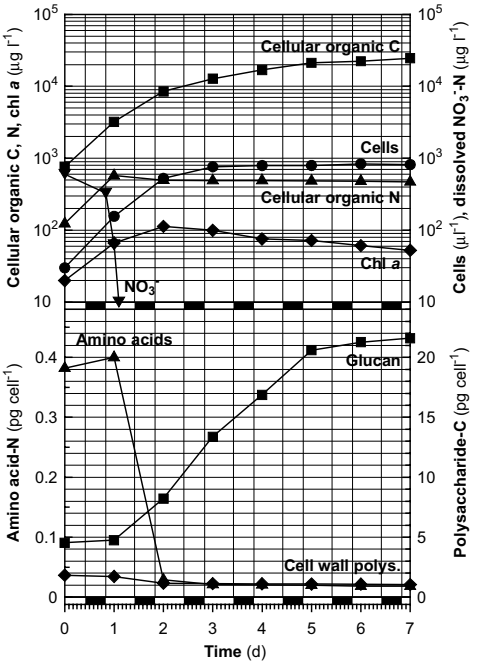

Fig. 2. Growth and chemical composition of *S. costatum* in 50 µM NO_3^- medium (case 2)

Table 1. Cellular and extracellular production rates of *S. costatum* during exponential (day 0-2) and stationary (day 5-7) 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

Table 2. Cellular chemical composition of *S. costatum* during exponential (day 0-2) and stationary (day 7) growth phase (sampled 4 h into the photophase, case 1).

	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

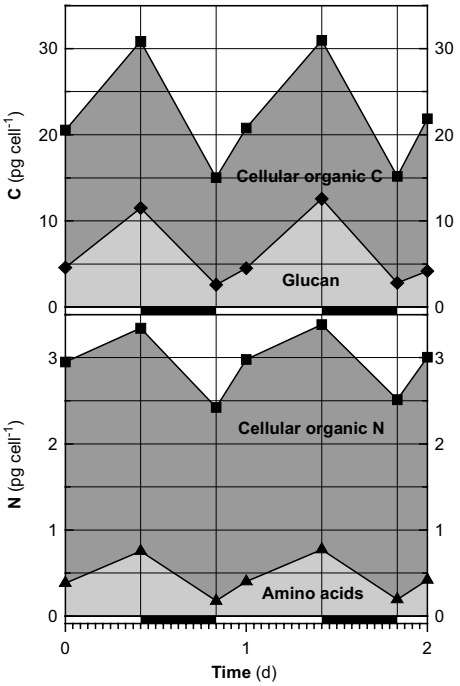

Fig. 3. Diel variation in cellular organic C and N and fractions of glucan and free amino acids during exponential growth of *S. costatum* (case 1, day 0-2)

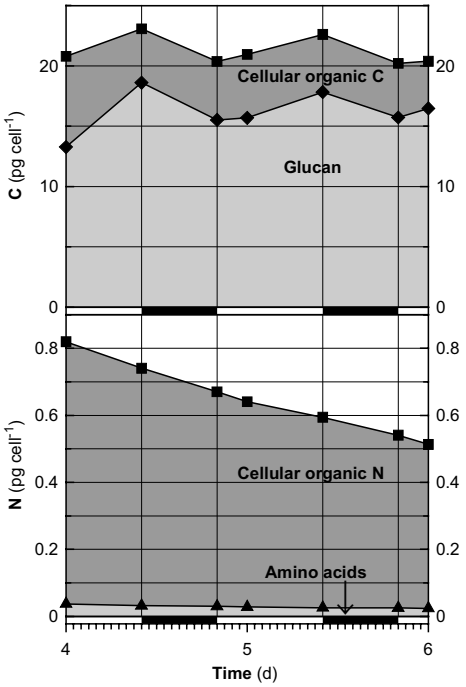

Fig. 4. Diel variation in cellular organic C and N and fractions of glucan and free amino acids during stationary phase of *S. costatum* (case 1, day 4-6)

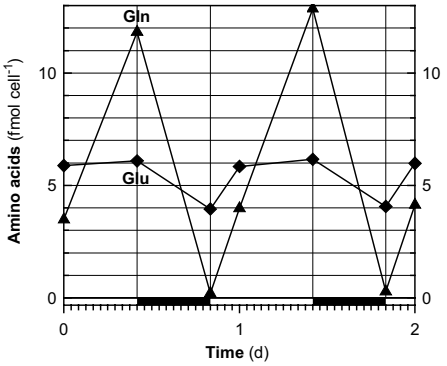

Fig. 5. Diel variation in cellular free Glu and Gln during exponential growth of *S. costatum* (case 1, day 0-2)

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

	Growth phase		
	Exponential		Stationary
	Dawn	Dusk	
Asp	22	9	4
Glu	36	17	10
Asn	1	2	-
Ser	2	2	1
Gln	2	34	1
Gly+Arg	5	5	1
Thr	2	3	1
Ala	11	8	4
Tyr	1	3	4
Met	-	-	1
Val	8	7	18
Phe	-	2	4
Ile	3	3	22
Leu	3	1	25
Lys	5	4	4

Extracellular production

During growth of the diatom, extracellular production was measured every 24 h (4 h into the photophase) as DOC, carbohydrates and free amino acids. Fig. 6 shows the production of DOC and fractions of mono- and polysaccharides and amino acids in case 1 and 2. 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 two cultures responded quite differently. In case 1 a transient high release, 3 pg C $cell^{-1}$ (28 PER), occurred between day 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 (day 5-7) at 0.16 pg C $cell^{-1}$ d^{-1}, corresponding to 4 PER. The sudden elevated release in 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, 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.

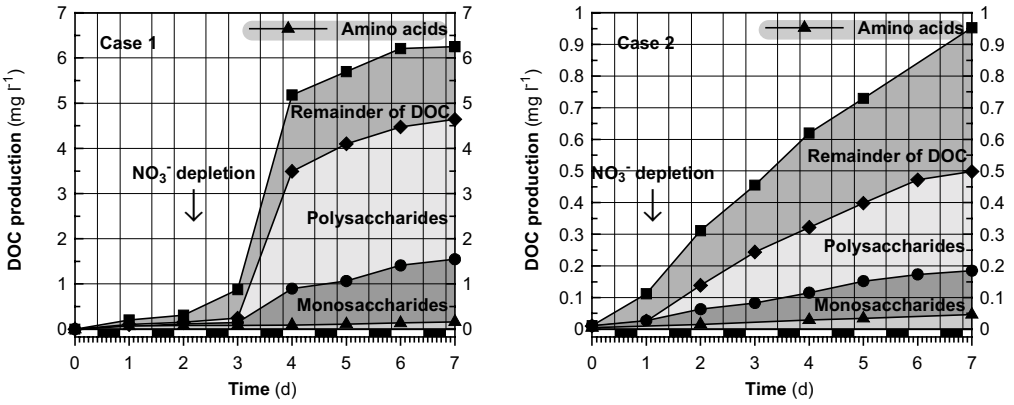

Fig. 6. Extracellular production of carbohydrates and free amino acids as fractions of total DOC during growth of *S. costatum* in 150 µM NO_3^- (case 1) and 50 µM NO_3^- (case 2) media

Table 4. Extracellular free amino acid composition (molar %) of *S. costatum* during exponential (day 0-2) and stationary (day 5-7) growth phase.

	Growth phase	
	Exponential	Stationary
Asp	19	10
Glu	32	18
Asn	-	1
Ser	4	1
Gln	-	4
Gly+Arg	13	6
Thr	-	2
Ala	10	5
Tyr	-	3
Met	-	5
Val	5	7
Phe	2	7
Ile	9	10
Leu	6	15
Lys	-	5

Radiocarbon fixation

Parallel cultures were radiolabeled with ^{14}C-bicarbonate, and samples were taken every 24 h (4 h into the photophase). Fig. 7 shows the cell density and ^{14}C fixation in cellular and extracellular fractions in case 1 and 2. Growth rates and cellular/extracellular production rates calculated from ^{14}C incorporation were similar to the rates based on chemical analysis (Table 1). However, the results after day 5 in case 1 were significantly lower, which was probably caused by dilution of the ^{14}C tracer with unlabeled bicarbonate (see Methods). Biochemical fractionation of cellular organic ^{14}C was performed at three different points (day 2, 3 and 7) during growth in case 1 (Fig. 8). The partitioning of organic ^{14}C shows the same trends as the chemical analyses, although the glucan fraction was 5-10% lower in the stationary phase. The dilution of ^{14}C-bicarbonate after day 5 probably contributes to this difference. The results also show that the cellular lipids increased significantly from exponential to stationary growth phase (from 5% to 9% of ^{14}C).

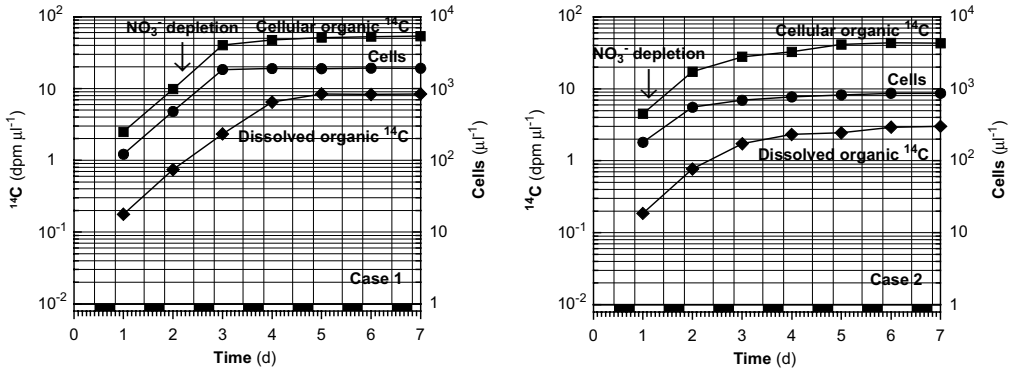

Fig. 7. ^{14}C fixation in cellular and extracellular (dissolved) fractions during growth of *S. costatum* in 150 µM NO_3^- (case 1) and 50 µM NO_3^- (case 2) media

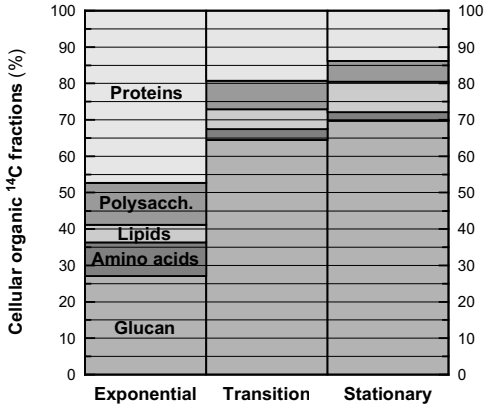

Fig. 8. Biochemical fractionation of cellular organic ^{14}C during exponential (day 2), transition (day 3), and stationary (day 7) growth phase of *S. costatum* (case 1)

DISCUSSION

Cellular production

The results show that *Skeletonema costatum* grows exponentially at 2.0 div. d^{-1} under the present conditions, which is close to the optimum growth rate for this alga (Figs. 1-2). Apart from diel changes in chemical composition, the alga undergoes balanced growth in the exponential phase.

The diel physiological variation is particularly characterized by the oscillations in cellular β-1,3-glucan, increasing >4-fold per cell during the photophase (Fig. 3). The glucan level increased most rapidly in the late part of the photophase, which is consistent with a study of the same alga by Vårum et al. (1986). Also, Hitchcock (1980) observed similar diel variations in cellular carbohydrate in *S. 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. 3). 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 went down 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 *S. 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 variations during exponential growth. Gln 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 induce increasing Gln/Glu 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 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:dark cycles. In a recent investigation we have demonstrated specific mobilization of β-1,3-glucan providing C skeletons for synthesis of amino acids and proteins in *S. costatum* (Granum & Myklestad 2001c).

Encountering NO_3^- exhaustion of the medium, cell numbers still increased 5-fold while the cellular N quota decreased accordingly (Figs. 1-2). 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 cells adapted to N limitation by reducing their cellular N contents to a minimum, entering a low activity stage. Dortch (1982) found similar effects of N limitation on N components in *S. costatum* and other diatoms including depletion of internal NO_3^- and NH_4^+. The concurrent reduction in growth rate and cell quota of N 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 3-4 d before it stabilized at 75-80% of cellular organic C (Figs. 1-2). The results indicate a high photosynthetic activity for several days after N depletion. During this period, biosynthesis of N-free carbohydrates was apparently not hampered. In the final stage, the diel variation in glucan was very low

(Fig. 4). The results suggest that the cellular pool of glucan was saturated within 3-4 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 *S. costatum* may accumulate up to 80% of cellular C under strong nutrient limitation (Myklestad 1974, Vårum & Myklestad 1984). Moreover, extensive accumulation of glucan under nutrient limitation has also 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 has been considered as nutrient deficiency. At the end of the photophase in nutrient-sufficient *S. costatum* cells, however, we obtained a slightly lower value than 1 in this study.

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 ^{14}C) after N depletion in addition to the large accumulation in β-1,3-glucan. However, both the glucan and lipid fractions were probably underestimated after day 5 due to significant dilution of the ^{14}C isotope (see Methods). Whereas new production of amino acids, proteins and cell wall polysaccharides was extremely low, significant metabolism of storage products still occurred in the late stationary phase.

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). Nutrient-limited cells in the euphotic zone are able to accumulate carbohydrates, causing increased cellular density and sinking below the nutricline. Upon nutrient replenishment and reversion of the carbohydrate/protein ratio, the cells migrate back to the surface waters. This vertical migration of algae with uncoupled C and N assimilation enhances transport of deep nutrients to the euphotic zone, thereby increasing oceanic primary production. Physiological changes induced by N depletion may also be connected to 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 particularly interesting as an ecological index of algal growth, as its occurrence in the sea is exclusively correlated with phytoplankton.

Extracellular production

Investigations of different diatoms have shown widely different levels of excretion, but *S. costatum* has generally been found in the low range (Myklestad 1974, 1977, Mague et al. 1980). This was confirmed in the present study, with only 4 PER in both exponential and stationary growth phase (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 1-2 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 polysaccharides released indicates cell lysis with leakage of β-1,3-glucan. The reason for this occurrence is uncertain, but similar reports of an abrupt increase in excretion at the transition between the exponential and stationary phases are found (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 *S. costatum* increased from 2-4% in exponentially growing cells to 14-65%

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 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 *S. 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. 2001).

Concluding remarks

In this study, typical cell quotas of organic C and N, carbohydrates and amino acids were established for the marine diatom *S. 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 substantial diel changes in cellular chemical composition, particularly the levels of β-1,3-glucan and free amino acids. NO_3^- exhaustion resulted in rapid accumulation of cellular glucan and concurrent reduction in cellular N quotas, especially free amino acids. When considering parameters such as C/N, protein/glucan and Gln/Glu as indicators of nutrient limitation, the diel changes in nutrient-sufficient cells must be taken into account.

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

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LITERATURE CITED

Admiraal W, Peletier H, Laane RWPM (1986) Nitrogen metabolism of marine planktonic diatoms; excretion, assimilation and cellular pools of free amino acids in seven species with different cell size. J Exp Mar Biol Ecol 98:241-263

Allan GG, Lewin J, Johnson PG (1972) Marine polymers. IV. Diatom polysaccharides. Bot Mar 15:102-108

Beattie A, Hirst EL, Percival E (1961) Comparative structural investigations on leucosin (chrysolaminarin) separated from diatoms and laminarin from the brown algae. Biochem J 79:531-537

Berges JA, Falkowski PG (1998) Physiological stress and cell death in marine phytoplankton: induction of proteases in response to nitrogen or light limitation. Limnol Oceanogr 43:129-135

Collos Y, Siddiqi MY, Wang MY, Glass ADM, Harrison PJ (1992) Nitrate uptake kinetics by two marine diatoms using the radioactive tracer ^{13}N. J Exp Mar Biol Ecol 163:251-260

Cuhel RL, Ortner PB, Lean DRS (1984) Night synthesis of protein by algae. Limnol Oceanogr 29:731-744

Dortch Q (1982) Effect of growth conditions on accumulation of internal nitrate, ammonium, amino acids, and protein in three marine diatoms. J Exp Mar Biol Ecol 61:243-264

Droop MR (1973) Some thoughts on nutrient limitation in algae. J Phycol 9:264-272

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350-356

Falkowski PG, Raven JA (1997) Aquatic photosynthesis. Blackwell, Malden, MA

Falkowski PG, Sukenik A, Herzig R (1989) Nitrogen limitation in *Isochrysis galbana* (Haptophyceae). II. Relative abundance of chloroplast proteins. J Phycol 25:471-478

Flynn KJ (1988) Some practical aspects of measurements of dissolved free amino acids in natural waters and within microalgae by the use of HPLC. Chem Ecol 3:269-293

Flynn KJ, Al-Amoudi OA (1988) Effects of N deprivation and darkness on composition of free amino acid pool in and on amino acid release from diatom *Phaeodactylum tricornutum* Bohlin. J Exp Mar Biol Ecol 119:131-144

Fogg GE (1983) The ecological significance of extracellular products of phytoplankton photosynthesis. Bot Mar 26:3-14

Ford CW, Percival E (1965a) The carbohydrates of *Phaeodactylum tricornutum*. Part I. Preliminary examination of the organism, and characterisation of low molecular weight material and of a glucan. J Chem Soc 1298:7035-7041

Ford CW, Percival E (1965b) The carbohydrates of *Phaeodactylum tricornutum*. Part II. A sulphated glucuronomannan. J Chem Soc 1299:7042-7046

Geider RJ, Osborne BA (1992) Using isotopes to measure gas exchange. In: Geider RJ, Osborne BA (eds) Algal photosynthesis: the measurement of algal gas exchange. Chapman & Hall, New York, p 32-70

Granum E, Myklestad SM (1999) Effects of NH_4^+ assimilation on dark carbon fixation and β-1,3-glucan metabolism in the marine diatom *Skeletonema costatum* (Bacillariophyceae). J Phycol 35:1191-1199

Granum E, Myklestad SM (2001a) A photobioreactor with pH control: demonstration by growth of the marine diatom *Skeletonema costatum*. J Plankton Res (Accepted)

Granum E, Myklestad SM (2001b) A simple combined method for determination of β-1,3-glucan and cell wall polysaccharides in diatoms. Hydrobiologia (Submitted)

Granum E, Myklestad SM (2001c) Mobilization of β-1,3-glucan and biosynthesis of amino acids induced by NH_4^+ addition to N-limited cells of the marine diatom *Skeletonema costatum* (Bacillariophyceae). J Phycol 37:772-782

Guerrero MG, Vega JM, Losada M (1981) The assimilatory nitrate-reducing system and its regulation. Ann Rev Plant Physiol 32:169-204

Guillard RRL, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. Can J Microbiol 8:229-239

Hama J, Handa N (1992) Diel variation of water-extractable carbohydrate composition of natural phytoplankton populations in Kinu-ura Bay. J Exp Mar Biol Ecol 162:159-176

Hama T, Honjo T (1987) Photosynthetic products and nutrient availability in phytoplankton population from Gokasho Bay, Japan. J Exp Mar Biol Ecol 112:251-266

Handa N (1969) Carbohydrate metabolism in the marine diatom *Skeletonema costatum*. Mar Biol 4:208-214

Haug A, Myklestad S (1976) Polysaccharides of marine diatoms with special reference to *Chaetoceros* species. Mar Biol 34:217-222

Haug A, Myklestad S, Sakshaug E (1973) Studies on the phytoplankton ecology of the Trondheimsfjord. I. The chemical composition of phytoplankton populations. J Exp Mar Biol Ecol 11:15-26

Hecky RE, Mopper K, Kilham P, Degens ET (1973) The amino acid and sugar composition of diatom cell-walls. Mar Biol 19:323-331

Hitchcock GL (1980) Diel variation in chlorophyll *a*, carbohydrate and protein content of the marine diatom *Skeletonema costatum*. Mar Biol 57:271-278

Holm-Hansen O, Lorenzen CJ, Holmes RW, Strickland JDH (1965) Fluorometric determination of chlorophyll. J cons Cons int explor mer 30:3-15

Ignatiades L, Fogg GE (1973) Studies on the factors affecting the release of organic matter by *Skeletonema costatum* (Greville) Cleve in culture. J Mar Biol Ass UK 53:937-956

Kirsten WJ (1979) Automatic methods for the simultaneous determination of carbon, hydrogen, nitrogen, and sulfur, and for sulfur alone in organic and inorganic materials. Anal Chem 51:1173-1179

Kuwata A, Hama T, Takahashi M (1993) Ecophysiological characterization of two life forms, resting spores and resting cells, of a marine planktonic diatom, *Chaetoceros pseudocurvisetus*, formed under nutrient depletion. Mar Ecol Prog Ser 102:245-255

Lindroth P, Mopper K (1979) High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with *o*-phthaldialdehyde. Anal Chem 51:1667-1674

Lomas MW, Glibert PM (2000) Comparisons of nitrate uptake, storage, and reduction in marine diatoms and flagellates. J Phycol 36:903-913

Mague TH, Friberg E, Hughes DJ, Morris I (1980) Extracellular release of carbon by marine phytoplankton; a physiological approach. Limnol Oceanogr 25:262-279

McConville MJ, Bacic A, Clarke AE (1986) Structural studies of chrysolaminaran from the ice diatom *Stauroneis amphioxys* (Gregory). Carbohydr Res 153:330-333

Morris I, Smith AE, Glover HE (1981) Products of photosynthesis in phytoplankton off the Orinoco River and in the Caribbean Sea. Limnol Oceanogr 26:1034-1044

Myklestad S (1974) Production of carbohydrates by marine planktonic diatoms. I. Comparison of nine different species in culture. J Exp Mar Biol Ecol 15:261-274

Myklestad S (1977) Production of carbohydrates by marine planktonic diatoms. II. Influence of the N/P ratio in the growth medium on the assimilation ratio, growth rate, and production of cellular and extracellular carbohydrates by *Chaetoceros affinis* var. *willei* (Gran) Hustedt and *Skeletonema costatum* (Grev.) Cleve. J Exp Mar Biol Ecol 29:161-179

Myklestad S, Haug A (1972) Production of carbohydrates by the marine diatom *Chaetoceros affinis* var. *willei* (Gran) Hustedt. I. Effect of the concentration of nutrients in the culture medium. J Exp Mar Biol Ecol 9:125-136

Myklestad S, Haug A, Larsen B (1972) Production of carbohydrates by the marine diatom *Chaetoceros affinis* var. *willei* (Gran) Hustedt. II. Preliminary investigation of the extracellular polysaccharide. J Exp Mar Biol Ecol 9:137-144

Myklestad S, Holm-Hansen O, Vårum KM, Volcani BE (1989) Rate of release of extracellular amino acids and carbohydrates from the marine diatom *Chaetoceros affinis*. J Plankton Res 11:763-774

Myklestad SM (1995) Release of extracellular products by phytoplankton with special emphasis on polysaccharides. Sci Total Environ 165:155-164

Myklestad SM (2000) Dissolved organic carbon from phytoplankton. In: Wangersky P (ed) The handbook of environmental chemistry [D]. Marine chemistry. Springer, Berlin, p 111-148

Myklestad SM, Skånøy E, Hestmann S (1997) A sensitive and rapid method for analysis of dissolved mono- and polysaccharides in seawater. Mar Chem 56:279-286

Myklestad SM, Vadstein O, Reinertsen H, Kirkvold S, Børsheim KY, Olsen Y (2001) Photosynthetic algal production, accumulation of soluble organic matter and bacterial production in mesocosms with varying nutrient supply. Mar Ecol Prog Ser (Submitted)

Parsons TR, Stephens K, Strickland JDH (1961) On the chemical composition of eleven species of marine phytoplankters. J Fish Res Bd Can 18:1001-1016

Paulsen BS, Myklestad S (1978) Structural studies of the reserve glucan produced by the marine diatom *Skeletonema costatum* (Grev.) Cleve. Carbohydr Res 62:386-388

Percival E, Rahman MA, Weigel H (1980) Chemistry of the polysaccharides of the diatom *Coscinodiscus nobilis*. Phytochemistry 19:809-811

Porter KG, Feig YS (1980). The use of DAPI for identifying and counting aquatic microflora. Limnol Oceanogr 25:943-948

Raimbault P, Mingazzini M (1987) Diurnal variations of intracellular NO_3^- storage by marine diatoms: effects of nutritional state. J Exp Mar Biol Ecol 112:217-232

Richardson TL, Cullen JJ (1995) Changes in buoyancy and chemical composition during growth of a coastal marine diatom: ecological and biogeochemical consequences. Mar Ecol Prog Ser 128:77-90

Schmid A-MM, Borowitzka MA, Volcani BE (1981) Morphogenesis and biochemistry of diatom cell walls. In: Kiermayer O (ed) Cytomorphogenesis in plants. Springer, Wien, p 63-97

Strickland JDH, Parsons TR (1972) A practical handbook of seawater analysis, 2nd ed. Bull Fish Res Bd Can 167, Ottawa

Sugimura Y, Suzuki Y (1988) A high-temperature catalytic oxidation method for the determination of non-volatile dissolved organic carbon in seawater by direct injection of a liquid sample. Mar Chem 24:105-131

Syrett PJ (1981) Nitrogen metabolism of microalgae. In: Platt T (ed) Physiological bases of phytoplankton ecology. Can Bull Fish Aquat Sci 210, Ottawa, p 182-210

Vanlerberghe GC, Schuller KA, Smith RG, Feil R, Plaxton WC, Turpin DH (1990) Relationship between NH_4^+ assimilation rate and *in vivo* phosphoenolpyruvate carboxylase activity. Regulation of anaplerotic carbon flow in the green alga *Selenastrum minutum*. Plant Physiol 94:284-290

Vergara JJ, Berges JA, Falkowski PG (1998) Diel periodicity of nitrate reductase activity and protein levels in the marine diatom *Thalassiosira weissflogii* (Bacillariophyceae). J Phycol 34:952-961

Villareal TA, Woods S, Moore JK, Culver-Rymsza K (1996) Vertical migration of *Rhizosolenia* mats and their significance to NO_3^- fluxes in the central North Pacific gyre. J Plankton Res 18:1103-1121

Volcani BE (1981) Cell wall formation in diatoms: morphogenesis and biochemistry. In: Simpson TL, Volcani BE (eds) Silicon and siliceous structures in biological systems. Springer, New York, p 157-200

Vårum KM, Myklestad S (1984) Effects of light, salinity and nutrient limitation on the production of β-1,3-D-glucan and exo-D-glucanase activity in *Skeletonema costatum* (Grev.) Cleve. J Exp Mar Biol Ecol 83:13-26

Vårum KM, Østgaard K, Grimsrud K (1986) Diurnal rhythms in carbohydrate metabolism of the marine diatom *Skeletonema costatum* (Grev.) Cleve. J Exp Mar Biol Ecol 102:249-256

Werner D (1977) Contributions of the study of diatoms to biology. In: Werner D (ed) The biology of diatoms. Blackwell, Oxford, p 1-3

Williams PJlB (1990) The importance of losses during microbial growth: commentary on the physiology, measurement and ecology of the release of dissolved organic material. Mar Microb Food Webs 4:175-206