PRIMARY PRODUCTION OF PARTICULATE PROTEIN AMINO ACIDS: ALGAL PROTEIN
METABOLISM AND ITS RELATIONSHIP TO
THE COMPOSITION OF PARTICULATE ORGANIC MATTER

by

STEVEN ERIC LOHRENZ

B.A., University of Oregon, Eugene
(1978)

SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE
DEGREE OF
DOCTOR OF PHILOSOPHY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY
AND
WOODS HOLE OCEANOGRAPHIC INSTITUTION
APRIL, 1985

© Steven Eric Lohrenz

The author hereby grants to M.I.T. and W.H.O.I. permission to reproduce
and distribute copies of this thesis document in whole or in part.

Signature of Author
~~Massachusetts Institute of Technology/Woods Hole~~
Oceanographic Institution. April 1985

Certified by
Craig D. Taylor, Thesis Supervisor

Accepted by
John J. Stegeman, Chairman
Joint Committee For Biological Oceanography,
Massachusetts Institute of Technology/
Woods Hole Oceanographic Institution

To my family, and George the dog

"If someone tells me that in making these conclusions I have gone beyond the facts I reply: 'This is true, that I have freely put myself among ideas which cannot be rigorously proved. That is my way of looking at things. Every time a chemist concerns himself with these mysterious phenomena and every time he has the luck to make an important step forward he will be led instinctively to attribute their prime cause to a class of reactions in harmony with the general results of his own researches. That is the logical course of the human mind, in all controversial matters.'"

Louis Pasteur, 1857

PRIMARY PRODUCTION OF PARTICULATE PROTEIN AMINO ACIDS: ALGAL PROTEIN
METABOLISM AND ITS RELATIONSHIP TO THE COMPOSITION OF
PARTICULATE ORGANIC MATTER

by
STEVEN ERIC LOHRENZ

Submitted to the Department of Biology on April 29, 1985
in partial fulfillment of the requirements for the
Degree of Doctor of Philosophy

ABSTRACT

The biochemical and physiological bases underlying primary production of particulate protein amino acids were investigated in an effort to understand the relationship between algal protein metabolism and particulate organic matter composition.

In order to examine biochemical processes associated with conversion of inorganic carbon and nitrogen into protein, the effects of NH_4^+ limitation on free amino acid and protein composition and incorporation of inorganic ^{14}C were studied in the marine chlorophyte, Nannochloris sp. (clone GSB Nanno). Free amino acid metabolism was sensitive to changes in steady state NH_4^+ -limited growth rates. Reduced carbon and nitrogen flux into protein resulting from nitrogen limitation of growth was associated with reductions in proportions of cellular carbon and nitrogen in the intracellular free amino acid (IFAA) pool. Growth rate-dependent changes in IFAA pool composition reflected changes in rate limiting steps which were intermediate between assimilation of inorganic nitrogen and the incorporation of nitrogen into macromolecules. The proportion of cellular carbon in both protein and pools of free amino acids decreased with decreasing growth rate. Distributions of incorporated inorganic ^{14}C among free amino acids and protein provided qualitative descriptions of growth related compositional variations. Saturation rates of (IFAA) carbon with dissolved inorganic ^{14}C did not significantly change as growth rates decreased. In contrast, saturation rates of free glutamate, glycine + alanine, and valine did decrease with growth rate. At low growth rate, specific activities of the newly assimilated glutamate, valine, and glycine + alanine in protein were higher than specific activities of their corresponding free amino acid pools. This was likely a consequence of metabolic segregation and more rapid saturation of protein precursor pools.

Enrichment of NH_4^+ -limited steady state cultures of Nannochloris sp. with NH_4^+ led to a dramatic time dependent increase in free glutamine concentrations accompanied by differential increases in other free amino acids. Patterns of isotope incorporation into the free amino acids reflected real changes in concentrations. Increases were associated with the diversion of photosynthetically fixed carbon from lipophilic material towards amino acid biosynthesis, and signalled the onset of increased protein synthesis. Preliminary investigations of two other marine phytoplankton species, Dunaliella tertiolecta (clone Dun) and Thalassiosira weissflogii (clone Actin), suggested that the nature and timing of the biochemical response to NH_4^+ enrichment is different among different species.

ABSTRACT (continued)

At high light intensity, increased NH_4^+ limitation of Nannochloris sp. was associated with decreases in cellular protein, protein to carbon ratios, and protein to chlorophyll a ratios. At low light intensities, cellular protein and protein to carbon ratios did not decrease with increasing NH_4^+ limitation. Chlorophyll a to protein ratios were generally higher at low light intensity and decreased with increasing NH_4^+ limitation, suggesting that nitrogen limitation suppressed low light enhancement of chlorophyll a production. Observed incorporation rates of inorganic ^{14}C exceeded predicted rates for glycine and alanine in protein under combined conditions of light and nitrogen limitation, an indication that protein turnover in excess of net synthesis was important under these conditions.

The characteristics of protein composition and incorporation of inorganic ^{14}C were examined in steady state NH_4^+ -limited cultures of three other species of marine phytoplankton. The species studied included Chaetoceros simplex (clone BBSM), Chattonella luteus (clone Ol1sth), and Chroomonas salina (clone 3C). Saturation of protein precursors was different for different species and for different protein amino acids. Nannochloris sp. displayed the most rapid and complete equilibration, while C. simplex exhibited relatively slow or incomplete saturation and high sensitivity to nitrogen depletion. Intermediate patterns were observed for the other species. For all species examined, protein glycine and alanine demonstrated relatively rapid and complete equilibration of precursor pools, and were least sensitive to nitrogen depletion.

With the knowledge that these selected amino acids consistently demonstrated relatively rapid and complete equilibration of precursor pools with ^{14}C -inorganic carbon in several taxonomically distinct marine algae, primary production of particulate protein amino acids and its relationship to the composition of particulate organic matter was investigated in the epilimnion of a semi-enclosed marine basin, Salt Pond, MA. Studies were conducted previous to and throughout a major bloom of Olisthodiscus magnus. Before the bloom, the ratio of particulate protein amino acid (PPAA) production to particulate organic carbon (POC) production was not significantly different from the relative proportions of PPAA and POC in the particulate organic matter. Comparisons between estimated production and observed concentration changes indicated residence times of POC and PPAA were similar (10 - 20 days). Thus with respect to POC and PPAA, particulate organic matter composition reflected the composition of organic matter being produced by photoautotrophs. During the bloom decline, the PPAA/POC production ratio, a reflection of the activities of the metabolically active algal population, was significantly less than the PPAA/POC ratio of the particulate organic matter. This discrepancy can be attributed either to increased turnover of protein associated with the low inorganic nitrogen concentrations and in situ light intensities, or selective removal of PPAA carbon by secondary transformational processes.

Thesis Supervisor: C. D. Taylor

Title: Associate Scientist

Woods Hole Oceanographic Institution

ACKNOWLEDGEMENTS

Because of a number of special people, I will fondly remember the years I've spent in Woods Hole. One of the people I was introduced to when I first came to Woods Hole was Russell L. Cuhel. He was integral in both my acclimation to this unique research environment, and in my choice of thesis research. I was then, and still am inspired by his dedication to excellence in both work and play.

My closest advisor and counselor throughout my thesis research was Craig D. Taylor. Craig's ability to laugh at inevitable mishaps helped me to place them in their proper perspective. An essential part of research is getting back up on one's feet after a fall. Craig made this part much easier. In addition, his insight helped me to make the most of my research efforts and explore aspects I would otherwise not have considered.

Another member of my thesis committee was Cindy Lee, whose advice helped considerably throughout my research. Her support was a lifesaver during troublesome times. Sallie W. Chisholm provided me the opportunity to explore some algal physiology during my stint at MIT. It was a refreshing change from classes, and the experience I gained helped me when I began working in Woods Hole. Both she and Graham Walker were my MIT "connections". Despite the obstacle of distance separating WHOI and MIT, their input as thesis committee members was integral in the evolution of my thesis. Joel Goldman contributed much information and many ideas which influenced my thesis research. He provided me the opportunity to conduct some experiments in his lab, and later supplied valuable equipment. The help of his lab members Mark Dennett and Carol Riley is also appreciated. John Waterbury formally joined my thesis committee late in the game, but he was always a member in spirit. He generously provided advice, equipment, supplies, and taught me alot about growing "little beasties". I am also grateful to Freddy Valois and Stan Watson.

One of the things I was most impressed by when I first came to Woods Hole was the cooperative spirit of the people who work here. This is exemplified by Holger Jannasch and the members of his lab, Carl Wirsen and Steve Molyneaux. Their help, both in terms of counsel and allowing me access to their lab, was very important. Others who generously provided assistance, information, and equipment were John Molongoski, Brian Howes, Dale Goehringer, Doug Nelson, Steve Boyd, Frank Carey, John Dacey, John Stegeman, Nancy Marcus, Don Anderson, Pat Glibert, Linda Graham, Diana Franks, Bonnie Woodward, and Debbie Wiebe.

ACKNOWLEDGEMENTS (continued)

Jane Peterson and Peggy Dimmock defended me against mechanical conspiracies. They were delightful and tolerant teachers, and friends. I am also grateful to the people who made being a student much easier: A. L. Peirson, III (Jake), Abbie Jackson, Dixie Berthel, and Connie Brackett.

Friendships come in many different shapes and sizes. I was lucky enough to have a few which I hope made me a better person, and definitely made my time in Woods Hole something to be cherished. Anne McElroy was and is a friend and scientific colleague. As co-classmates, we were both sympathetic and enthusiastic research partners. My sole, single, and only officemate was Melissa Lakich. She was tolerant of my constant requests for reviews and she frequently entertained me with welcome distractions. My gifted summer student, Michael Knapp, was an excellent and devoted associate in field endeavours. Maura Connor discussed the thesis with me at length throughout the writing stages, and her continued support and help is greatly appreciated. My housemates, Dave Kulis and Sheri Schmaltz, were extremely tolerant of my bizarre schedule and were all round good people to live with. My family was and is a constant source of love and support.

There are many others whom I have not mentioned. To all of you, thanks.

Financial support for this research was provided by the Coastal Research Center (25/67.10) through a grant from the Andrew W. Mellon Foundation, by the National Science Foundation (OCE-82-15854), and by the Education Department.

TABLE OF CONTENTS

TITLE PAGE.....	1
ABSTRACT.....	4
ACKNOWLEDGEMENTS.....	6
TABLE OF CONTENTS.....	8
LIST OF FIGURES.....	15
LIST OF TABLES.....	24
 CHAPTER 1. GENERAL INTRODUCTION.....	 28
 THE COMPOSITIONAL DYNAMICS OF ORGANIC MATTER IN AQUATIC ENVIRONMENTS.....	 29
BACKGROUND:	
The Marine Food Chain and the Composition of Organic Matter.....	30
Biochemical Cycles and the Distribution of Organic Matter.....	35
The Chemical Composition of Natural Populations.....	39
Isotope Tracing of Microbial Activities.....	42
 CHAPTER 2. METHODS AND MATERIALS.....	 47
 CULTURE METHODS.....	 48
Media Composition.....	48
Growth Conditions.....	48

TABLE OF CONTENTS (continued)

CHEMICAL ANALYSES.....	52
Chlorophyll <u>a</u> Analysis.....	52
Elemental Carbon and Nitrogen Analyses.....	53
Nutrient Analyses.....	53
Dissolved Inorganic and Organic Carbon Analyses.....	53
Protein Assay.....	56
LABORATORY INCUBATION PROCEDURES.....	56
Sampling Procedures.....	56
Radioassay Procedures.....	57
SUBCELLULAR FRACTIONATION PROCEDURES.....	58
Modification of Existing Procedures.....	58
Comparison of Subcellular Fractionation Procedures.....	60
AMINO ACID ANALYSIS.....	64
Amino Acid Derivatization Procedure.....	64
Column Packing Procedure.....	71
Separation and Collection.....	72
EXTRACTION AND RECOVERY.....	72
Free Amino Acid Extraction and Recovery.....	72
Recovery from Hydrolysis.....	73
Recovery of Protein.....	79
Recovery of Other Subcellular Fractions.....	82
SALT POND PRODUCTION INCUBATIONS.....	82
Summer 1982.....	82
Summer 1983.....	84
LIGHT INTENSITY MEASUREMENTS.....	85

TABLE OF CONTENTS (continued)

CHAPTER 3. RELATIONSHIPS BETWEEN FREE AMINO ACID AND PROTEIN COMPOSITION AND INCORPORATION OF INORGANIC ^{14}C IN NH_4^+ -LIMITED STEADY STATE CULTURES OF MARINE PHYTOPLANKTON.....	93
INTRODUCTION.....	94
Relationships Between Cellular Chemical Composition and Nitrogen Limited Growth Rate.....	94
Relationships of the Distribution of Assimilated Inorganic ^{14}C to Cellular Chemical Composition.....	96
Effects of Nitrogen limitation on Patterns of Inorganic ^{14}C Incorporation into Cell Material.....	98
I. DYNAMICS OF FREE AMINO ACID AND PROTEIN COMPOSITION AND INCORPORATION OF INORGANIC ^{14}C IN NH_4^+ -LIMITED STEADY STATE CULTURES OF THE MARINE CHLOROPHYTE, <u>NANNOCHLORIS</u> SP.....	103
METHODS.....	103
RESULTS AND DISCUSSION.....	103
Cellular Chemical Composition as a Function of Relative Growth Rate.....	103
Nitrogen Specific Turnover of Nitrogen Transport Mediators...	111
Saturation With Inorganic ^{14}C : Relative Specific Activities of Intracellular Free Amino Acids.....	115
Relationships Between Free and Protein Amino Acid Saturation Rates.....	138

TABLE OF CONTENTS (continued)

Time Course Distribution of ^{14}C -label Among Cellular Materials and its Relationship to Cellular Chemical Composition.....	158
Comparisons Between the Composition of Free Amino Acids and the Distribution of ^{14}C	159
Time Course Distributions of ^{14}C Among Subcellular Fractions as a Function of Relative Growth Rate.....	171
II. EFFECTS OF NH_4^+ ENRICHMENT ON AMINO ACID AND TOTAL CARBON METABOLISM IN AN NH_4^+ -LIMITED STEADY STATE CULTURE OF <u>NANNOCHLORIS</u> SP.	
METHODS.....	180
RESULTS AND DISCUSSION.....	181
Increases in Intracellular Free Amino Acids in Response to Nitrogen Enrichment.....	181
Saturation of Free Amino Acids with ^{14}C in Control and Enriched Cultures.....	198
Effects of NH_4^+ Enrichment on the Time Course Distributions of ^{14}C in Cellular Material.....	200
III. PRELIMINARY OBSERVATIONS OF NH_4^+ ENRICHMENT EFFECTS ON CARBON METABOLISM IN TWO OTHER SPECIES.....	
METHODS.....	209
Response of Intracellular Free Amino Acids of <u>Dunaliella</u> <u>tertiolecta</u> to Nutrient Enrichment.....	209
Effects of NH_4^+ Enrichment on Time Course Distributions of ^{14}C in Cellular Material.....	210
Experiment 1.....	210

TABLE OF CONTENTS (continued)

Experiment 2.....	211
Experiment 3.....	212
RESULTS AND DISCUSSION.....	212
<u>Dunaliella tertiolecta</u>	212
<u>Thalassiosira weissflogii</u>	215
CONCLUSIONS.....	235

CHAPTER 4. RELATIONSHIPS BETWEEN NET SYNTHESIS RATES OF PROTEIN AMINO
ACIDS AND INCORPORATION OF INORGANIC ¹⁴C IN MARINE PHYTOPLANKTON.... 238

INTRODUCTION.....	239
BACKGROUND:	
Tracing Production of Particulate Protein Amino Acids.....	240
Radioisotopic Tracing of Microbial Activities.....	242
METHODS.....	244
Comparison of Protein Assay Methodologies.....	244
RESULTS AND DISCUSSION.....	246
Effects of Growth Conditions on Chemical Composition of	
<u>Nannochloris</u> sp.....	246
Interspecies Comparisons of Chemical Composition	249
Kinetics of Isotope Incorporation: Agreement Between Predicted and	
Observed Carbon Incorporation Rates.....	256
High Light Adapted Steady State Cultures of Nannochloris sp.:	
Isotope Kinetics at High and Low Relative Growth Rates.....	258

TABLE OF CONTENTS (continued)

POC and PPAA.....	258
Carbon Incorporation into Individual	
Protein Amino Acids.....	260
Elucidation of Nitrogen Depletion Effects Using Valine	
as a Model Protein Amino Acid.....	296
Comparisons of Carbon Incorporation and Net Protein Synthesis	
Rates in <u>Nannochloris</u> sp. During Growth under Combined	
Conditions NH ₄ ⁺ -limitation and Low Light.....	302
POC Predicted versus Observed Production.....	302
Total PPAA Carbon Predicted versus Observed	
Production.....	302
Individual Protein Amino Acid Observed versus	
Predicted Carbon Incorporation.....	302
Protein Turnover.....	318
Comparisons of Observed and Predicted Carbon Incorporation in	
Other Species of Marine Algae.....	324
Observed and Predicted Carbon Incorporation Into	
Particulate Organic Carbon.....	324
PPAA Observed versus Predicted Carbon Incorporation.....	324
Observed versus Predicted Carbon Incorporation into	
Individual Protein Amino Acids.....	325
<u>Chattonella luteus</u>	325
<u>Chroomonas salina</u>	337
<u>Chaetoceros simplex</u>	343
CONCLUSIONS.....	352

TABLE OF CONTENTS (continued)

CHAPTER 5. PRIMARY PRODUCTION OF PARTICULATE ORGANIC CARBON AND PARTICULATE PROTEIN AMINO ACIDS IN SALT POND, MA: THE RELATIONSHIP TO THE COMPOSITION OF PARTICULATE ORGANIC MATTER.....	361
INTRODUCTION.....	363
RESULTS AND DISCUSSION.....	370
Compositional Dynamics of Particulate Organic Matter in a Well Mixed Euphotic System.....	370
Incubation Procedures.....	378
Primary Production of Particulate Organic Carbon.....	380
Integrated POC Production Estimates.....	381
Particulate Protein Amino Acid Production.....	393
Relationship Between Production and Concentration.....	404
Prebloom Comparisons of POC and PPAA Dynamics.....	405
POC and PPAA Dynamics During Bloom.....	419
Production Characteristics and Population Metabolism.....	421
Net Removal of PPAA Relative to POC During the Bloom.....	427
CONCLUSIONS.....	432
REFERENCES.....	434
BIOGRAPHY.....	452

LIST OF FIGURES

	Page
Figure 2-1. Schematic of continuous culture system.....	54
Figure 2-2. Representative chromatogram showing elution pattern of dansylated amino acids separation by reverse phase high pressure liquid chromatography.....	74
Figure 2-3. Schematic of incubation system used in Salt Pond 1982 experiments.....	86
Figure 2-4. Schematic of single chamber SID (sampler incubation device).....	88
Figure 2-5. Schematic of dual chamber hydraulically operated SID....	90
Figure 3-1. Proportions of intracellular free amino acid carbon associated with individual amino acids of NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp. at high and low relative growth rates.....	108
Figure 3-2. Time course of the relative specific activity of intracellular free amino acid carbon in batch and NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	118
Figure 3-3. Time course of the relative specific activity of intracellular free glutamate at high and low relative growth rates in NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	120
Figure 3-4. Extended time course of the relative specific activity of intracellular free glutamate in batch and NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	122
Figure 3-5. Time course of the relative specific activity of intracellular free glutamine in batch and NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	124
Figure 3-6. Time course of the relative specific activity of intracellular free glycine + alanine in batch and NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	126
Figure 3-7. Time course of the relative specific activity of intracellular free aspartate in batch and NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	128
Figure 3-8. Time course of the relative specific activity of intracellular free valine in batch and NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	130

	LIST OF FIGURES (continued)	Page
Figure 3-9.	Time course of the relative specific activity of intracellular free proline in batch and NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	134
Figure 3-10.	Simplified diagram showing the major pathways associated with the biosynthesis of glycine, serine, glutamine, glutamate, alanine and aspartate.....	136
Figure 3-11.	Time course of the relative specific activity of intracellular free glutamate and nascent protein glutamate in batch and NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	140
Figure 3-12.	Time course of the relative specific activity of intracellular free glutamate and nascent protein glutamate in an NH_4^+ -limited steady state culture of <u>Nannochloris</u> sp. at low relative growth rate.....	142
Figure 3-13.	Time course of relative specific activity of nascent protein glutamate at high and low relative growth rate in NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	146
Figure 3-14.	Time course of the relative specific activity of intracellular free valine and nascent protein valine in an NH_4^+ -limited steady state culture of <u>Nannochloris</u> sp. at high relative growth rate.....	148
Figure 3-15.	Time course of the relative specific activity of intracellular free aspartate and nascent protein aspartate in an NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp. at high relative growth rate.....	150
Figure 3-16.	Time course of the relative specific activity of intracellular free valine and nascent protein valine in an NH_4^+ -limited steady state culture of <u>Nannochloris</u> sp. at low relative growth rate.....	152
Figure 3-17.	Time course of the relative specific activity of nascent protein proline + arginine in NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp. at high and low relative growth rates.....	156
Figure 3-18.	Time course of distribution of incorporated inorganic ^{14}C relative to equilibrium distribution among subcellular fractions in a batch culture of <u>Nannochloris</u> sp.....	160

LIST OF FIGURES (continued)

Page

- Figure 3-19. Time course of the distribution of incorporated inorganic ^{14}C in alcohol soluble material from batch cultures of Nannochloris sp..... 162
- Figure 3-20. Time course of the distribution of incorporated inorganic ^{14}C in intracellular free amino acids in NH_4^+ -limited steady state cultures of Nannochloris sp. at high and low relative growth rates.. 164
- Figure 3-21. Proportions of intracellular free amino acid carbon and ^{14}C associated with individual amino acids of NH_4^+ -limited steady state cultures of Nannochloris sp. at high and low relative growth rates..... 172
- Figure 3-22. Time course of the distribution of incorporated inorganic ^{14}C among subcellular fractions in NH_4^+ -limited steady state cultures of Nannochloris sp. at high and low relative growth rates.. 174
- Figure 3-23. Distribution of total cellular carbon and total ^{14}C uptake among intracellular free amino acids and protein in NH_4^+ -limited steady state cultures of Nannochloris sp. at high and low relative growth rates.. 176
- Figure 3-24. Increase in intracellular free glutamine concentrations in response to NH_4Cl enrichment (200 micromolar) of an NH_4^+ -limited steady state culture of Nannochloris sp..... 182
- Figure 3-25. Increase in intracellular free glutamate concentrations in response to NH_4Cl enrichment (200 micromolar) of an NH_4^+ -limited steady state culture of Nannochloris sp..... 184
- Figure 3-26. Increase in intracellular free proline concentrations in response to NH_4Cl enrichment (200 micromolar) of an NH_4^+ -limited steady state culture of Nannochloris sp..... 186
- Figure 3-27. Increase in intracellular free aspartate concentrations in response to NH_4Cl enrichment (200 micromolar) of an NH_4^+ -limited steady state culture of Nannochloris sp..... 188
- Figure 3-28. Increase in intracellular free alanine concentrations in response to NH_4Cl enrichment (200 micromolar) of an NH_4^+ -limited steady state culture of Nannochloris sp..... 194

	LIST OF FIGURES (continued)	Page
Figure 3-29.	Time course of the relative specific activity of total intracellular free amino acid carbon in control and enriched NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	196
Figure 3-30.	Time course of total inorganic ^{14}C uptake by control and enriched NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	202
Figure 3-31.	Time course of the incorporation of inorganic ^{14}C into subcellular fractions in control and enriched NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp. growing at 24°C	204
Figure 3-32.	Time course of the incorporation of inorganic ^{14}C into subcellular fractions in control and enriched NH_4^+ -limited steady state cultures of <u>Dunaliella tertiolecta</u> growing at 24°C	216
Figure 3-33.	Time course of the incorporation of inorganic ^{14}C into subcellular fractions in control and enriched NH_4^+ -limited steady state cultures of <u>Dunaliella tertiolecta</u> growing at 16°C	218
Figure 3-34.	Time course of total inorganic ^{14}C uptake by control and enriched NH_4^+ -limited steady state cultures of <u>Dunaliella tertiolecta</u> growing at 16°C	220
Figure 3-35.	Time course of the distribution of incorporated inorganic ^{14}C among subcellular fractions in control and enriched NH_4^+ -limited steady state cultures of <u>Dunaliella tertiolecta</u> growing at 12°C	222
Figure 3-36.	Time course of total inorganic ^{14}C uptake by control and enriched NH_4^+ -limited steady state cultures of <u>Dunaliella tertiolecta</u> growing at 12°C	224
Figure 3-37.	Time course of total inorganic ^{14}C uptake by control and enriched NH_4^+ -limited steady state cultures of <u>Thalassiosira weissflogii</u> growing at 16°C	226
Figure 3-38.	Time course of total inorganic ^{14}C uptake by control and enriched NH_4^+ -limited steady state cultures of <u>Thalassiosira weissflogii</u> growing at 12°C	228
Figure 3-39.	Time course of the distribution of incorporated inorganic ^{14}C among subcellular fractions in control and enriched NH_4^+ -limited steady state cultures of <u>Thalassiosira weissflogii</u> growing at 16°C	230

LIST OF FIGURES (continued)

Page

- Figure 3-40. Time course of the distribution of incorporated inorganic ^{14}C among subcellular fractions in control and enriched NH_4^+ -limited steady state cultures of Thalassiosira weissflogii growing at 12°C . 232
- Figure 4-1. Comparisons of observed total ^{14}C uptake rates with theoretical particulate organic carbon production rates of high light NH_4^+ -limited steady state cultures of Nannochloris sp..... 262
- Figure 4-2. Comparisons of observed particulate protein amino acid isotope incorporation rates with theoretical rates in high light NH_4^+ -limited steady state cultures of Nannochloris sp..... 264
- Figure 4-3. Observed and predicted 1.5 hour time courses of isotope incorporation into particulate protein amino acids where flow was interrupted during the incubation of high light NH_4^+ -limited steady state cultures of Nannochloris sp..... 266
- Figure 4-4. Observed and predicted 1.0 hour time courses of isotope incorporation into particulate protein amino acids where flow was not interrupted during the incubation of a high light NH_4^+ -limited steady state culture of Nannochloris sp..... 268
- Figure 4-5. Observed and predicted 3.0 hour time courses of isotope incorporation into particulate protein amino acids where flow was interrupted during the incubation of high light NH_4^+ -limited steady state cultures of Nannochloris sp..... 270
- Figure 4-6. Observed and predicted 6.0 hour time courses of isotope incorporation into particulate protein amino acids where flow was not interrupted during the incubation of a high light NH_4^+ -limited steady state culture of Nannochloris sp..... 272
- Figure 4-7. Observed and predicted 66 hour time courses of isotope incorporation into particulate protein amino acids where flow was not interrupted during the incubation of a high light NH_4^+ -limited steady state culture of Nannochloris sp..... 274
- Figure 4-8. Theoretical and observed isotopic carbon incorporation rates into alanine, glycine, glutamate, and aspartate in protein of high light NH_4^+ -limited steady state cultures of Nannochloris sp..... 276

	LIST OF FIGURES (continued)	Page
Figure 4-9.	Simplified diagram showing major pathways associated with the biosynthesis of glycine, serine, glutamine, glutamate, alanine, and aspartate.....	278
Figure 4-10.	Observed and predicted time courses of isotope incorporation into glycine, alanine, glutamate, and aspartate in protein of high light NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	280
Figure 4-11.	Diagram showing proposed routes for intrachloroplast production and utilization of phosphoenol pyruvate and pyruvate.....	282
Figure 4-12.	Pathways associated with the biosynthesis of tryptophan, tyrosine, and phenylalanine.....	286
Figure 4-13.	Observed and predicted time courses of isotope incorporation into phenylalanine in protein of high light NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	288
Figure 4-14.	Pathways associated with the biosynthesis of methionine, threonine, isoleucine, lysine, valine, and leucine.....	290
Figure 4-15.	Theoretical and observed rates of isotopic carbon incorporation into isoleucine + leucine, proline + arginine, valine, phenylalanine, lysine, and threonine in protein of high light NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	292
Figure 4-16.	Observed and predicted time courses of isotope incorporation into threonine, valine, isoleucine + leucine, and lysine in protein of high light NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	294
Figure 4-17.	Comparisons of observed isotopic carbon incorporation into valine in protein with that predicted on the assumption that all carbon flux through the free intracellular valine pool was associated with synthesis of protein valine.....	300
Figure 4-18.	Observed total ^{14}C uptake rates and theoretical particulate organic carbon production rates of low light NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	304
Figure 4-19.	Theoretical and observed rates of isotopic carbon incorporation into particulate protein amino acids in low light NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	306

LIST OF FIGURES (continued)

Page

- Figure 4-20. Observed and predicted time courses of isotope incorporation into particulate protein amino acids of low light NH_4^+ -limited steady state cultures of Nannochloris sp..... 308
- Figure 4-21. Theoretical and observed rates of isotopic carbon incorporation into alanine, glycine, glutamate, and aspartate in protein of low light NH_4^+ -limited steady state cultures of Nannochloris sp..... 310
- Figure 4-22. Observed and predicted time courses of isotope incorporation into glycine, alanine, glutamate, and aspartate in protein of low light NH_4^+ -limited steady state cultures of Nannochloris sp..... 312
- Figure 4-23. Theoretical and observed rates of isotopic carbon incorporation into isoleucine + leucine, proline, phenylalanine, lysine, and threonine in protein of low light NH_4^+ -limited steady state cultures of Nannochloris sp..... 314
- Figure 4-24. Observed and predicted time courses of isotope incorporation into threonine, isoleucine + leucine, phenylalanine, and lysine in protein of low light NH_4^+ -limited steady state cultures of Nannochloris sp..... 316
- Figure 4-25. Observed total ^{14}C uptake and theoretical particulate organic carbon production rates of high light NH_4^+ -limited steady state cultures of four species of marine phytoplankton..... 326
- Figure 4-26. Theoretical and observed rates of isotopic carbon incorporation into particulate protein amino acids of high light NH_4^+ -limited steady state cultures of four species of marine phytoplankton..... 328
- Figure 4-27. Observed and predicted time courses of isotope incorporation into particulate protein amino acids of high light NH_4^+ -limited steady state cultures of Chattonella luteus, Chroomonas salina, and Chaetoceros simplex..... 330
- Figure 4-28. Theoretical and observed rates of isotopic carbon incorporation into alanine, glycine, glutamate, aspartate, and serine in protein of high light NH_4^+ -limited steady state cultures of Chattonella luteus, Chroomonas salina, and Chaetoceros simplex..... 332
- Figure 4-29. Observed and predicted time courses of isotope incorporation into glycine, alanine, glutamate, and aspartate in protein of a high light NH_4^+ -limited steady state culture of Chattonella luteus 334

LIST OF FIGURES (continued)

Page

Figure 4-30.	Theoretical and observed rates of isotopic carbon incorporation into isoleucine + leucine, valine + arginine, proline, phenylalanine, lysine, and threonine in protein of high light NH_4^+ -limited steady state cultures of <u>Chattonella luteus</u> , <u>Chroomonas salina</u> , and <u>Chaetoceros simplex</u>	338
Figure 4-31.	Observed and predicted time courses of isotope incorporation into threonine, valine + arginine, isoleucine + leucine, lysine, phenylalanine, and proline in protein of high light NH_4^+ -limited steady state cultures of <u>Chattonella luteus</u>	340
Figure 4-32.	Observed and predicted time courses of isotope incorporation into glycine, alanine, glutamate, and aspartate in protein of high light NH_4^+ -limited steady state cultures of <u>Chroomonas salina</u>	344
Figure 4-33.	Observed and predicted time courses of isotope incorporation into threonine, valine + arginine, isoleucine + leucine, lysine, phenylalanine, and proline in protein of high light NH_4^+ -limited steady state cultures of <u>Chroomonas salina</u>	346
Figure 4-34.	Observed and predicted time courses of isotope incorporation into glycine, alanine, glutamate, and aspartate in protein of high light NH_4^+ -limited steady state cultures of <u>Chaetoceros simplex</u>	348
Figure 4-35.	Observed and predicted time courses of isotope incorporation into threonine, valine + arginine, isoleucine + leucine, lysine, phenylalanine, and proline in protein of high light NH_4^+ -limited steady state cultures of <u>Chaetoceros simplex</u>	350
Figure 5-1.	Processes involved in production and removal of organic matter.....	364
Figure 5-2.	Simplified diagram illustrating the influence of primary inputs and net removal of organic matter on particulate organic matter composition.....	368
Figure 5-3.	Depth profiles of chlorophyll <u>a</u> on days during which incubations were conducted in Salt Pond.....	372
Figure 5-4.	Depth profiles of specific gravity during study period in Salt Pond.....	374
Figure 5-5.	Representative incubation (5 Aug 83) showing particulate organic carbon production in conjunction with surface light regime.....	382

	LIST OF FIGURES (continued)	Page
Figure 5-6.	Particulate organic carbon production during sampling period shown in relationship to dynamics of chlorophyll <u>a</u> and particulate organic carbon.....	384
Figure 5-7.	Diel light regime from 1982 during the same time of year as the period of study in Salt Pond.....	386
Figure 5-8.	Time course of carbon fixation by surface sample from Salt Pond incubated during midday under natural sunlight attenuated to different intensities.....	394
Figure 5-9.	Proportion of surface light reaching 1 meter during study period in Salt Pond.....	396
Figure 5-10.	Particulate organic carbon, chlorophyll <u>a</u> , and particulate protein amino acid concentrations in the Salt Pond mixed layer during the study period.....	406
Figure 5-11.	Comparison of observed concentrations of particulate organic carbon and particulate protein amino acids to the projected concentrations based on mean production rates.....	408
Figure 5-12.	Comparison of production and concentration ratios of particulate protein amino acids and protein glycine relative to particulate organic carbon.....	410
Figure 5-13.	Orthophosphate, ammonium, nitrate, and nitrite concentrations at the one meter sampling depth in relationship to particulate organic carbon and chlorophyll <u>a</u> concentrations during the period of study in Salt Pond.....	422
Figure 5-14.	Particulate organic carbon to chlorophyll <u>a</u> ratios during the study period in Salt Pond.....	424
Figure 5-15.	Proportions of total ¹⁴ C incorporation associated with subcellular fractions of natural phytoplankton populations during the study period in Salt Pond.....	428

LIST OF TABLES

	Page
Table 2-1. Mineral concentrations in artificial seawater.....	49
Table 2-2. Concentrations of nutrients and trace elements in standard culture media.....	50
Table 2-3. Species list of marine algae obtained from the culture collection of marine phytoplankton.....	51
Table 2-4. Outline of fractionation procedures.....	62
Table 2-5. Comparison of results obtained using different fractionation procedures.....	63
Table 2-6. Response factors for integrated absorbance by dansylated amino acids at 254nm.....	66
Table 2-7. Analytical variations (95% confidence intervals) for individual amino acids resulting from the combination of errors associated with injection, storage, variation in derivatization efficiency, and peak integration.....	67
Table 2-8. Comparison of derivatization incubation periods of one and four hours at 37°C.....	68
Table 2-9. Correlations between response factors and molar ratio of amino groups/dansyl chloride (range 0.001-0.12) in the derivatization mixture.....	69
Table 2-10. Dependence of response factor on time of storage at -6°C.....	70
Table 2-11. Proportion of labeled free amino acids recovered in subcellular fractions.....	76
Table 2-12. Free amino acid recovery in aqueous alcohol fraction....	77
Table 2-13. Recovery of amino acids from hydrolysis.....	78
Table 2-14. Comparison of different methodologies for measurement of protein in <u>Nannochloris</u> sp.....	80
Table 2-15. Recovery of protein amino acids after storage in 80% methanol at -6°C.....	81
Table 2-16. Stability of subcellular fractions with storage at -20°C relative to storage in liquid N ₂	83

LIST OF TABLES (continued)

Page

Table 3-1.	Proportions of total cellular carbon and nitrogen in free amino acids of <u>Nannochloris</u> sp. as a function of relative growth rate.....	105
Table 3-2.	Proportions of total cellular carbon and nitrogen in protein of <u>Nannochloris</u> sp. as a function of relative growth rate.....	106
Table 3-3.	Proportions of the intracellular free amino acid carbon pool associated with individual amino acids as a function of relative growth rate of <u>Nannochloris</u> sp.....	107
Table 3-4.	Nitrogen specific turnover rates of nitrogen transport mediators in relationship to relative growth rate of <u>Nannochloris</u> sp.....	113
Table 3-5.	Relative specific activities of nascent protein amino acids in <u>Nannochloris</u> sp.....	154
Table 3-6.	Distributions of total cellular carbon and total ¹⁴ C uptake among the intracellular free amino acid pool of <u>Nannochloris</u> sp. at high relative growth rate.....	166
Table 3-7.	Distributions of total cellular carbon and total ¹⁴ C uptake among free amino acids in <u>Nannochloris</u> sp. at low relative growth rate.....	167
Table 3-8.	Proportions of intracellular free amino acid carbon and ¹⁴ C associated with individual amino acids of <u>Nannochloris</u> sp. at high relative growth rate.....	169
Table 3-9.	Proportions of intracellular free amino acid carbon and ¹⁴ C associated with individual amino acids of <u>Nannochloris</u> sp. at low relative growth rate.....	170
Table 3-10.	Proportions of the intracellular free amino acid carbon pool associated with individual amino acids in control and NH ₄ ⁺ enriched cultures of <u>Nannochloris</u> sp.....	190
Table 3-11.	Comparisons of intracellular free amino acid concentrations in control and NH ₄ ⁺ enriched cultures of <u>Nannochloris</u> sp.....	191
Table 3-12.	Relative specific activities of intracellular free amino acids in control and enriched cultures of <u>Nannochloris</u> sp.....	199

	LIST OF TABLES (continued)	Page
Table 3-13.	Distributions of total ^{14}C uptake among intracellular free amino acids in control and enriched cultures of <u>Nannochloris</u> sp.....	206
Table 3-14.	Distributions of ^{14}C uptake within the intracellular free amino acids of control and enriched cultures of <u>Nannochloris</u> sp.....	208
Table 3-15.	Intracellular free amino acid concentrations in control and enriched cultures of <u>D. tertiolecta</u>	213
Table 4-1.	Bulk chemical composition parameters for high light batch cultures of <u>Nannochloris</u> sp.....	245
Table 4-2.	Bulk chemical composition parameters for high light NH_4^+ -limited steady state growth of <u>Nannochloris</u> sp.	247
Table 4-3.	Bulk chemical composition parameters for low light batch and NH_4^+ -limited steady state growth of <u>Nannochloris</u> sp.....	248
Table 4-4.	Bulk chemical composition parameters for high light NH_4^+ -limited steady state cultures of selected marine algae.....	251
Table 4-5.	Bulk chemical composition parameters for high light batch and NH_4^+ -limited steady state cultures of <u>Chattonella luteus</u>	252
Table 4-6.	Cellular chemical composition parameters of high light batch and NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	253
Table 4-7.	Cellular chemical composition parameters of low light batch and NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	254
Table 4-8.	Cellular chemical composition parameters of NH_4^+ -limited steady state cultures of selected marine algae.....	255
Table 4-9.	Turnover rate constants for light and NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	322
Table 4-10.	Intracellular free amino acid carbon concentrations in high light NH_4^+ -limited steady state cultures of <u>Nannochloris</u> sp.....	355
Table 4-11.	Relative abundance of glycine and alanine in protein of different algal sources.....	357

	LIST OF TABLES (continued)	Page
Table 4-12.	Relative abundance of glycine and alanine in protein....	358
Table 4-13.	Comparison of observed, predicted and extrapolated protein carbon incorporation rates.....	359
Table 5-1.	Dissolved organic carbon concentrations from ground water, rain water, and Salt Pond surface water.....	376
Table 5-2.	Comparison of inorganic ^{14}C fixation as determined by acidification/sparging and filtration.....	388
Table 5-3.	Coefficients of variation for replicate production estimates.....	389
Table 5-4.	Particulate organic carbon production rates for consecutive incubations in Salt Pond, summer 1983.....	390
Table 5-5.	Product-moment correlation coefficients.....	391
Table 5-6.	Simulated <u>in situ</u> production rates for Salt Pond, 1982..	398
Table 5-7.	Light extinction in the mixed layer of Salt Pond, summer 1983.....	399
Table 5-8.	Surface incident and atmosphere corrected solar radiation: 10 Aug - 1 Sept 83, Salt Pond.....	400
Table 5-9.	Particulate protein amino acid production rates.....	401
Table 5-10.	Proportion of glycine and alanine carbon in protein recovered from hydrolysis.....	403
Table 5-11.	Production and net removal of POC and PPAA.....	412
Table 5-12.	Changes in dissolved inorganic and organic carbon, dissolved organic nitrogen, and NH_4^+ concentrations below three meters in Salt Pond, summer 1983.....	414
Table 5-13.	Dark uptake rates of inorganic ^{14}C : Salt Pond, summer 1983.....	417
Table 5-14.	Comparisons of morning and midday production and composition ratios, Salt Pond, 1 Sept 83.....	420

CHAPTER 1. GENERAL INTRODUCTION

THE COMPOSITIONAL DYNAMICS OF ORGANIC MATTER IN AQUATIC ENVIRONMENTS

Planktonic photoautotrophs account for the greatest proportion of primary productivity in the world's oceans (e.g. Whittle, 1977; Woodwell et al., 1978; Peterson, 1980; Neinhuis, 1981). Through their ability to synthesize organic matter from primary substrates using the sun's energy, phytoplankton provide the source of energy and building blocks for growth of virtually all other marine organisms. Yet phytoplankton carbon is a small proportion of the ocean's total particulate carbon (Cauwet, 1981; Neinhuis, 1981). Even if only the euphotic layer particulate organic matter is considered, phytoplankton carbon may often account for less than half the particulate carbon, especially in oligotrophic systems (e.g. Hobson, 1971; Hobson et al., 1973; Beers et al., 1975; Eppley et al., 1977; Wangersky, 1977; Whittle, 1977; Sharp et al., 1980).

The processes whereby phytoplankton derived primary organic matter is converted, either directly or indirectly, into other organisms of the marine food chain and into non-living reservoirs of organic matter have been the subjects of study since the very earliest stages of oceanographic research. Despite the intensity and duration of these investigations, and largely as a result of methodological limitations, researchers have only just begun to unravel the complexities of organic matter transformations and the roles of biological processes which mediate them.

The remainder of this introduction reviews the major processes which influence the composition of organic matter in the oceans as they are currently understood, and specifically considers the relative importance of primary inputs by phytoplankton in influencing the particulate organic matter composition in surface waters of the oceans. Evidence is cited

suggesting that processes other than primary production of organic matter may significantly influence particulate organic matter composition, and lead to ambiguities in attempts to use chemical and biochemical measurements as assays of phytoplankton physiological state and biosynthetic activity.

BACKGROUND

The Marine Food Chain and the Composition of Organic Matter

The composition of particulate organic matter is a function of the interactive roles of microbial autotrophic and heterotrophic processes which are the basis for the marine food web. Recent investigations have begun to recognize the importance of the behaviour of individual organisms and individual organic compounds in controlling this level of marine trophodynamics. The classical view of the marine food chain is that phytoplankton pass through herbivores on to primary carnivores, and it is through this stepwise transfer of organic matter that production of commercially important species is realized (e.g. Issacacs, 1969; Anikouchine and Sternberg, 1973; Steele, 1974). This paradigm has since been re-examined and the importance of non-phytoplanktonic microorganisms in food web processes is now being evaluated (Pomeroy, 1974; Sieburth et al., 1977, 1978; Landry, 1977; Joiris, 1977; Porter et al., 1979; Sorokin, 1981; Williams, 1981; Azam et al., 1983). One of the major problems plaguing the classic paradigm is that it fails to account for the fate of the large portion of primary organic matter which passes directly to the dissolved organic pool (Williams, 1981). In the rest of this section, I discuss how production and utilization of dissolved organic matter can influence particulate organic matter composition.

The highest concentration of dissolved organic carbon in the oceans is in the euphotic zone (e.g. Menzel and Ryther, 1970; Handa, 1970; Degens, 1970; Gagosian and Lee, 1981) where the vast majority of primary production occurs. Dissolved organic matter may be produced by a variety of mechanisms including phytoplankton excretion (e.g. Fogg, 1962, 1977; Hellebust, 1965; Sharp, 1977; Mague et al., 1980), cell autolytic processes (e.g. Golterman, 1964; Bell and Mitchell, 1972; Dawson and Duursma, 1981), release of soluble material during ingestion (e.g. Whittle, 1977), and zooplankton excretion (Webb and Johannes, 1967). Williams (1981) cited evidence that as much as 50% of primary production may pass through the dissolved organic pool. Poulet and Martin-Jezequel (1983) and Hammer and Brockman (1983) observed highest release of dissolved free amino acids in algal cultures nearing stationary phase. This would suggest that higher proportions of primary organic matter may be transferred to the dissolved organic matter in algal populations which are stressed (e.g. the latter portion of a bloom).

A number of studies have observed significant increases in dissolved organic compounds associated with blooms. These include amino acids (Jeffries, 1969; Ittekkot, 1982), fatty acids (Jeffries, 1970; Kattner et al., 1983), and carbohydrates (Handa, 1970; Ittekkot et al., 1981, 1982). Stress related compensations in algal metabolism result in significant changes in biosynthetic patterns (e.g. Syrett, 1953; Thomas and Krauss, 1955; Fogg, 1956, 1959; Handa, 1969; Caperon and Meyer, 1972; Myklestad and Haug, 1972; Thomas and Dodson, 1972; Rhee, 1973, 1978; Morris et al., 1974; Conover, 1975; Harrison et al., 1976; Myklestad, 1977; Edge and Ricketts, 1977; Laws and Wong, 1978; Goldman and Peavey, 1979; Goldman, 1980; Laws and Bannister, 1980; Hitchcock, 1981; Shifrin

and Chisholm, 1981; Konopka and Schnur, 1981). However, the effect of such changes in algal biosynthetic patterns on particulate organic matter composition could be obscured by production and utilization of dissolved organic material.

Investigations of the relative contribution of specific organic compounds to particulate organic matter indicate selective depth-dependent loss of biogenic materials such as amino acids (Degens, 1970; Siezen and Mague, 1978; Lee and Cronin, 1982, 1984; Lee et al., 1983), lipid (Wakeham et al., 1980; Gagosian et al., 1982; Lee et al., 1983), and carbohydrates (Handa, 1970). Lee and Cronin (1982) constructed a model based on the composition of sediment trap material showing 80% of the particulate amino acids produced during photosynthesis are decomposed or released in the upper 14m in a Peru upwelling area. Relative depth dependent variations among particulate amino acids are different for different amino acids, suggesting different production mechanisms and susceptibility to heterotrophic activity (Siezen and Mague, 1978; Lee and Cronin, 1982, 1984).

Bacterial growth can be supported by both dissolved and particulate organic material. An operational distinction of particulate material is any substance retained on an 0.45 micron filter. This is an adequate definition for the purposes of this discussion, as it is likely that larger material is an obligatory intermediate in the conversion of primary substrates to upper trophic levels. On the other hand, dissolved organic matter accounts for by far the largest proportion of organic material in the marine environment (Neinhaus, 1981; Cauwet, 1981). It has been suggested that bacterial utilization of dissolved organic material and subsequent grazing of the bacteria by microzooplankton may

provide a route for the dissolved organic reservoir to re-enter the particulate organic phase, and hence the food web (e.g. Beers and Stewart, 1969 in Landry, 1977; Sorokin, 1981; Williams, 1981; Azam et al., 1983). Such a mechanism for production of particulate organic material could potentially influence particulate organic matter composition independently of primary production.

There is substantial evidence suggesting that bacteria may indeed efficiently assimilate specific dissolved organic substrates including glucose, (Andrews and Williams, 1971; Derenbach and Williams, 1974), amino acids (Hobbie et al., 1968; Andrews and Williams, 1971; Crawford et al., 1974; Williams et al., 1976; Dawson and Gocke, 1978; Hollibaugh et al., 1980; Williams, 1981; Hagström et al., 1984), and protein (Hollibaugh and Azam, 1983). Direct utilization by bacteria of dissolved organic matter released by phytoplankton has been demonstrated using selective filtration of natural samples incubated with $^{14}\text{C-HCO}_3$ (Azam and Holm-Hansen, 1973; Derenbach and Williams, 1974; Larsson and Hagström, 1979; Cole et al., 1982). Although the selective filtration methods used in these studies result in ambiguities in production and utilization rates (see critique in Smith and Horner, 1981), bacterial incorporation of label was consistently observed. A variety of attempts have been made to distinguish the production and heterotrophic utilization of photosynthesized dissolved organic matter including kinetic analyses of isotope distribution between dissolved and particulate phases (Wiebe and Smith, 1977; Bell, 1980, 1983; Bell and Sakshaug, 1980; Li, 1983) and use of antibiotics (e.g. Jensen, 1984).

Bell and Mitchell (1972) have shown that microbial association with algal cultures seems to be greatest as the algal culture nears

senescence, and it is at this time that, through cell autolysis, the largest amounts of dissolved organic matter are released. Other evidence for close coupling between production and utilization is the diel periodicity in concentrations of some dissolved substrates. Diel variations have been observed in the concentrations of amino acids (Mopper and Lindroth, 1982) and other compounds (e.g. Sieburth et al., 1977). Consistent with this was the observations by Hammer and Brockman (1983) of a rhythmic release of dissolved free amino acids in partially synchronized outdoor cultures of Thalassiosira rotula. Harvey (1983) also observed rapid fluctuations in dissolved carbohydrates in the New York Bight and attributed it to rapid cycles of production and utilization. Ittekkot (1982) noted that changes in dissolved carbohydrates and amino acids during a spring plankton bloom in the northern North Sea were dissimilar when both were analyzed in the same samples, demonstrating the unique behaviour of different types of organic material. Mopper and Lindroth (1982) suggested on the basis of their studies of diel variations in dissolved free amino acids in the Baltic Sea that the differential behaviour of individual amino acids provided insights as to the production and utilization processes which dominated during different periods in a diel cycle.

Because processes controlling the composition and distribution of bulk organic matter in seawater involve differential rates of production and consumption of individual organic compounds, understanding these processes and their influence on particulate organic matter composition will require rate measurements of individual classes of organic material. The relatively low concentrations of identified biogenic compounds shows that organic carbon is being removed at a rate comparable

to production (Gagosian and Lee, 1981). Although determinations of concentrations of compounds by themselves cannot provide estimates of rates of production or utilization, the rapid depth- and time-dependent fluctuations of these biogenic compounds is consistent with an interactive role of autotrophic and heterotrophic processes in influencing the composition and cycling of dissolved and particulate organic material in the ocean.

Biochemical Cycles and the Distribution of Organic Matter

Menzel and Ryther (1970) noted that it is "increasingly obvious that biochemical cycling of organic matter is not closely coupled with that of plants and animals which produce and immediately consume organic matter in the familiar food cycle of the sea". Recycling of organic matter relies on the role of non-phytoplanktonic organisms to transform the primary organic matter back to substrates utilizable by phytoplankton. Heterotrophic organisms may significantly influence particulate organic composition simply by the presence of their biomass. Perhaps an even greater impact on composition may be through their activities as catalysts of organic matter transformations independent of algal metabolism.

The recycling of a variety of materials in euphotic systems has been investigated. Estimates of losses of organic material from the euphotic layer indicate that in oligotrophic systems, as little as 1- 10% of the primary production escapes into the deep ocean (Bishop, 1977, 1978; Whittle, 1977; Parsons et al., 1977; Harrison, 1980). Rapid cycles of nutrient utilization and remineralization have been demonstrated (e.g. Glibert et al., 1982; Caperon et al., 1979; Harrison et al., 1978;

Harrison, 1980; Garside and Glibert, 1984; Garside, 1984). Eppley and Peterson (1979) argue that 80% of nitrogen used by open ocean phytoplankton is recycled. Williams (1981), citing evidence from various works, suggested that zooplankton metabolism cannot account for the majority of nitrogen regeneration and bacteria may not be primarily responsible for remineralization since they tend to assimilate organic substrates with high efficiency. Although others have shown that bacteria are capable of remineralizing nutrients from decomposing algae, turnover of particulate matter in seawater is apparently more rapid than possible from microbial activity alone (Harrison, 1980).

These observations have stimulated interest in the role of microzooplankton in remineralization of organic matter. Johannes (1965) demonstrated that remineralization is enhanced by the presence of both bacteria and protozoa, compared to just bacteria alone. Evidence as to the importance of these microorganisms in organic matter cycling is steadily accumulating (e.g. Johannes, 1965; Stout, 1980; Sorokin 1981; Williams, 1981; Banse, 1982; Azam et al., 1983; Azam and Ammerman, 1984).

The inclusion of bacteria and microzooplankton in chemical analyses of particulate organic material could bias the results since their composition may be significantly different from phytoplankton (e.g. Beers, 1966; Banse, 1974, 1977). Frequent efforts have been made to estimate the proportion of particulate carbon associated with phytoplankton and other living and nonliving reservoirs using microscopically and electronically derived particulate volumes to estimate carbon content (Strathman, 1967; Zeitzchel, 1970; Hobson et al., 1971; Hobson et al., 1973; Beers et al., 1975; Whittle, 1977; Eppley et

al., 1977; Sharp et al., 1980; Sorokin, 1981; Hendrikson et al., 1982; Holligan et al., 1984). Some of these studies have been criticized since they are based on carbon to volume ratios which are clearly subject to variations as a result of differences in species and physiological state (Chan, 1978, 1980), and preservation methods can destroy or morphologically alter organisms (e.g. Paerl, 1984). Nevertheless, these studies indicate almost exclusively that phytoplankton do not account for the majority of particulate organic material, and heterotrophic biomass may be comparable to phytoplankton (e.g. Sorokin, 1981; Caron, pers. comm.). Frequently, the detrital material (generally including some bacteria) accounts for the majority of particulate carbon (e.g. Zeitzschel, 1970; Beers et al., 1975).

The importance of detrital material as a reservoir of particulate organic material and a potential food source (e.g. Alldredge, 1979; Wangersky, 1977) has provoked interest in its chemical composition and mechanisms of formation. Suggested modes of formation include aggregation of colloidal material associated with collapse of bubbles (e.g. Baylor and Sutcliffe, 1963; Johnson, 1976), bacterial clumping (e.g. Pearl, 1974; Sorokin, 1981), and adsorption of dissolved organic matter onto colloidal material (e.g. Wangersky, 1977; Cauwet, 1978, Gagosian and Lee, 1981). Histochemical analyses of aggregates has shown that they may contain protein and carbohydrate (Nemoto and Ishikawa, 1969; and Gordon, 1970; in Parsons et al., 1977). Fallon and Brock (1979) observed that the composition of particles during the latter portion of a bloom indicated partially autolyzed or decomposed algal cells may also be an important nonliving source of particles.

There have been a variety of attempts to chemically distinguish living particulate carbon from non-living detrital carbon. Frequently, the concentration of total particulate carbon is regressed against chlorophyll concentrations in field samples from a particular water mass over some period of time. The assumptions are that chlorophyll not associated with living plant material is rapidly degraded, so that the intercept of such a regression provides an average value for nonphytoplanktonic carbon in the particulate material, and the slope is an average value of the carbon to chlorophyll ratio of the phytoplankton population (Banse, 1977). Packard and Dortch (1975) used a similar approach comparing regressions of protein-N against chlorophyll in oceanic and upwelling communities. They estimated that anywhere from 35% (upwelling) to 80% (oceanic) of the particulate protein-N may be nonphytoplanktonic. Barlow (1982b) citing evidence that chlorophyll may be associated with nonliving particulate matter, questioned its value as a tracer of living plant material. He used glucan, a labile reserve polysaccharide in diatoms, in place of chlorophyll to estimate detrital (including bacteria) content of protein, ATP, and total carbohydrate. The validity of such extrapolations is dependent on the constancy of the relative biochemical composition of living organic matter (e.g. Steele and Baird, 1962; Donaghay et al., 1978; Karl, 1980; Goldman, 1980), and independence of the behaviour of different organic reservoirs over time (Banse 1977). Despite the limitations, the results consistently indicate a substantial amount of carbon and biogenic compounds such as protein and carbohydrate may not be associated with phytoplankton.

Wangersky (1977) suggested that only during a bloom are phytoplankton a major portion of particulate material, and that yearly cycles of particulate organic carbon and productivity are not necessarily correlated in time. Microautoradiographic techniques have shown that even if phytoplankton biomass dominates the particulate organic material, proportions of production associated with different species may not be representative of their biomass (eg. Watt, 1971; Maguire and Neill, 1971; Gutel'makher, 1973; Davenport and Maguire, 1984). The presence of significant amounts of particulate organic matter not directly associated with the active phytoplankton population raises doubts as to whether primary production is the dominant factor influencing the composition of particulate organic matter. The possibility that other processes may be more important in controlling particulate organic matter composition makes it less feasible to use the chemical and biochemical composition of particulate organic matter as an index of phytoplankton physiological state.

The Chemical Composition of Natural Populations

Phytoplankton possess the capacity for physiological interaction with their environment, which can lead to changes in their metabolism and resulting biochemical composition. Consequently, it seems reasonable to expect phytoplankton generated variations in the composition of particulate organic material. But to what extent can we attribute the composition of particulate organic material to the activities of phytoplankton? Various investigators have suggested the use of chemical ratios as an indication of a natural phytoplankton population's physiological state. The C:N ratio has been one of the most commonly

referred to in the literature largely because it is a straightforward measurement and, in the laboratory, demonstrates a reasonably uniform correlation with relative growth rate for a variety of species (Donaghay et al., 1978; Goldman et al., 1979; Goldman, 1980). However, its value as a precise indicator of the physiological state of natural populations has been criticized on the basis that it is sensitive only at low relative growth rates (Donaghay et al., 1978), and is subject to interference by nonphytoplankton material (Banse, 1974, 1977; Goldman, 1980). Similar criticisms can be raised against carbon to phosphorus ratios, and carbon to chlorophyll ratios as indices of relative growth rate (c.f. Goldman, 1980).

Another chemical ratio which has been suggested as a useful relative indicator of population physiological state is the protein/carbohydrate ratio (Myklestad and Haug, 1972; Haug et al., 1973; Barlow, 1982a,b; Barlow and Henry, 1982; Konopka, 1982). As is the case for other ratios, it is subject to interference by nonphytoplankton material. Moreover, results of its application to mixed natural populations may often be ambiguous since different species have different protein content even if they are growing under similar conditions (Chan, 1978, 1980). Konopka (1982) compared several biochemical indices in a relatively pure natural population of Oscillatoria rubescens including protein to chlorophyll, carbohydrate to protein, and RNA to protein ratios. Despite the relative uniformity of the natural community, results led to conflicting conclusions illustrating the difficulties in interpreting biochemical measurements. A fundamental criticism of such biochemical approaches is their use of colorimetric assays on natural mixtures of particulate organic material. Such assays provide an adequate relative index when

used with pure cultures in controlled laboratory situations, but are less meaningful when applied to complex organic matrices which make up particulate organic matter in natural systems (Cauwet, 1981).

An additional problem which is independent of the interference of nonphotosynthetically derived material is that diel variations in biosynthetic patterns can substantially influence the results of chemical and biochemical assays. Goldman (1980) noted that average carbon to nitrogen and carbon to chlorophyll a ratios for cultures grown in light/dark cycles were lower than values for cultures grown in constant light regimes. The discrepancy was most pronounced at relative growth rates of approximately $0.7u_{\max}$ and below, and would consequently affect interpretations of the relationship between these ratios and relative growth rate. Hitchcock (1980) investigated time-dependent variations in biochemical parameters throughout a light/dark cycle. He observed significant increases during the photoperiod in cellular chlorophyll a and carbohydrate of Skeletonema costatum, particularly at higher temperatures and lower light intensities. Cellular protein seemed to be the least variable of these parameters. Hitchcock noted that the protein/carbohydrate ratio showed a diel range comparable to that in a culture progressing from a nutrient sufficient to deficient state and cautioned the use of this ratio as an indication of nutrient status of natural populations. Van Liere et al. (1979) also observed higher ratios of carbohydrate production during the photoperiod in continuous cultures of Oscillatoria agardhii grown on a light/dark cycle. Other biopolymers were produced at a relatively constant rate throughout the light and dark period, presumably with dark synthesis occurring at the expense of carbohydrate. This general pattern of storage product accumulation

during the photoperiod has been observed by other investigators in laboratory situations (Darley et al., 1976; also Tamiya (1957) and Kanazawa (1964) in Morris, 1981). Despite the well recognized dependence of biosynthetic processes on the diel cycle, it has rarely been considered in field investigations which used chemical and biochemical ratios in efforts to physiologically characterize phytoplankton populations.

In light of the variety of criticisms which have been raised, it is apparent that conclusions about the activities of microorganisms based solely on determinations of the concentrations of chemical substances are often ambiguous and provide little information about the organisms' respective roles in organic matter cycling. A more direct approach to estimating microbial activity and biochemical composition involves the use of isotopic tracers. Although such an approach is subject to significant methodological constraints which will be discussed, it provides the most definitive means of probing the composition of living microbial biomass and tracing production of specific organic materials.

Isotope Tracing of Microbial Activities

Isotopic methods avoid problems of detrital interference by directly tracing microbial metabolism. However, they are subject to other potential artifacts. I will limit this discussion to the use of C and N isotopes as tracers for the sake of brevity. Probably the most commonly used isotope is $^{14}\text{C-HCO}_3$ after the method of Steeman-Neilsen (1952). However, the history of this technique has been one of controversy since its inception (Yentsch and Menzel, 1974; Lean and Burnison, 1979; Peterson, 1980; Dring and Jewson, 1982). Some of the problems are

associated with sample manipulation and include effects of containment, potential contamination and the use of endpoint incubations which must assume linear uptake (e.g. Carpenter and Lively, 1980; Fitzwater et al., 1982; Geiskes et al., 1979; Goldman et al., 1981a; Venrick et al., 1977; Ferguson et al., 1984; Ferguson and Sunda, 1984). Other problems are associated with the lack of a clear understanding of the integrated effects of biological processes of carbon fixation, respiration and photorespiration, and their relationship to the net production of particulate carbon (e.g. Harris et al., 1979; Peterson, 1980; Li and Goldman, 1981; Dring and Jewson, 1982).

The introduction of the ^{15}N method (Neess et al., 1962; Dugdale and Goering, 1967) provided another tool for tracing the utilization of a primary substrate by phytoplankton. In addition to problems associated with sample manipulation mentioned for $^{14}\text{C-HCO}_3$, there are other potential artifacts unique to this method. The relatively low concentration of inorganic nitrogen in seawater increases the chance for perturbing the system by the addition of tracer and can result in artificially enhanced uptake rates (Dugdale and Goering, 1967; Fisher et al., 1981; McCarthy and Goldman, 1979; Glibert and Goldman, 1981; Goldman et al., 1981a,b). Conversely, if ambient nitrogen levels are low, depletion of substrate could lead to underestimates of uptake if endpoint incubations are extended over too long a period (e.g. Goldman et al., 1981a). Freezing of samples can alter ambient concentrations, particularly at low levels (Carpenter and McCarthy, 1975). Other potential artifacts include dilution of added tracer by remineralization (Harrison et al., 1978; Caperon et al., 1979; Glibert et al., 1982) and uptake of organic nitrogen sources not traced (e.g. Remsen, 1971;

McCarthy, 1972; Schell, 1974; Wheeler et al., 1974; Wheeler and Stephens, 1977) both leading to underestimates of uptake.

Despite their limitations, the combined use of ^{14}C -inorganic carbon and ^{15}N -inorganic nitrogen permitted comparisons of phytoplankton uptake ratios of carbon and nitrogen (i.e. $^{14}\text{C-HCO}_3$ and ^{15}N) with the carbon to nitrogen ratio in particulate matter (Slawyk et al., 1977; Collos and Slawyk, 1979; Eppley et al., 1977; Caperon et al., 1979; Sharp et al., 1980; Slawyk et al., 1980; Harrison et al., 1983). These investigations provided a comparison of measured activities of primary producers and the composition of particulate organic matter. The results of these investigations have frequently demonstrated a positive correlation between C:N uptake ratios and the C:N ratio in the particulate material, but quantitative agreement was generally poor and uptake ratios varied over a much larger range than particulate ratios. In addition to potential interference by detritus and non-algal biomass, some of the discrepancies may have been due to the fact that nitrogen and carbon uptake are not necessarily coupled (Bassham and Kirk, 1964; Collos and Slawyk, 1979; Goldman et al., 1981a). Consequently, the C:N uptake ratios may not have been a reflection of the relative composition or biosynthesis of particulate carbon and nitrogen.

The ubiquitous nature of carbon in living biomass, and the specificity of the association of inorganic carbon fixation with photosynthetic organisms provide a basis for using inorganic ^{14}C to trace the biochemical composition and metabolism of phytoplankton populations. Through precise rate measurements of primary production of chemically distinct biogenic materials, it is possible to determine the extent to which changes in particulate organic matter composition are

attributable to phytoplankton production as opposed to transformations mediated by other biological processes. Information about rates of primary production of specific types of organic material will help to define the role of phytoplankton in biochemical cycling of organic matter, as well as provide a direct assessment of growth-related phytoplankton activity.

Amino acids are the building blocks of protein and several investigators have pointed out the utility of protein as a model biopolymer reflecting growth (Cuhel, 1981; Cuhel et al., 1981a,b,c, 1982a,b,1983; Bates, 1981; Li and Harrison, 1982). Amino acids and protein are nutritionally important to heterotrophs, and make up a substantial proportion of primary production.

* * * *

This thesis describes a series of studies conducted in order to evaluate the use of inorganic ^{14}C as a probe of composition and rates of biosynthesis of free amino acids and protein in marine phytoplankton. The investigative approach consisted of three main parts. Initially, the growth rate-related steady state composition of free amino acid and protein and their kinetics of ^{14}C incorporation were determined in NH_4^+ -limited cultures of the marine chlorophyte, Nannochloris sp. In addition, the dynamics of free amino acid composition and isotope incorporation associated with NH_4^+ enrichment were examined. The results provided a basis for relating the distribution of incorporated inorganic ^{14}C to cellular chemical composition and metabolism. Differences between species in their responses to enrichment were also evaluated.

Secondly, isotope incorporation rates were compared to net synthesis rates of protein amino acids in NH_4^+ -limited steady state cultures of Nannochloris sp. at high and low light intensities. Intercomparisons were also made among the incorporation characteristics in NH_4^+ -limited steady state cultures of three other marine phytoplankton species. The results revealed that isotope incorporation into the protein amino acids glycine and alanine were generally in good agreement with net synthesis rates. This observation provided a foundation for estimating net protein synthesis rates in natural populations.

Finally, with the aid of an extensive framework of laboratory results, I examined the relationship of primary production of particulate organic carbon and particulate protein amino acids to observed concentration changes and relative abundances of particulate organic carbon and protein amino acids in a well mixed euphotic system. The studies were conducted over a wide range of environmental conditions and population physiological states. The objectives were to assess the degree to which phytoplankton metabolism is reflected by particulate organic matter composition, and determine the relative importance of primary inputs and secondary transformations in influencing the particulate protein amino acid content of particulate organic matter. This investigation provided an unprecedented opportunity to explore the relationship between phytoplankton as a primary source of particulate amino acids, and the behaviour of this important class of organic compounds in the marine environment.

CHAPTER 2. METHODS AND MATERIALS

CULTURE METHODS

Media Composition

Cultures were maintained in an artificial seawater medium (Guillard, 1975; Guillard and Ryther, 1962; Goldman and McCarthy, 1978). The mineral composition of the mixture is given in Table 2-1. The amounts shown were dissolved in deionized water (Barnstead Nannopure water purification system). Before use, the solution was filtered through 47mm Whatman GF/F filters and autoclaved in teflon containers. The artificial seawater was subsequently enriched with nutrients, individually sterilized in teflon containers (with the exception of the vitamin mixture which was filter sterilized and stored in screw cap pyrex test tubes). Media concentrations and working stock concentrations of nutrients are given in Table 2-2. Formation of a reddish precipitate was frequently observed after nutrient enrichment. Guillard (1975) noted that a reddish precipitate containing phosphorus appeared after autoclaving the artificial seawater media together with the nutrients and trace elements. The reddish color suggests that iron hydroxides may also have been present. This could be removed by sterile filtration through a 0.22 μ Millipore membrane filter.

Growth Conditions

Axenic stock cultures were obtained from R. R. L. Guillard, currently associated with the Culture Collection of Marine Phytoplankton (CCMP) at Bigelow Laboratories in Maine, USA. A list of species used in this thesis is given in Table 2-3.

Table 2-1. Mineral concentrations in artificial seawater.

Nutrient	Concentration (mM)
NaCl	400
MgSO ₄ (anhydr.)	20
MgCl ₂ · 6H ₂ O	20
CaCl ₂ (anhydr.)	10
KCl	10
KBr	.80 ^a
H ₃ BO ₄	.20 ^b

^aPrepared as a stock solution of 1.6M and diluted 0.5ml per liter.

^bPrepared as a stock solution of 0.4M and diluted 0.5ml per liter.

Table 2-2. Concentrations of nutrients and trace elements in standard culture media.

Nutrient	Media Concentration		Working Stock Concentration (mM)
	(μ M)	(mg/l)	
NaH ₂ PO ₄ • H ₂ O	40	5.5	40
Na ₂ SiO ₃ • 9H ₂ O	106	30	106
Fe Sequestrene	11.7	5	11.7
CuSO ₄ • 5H ₂ O	0.04	0.01	0.04 ^a
ZnSO ₄ • 7H ₂ O	0.08	0.022	0.08 ^a
CoCl ₂ • 6H ₂ O	0.5	0.01	0.5 ^a
MnCl ₂ • 4H ₂ O	0.9	0.18	0.9 ^a
Na ₂ MoO ₄ • 2H ₂ O	0.03	0.006	0.03 ^a
NH ₄ Cl	500 ^b	26.5	500
Vitamins:			
Thiamin-HCl	0.3	0.1	0.3
Biotin	2.0x10 ⁻³	0.5 μ g/l	2.0x10 ⁻³
B ₁₂	3.7x10 ⁻⁴	0.5 μ g/l	3.7x10 ⁻⁴

^aPrepared as individual primary stocks; working stock consisted of a mixture of each metal solution diluted 1000 times plus Fe sequestrene. This mixture was autoclaved in teflon, followed by sterile filtration through a 0.22 μ Millipore membrane filter.

^bFor continuous culture experiments, NH₄Cl was added to 125 μ M.

^cPrepared as a primary stock at a concentration of 0.1mg/ml.

^dPrepared as a primary stock at 1.0mg/ml.

Table 2-3. Species list of marine algae obtained from the culture collection of marine phytoplankton.

Clone	Species	Isolator	Location	Date
Dun	<u>Dunaliella tertiolecta</u>			
Actin	<u>Thalassiosira weissflogii</u>	Guillard	Gardiner's Island Pond, N. Y.	
GSB Nanno	<u>Nannochloris</u> sp.		Great South Bay, N. Y.	
Olisth	<u>Chattonella luteus</u> (previously <u>Olisthodiscus</u>) Loeblich and Fine, also Gibbs et al.	Conover	Long Island Sound, USA	
3C	<u>Chroomonas salina</u> (Wislouch) Butcher	Guillard	(Tank) Milford, CT	1956
BBsm	<u>Chaetoceros simplex</u>	Guillard	32°10'N 64°30'W	14 Apr 60

Cultures were maintained at 20°C in a 12:12 light:dark cycle regime in 15 x 150 mm borosilicate tubes capped with white polypropylene caps. Prior to inoculation tubes were extracted with 1.5N HCl overnight, rinsed and subsequently autoclaved with approximately 10 mls deionized water. The water was poured off and media was added aseptically.

Flasks used for continuous culture experiments (Fig. 2-1) were constructed by Anderson Glass. The 0.5 liter jacketed flasks were similar to those used by Goldman and McCarthy (1978). Temperature was maintained within 1°C of 21°C by a Haake D2 heating-circulation water bath cooled with tap water. Cultures were illuminated using three parallel Vita-Lite fluorescent bulbs which provided an average quantum density of approximately 176 ± 70 (95% confidence interval) $\mu\text{E}/\text{m}^2/\text{s}$ (range 90 - 200 $\mu\text{E}/\text{m}^2/\text{s}$ from top to bottom of culture vessel). Light intensities were determined in flasks containing deionized water using a Biospherics submersible 4pi probe. Cultures were mixed by a combination of aeration (Second Nature Whisper 600 aquarium type air pump) and magnetic stirring (Fisher Cat. No. 14-511-IV2 magnetic stirrer). Air was pumped through a combined mixture of sterile glass wool and cotton, followed by sparging through sterile distilled water, and finally passed through an additional sterile cotton filter before being blown into the culture. Media was dispensed to the cultures via Harvard Apparatus Peristaltic Pumps. Tubing was silicon and glass.

CHEMICAL ANALYSES

Chlorophyll a analyses

Chlorophyll concentrations were determined by the fluorometric method of Yentsch and Menzel (1963), as described by Smith et al. (1981).

Samples were filtered on a Millipore type HA (nominal 0.45um pore size) and the filter dissolved in 90% aqueous acetone. Samples were incubated for 10 minutes and fluorescence read on a Turner designs Model 10-005R fluorometer equipped with filters for chlorophyll a fluorescence. Readings were corrected for phaeopigment fluorescence by subtracting readings for acidified samples (two drops of 0.75N HCl) from those for unacidified samples.

Elemental Carbon and Nitrogen Analyses

Samples were filtered on combusted (500°C overnight) Whatman GF/F 25mm glass fiber filters using a Millipore filtration apparatus, and filters were dried in an oven at 55°C. Dried filters were stored dessicated until they were analysed for elemental carbon and nitrogen using a Perkin-Elmer 240 elemental analyzer. Philip Klarner and Bonnie Woodward performed the analyses.

Nutrient Analyses

Samples for nutrient analysis were filtered through combusted Whatman GF/F filters and stored frozen at -20°C until they were analyzed. Nutrients (ammonium, nitrate, nitrite, silicate, and orthophosphate) were analyzed using a Technicon autoanalyzer. All nutrient analyses were made by Zophia Mlodzinksa.

Dissolved Inorganic and Organic Carbon Analyses

Dissolved inorganic carbon analyses were performed by adding 3ml samples, previously filtered through combusted Whatman GF/F filters, into 15ml serum bottles stoppered with butyl rubber. Samples were acidified

Figure 2-1. Schematic of continuous culture system. Abbreviations: CF, cotton filter; GW, glass wool; AP, air pump; OR, overflow reservoir; CB, circulating bath flow; SS, sterile syringe; LLV, Luer-lok valve; S, magnetic stirrer; MR, media reservoir; PP, peristaltic pump.

with 0.15mls 18N H_2SO_4 and stirred for a minimum of 3 hours. Gaseous CO_2 was then analyzed by infrared spectroscopy and corrected for air blank. Dissolved organic carbon was determined by the method of Menzel and Vaccaro (1964). Analyses were made by Brian Howes and Dale Goehring.

Protein Assay

Bulk protein analyses were made using the method of Bradford (1976). The method uses Coomassie Brilliant Blue dye binding reagent which is commercially available from Bio-Rad. Standard curves were determined using egg ovalbumin (Sigma) which has a response factor representative of average proteins. Extraction for this assay involved grinding the filtered sample in 0.5 N NaOH and subsequently extracting two times for 10 minutes at $80^{\circ}C$, centrifuging at 4000g for 20 minutes and combining the supernates (Rausch, 1981). The assay solution consisted of 0.5ml sample in 0.5N NaOH, 0.5 ml 0.5M H_3PO_4 , and 0.25 ml concentrated Bio-Rad reagent solution of Coomassie Brilliant Blue. The solution was allowed to incubate for 10 minutes and absorbance was read at 595 nm. I have found that the response factor for this assay is highly sensitive to the pH of the solution. The solution composition described here gives adequate sensitivity in the 2-25 $\mu g/ml$ sensitivity range (in the extracted solution).

LABORATORY INCUBATION PROCEDURES

Sampling Procedure

For culture experiments, steady state was defined as the time when chlorophyll a concentration and cell density remained constant for at

least 2 generations. For the lowest growth rate under low light conditions, steady state was maintained only 1.1 generations. Immediately preceding isotope incubations, samples were removed for particulate elemental carbon and nitrogen analyses. Filtrates were collected and stored refrigerated for subsequent analysis of dissolved inorganic carbon. Cells were counted on a Spencer Bright-line hemocytometer. In addition, chlorophyll a concentration and pH were determined. For these analyses, no more than 15% of the culture volume was removed from the full steady state chemostat, and the isotope incubation was begun within 20 minutes of initial sampling. Immediately before introducing the radiolabel (C^{14} -inorganic carbon, New England Nuclear), aeration was stopped.

Time series sampling during the incubations included filtration of particulates on combusted Whatman GF/F glass fiber filters. For radiolabeled samples, filters were cut to a diameter of 13mm using a template (Cuhel et al., 1981a) and housed in Millipore SX00 13 syringe filter holders. A Gast Manufacturing Corp. Series #1077 vacuum pump was used. Filtration pressures did not exceed 150mm Hg. Filtered samples for amino acid analyses were placed in acid washed 20ml glass scintillation vials containing 1.5ml 80% methanol and stored at $-6^{\circ}C$ until analysis.

Radioassay Procedures

For estimates of total particulate C^{14} fixation, filters were placed in the bottom of a 20ml scintillation vial, covered with 0.1ml 1N HCl, and dried under a gentle air flow at $55^{\circ}C$. The dried filters were moistened with a drop of deionized water, and 10 ml scintillation fluor

was added. Aquasol was used for all high light intensity experiments with Nannochloris sp., and Salt Pond experiments prior to 13 August 83. Scintiverse II (Fisher) was used for all other experiments. Comparisons between Aquasol and Scintiverse II indicated their quench characteristics were quite similar for the materials being counted (data not shown).

Total acid non-volatile ^{14}C was assayed by a method similar to that described by Schindler and Holmgren (1971) and Li and Goldman (1981). One ml of the culture was acidified to a concentration of 0.32N H_2SO_4 (pH 1.2) in a 7 ml scintillation vial, sparged with air for 15 minutes, and 6 ml Scintiverse II added. Counts per minute were measured with a Beckman LS-100C liquid scintillation counter. Measured counts were quench corrected by the channels ratio method (Kobayashi and Maudsley, 1974).

SUBCELLULAR FRACTIONATION PROCEDURES

Modification of Existing Procedures

Subcellular fractionation procedures involve sequential solvent extraction of cellular material into fractions whose chemical characteristics are operationally defined by the solvent characteristics. The procedure described here involves several modifications of procedures previously described by Roberts et al. (1963), Morris et al. (1974), Li et al. (1980), Larsen et al. (1981), Cuhel et al. (1981b), and Hitchcock (1981).

Initially, samples containing glass fiber filters were transferred to Bellco 12ml glass tissue homogenizers. Homogenizer tubes were cut down to a 10 ml volume using a glass saw so that they would fit in the HS-4 rotor. The contents were ground in approximately 0.1ml 80% aqueous

methanol, which had previously been filtered through a combusted Whatman GF/F filter. In cases where the sample had been stored in 80% aqueous methanol in scintillation vials, the excess liquid was subsequently transferred to the grinder, along with an 80% aqueous methanol rinse of the scintillation vial. The resulting homogenous suspension was vortexed, and subsequently centrifuged at 4000g for 20 minutes in a Sorvall RC2B refrigerated centrifuge with an HS-4 swinging bucket rotor. Temperature was maintained at 15°C. The supernatant was removed, and temporarily held aside in a small test tube, while the pellet was subsequently resuspended and extracted in 1.5 ml 80% aqueous ethanol at 60°C for 20 minutes. This mixture was again centrifuged, and the supernates combined. The pellet was extracted two additional times with a 1:1 mixture of 80% aqueous ethanol and diethyl ether at 60°C for 20 minutes and 10 minutes respectively, again combining the supernates. The combined supernate included intracellular free amino acids, other low molecular weight metabolites, and lipophilic polymers. The lipophilic material was separated by concentrating the combined supernatant to 3 ml at 60°C under air, adding 1 ml deionized water, and extracting this solution two times with 5 ml diethyl ether. For scintillation counting of the aqueous alcohol soluble and the lipophilic fractions, the entire fraction or an aliquot was transferred to either a 20ml or a 7ml scintillation vial, evaporated to near dryness, and scintillation fluor added in a ratio of less than 1:6 sample to fluor volume. In the case of the aqueous alcohol fraction, 0.1ml 1N HCl was added before evaporative drying to insure complete removal of inorganic ¹⁴C.

The remaining pellet was then subjected either to hydrolysis for high pressure liquid chromatographic separation, quantitation, and collection of protein amino acids (see **Amino Acid Analysis**), or was further fractionated into hot (95°C) trichloroacetic acid soluble (largely carbohydrate and nucleic acid material) and insoluble material (largely residual protein) (e.g. Roberts et al., 1963; Cuhel et al., 1981b; Morris et al., 1974; Konopka and Schnur, 1980, 1981). For the latter procedure, the pellet was resuspended in 1.5 ml trichloroacetic acid and extracted two times for 20 minutes and 10 minutes respectively at 95°C. The acid supernatants were combined and 1 ml mixed with 0.75 ml Tris-hydroxyethanolamine (pH 13) and transferred to a 10 ml Nalgene filmware scintillation envelope. Scintillation fluor (6 ml) was added to form a gel. Alternatively, the entire acid fraction (approximately 3ml) was added to a 20 ml glass scintillation vial and 17 ml scintillation fluor added. The remaining pellet was transferred with sequential rinses of 0.75 ml 0.1N NaOH, 1.0 ml deionized water, and 0.75 ml 1M Tris (pH 7) into either a 10 ml filmware bag or a 20 ml glass scintillation vial and combined with 6-10 ml scintillation fluor.

Comparison of Subcellular Fractionation Procedures

The prolific use of subcellular fractionation procedures over the past 2 decades has resulted in the development of a variety of techniques. The lack of clear chemical definitions for the operationally defined fractions has limited the extent to which results using different procedures can be directly compared. Hitchcock (1981) compared the ability of different fractionation procedures to recover standards representative of the operationally defined fractions. He found

substantial differences between the different methods. He also compared the carbon content of different subcellular fractions as determined by chemical analysis with that derived from ^{14}C in subcellular fractions of Gonyaulax tamarensis labelled for 6 hours. He found good agreement between the amount of lipid carbon derived from chemical composition and lipophilic ^{14}C . In contrast, hot trichloroacetic acid extractable ^{14}C underestimated that determined from chemical analyses, while the residual ^{14}C in the pellet was higher than the estimate of protein carbon.

In view of these observations, I felt it was important to compare my fractionation method with two procedures described in the literature. One of these methods, that of Li et al. (1980), was also described in the Hitchcock study so that intercomparison of results was possible. The method of Cuhel et al. (1981b) is very similar to the fractionation procedure described by Roberts et al. (1963). Flow diagrams outlining the procedures are shown in Table 2-4. Material for fractionation was an axenic batch culture of Nannochloris sp. which was grown in the presence of inorganic ^{14}C for 4 generations to ensure approximate equilibrium label distribution. Amounts of carbon, as calculated from the levels of ^{14}C label associated with comparable fractions for each method are shown in Table 2-5. All three procedures gave very similar results for the cold acid/aqueous alcohol, and lipophilic fractions, and agreement between the Cuhel et al. and my method were quite similar for all fractions. However, the Li et al. procedure, which uses filtration rather than centrifugation to separate soluble and insoluble material, tended to give lower proportions of label in the hot trichloroacetic acid fraction, and higher estimates in the residue fraction.

Table 2-4. Outline of fractionation procedures.

^amethanol

^btrichloroacetic acid

^clow molecular weight metabolites

^dethanol

^eFor the Roberts et al. method, this fraction contains alcohol soluble protein.

Table 2-5. Comparison of results using different fractionation procedures.

Method:	Li et al.	Roberts et al./ Cuhel et al.	My method
Fraction	(µgC/l)		
Alcohol or Cold Acid Soluble	1500 (104) ^a	1710 (156)	2010 (381)
Lipophylic	3650 (322)	3580 (50)	3610 (248)
Hot Acid Soluble	1050 (24)	1380 (547)	1840 (149)
Residue	5960 (248)	4010 (348)	3990 (199)
Sum of Fractions	12060 (419)	10680 (668)	11450 (518)
Actual Total	12376 (414)		

^aErrors in parentheses represent 95% confidence limits (n=3).

The residue is considered to be largely protein. More efficient recovery of protein by the method of Li et al. is unlikely since comparisons of different protein assay methodologies indicated relatively complete recovery of protein in the hot trichloroacetic acid insoluble fraction (see Recovery of Protein by Extraction). Rather, it seems the Li et al. method results in a less complete separation of hot acid soluble and insoluble fractions. It may be that the resuspension associated with vortexing used in my method and that of Cuhel et al. leads to more effective solubilisation than the filtration procedure used by Li et al.

AMINO ACID ANALYSIS

Amino Acid Derivatization Procedure

Free or hydrolyzed amino acids were derivatized with dansyl chloride (5-(dimethylamino)-1-naphthalene-sulfonyl chloride) in a procedure which was a modification of a number of published literature methods (Wiedmier et al., 1982; DeJong et al., 1982; Tapuhi et al., 1981; Parris and Gallelli, 1984; MacNicol, 1978; Bayer et al., 1976). Dansyl chloride (Sigma) was dissolved in acetonitrile (Spectra UV Grade, Fisher) to a concentration of 6 mg/ml. This solution was stored dessicated under argon or nitrogen at -20°C . Extracted amino acid samples were dried under air at 60°C and resuspended in equal volumes of 0.5M NaHCO_3 (pH 8.5), KMnO_4 distilled H_2O , and the dansyl chloride solution. It was found that this suspension could be effectively mixed by brief sonication. The reaction mixture was incubated in darkness for 1 hour at 37°C . The reaction was terminated by addition of KMnO_4 distilled H_2O and 0.1M H_3PO_4 (HPLC Grade, Fisher) in final volume proportions of 7.5:3:2 (H_2O :reaction mixture: H_3PO_4).

Potential sources of error in determining amino acid concentrations included injection error, storage losses, variations in derivatization efficiency, error in peak integration, and error in preparation of standards. Mean response factors (integrated peak area/nanomoles injected) are shown along with 95% confidence intervals in Table 2-6. The 95% confidence intervals include all previously mentioned sources of error. This error was propagated in all subsequent calculations. Analytical variations resulting from errors other than that associated with preparation of standards are shown in Table 2-7. These errors were smaller than those in Table 2-6, indicating that the standard preparation error was significant.

Various conditions which might affect the efficiency of derivatization were evaluated. No significant increases in response factors were observed if the derivatization mixture was incubated 4 hours versus 1 hour (Table 2-8). Correlations between response factors and the reaction mixture molar ratio of amino groups/dansyl chloride (range 0.001-0.12) were examined. Correlation coefficients are given in Table 2-9, and were not significant at the 0.05 probability level except in the case of serine and threonine. This indicates that adequate derivatization can generally be achieved provided dansyl chloride is present in a 10-fold molar excess relative to amino nitrogen (c.f. Bayer et al., 1976). The dependence of response factors on time of storage at -6°C is shown in Table 2-10. There did not appear to be any consistent decreases up to 90 days after derivatization. Samples in this study were analyzed within 2 weeks of derivatization. DeJong et al. (1981) noted that Dns-tyrosine was relatively unstable. However, it was not included in my analyses.

Table 2-6. Response factors for integrated absorbance by dansylated amino acids at 254nm.

Amino Acid	Response factor (V sec nmole ⁻¹)	95% Conf. Int. ^a	n	%
asp	0.588	0.111	8	18.9
glu	0.570	0.113	9	19.8
gln	0.474	0.170	8	35.9
ser	0.715	0.170	8	23.8
thr	0.572	0.074	6	12.9
gly	0.748	0.149	7	19.9
ala	0.658	0.121	6	18.4
Baba	0.365	0.176	1	48.2
Gaba	0.614	0.236	5	38.4
arg	0.585	0.232	5	39.6
pro	0.615	0.202	4	32.8
val	0.554	0.145	6	26.2
ile	0.361	0.171	4	47.3
leu	0.602	0.120	4	19.9
trp	0.451	0.280	3	62.1
phe	0.621	0.241	3	38.8
lys	0.945	0.436	3	46.1
his	1.09	0.264	3	24.2
orn	0.884	0.318	3	36.0

^aError due to injection error, storage losses, variation in derivatization efficiency, peak integration error, and standard preparation error.

Table 2-7. Analytical variations (95% confidence intervals) for individual amino acids resulting from the combination of errors associated with injection, storage, variation in derivatization efficiency, and peak integration.

Amino Acid	% ^a	n
asp	5.2	36
glu	6.2	36
gln	5.7	32
ser	5.0	31
thr	5.5	23
gly	8.1	30
ala	8.4	20
gaba	7.4	24
arg	8.7	20
pro	7.0	16
val	10.2	26
ile	10.5	18
leu	4.4	18
trp	14.2	13
phe	7.5	14
lys	9.7	13
his	5.1	13
orn	8.8	13

^a95% confidence interval expressed as percentage of the response factor.

Table 2-8. Comparison of derivatization incubation periods of one and four hours at 37°C.

Amino Acid	Incubation Period (0.125 V sec, 50 μ l injection)	
	1 hour	4 hours
asp	0.933	0.922
glu	0.897	0.944
gln	0.964	0.968
ser	1.50	1.65
thr	1.47	1.46
gly	1.51	1.50
ala	1.35	1.28
bala	1.62	1.64
baba	1.11	1.09
aaba	0.788	0.780
gaba	1.60	1.53
pro	1.35	1.35
arg	1.57	1.52
val	0.902	0.824
met/cys	0.905	0.785
ile	0.614	0.531
leu	1.21	1.10
trp	0.988	0.891
phe	1.46	1.33
orn	1.96	2.29
lys	2.30	2.48
his	2.47	2.68

Table 2-9. Correlations between response factors and molar ratio of amino groups/dansyl chloride (range 0.001-0.12) in the derivatization mixture.

Amino Acid	Correlation Coefficient	n
asp	0.0493	10
glu	-0.580	11 ^a
gln	-0.546	11 ^a
ser	-0.726	11 ^b
thr	-0.856	10 ^c
gly	0.175	10
ala	-0.162	8
arg	-0.696	8 ^a
pro	-0.328	8
val	-0.212	10
ile	-0.150	10
leu	-0.172	10
trp	0.016	9
phe	0.185	9
lys	-0.147	9
his	-0.463	9
orn	-0.360	9

^aSignificant at P=0.10 level.

^bSignificant at P=0.02 level.

^cSignificant at P=0.01 level.

Table 2-10. Dependence of response factor^a on time of storage at -6°C.

Date of derivatization:	14 Aug 83	15 Mar 83	14 Feb 83
Days of storage:	0 87	0 81	0 95
Amino Acid			
asp	3.49 3.15	4.80 6.31	4.98 5.30
glu	5.53 5.03	4.10 7.32	3.60 3.60
gln	3.55 3.71	3.23 3.72	3.59 2.65
ser	4.96 5.22	8.29 6.70	6.00 6.80
thr	4.54 5.11	4.98 4.49	4.77 4.58
gly	6.26 5.42	5.62 4.92	5.70 6.30
ala	n.d. n.d.	5.60 4.9	4.94 5.40
pro	3.56 3.73	6.37 n.d.	4.80 5.70
arg	6.87 4.80	6.15 5.40	6.00 4.80
val	5.34 4.28	3.99 5.50	3.37 3.53
ile	4.52 2.95	2.50 3.10	1.94 1.80
leu	5.84 5.03	4.98 6.63	3.92 3.86
trp	5.63 3.47	3.32 4.23	2.86 2.66
phe	4.91 4.84	5.89 8.50	4.43 4.98
orn	9.33 6.84	7.90 9.00	5.89 6.06
lys	8.80 6.01	9.50 10.0	6.51 6.62
his	9.26 8.11	9.50 8.56	8.00 8.37

^aValues represent mean of 2 or more injections.

Column Packing Procedure

The dansylated amino acids were separated by high pressure liquid chromatography. All columns were slurry-packed. The dimensions of the column were: inner diameter 4.1mm, outer diameter 1/4 inch, length 25cm with an additional 5cm guard column (Alltech Associates). The packing procedure involved connecting an SS1 slurry reservoir to a well cleaned analytical column containing a 2 μ m frit at the bottom and plumbed so that waste solvent would drain into a beaker. The column was initially filled with dibromomethane, and an amount of packing material adequate to fill the column with a slight excess was suspended in a 1:1 mixture of methylene chloride and dibromomethane and gently layered onto the dibromomethane. Finally, the slurry was layered with acetonitrile or acetone until the reservoir was filled. At this point an air driven fluid pump (Haskel Engineering and Supply Co.) well primed with the mobile phase solvent was connected to the reservoir. Back pressure was increased to approximately 8000-10,000 psi and rapidly released onto the reservoir/analytical column system. Once a volume adequate to flush the system several times had passed through, pressure was reduced. The analytical column was disconnected from the reservoir and excess packing material was carefully smoothed off the top and a frit and endcap firmly screwed on. The column was rinsed well with the mobile phase carrier (acetonitrile) before analyses were made. Packing material used was always 5 micron octyldecasilane (ODS), however, two different brands were used: Spherisorb S5-ODS and Nucleosil 5 C₁₈ both available from Alltech Associates.

Separation and Collection

Separation was accomplished using a gradient system driven by a Waters Associates 6000A pump assisted by an M-45 pump. Both were interfaced with a Waters Associates M660 Solvent Programmer. A Waters Associates UK6 injector was used with a 1 ml sample loop. Detection was by ultraviolet absorbtion at 254nm on a Waters Associates Model 440 absorbance detector. Integration of curve signals was accomplished by either an Hewlett Packard 3390A integrator or an SP4270 Spectrophysics integrator. A Linseis chart recorder was also used. The gradient conditions for Spherisorb were 12-55% acetonitrile in 0.03M phosphate buffer (pH 6.25) over a convex gradient (curve 7, Waters M660 Solvent Programmer) of 75 minutes duration. For Nucleosil packing material, comparable separation could be achieved with a convex gradient (curve 7) of 15-55% acetonitrile in 0.03M phosphate buffer (pH 6.25) over 40 minutes. A representative elution scheme is shown in Fig. 2-2.

Fractions for isotope quantitation were collected directly from the detector outflow. Scintillation fluor was added directly to the fractions, and fraction volume generally did not exceed 35% of fluor volume. Quench corrections were made by the channels ratio method.

EXTRACTION AND RECOVERY

Free Amino Acid Extraction and Recovery

Recovery of free amino acids in the aqueous alcohol fraction was determined by two different techniques. In the first procedure, aliquots of an isotopically labeled mixture of amino acids were added to a combusted Whatman GF/F glass fiber filter (cut to a diameter of 13 mm and

combusted overnight at 500°C). The filters were subsequently extracted and the proportion of label associated with each solvent fraction was quantified by liquid scintillation counting. The results are shown in Table 2-11 and indicate that the free amino acids will be relatively completely extracted into the aqueous alcohol fraction, provided they are released from cell material during grinding.

Recovery of individual free amino acids was also determined by adding an aliquot of a standard mixture (prepared from amino acids purchased from Sigma) to a combusted Whatman GF/F glass fiber filter and extracting. Recoveries for the individual amino acids, shown in Table 2-12, were uniformly high.

Recovery from Hydrolysis

Conditions for hydrolysis of protein into individual protein amino acid residues (6N HCl, 20 hours at 110°C under N₂) results in loss of amide nitrogen from asparagine and glutamine, and nearly complete destruction of tryptophan (DeJong et al., 1981). The effects of such treatment on the individual amino acids was investigated by adding aliquots of a standard mixture of free amino acids to a combusted Whatman GF/F filter (13mm) in a 10 ml ampule and hydrolyzing under conditions as described above. Recoveries of individual amino acids are given in Table 2-13. In addition to the losses referred to above, my results indicate substantial loss of serine and threonine, and some loss of methionine + cysteine, phenylalanine and isoleucine. Possibly, the presence of low levels of residual oxygen led to oxidative destruction of these amino acids during hydrolysis.

Figure 2-2. Representative chromatogram showing elution pattern of dansylated amino acids separated by reverse phase high pressure liquid chromatography. Column contained Spherisorb S5-ODS 5 micron octyldecasilane. Mobile phase consisted of a 75 minute exponential gradient of 15-55% acetonitrile in 0.03M NaH_2PO_4 (pH 6.25).

Table 2-11. Proportion of labeled free amino acids recovered in subcellular fractions.

Cell Fraction	% Recovered Label
Alcohol Soluble	93.7 (92.7 - 94.6, n=3)
Lipophylic	1.7 (1.52 - 1.90, n=3)
Residue	5.17 (4.73 - 5.64, n=3)

Table 2-12. Free amino acid recovery in the aqueous alcohol fraction.

Amino Acid	% ^a
asp	n.d.
glu	103.0
gln	93.9
ser	110.0
thr	98.4
gly	104.0
ala	108.3
baba	83.9
aaba	n.d.
gaba	110.1
pro	110.0
arg	101.6
val	103.5
met/cys	n.d.
ile	118.4
leu	86.8
trp	114.1
phe	107.0
orn	100.7
lys	101.6
his	103.1

^aValues represent the mean of three replicates.

Table 2-13. Recovery of amino acids from hydrolysis.

<u>Amino acid</u>	<u>%^a</u>
asp	87.6
glu	158.5
gln	10.1
ser	5.0
thr	25.7
gly	92.6
ala	91.8
baba	81.6
aaba	88.9
gaba	89.4
pro	84.6
arg	69.2
val	102.0
met/cys	58.2
ile	74.1
leu	94.6
trp	-
phe	73.2
orn	103.5
lys	99.2
his	95.7

^aValues represent the mean of three replicates.

No attempt was made to correct for these losses. Losses were assumed to be internally consistent among different experiments. In the next section, recoveries of hydrolyzed protein are compared to those of other assay and extraction procedures.

Recovery of Protein

Recoveries of protein using different assay and extraction procedures are shown in Table 2-14, expressed as a percent of total particulate carbon. For the purposes of this comparison, it was assumed that protein was 53% carbon by weight based on the average amino acid residue composition (Jukes et al., 1975; Dolittle, 1981). Differences between recoveries of protein assayed by the Bradford method before and after extraction of free amino acids were slight. Direct chemical analysis of hydrolyzed protein amino acids yielded a slightly lower recovery, probably due to destruction of some protein amino acids (see previous section). Recovery of C¹⁴-label in the hot trichloroacetic acid insoluble fraction of equilibrium labeled cells gave the highest recovery as might be expected since nonprotein carbon may be included in this material.

Overall, there was good agreement between the different procedures. There was apparently little loss of protein during extraction of free amino acids and lipophilic material, and most of the carbon in the hot trichloroacetic acid insoluble material was in protein. Recovery of hydrolyzed protein was good despite specific losses.

In general, the recoveries of individual protein amino acids did not decline over an approximately 22 month storage period in 80% methanol at -6°C (Table 2-15). In fact, in some cases recoveries increased,

Table 2-14. Comparison of different methodologies for measurement of protein in Nannochloris sp.

Method	% Carbon in Protein
Bradford Assay (Bio-Rad Coomassie Blue):	
before extraction ^a	21.4
after extraction ^b	22.5
Sum of hydrolyzed protein amino acids ^c	18.9
Equilibrium ¹⁴ C residual carbon ^d	24.2

^aProtein extracted from whole cells with 0.5N NaOH (2 x 10 minutes @ 80°C) and assayed using Coomassie Blue dye binding reagent.

^bProtein extracted and assayed as above from cell material previously extracted for alcohol soluble and lipophylic fractions.

^cSum of hydrolyzed protein amino acids from cell material previously extracted for alcohol soluble and lipophylic fractions; individual amino acids were quantified by dansylation.

^dResidual hot trichloroacetic acid insoluble ¹⁴C from equilibrium labeled cell material.

Table 2-15. Recovery of protein amino acids after storage in 80% methanol at -6°C.

Amino Acid	Days of storage: 16			84 (0.5 V sec)			662		
	mean	SE ^a	n	mean	SE	n	mean	SE	n
asp	0.98	0.01	2	1.32	0.08	3	1.18	0.12	3
glu	2.04	0.11	2	2.51	0.17	3	2.92	0.50	3
thr	0.47	0.06	2	1.22	0.06	3	1.03	0.23	3
gly	5.74	0.01	2	5.79	0.38	3	5.52	0.17	3
ala	3.89	0.20	2	5.15	0.51	3	4.92	0.22	3
pro	2.15	0.01	2	2.35	0.06	3	2.33	0.08	3
arg	2.18	0.06	2	1.78	0.09	3	2.69	0.12	3
val	3.60	0.25	2	3.64	0.12	3	2.91	0.05	3
ile + leu	6.61	0.31	2	6.85	0.12	3	5.75	0.15	3
lys	4.54	0.30	2	3.53	0.30	3	4.64	0.20	3
his	0.96	0.07	2	0.65	0.03	3	1.04	0.02	3

^astandard error

probably reflecting an improvement in analytical technique. Independent observations support the view that the protein fraction is relatively stable during storage (see, for example, next section). These observations suggest that specific losses which may have occurred would not significantly affect interpretation of the results.

Recovery of Other Subcellular Fractions

Table 2-16 shows the results of a comparison of ^{14}C associated with different subcellular fractions as a function of time of storage at -20°C . These values are compared to label distributions associated with material which had been stored in liquid N_2 . For the latter, there was little variation with time, and so all values were pooled. The only fraction showing a consistent difference is the lipophylic fraction, which was low for samples stored at -20°C even after only 13 days storage. Lipid is known to be susceptible to lipase activity, even at this low temperature (Christie, 1973). It was not feasible to store all samples in liquid N_2 . Therefore, in order to minimize the possibility of enzymatic degradation, samples were initially fixed by placing in 80% aqueous methanol in 20ml glass scintillation vials, and subsequently stored at -6°C .

SALT POND PRODUCTION INCUBATIONS

Summer 1982

During summer of 1982 two experiments were conducted in which the light intensity dependence of ^{14}C incorporation into subcellular fractions was determined in surface samples from Salt Pond. Samples were obtained from a depth of one meter approximately 25 yards from shore and

Table 2-16. Stability of subcellular fractions with storage at -20°C relative to storage in liquid N_2 ^a.

Fraction	Days Stored @ -20°C :			Liquid N_2
	13	120	164	
Alcohol Soluble	1980 (149) ^b	2120 (99)	2220 (130)	1927 (118, n=7)
Lipohylic	3210 (149)	3050 (248)	3040 (20)	3651 (109, n=7)
Hot Acid Soluble	1840 (149)	1760 (99)	2130 (230)	1720 (549, n=8)
Residue	4380 (149)	4910 (224)	4360 (90)	4269 (287, n=8)

^aFractions stored in liquid N_2 were relatively constant and values given here represent overall mean.

^bErrors in parentheses represent 95% confidence intervals.

incubated on shore in a series of syringe-like cylindrical incubation chambers provided by John Waterbury (Fig. 2-3). The chambers were shaded by neutral density fiberglass screening and cooled by circulating Vineyard Sound seawater (approx. temp 19°C). Gentle rolling of a small teflon ball inside the chambers maintained adequate mixing. In order to assess effects of nitrogen enrichment on primary production and algal metabolic characteristics, NH_4Cl was added to selected chambers to a concentration of 100 μM . Sampling methods differed slightly from the laboratory procedures outlined previously. Samples (25ml) were taken in a time course and filtered through uncombusted Whatman GF/F filters which had been cut to 13 mm diameter and housed in Millipore SX00-13 syringe filter holders. Filters were rinsed with 5 ml filtered seawater, heat sealed in Nalgene polypropylene filmware bags, and placed on dry ice until return to the laboratory where they were then stored at -20°C until analysis.

Summer 1983

During summer of 1983, all field production measurements were made in situ. Incubations conducted from 20 July 83 through 10 Aug 83 were conducted using a spring loaded sampler incubation device (Fig. 2-4). A prototype design is described by Taylor et al. (1983). For incubations conducted from 26 Aug 83 through 3 October 83, a dual chamber hydraulic sampling and subsampling device was used (Fig. 2-5). All aspects of isotope addition and subsample collection were executed in situ without vertical movement of the incubation chamber. With the exception of some stainless steel components, metal surfaces of the device were coated with teflon so as to minimize metal contamination. The devices were suspended

at the one meter depth from a wooden 2x4 beam mounted on an aluminum rowboat. Mixing during the incubation was accomplished by the natural wave action on the boat, and by periodic manual agitation of the chamber. Time course sampling was accomplished by means of teflon tubing which extended to the surface. Samples were drawn into shaded Luer-lok plastic syringes and contents were subsequently filtered. The filtration apparatus was shaded from direct sunlight. A vacuum was generated by a Nalgene Mytovac vacuum pump equipped with a pressure gauge. Pressures did not exceed 150mm Hg. Sample fixation and storage was as described for the laboratory experiments. Water samples for chlorophyll a, particulate elemental carbon and nitrogen, nutrients, and dissolved inorganic carbon were collected into polypropylene bottles using a peristaltic pump with a silicon tube incremented in 0.5m intervals. These were returned to the laboratory and stored at 5°C until they were processed (within two hours of collection).

LIGHT INTENSITY

Surface incident light intensity during field incubations was continuously monitored with a Biospherics 4pi quantum sensor interfaced with a calibrated chart recorder. Depth profiles of light intensity were determined using a submersible Li-Cor 2pi quantum sensor. Intensity was measured at 0.5m intervals and extinction was estimated by taking the mean over the mixed layer of $\ln(P_z/P_0)$ where P_0 is surface quantum flux and P_z , the quantum flux at depth z .

Figure 2-3. Schematic of incubation system used in Salt Pond 1982 experiments. A. Diagram of polycarbonate incubation chamber.

Abbreviations: SV, sampling valve; FP, fill port; TB, teflon ball (for mixing); P, piston. Piston was pushed inward during sampling operation using a screw-crank device. B. Diagram of chambers in plexiglas incubation case. Chambers were attenuated to different light intensity using fiberglass screening. Arrows indicate direction of flow of surface temperature seawater. Abbreviations: L, latch.

Figure 2-4. Schematic of single chamber SID (sampler incubation device). Lower sequence shows principle of operation. Abbreviations: P, piston; PS, pressure spring; CV, check valve; ip, isotope injection port; Sp, spring; cl, spring retainer clip; R, isotope reservoir.

Figure 2-5. Schematic of dual chamber hydraulically operated SID.

Principle of operation is similar to design shown in Fig. 2-4, except that endcap (EC) with check valve (CV) remains stationary throughout sampling and incubation, while piston (P) is moved hydraulically for both initial sample introduction, and subsequent subsampling operation. Other symbols: T, teflon sampling tube; SP, spring; IC, incubation chamber; PU, pump (only front pump shown). Pumps were controlled from surface (wires not shown).

CHAPTER 3. RELATIONSHIPS BETWEEN FREE AMINO ACID AND PROTEIN
COMPOSITION AND INCORPORATION OF INORGANIC ^{14}C IN
 NH_4^+ -LIMITED STEADY STATE CULTURES OF
MARINE PHYTOPLANKTON

INTRODUCTION

Relationships Between Cellular Chemical Composition and Nitrogen Limited Growth Rate

The potential importance of nitrogen in limiting phytoplankton growth has long been recognized (e.g. Chu, 1943, in Conover, 1975). The supply of nitrogen is also known to substantially affect plant metabolism and cellular chemical composition. Changes in metabolism associated with nitrogen limitation affect the relative abundances of carbon associated with free and protein amino acids (Rhee, 1978, 1980; Demanche et al., 1979; Demanche, 1980; Dortch, 1982; Maske, 1982; Wheeler, 1983). Growth rate-dependent changes in the levels of free amino acids reflect changes in rate limiting steps of nitrogen assimilation and incorporation into macromolecules (Wheeler, 1983). Such growth rate-related changes in mechanisms of processing intracellular nitrogen may be responsible for the transient rapid NH_4^+ uptake capabilities of laboratory and natural phytoplankton populations (McCarthy and Goldman, 1979; Glibert and Goldman, 1981; Goldman and Glibert, 1982; Wheeler, 1983).

The first step in assimilation of inorganic nitrogen is the glutamine synthetase/glutamine oxoglutarate glutamate aminotransferase or GS/GOGAT enzyme couple (e.g. Miflin and Lea, 1977; Miflin et al., 1980; Syrett, 1981). A variety of studies have documented differential increases in intracellular free glutamine and other selected amino acids upon NH_4^+ enrichment of nitrogen deficient algal cultures (Syrett, 1953; Hattori, 1958 in Syrett, 1981; Riesner et al., 1960; Turpin and Harrison, 1978). These responses are analogous to observed patterns of assimilation of inorganic ^{14}C into intracellular free amino acids in response to NH_4^+ enrichment in algae (e.g. Holm-Hansen et al., 1959; Kanazawa et

al., 1970, 1972) and higher plants (Platt et al. 1977; Paul et al., 1978; Larsen et al., 1981). With the exception of the study by Turpin and Harrison (1978), the pre-enrichment physiological states and growth rates were not precisely defined in the investigations cited above. This precludes an evaluation of the relationship between growth rate and the steady state levels of individual free amino acids. In view of the observed responses to enrichment, it is likely that growth rate dependent changes in total free amino acid pool size are accompanied by changes in the relative abundances of individual free amino acids. This would reflect changes in rate limiting steps intermediate between uptake of inorganic nitrogen, and nitrogen assimilation into macromolecules. Descriptions of such compositional changes may serve as an index of the nitrogen status of phytoplankton populations.

Goldman (1980) noted that C:N ratios were uniformly related to relative growth rate for many different algae. However, the presence of other microbes and non-living organic matter leads to an ambiguous relationship between algal primary production and particulate organic matter composition (e.g. Beers et al., 1975; Banse, 1974, 1977; Wangersky, 1977). The ability to probe compositional changes in free amino acids and protein using inorganic ^{14}C as a tracer may permit selective characterization of the nitrogen status of natural algal populations, and perhaps, a relative index of growth rates. Such an approach would be free of problems relating to interference by nonalgal biomass, thereby providing an unbiased assay of the metabolism and composition of the active autotrophic population. A prerequisite to relating ^{14}C distributions to relative abundances of carbon in free and

protein amino acids will be to characterize the effects of nitrogen limitation on the kinetics of isotope incorporation.

Relationships of the Distribution of Assimilated Inorganic ^{14}C to Cellular Chemical Composition

Radioisotopic carbon can be used to trace various levels of algal metabolism. On the basis of kinetic analyses of ^{14}C distributions among specific biological molecules in Chlorella, Calvin and co-workers postulated a mechanism for the photosynthetic carbon reduction cycle (e.g. Bassham et al., 1954). The rapid saturation characteristics of the photosynthetic carbon reduction cycle intermediates made it possible to equate steady state label distributions to the carbon pool sizes of functioning intermediate reservoirs (Calvin and Massini, 1952). In this manner, it was possible to determine their responses to changes in growth conditions. It was later recognized that some molecules from the photosynthetic carbon reduction cycle were used directly in the biosynthesis of intracellular free and protein amino acids (Smith et al., 1961; Bassham and Kirk, 1964; Bassham et al., 1964). Studies with isolated chloroplasts have since demonstrated the ability of chloroplasts to synthesize a variety of amino acids (e.g. Kirk and Leech, 1972; Leech and Murphy, 1976; Mills et al., 1980; Schulze-Seibert et al., 1984). Despite the close relationship, saturation of intracellular free amino acids with inorganic ^{14}C was generally observed to be slower than that of the photosynthetic carbon reduction cycle intermediates, and may be incomplete even after a few hours of incubation with inorganic ^{14}C (Smith et al., 1961; Bassham and Kirk, 1964; Bassham et al., 1964; Hellebust and Bidwell, 1964; Larsen et al., 1981). Furthermore,

different amino acids have different isotope incorporation kinetics due to differences in the degree to which their biosynthesis is coupled to the metabolic flux of photosynthetic carbon. As a result of the relatively slow saturation characteristics, the specific activity of the free amino acids may be significantly less than that of the dissolved inorganic carbon pool during the initial period of an incubation. The relationship between the amount of label associated with the free amino acids and their carbon pool sizes will be time dependent. It is likely that growth rate and physiological state will affect the saturation rates of free amino acids. Because growth rates were not precisely defined in previous investigations, it has not been possible to evaluate the effect of growth rate variations on free amino acid saturation rates.

Incorporation of ^{14}C into protein amino acids does not necessarily reflect the incorporation characteristics of the free amino acids. Bassham et al. (1964) found that ^{14}C incorporation into some protein amino acids of Chlorella rapidly became linear, despite the fact that corresponding free amino acid pools had not yet fully equilibrated. The interpretation was that protein amino acid precursor pools were metabolically segregated, and turning over faster than the total intracellular free amino acid pools. The concept of metabolic segregation has been supported by the work of other researchers (Hellebust and Bidwell, 1964; Oaks and Bidwell, 1970; Wheeler and Stephens, 1977). One explanation for the observed segregation is that the actively turning over pools represented the chloroplast pools, the synthesis of which were closely coupled to the photosynthetic carbon

reduction cycle. However, metabolic segregation may not necessarily require physical separation of pools by membranes (Mifflin and Lea, 1977). If rapidly saturating pools exist, and serve as the exclusive source of precursors for cellular protein synthesis, then carbon incorporation into protein amino acids should rapidly reflect net biosynthetic rates provided there is little or no turnover of protein in excess of net synthesis. Furthermore, the proportion of total ^{14}C uptake associated with protein would correspond to the proportion of cellular carbon in protein.

Effects of Nitrogen Limitation on Patterns of Inorganic ^{14}C Incorporation into Cellular Material

A number of investigators have described effects of nitrogen limitation or deficiency on inorganic carbon incorporation into algal cell material (Fogg, 1956; Morris et al., 1974; Konopka and Schnur, 1981). In these studies, the labeled cell material was fractionated using sequential solvent extraction techniques. In general, increased nitrogen limitation led to increased proportions of incorporated ^{14}C in storage materials such as carbohydrate or lipid, and decreased proportions associated with protein. This is consistent with independent observations of biochemical variations associated with nitrogen starvation (e.g. Syrett, 1953; Thomas and Krauss, 1955; Fogg, 1959; Handa, 1969; Myklestad and Haug, 1972; Conover, 1975; Edge and Ricketts, 1977; Myklestad, 1977; Hitchcock, 1981; Shifrin and Chisholm, 1981) and nitrogen limitation (e.g. Thomas and Dodson, 1972; Caperon and Meyer, 1972; Rhee, 1973, 1978, 1980; Harrison et al., 1976; Laws and Wong, 1978; Goldman and Peavey, 1979; Goldman, 1980; Laws and Bannister, 1980;

Konopka and Schnur, 1981; Dortch, 1982; Maaske, 1982; Wheeler et al., 1983). An exception was the observations of Morris et al. (1974) that ^{14}C incorporation into hot trichloroacetic acid insoluble material (protein) was enhanced in nitrogen limited continuous cultures of Phaeodactylum tricornutum.

Fractions of cellular material derived by solvent extraction techniques are operationally characterized. The lack of precise chemical definitions of the subcellular fractions makes their relationship to cellular metabolism somewhat ambiguous. Comparisons of different fractionation procedures by Hitchcock (1981) demonstrated the potential for variations associated with the different methodologies. Changes in species composition can also influence the chemical contents of subcellular fractions even when obtained by identical procedures. Hitchcock (1981) also noted a lack of correlation between the distribution of ^{14}C among subcellular fractions after a 6 hour incubation and the amount of carbon estimated by chemical assays of constituents. Although Konopka and Schnur (1981) did conduct biochemical analyses, the data presented were not adequate to make direct comparisons to inorganic ^{14}C incorporation patterns and the influence of nitrogen status on this relationship. It is obvious that solvent extractions which are not further chemically characterized can at best be classified as operational definitions of a "type" of cell material. I have avoided this problem by isolating, quantifying, and radioassaying individual free and protein amino acids, so that my results can be interpreted on clearly defined chemical and physiological bases.

Another problem with current fractionation methods is the lack of proper consideration of potential containment artifacts and effects of incubation period on isotope distribution. Olive and Morrison (1967) and Olive et al. (1969) employed endpoint incubations from sunrise to sunset, while Hitchcock (1978) incubated his samples for 24 hours. Morris and Skea (1978), Smith and Morris (1980a,b), Morris et al. (1981), and Priscu and Goldman (1983) sampled in a time course over a light/dark cycle. Because of the problems associated with extended containment (Vollenweider and Nauwerck, 1961; Venrick et al., 1977; Goldman et al., 1981a; Peterson, 1980; Ferguson et al., 1984), the degree to which the behaviour of samples from long term incubations reflects the activity of the natural populations is questionable. This has led to use of shorter incubation periods.

Barlow (1982), Barlow and Henry (1982), Konopka (1982), and Priscu and Goldman (1983) used endpoint incubations of approximately 4 hours. Li and Platt (1982) conducted endpoint incubations of 2 hour duration. Li et al. (1980) compared distribution of ^{14}C among subcellular fractions from 2 and 4 hour incubations of samples of natural populations of Oscillatoria. They found significant time dependent changes which were influenced by photosynthetic rate.

Although short incubation periods minimize containment artifacts, they are more subject to the effects of isotopic disequilibrium. Hitchcock (1981) characterized the time dependence of isotopic distribution into different cell fractions of dinoflagellate cultures. His results demonstrated an initial accumulation of label in the low molecular weight pool, which subsequently diminishes as label is distributed into other cell fractions. He noted that up to four hours

were required to reach plateaus in distributions. Cuhel and Waterbury (1984) compared equilibrium distributions of isotopes with short term distributions in the cyanobacterium Synechococcus sp. and found that even after 6 hours, the short term distribution of isotopic carbon in the hot TCA insoluble material (protein) underestimated the equilibrium distribution by about 10%.

These studies illustrated the importance of understanding the dynamics of ^{14}C incorporation in order to relate it to cellular chemical composition. A major objective of this investigation was to characterize the effects of nitrogen limitation the steady state composition of free amino acids and protein, and their kinetics of inorganic ^{14}C incorporation.

As pointed out by Goldman and Glibert (1983), a direct relationship between growth and nitrogen uptake only occurs when a population is in steady state balanced growth. Although steady state continuous cultures are not representative of the natural populations (Jannasch, 1974), they do provide a means for studying characteristics of populations of well defined physiological state where growth rate, dilution rate, and specific nutrient uptake rates (V_m) are equal. Initially, results from NH_4^+ -limited steady state populations will be considered, where growth rate and nitrogen uptake are coupled and precisely defined. Subsequently, I will describe the metabolic dynamics associated with enrichment of NH_4^+ -limited cultures with saturating levels of NH_4^+ .

Specifically, this study addressed the following questions:

- 1) What is the effect of growth rate on steady state levels of intracellular free amino acids?
- 2) What is the effect of growth rate on the time course of saturation of free amino acids with inorganic ^{14}C ?
- 3) What is the relationship between nitrogen turnover of nitrogen transport mediators, and saturation rates with ^{14}C ?
- 4) What is the relationship between the specific activities of free amino acids and newly assimilated protein? Does segregation of precursor pools result in rapid saturation such that the incorporation of ^{14}C into protein reflects actual protein carbon production?
- 5) To what extent does the distribution of ^{14}C among free amino acids reflect their proportion of total cellular carbon?
- 5) How is the relationship of ^{14}C distribution among other cellular material influenced by growth rate?

Important considerations in the NH_4^+ enrichment studies were:

- 1) What are the transient responses of both composition and isotope distribution to NH_4^+ enrichment of an NH_4^+ limited steady state culture?
- 2) To what extent can metabolic responses of NH_4^+ enrichment be generalized among species?

Most of the results involve studies conducted with the marine chlorophyte, Nannochloris sp. (clone GSB Nanno). This species was chosen because it commonly occurs in near shore environments, and is easily cultured. It is a small organism (approximately 2 micron diameter), and

contains only one chloroplast. Its relative simplicity makes it an ideal model organism for these studies. Subsequently, results of preliminary investigations on several other species will be discussed.

The potential impact of nitrogen supply on algal growth and cellular chemical composition has important implications for the abundance and composition of primary organic matter, and its incorporation into the marine food web. This study provides information about relationships between carbon assimilation patterns and changes in cellular chemical composition associated with nitrogen limitation. The information will provide a foundation for using inorganic ^{14}C to probe chemical composition and quantify biosynthetic rates in natural autotrophic populations. Such an investigative approach will be useful in characterizing the metabolism of primary producers, and defining their role in the flux and compositional dynamics of particulate organic matter.

I. DYNAMICS OF FREE AMINO ACID AND PROTEIN COMPOSITION AND INCORPORATION OF INORGANIC ^{14}C IN NH_4^+ -LIMITED STEADY STATE CULTURES OF THE MARINE CHLOROPHYTE, NANNOCHLORIS SP.

METHODS

All methods used in this section were described in Chapter 2.

RESULTS AND DISCUSSION

Cellular Chemical Composition as a Function of Relative Growth Rate

The relationship between relative growth rate (μ/μ_{max}) of NH_4^+ -limited cultures of Nannochloris sp. and distributions of

cellular carbon and nitrogen among intracellular free amino acids (IFAA) and protein are shown in Tables 3-1 and 3-2 respectively. Relative growth rate is defined as the dilution rate of the chemostat (μ , absolute growth rate) normalized to the maximum growth rate ($\mu_{\max}$ = batch culture growth rate under the same conditions of light and temperature (e.g. Goldman, 1980). Percentages of cellular carbon associated with the intracellular free amino acids (IFAA) decreased with relative growth rate as shown in Table 3-1. Similarly, the percentage of cellular carbon associated with protein decreased (Table 3-2). There was a decline in the percentage of cellular nitrogen associated with the free amino acids, but no significant change in the percentage of cellular nitrogen associated with protein as a function of relative growth rate (Table 3-2) as has been previously reported (Maske, 1982). Maske (1982) observed a decrease of from 8.0 - 2.5% of the cellular nitrogen associated with free amino acids in continuous cultures of the diatom, Skeletonema costatum, with increasing NH_4^+ limitation. This was within the range reported in this study (Table 3-1). Maske (1982) also reported a decrease in the proportion of cellular nitrogen associated with protein from 82 - 52% as growth rate increased, which was slightly higher than the range observed for Nannochloris sp. (Table 3-2). Rhee (1978) found 44% of the cellular nitrogen was associated with protein in nitrate limited continuous cultures of Scenedesmus sp., similar to the value I observed for Nannochloris sp.

Significant changes in the IFAA pool composition accompanied the growth rate-related decline in pool size (Table 3-3, Figure 3-1). Individual amino acids were identified by comparison of retention times with authentic standards. In general, the separation was adequate so as

Table 3-1. Proportions of Total Cellular Carbon and Nitrogen in Free Amino Acids of Nannochloris sp. as a Function of Relative Growth Rate

Relative Growth Rate ^a	% Carbon	% Nitrogen
1.00 (0.037) ^b	6.8 (1.7)	12.0 (3.2)
.965 (0.098)	5.0 (1.5)	8.5 (3.3)
.933 (0.031)	4.5 (0.90)	7.0 (1.7)
.458 (0.014)	2.7 (0.60)	5.8 (1.5)
.229 (0.016)	0.31 (0.07)	1.4 (0.34)

^a $\mu/\mu_{\max}$ where $\mu_{\max} = 1.44 (0.04) \text{ day}^{-1}$.

^bValues in parentheses represent 95% confidence intervals.

Table 3-2. Proportions of Total Cellular Carbon and Nitrogen in Protein of Nannochloris sp. as a Function of Relative Growth Rate

Relative Growth Rate ^a	% Carbon	% Nitrogen
1.00 (0.037) ^b	22.5 (3.7)	59.0 (12.5)
0.965 (0.098)	21.9 (3.9)	42.8 (9.5)
0.933 (0.031)	18.9 (4.9)	31.2 (21.0)
0.458 (0.014)	16.8 (3.0)	36.8 (8.4)
0.229 (0.016)	8.8 (2.0)	42.0 (7.3)

^a $\mu/\mu_{\max}$ where $\mu_{\max} = 1.44 (0.04) \text{ day}^{-1}$.

^bValues in parentheses represent 95% confidence intervals.

Table 3-3. Proportions of the Intracellular Free Amino Acid Carbon Pool Associated with Individual Amino Acids as a Function of Relative Growth Rate of *Nannochloris* sp.

Relative ^a Growth Rate:		% Intracellular Free Amino Acid Pool Carbon				
		1.00 ^b	0.965	0.933	0.458 ^b	0.229
Amino Acid:	ASP	1.7 (0.93) ^c	1.2 (0.64)	1.05 (0.25)	2.1 (1.8)	1.3 (1.6)
	GLU	9.2 (2.6)	9.1 (3.8)	9.2 (2.3)	18.7 (4.9)	53.4 (14.9)
	GLN	4.4 (1.6)	7.8 (3.1)	11.5 (3.6)	1.5 (0.61)	3.7 (1.6)
	SER	3.3 (1.9)	0.52 (0.19)	0.38 (0.38)	2.3 (1.1)	0.92 (0.69)
	THR	0.49 (0.29)	0.14 (0.14)	0.21 (0.04)	1.1 (1.0)	0.71 (0.31)
	GLY	0.74 (0.20)	0.24 (0.12)	0.22 (0.08)	0.72 (0.44)	0.50 (0.22)
	ALA	3.0 (1.4)	0.43 (0.29)	1.06 (0.82)	13.7 (7.2)	8.3 (3.1)
	PRO	56.7 (22.1)	70.5 (30.0)	75.6 (16.5)	38.5 (14.4)	21.4 (8.6)
	VAL	1.6 (0.42)	0.58 (0.84)	n.d.	2.8 (1.6)	3.7 (1.3)
	ARG	7.8 (3.6)	6.0 (11.0)	n.d.	7.8 (3.4)	n.d.
	BABA	n.d.	0.72 (0.58)	0.77 (0.20)	2.0 (1.4)	6.0 (1.7)
	IRP	n.d.	n.d.	n.d.	6.6 (3.9)	n.d.
	PHE	n.d.	0.13 (0.06)	n.d.	n.d.	n.d.
	LYS	n.d.	0.63 (0.34)	n.d.	1.4 (0.85)	n.d.
	HIS	n.d.	1.4 (1.2)	n.d.	0.82 (0.26)	n.d.
	ORN	2.2 (1.8)	0.71 (0.32)	n.d.	n.d.	n.d.

^a $\mu/\mu_{\max}$ where $\mu_{\max} = 1.44 (0.04) \text{ day}^{-1}$.

^bThese cultures were not axenic.

^cValues in parentheses represent 95% confidence intervals.

Figure 3-1. Proportions of intracellular free amino acid carbon associated with individual amino acids of NH_4^+ -limited steady state cultures of Nannochloris sp. at high ($\mu/\mu_{\text{max}} = 0.933$) and low ($\mu/\mu_{\text{max}} = 0.229$) relative growth rates.

to make identification unambiguous. I was not able to resolve beta-glutamic and glutamic acid, however. Furthermore, proline, gamma-aminobutyric acid, and arginine all eluted closely, making distinctions difficult. In the remainder of the thesis, I designate derivatizable compounds of interest according to the elution characteristics of standards. However, these identifications have not been verified by an alternate separation procedure, or more rigorous procedures such as mass spectrometry.

It has been suggested that the relative proportions of cellular nitrogen associated with free amino acids may be a means of indexing nitrogen status of the phytoplankton population (e.g. Rhee, 1978; Dortch, 1982). My results suggest even more information may be revealed by characterizing the composition of individual intracellular free amino acids. Knowledge about variations in individual free amino acids as a function of nitrogen supply may also help in understanding the relative importance of rate limiting steps in the metabolic flux of nitrogen.

Glutamate and alanine increased in proportion to IFAA pool carbon as relative growth rate decreased (Table 3-3). The proportion associated with proline decreased, and there was some indication of a decrease in glutamine, although proportions were variable. The decrease in the proportion of proline carbon in IFAA carbon as growth rate decreased suggests its production/utilization ratio decreased more than that of other IFAA's. The accumulation of proline as a free amino acid in a microalga has not been observed to my knowledge. Wheeler and Stephens (1977) noted the presence of a large storage pool of arginine in Platymonas sp. Arginine elutes close to proline, and I recognize the possibility that what I have identified as proline is actually arginine.

On the basis of extensive comparisons of retention times to proline standards, I believe the compound was proline and shall continue to refer to it as such. In Nannochloris sp., the proline pool serves as a reserve nitrogen source during nitrogen limitation (Table 3-3). Levels of free proline in some higher plants are sensitive to water stress (Stewart et al., 1977). Proline is a major dissolved free amino acid associated with salt marsh grasses, thought to be an osmoregulator. In view of the range of halotolerance by Nannochloris sp., which can be inferred from its broad distribution in coastal environments, it's possible that proline serves as an osmoregulator in this organism as well.

The proportion of IFAA carbon associated with other free amino acids increased slightly or remained constant as relative growth rate decreased, an indication that their utilization was coupled to the decrease in nitrogen supply. The fact that glutamate concentration remained relatively high would suggest that transamination involved with the formation of other amino acids was inhibited. Possibly, reductions in supply of carbon skeletons for transamination occurred as a result of regulatory effects of nitrogen on the storage of carbon (e.g. Mohamed and Gnaman, 1979; Hipkin and Syrett, 1977).

Nitrogen Specific Turnover Rates of Nitrogen Transport Mediators

Obviously, in a nitrogen limited steady state culture, the ultimate rate limiting step in nitrogen assimilation and growth rate is the supply of nitrogen to the culture. Metabolically, the reaction which limits growth may vary as a result of changes in nitrogen supply. The relative importance of different metabolic steps in limiting the assimilative flux

of nitrogen was examined by comparing the specific flux of nitrogen through intracellular pools of nitrogen transport intermediates. Rates of nitrogen uptake were estimated based on the dilution rates of the steady state cultures and measured particulate organic nitrogen concentrations according to the equation:

$$R_N = DN_O \quad (3-1)$$

where R_N is the net nitrogen uptake rate ($\mu\text{M N l}^{-1} \text{ hr}^{-1}$), D is the dilution rate of the continuous culture (hr^{-1}), and N_O is the steady state particulate organic nitrogen concentration ($\mu\text{M l}^{-1}$). Nitrogen specific turnover rates of nitrogen transport mediators were determined by normalizing R_N to the concentrations of IFAA nitrogen, glutamine amide nitrogen (initial site of nitrogen assimilation via glutamine synthetase, GS), and glutamate nitrogen (obligatory intermediary transport pool via glutamine-oxoglutarate glutamate aminotransferase, GOGAT). There were dramatic differences between high and low relative growth rates in turnover rates for glutamine amide nitrogen and glutamate nitrogen (Table 3-4). Glutamine amide nitrogen had a much higher turnover rate at the low relative growth rate, while in contrast, glutamate nitrogen specific turnover decreased with decreasing relative growth rate. At high relative growth rate, glutamine amide nitrogen, and glutamate nitrogen turn over at approximately the same rate. This indicates that these rates are coupled under conditions of nitrogen saturation. The decline in levels of glutamine relative to glutamate as relative growth rate decreases (Table 3-3) and the high specific turnover of glutamine amide nitrogen at low relative growth rate

Table 3-4. Nitrogen Specific Turnover Rates of Nitrogen Transport Mediators in Relationship to Growth Rate of Nannochloris sp.

Relative ^a Growth Rate	Nitrogen Specific Turnover Rates (hr ⁻¹) ^b		
	Free Amino Acid Nitrogen	Glutamine Amide Nitrogen	Glutamate Nitrogen
0.933 (0.031) ^c	0.74 (0.17)	7.8 (2.6)	9.8 (2.8)
0.229 (0.016)	1.00 (0.25)	30.2 (14)	2.1 (0.66)

^a $\mu/\mu_{\max}$ where $\mu_{\max} = 1.44 (0.04) \text{ day}^{-1}$.

^bTotal nitrogen uptake rate normalized to transport pool nitrogen concentrations.

^cValues in parentheses represent 95% confidence intervals.

(Table 3-4) are an indication of a high utilization/production ratio. This would be the case if the glutamine synthetase (GS) reaction becomes increasingly rate limiting with decreasing nitrogen supply, and is in agreement with the conclusions of Wheeler (1983) based on studies assaying the activities of enzymes involved in nitrogen assimilation. GOGAT, which uses glutamine and alpha-ketoglutarate as substrates, is competitively inhibited by glutamate (Mifflin et al., 1980). Thus a decline in the glutamine/glutamate ratio would tend to reduce the rate of nitrogen flux through the GOGAT reaction as well. The reduced flux of nitrogen ultimately contributed to the decline in proportions of other free amino acids, and the suppression of growth.

There were no significant differences in IFAA nitrogen specific turnover rates at high and low relative growth rates (Table 3-4). Although turnover of glutamine amide nitrogen increased with decreasing relative growth rate, its relative abundance decreased, thereby diminishing its impact on total IFAA nitrogen turnover. In contrast, the turnover rate of glutamate nitrogen decreased and the proportion of IFAA nitrogen in glutamate increased. It is not possible to directly assess the nitrogen turnover rates for proline, glycine + alanine, and aspartate with my data. Apparently the decline in other free amino acids such as proline, which are probably turning over more slowly, compensated for the decreased glutamate nitrogen turnover rate. This resulted in little or no change in bulk free amino acid nitrogen turnover rates with growth rate.

Saturation With Inorganic ^{14}C : Relative Specific Activities of Intracellular Free Amino Acids

Time course descriptions of specific activities of free amino acids were determined by both quantifying the concentration of the individual free amino acids and radioassaying their incorporation of ^{14}C . The absolute specific activity of an amino acid is given as:

$$\text{SA}_{\text{AA}} = \text{C}^*(t)/\text{C}_{\text{AA}} \quad (3-2)$$

where SA_{A} is the absolute specific activity, $\text{C}^*(t)$ is the dpms of ^{14}C in the amino acid pool at time t , and C_{AA} is the steady state carbon pool concentration of the amino acid. In order to facilitate comparisons among different experiments, absolute specific activities were normalized to the specific activity of the dissolved inorganic carbon pool:

$$\text{SA}_{\text{DIC}} = \text{C}^*_{\text{total}}/\text{C}_{\text{DIC}} \quad (3-3)$$

where $\text{C}^*_{\text{total}}$ equals total ^{14}C dpms in the dissolved inorganic carbon pool, and C_{DIC} is the concentration of dissolved inorganic carbon (both of which are assumed to remain constant during the incubation). The relative specific activity, $\text{SA}_{\text{AA}}/\text{SA}_{\text{DIC}}$, is equal to one when the specific activities of the free amino acid and dissolved organic carbon pools are equal.

The time dependence of the relative specific activity of IFAA carbon is shown in Figure 3-2. Arrows indicate times when flow was interrupted. There was no apparent effect of variations in relative growth rate on total IFAA carbon saturation rate. Saturation remained

below 50% throughout the three hour incubation period for both high and low relative growth rates. The turnover rate of intracellular free amino acid nitrogen was also unaffected by changes in relative growth rate (Table 3-4). The constancy of saturation rates over varying conditions of nitrogen limitation means that assimilative flux of carbon and nitrogen through IFAA's is proportional to the total pool levels. That is, given relatively constant turnover rates, pool size provides an index of flux. Turnover rates for IFAA carbon, as derived from the time course variations in relative specific activity, were substantially lower than those for nitrogen (approximately 0.2 hr^{-1} for carbon as opposed to $0.7 - 1.0 \text{ hr}^{-1}$ for nitrogen). This reflects the independence of carbon and nitrogen flux through the IFAA pool, and is consistent with the results of Bassham and Kirk (1964), who found that labelling rates of free amino acids with ^{15}N derived from NH_4^+ were higher than incorporation rates of inorganic ^{14}C .

Despite the apparent uniformity in total IFAA carbon saturation rates, saturation characteristics of individual free amino acids were differentially affected by changes in relative growth rate. Comparisons between time course variations in glutamate relative specific activities revealed a higher saturation rate associated with high relative growth rate (Figure 3-3). The extended time course in Figure 3-4 includes results from batch cultures and another chemostat experiment at an intermediate relative growth rate. Batch cultures would not be subject to nutrient depletion effects which may result from the interruption of media input in the continuous cultures (indicated by arrows in Figs. 3-3 and 3-4). Flow was not interrupted in the intermediate growth rate continuous culture. It is apparent that the batch and high relative

growth rate patterns were similar and higher than the intermediate and low relative growth rate patterns. A similar trend was observed in the growth rate dependence of glutamate nitrogen turnover (Table 3-4). Again, the turnover of glutamate nitrogen was considerably higher than that of carbon (c.f. Fig. 3-3 and Table 3-4), an illustration of the importance of glutamate as a mediator of intracellular nitrogen transport.

Growth rate dependence of glutamine carbon saturation rates was less clear (Fig. 3-5). Batch culture saturation rates were higher, while no significant differences could be shown for high and low relative steady state growth rates. It may be that saturation of glutamine is highly sensitive to nitrogen depletion as a result of cessation of nutrient supply (time of which is indicated by the arrows in Figure 3-4).

In contrast to the relatively slow saturation of glutamate, glutamine, and IFAA carbon with inorganic ^{14}C , the specific activities of glycine + alanine (Figure 3-6) and aspartate (Figure 3-7) rapidly approached unity at high relative growth rate. This suggests their biosynthesis is closely coupled to the production of photosynthetically derived carbon skeletons. As in the case of glutamate, saturation of glycine + alanine was slower at low relative growth rate and may have been sensitive to the interruption of media inflow (indicated by arrows). Data were insufficient to make any such conclusions for aspartate. Valine saturation characteristics resembled glutamate, displaying a higher saturation rate at the high relative growth rate (Figure 3-8). As for glycine+alanine, saturation at the low relative growth rate appeared to be sensitive to the interruption of media inflow. Proline displayed slow saturation kinetics at both high and low

Figure 3-2. Time course of the relative specific activity of intracellular free amino acid carbon in batch and NH_4^+ -limited steady state cultures of Nannochloris sp. Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols correspond to different relative growth rates (μ/μ_{max}): triangle, 1.00 (batch culture); X, .965 (batch culture); square, .933; cross, .229. Errors represent propagated estimates of analytical error based on 95% confidence intervals. Media inflow was stopped at 0.33 hours for $\mu/\mu_{\text{max}} = 0.933$, and at 1.0 hour for $\mu/\mu_{\text{max}} = 0.229$ (indicated by arrows).

Figure 3-3. Time course of the relative specific activity of intracellular free glutamate at high and low relative growth rate in NH_4^+ -limited steady state cultures of Nannochloris sp. Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols correspond to different relative growth rates (μ/μ_{max}): square, 0.933; cross, 0.229 day^{-1} . Errors represent propagated estimates of analytical error based on 95% confidence intervals. Media inflow was stopped at 0.33 hours for $\mu/\mu_{\text{max}} = 0.933$, and at 1.0 hour for $\mu/\mu_{\text{max}} = 0.229$ (indicated by arrows).

Figure 3-4. Extended time course of the relative specific activity of intracellular free glutamate in batch and NH_4^+ -limited steady state cultures of Nannochloris sp. Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols correspond to different relative growth rates (μ/μ_{max}): triangle, 1.00 (batch culture); X, 0.965 (batch culture); square, 0.933; diamonds, 0.458; cross, 0.229. Errors represent propagated estimates of analytical error based on 95% confidence intervals. Media inflow was stopped at 0.33 hours for $\mu/\mu_{\text{max}} = 0.933$, and at 1.0 hour for $\mu/\mu_{\text{max}} = 0.229$ (indicated by arrows).

Figure 3-5. Time course of the relative specific activity of intracellular free glutamine in batch and NH_4^+ -limited steady state cultures of *Nannochloris* sp. Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols correspond to different relative growth rates (μ/μ_{max}): triangle, 1.00 (batch culture); X, 0.965 (batch culture); square, 0.933; diamonds, 0.458; cross, 0.229. Errors represent propagated estimates of analytical error based on 95% confidence intervals. Media inflow was stopped at 0.33 hours for $\mu/\mu_{\text{max}} = 0.933$, and at 1.0 hour for $\mu/\mu_{\text{max}} = 0.229$ (indicated by arrows).

Figure 3-6. Time course of the relative specific activity of intracellular free glycine + alanine in batch and NH_4^+ -limited steady state cultures of Nannochloris sp. Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols correspond to different relative growth rates (μ/μ_{max}): X, 0.965 (batch culture); square, 0.933; cross, 0.229. Errors represent propagated estimates of analytical error based on 95% confidence intervals. Media inflow was stopped at 0.33 hours for $\mu/\mu_{\text{max}} = 0.933$, and at 1.0 hour for $\mu/\mu_{\text{max}} = 0.229$ (indicated by arrows). Curves drawn by eye.

Figure 3-7. Time course of the relative specific activity of intracellular free aspartate in batch and NH_4^+ -limited steady state cultures of Nannochloris sp. Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols correspond to different relative growth rates (μ/μ_{max}): triangle, 1.00 (batch culture); X, 0.965; square, 0.933; diamonds, 0.458; cross, 0.229. Errors represent propagated estimates of analytical error based on 95% confidence intervals. Curve drawn by eye.

Figure 3-8. Time course of the relative specific activity of intracellular free valine in batch and NH_4^+ -limited steady state cultures of Nannochloris sp. Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols correspond to different relative growth rates (μ/μ_{max}): triangle, 1.00 (batch culture); X, 0.965 (batch culture); square, 0.933; diamonds, 0.458; cross, 0.229. Errors represent propagated estimates of analytical error based on 95% confidence intervals. Media inflow was stopped at 0.33 hours for $\mu/\mu_{\text{max}} = 0.933$, and at 1.0 hour for $\mu/\mu_{\text{max}} = 0.229$ (indicated by arrows). Curves drawn by eye.

relative growth rates (Figure 3-9), supporting the hypothesis of its function as a reserve nitrogen source or osmoregulator, as opposed to a central biosynthetic intermediate.

A diagram of the biosynthetic pathways of glutamate, glutamine, aspartate, alanine, glycine and serine is shown in Figure 3-10. Glutamate and glutamine are products of the reductive amination of alpha-ketoglutarate, which is a carbon skeleton derived from the tricarboxylic acid cycle. This may explain their relatively slow equilibration rates, which involve saturation of both the large intracellular free glutamate and glutamine pools, as well as the respiratory intermediate, alpha-ketoglutarate.

The biosynthesis of glycine and serine can be derived from 3-phosphoglycerate produced by the RuDP carboxylase reaction of the Calvin cycle, and consequently would be expected to rapidly saturate. However their formation may also involve reactions of the photorespiratory product glycolate which are catalyzed by enzymes localized in the peroxisomes and mitochondria (Keys et al., 1978 in Mifflin et al., 1980). If the latter biosynthetic pathway is important, it may involve saturation of a larger carbon precursor pool. There is evidence from studies with Chlorella to suggest that the glycolate photorespiratory pathway is less favored under conditions where ammonia is the sole nitrogen source (Beudeker and Tabita, 1984). Alanine is derived from pyruvate, and aspartate from oxaloacetate. Both pyruvate and oxaloacetate are produced outside the chloroplast by enzymes of the glycolysis cycle. The fact that glycine + alanine and aspartate rapidly saturated at the high relative growth rate would require either that the entire cytosolic pools of their carbon skeletons equilibrated with

inorganic ^{14}C , or that production of their carbon skeletons was isolated from more slowly turning over pools. Studies with spinach chloroplasts have indicated that pyruvate and oxaloacetate may be produced within the chloroplast (Schulze-Siebert et al., 1984) which could explain the rapid saturation of aspartate and alanine at the high relative growth rate.

Proline is derived from glutamate. Its low saturation rate is likely a consequence of its high intracellular pool concentration and the slow saturation of glutamate. Valine is derived from pyruvate. Its saturation characteristics are discussed further in Chapter 4.

IFAA carbon and nitrogen turnover rates were relatively uniform over a wide range of nitrogen limited growth rates, despite the diverse responses of nitrogen and carbon turnover of individual amino acids to changes in relative growth rate. This is apparently a consequence of adjustments in relative abundances, as well as saturation rates. The fact that total IFAA carbon saturation was unaffected by growth rate can be explained at least partially by the fact that the proportion of the slowly turning over proline declined as growth rate declined. Hence, although the turnover of other amino acids such as glutamate, glycine + alanine, and valine were lower for the low relative growth rate, the decline in proline had a buffering effect on the saturation rate of total pool carbon.

Obviously, IFAA carbon metabolism is highly sensitive to growth rate, and responses of individual free amino acids are diverse. Free amino acids are intermediates in the synthesis of protein. However, protein amino acid precursor pools may be segregated from the bulk intracellular free amino acid pools. In the next section I discuss the extent to which

Figure 3-9. Time course of the relative specific activity of intracellular free proline in batch and NH_4^+ -limited steady state cultures of Nannochloris sp. Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols correspond to different relative growth rates (μ/μ_{max}): triangle, 1.00 (batch culture); X, 0.965; square, 0.933; diamonds, 0.458; cross, 0.229. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 3-10. Simplified diagram showing the major pathways associated with the biosynthesis of glycine (GLY), serine (SER), glutamine (GLN), glutamate (GLU), alanine (ALA), and aspartate (ASP). After Miflin and Lea (1977) and Miflin et al. (1980). PGA = phosphoglycerate, PEP = phosphoenolpyruvate, PYR = pyruvate, OAA = oxaloacetate, TCA = tricarboxylic acid cycle, KGA = alpha-ketoglutarate, RuDP = ribulose-1,5-diphosphate, FDP = fructose-1,6-diphosphate; G6P = glucose-6-phosphate. Formation of glycine and serine from glycolate may involve participation of peroxisomes and mitochondria (not indicated, see Keys et al., 1978, in Miflin et al., 1980). The proposed direct formation of phosphoenolpyruvate and pyruvate within the chloroplast (Schulze-Siebert et al., 1984) is also not shown.

observed saturation characteristics of free amino acids reflect that of newly assimilated protein amino acids. Such information is crucial in attempts to understand the relationship of isotopic labelling of protein to actual net protein synthesis, and the path of photosynthetically fixed carbon in the biosynthesis of protein amino acid precursors.

Relationships Between Free and Protein Amino Acid Saturation Rates

The production of nascent protein amino acids in a steady state continuous culture will be:

$$dP/dt = DP_0 \quad (3-4)$$

where D is the dilution rate of the chemostat, and P_0 is the steady state protein amino acid carbon concentration. dP/dt is also equal to the rate of dilution of protein carbon as a result of media inflow. Now let a source of radioactive substrate (e.g. inorganic ^{14}C) be added at time zero. For short incubation times and low growth rates (Dt less than 0.05), the dilution of nascent isotopically labeled protein can be ignored, and the chemostat can be effectively treated as a batch culture with growth rate D . In the cultures which will be considered, flow was interrupted during the incubation, at which point this assumption becomes even more valid. For further discussion, refer to Chapter 4. Given that the concentration of nascent protein, $P_n(t)$, is zero at time zero, then integration of equation 3-4 yields:

$$P_n(t) = P_0 (e^{Dt} - 1) \quad (3-5)$$

The specific activity of the nascent protein is:

$$P^*(t)/P_n(t) = (P^*(t)/P_o) (1/(e^{Dt} - 1)) \quad (3-6)$$

where $P^*(t)$ is the amount of isotope incorporated into the protein amino acid at time t (c.f. Wheeler and Stephens, 1977). $P^*(t)/P_o$ would be the specific activity of the total protein amino acid. As for the intracellular free amino acids, the specific activities were expressed as a relative specific activity, by normalizing to the specific activity of the dissolved inorganic carbon pool, SA_{DIC} (equation 4-3). A value of one indicates complete saturation with inorganic carbon.

Glutamate is one of the most abundant intracellular free amino acids, and is also an important component of protein. It should be noted that protein "glutamate" includes both glutamate and hydrolyzed glutamine. However, in view of the similarity in the saturation characteristics of free glutamate and glutamine, and the fact that glutamine is present in lower abundance relative to glutamate in average protein (mole % is approximately 5.5 for glutamate, and 3.5 for glutamine, Jukes et al., 1975; Dolittle, 1981), I feel it is reasonable to compare specific activities of protein glutamate and free glutamate. Comparisons between the relative specific activities of nascent protein glutamate and intracellular free glutamate indicated that they were generally similar at high relative growth rate with the exception of one transient point (Figure 3-11). This suggests that intracellular pools of glutamate were in equilibrium with the source of precursors to protein biosynthesis. At low relative growth rate, nascent protein glutamate had a higher specific activity than the intracellular free amino acid pool (Figure 3-12), a

Figure 3-11. Time course of the relative specific activity of intracellular free glutamate and nascent protein glutamate in batch and NH_4^+ -limited steady state cultures of Nannochloris sp. Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols for intracellular free amino acids (μ/μ_{max}): triangle, 1.00 (batch culture); X, 0.965; square, 0.933. Cross represents relative specific activity of nascent protein glutamate in culture growing at 0.933. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 3-12. Time course of the relative specific activity of intracellular free glutamate and nascent protein glutamate in an NH_4^+ -limited steady state culture of Nannochloris sp. at low relative growth rate ($\mu/\mu_{\text{max}} = 0.229$). Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols: square, nascent protein glutamate specific activity; cross, intracellular free glutamate specific activity. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

possible indication that a semi-isolated more rapidly turning over pool was preferentially used as the precursor source for protein synthesis. Despite this apparent preferential use of photosynthetically derived amino acid carbon, nascent specific activities of protein glutamate were still higher at high relative growth rate (Figure 3-13).

There was consistent agreement between protein and free amino acid specific activities of valine (Figure 3-14) and aspartate (Figure 3-15) at high relative growth rates. As for glutamate, nascent protein valine specific activity tended to exceed the free amino acid specific activity at the low relative growth rate (Figure 3-16). There was insufficient data to evaluate this relationship for aspartate at the low relative growth rate. For glycine + alanine, nascent protein amino acids appeared to rapidly saturate (Table 3-5). Again, at the low relative growth rate free amino acid activities were considerably below the specific activities of these nascent protein amino acids (c.f. Figs. 3-6 and 3-5).

Likely explanations for discrepancies between the specific activities of the amino acids in the intracellular pool and the nascent protein include compartmentalization of free amino acid pools. A separate more rapidly saturating pool of free amino acid precursors for protein could result in differences between specific activities of intracellular free and nascent protein amino acids. The existence of multiple metabolic pools has been reported by a number of investigators (Smith et al., 1961; Bassham and Kirk, 1964; Oaks and Bidwell, 1970; Wheeler and Stephens, 1977). I observed significant differences between the free and nascent protein specific activities only under conditions of nitrogen limitation. This would suggest that as nitrogen limitation

increased, an increased proportion of protein amino acid precursors were drawn from the rapidly saturating pool. Previous studies where compartmentalization has been demonstrated have generally not considered effects of variations in the physiological state. Unlike glycine and alanine, nascent protein glutamate and valine do not fully equilibrate. Therefore, these protein amino acids cannot be derived exclusively from rapidly saturating precursor pools. The close relationship between free and protein amino acid specific activities of glutamate and valine at high relative growth rate suggests there is some interequilibration of their different metabolic pools.

Another possible explanation for differences between free and nascent protein amino acid specific activities is protein turnover (e.g. Huffaker and Peterson, 1974; Cuhel et al., 1984; Trevawas et al., 1972; Bidwell et al., 196 ; Bates, 1981). Turnover of protein could result in a significantly greater gross rate of protein synthesis than the net rate which was estimated. If isotopic assimilation reflected this gross rate, which would be the case if respired protein was not immediately equilibrated with nascent protein, then this could lead to an overestimate of nascent protein specific activity by our methods. Such a discrepancy would be larger under conditions of greater protein turnover, such as might be expected under conditions of nitrogen depletion (e.g. Trevawas et al., 1972). If turnover of protein was the predominant cause of the discrepancy between free and nascent protein specific activities at the low relative growth rate, then there was a fortuitous mixing of the isotopic inputs into glycine and alanine precursor pools such that enhancement due to turnover offset the low saturation rate of the intracellular free amino acid pools. However, this seems unlikely.

Figure 3-13. Time course of relative specific activity of nascent protein glutamate at high and low relative growth rate in NH_4^+ -limited steady state cultures of Nannochloris sp. Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols (μ/μ_{max}): square, .933; cross, .229. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 3-14. Time course of the relative specific activity of intracellular free valine and nascent protein valine in an NH_4^+ -limited steady state culture of Nannochloris sp. at high relative growth rate ($\mu/\mu_{\text{max}} = 0.933$). Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols: square, nascent protein valine specific activity (connected by line); cross, intracellular free valine specific activity. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 3-15. Time course of the relative specific activity of intracellular free aspartate and nascent protein aspartate in an NH_4^+ -limited steady state cultures of Nannochloris sp. at high relative growth rate ($\mu/\mu_{\text{max}} = 0.933$). Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols: square, nascent protein aspartate specific activity; cross, intracellular free aspartate specific activity. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 3-16. Time course of the relative specific activity of intracellular free valine and nascent protein valine in an NH_4^+ -limited steady state culture of Nannochloris sp. at low relative growth rate ($\mu/\mu_{\text{max}} = 0.229$). Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols: square, nascent protein valine specific activity; cross, intracellular free valine specific activity. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Table 3-5. Relative Specific Activities of Nascent Protein Amino Acids in *Nannochloris* sp.

Relative ^a Growth Rate	Incubation Time (hr)	Amino Acid:	Relative Specific Activities ^b			
			GLY	ALA	ILE + LEU	PHE
0.933 (0.031) ^c	0.25	0.82 (0.19) ^d	0.94 (0.20)	0.52 (0.12)	1.09 (0.45)	
	0.75	0.99 (0.22)	0.76 (0.16)	0.57 (0.13)	1.21 (0.49)	
	1.33	1.05 (0.23)	0.84 (0.17)	0.63 (0.14)	1.44 (0.58)	
	1.67	1.04 (0.23)	0.99 (0.20)	0.59 (0.13)	1.38 (0.55)	
	3.0	1.07 (0.24)	1.11 (0.23)	0.63 (0.14)	1.02 (0.41)	
0.229 (0.016)	0.25	1.20 (0.32)	1.43 (0.39)	0.32 (0.08)	0.39 (0.17)	
	0.50	1.26 (0.32)	1.10 (0.25)	0.64 (0.16)	0.68 (0.28)	
	1.1	1.32 (0.33)	1.05 (0.25)	1.06 (0.26)	1.37 (0.57)	
	1.7	1.22 (0.31)	1.42 (0.34)	0.89 (0.22)	n.d. ^e	
	2.87	1.05 (0.26)	0.80 (0.19)	0.59 (0.14)	0.58 (0.24)	

^a $\mu/\mu_{\max}$ where $\mu_{\max} = 1.44 (0.04) \text{ day}^{-1}$.

^bRelative specific activity = dpm/a amino acid carbon normalized to specific activity of the dissolved inorganic carbon pool.

^cGrowth rate errors (in parentheses) represent 95% confidence intervals.

^dSpecific activity errors (in parentheses) represent propagated estimates of analytical error based on 95% confidence intervals.

^en.d. = no data.

Analytical methods were not adequate to resolve proline and arginine from hydrolyzed protein, so it is not possible to compare nascent protein specific activities directly to intracellular free proline specific activities. However, nascent protein arginine and proline specific activities were low as were those for free proline (Figure 3-17), which does not support the existence of a segregated proline precursor pool.

The characteristics of nascent protein isoleucine + leucine and phenylalanine were also examined. Differences between high and low relative growth rate were not significant (Table 3-5). Intracellular free amino acid concentrations were close to the limit of sensitivity for detection, so it was not possible to compare free amino acid specific activities with protein.

Thus far, I have discussed the characteristics of saturation of the free and protein amino acids with inorganic carbon. In the case of a few amino acids (glycine, alanine, aspartate, and valine), their saturation rate is rapid enough that their specific activity can be related directly to that of the dissolved inorganic carbon pool. However, this was only the case at the high relative growth rate. Nascent protein glycine and alanine, appear to rapidly equilibrate, irrespective of growth rate. As will be demonstrated in Chapter 4, such rapid saturation kinetics make these amino acids ideal model compounds for tracing protein synthesis.

For the remaining amino acids, determining their absolute concentrations based on ^{14}C incorporation is difficult for short incubations. However, it is possible to obtain qualitative descriptions of the free amino acid and protein composition of the algal biomass by examining the proportions of total ^{14}C uptake associated with these biogenic materials. This information may be useful in providing an indication of the nitrogen status of an algal population.

Figure 3-17. Time course of the relative specific activity of nascent protein proline + arginine in NH_4^+ -limited steady state cultures of Nannochloris sp. at high and low relative growth rate. Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols (μ/μ_{max}): square, 0.933; cross, 0.229. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Time Course Distribution of ^{14}C -label Among Cellular Materials and its Relationship to Cellular Chemical Composition

In Figure 3-18, the short term time course distributions of inorganic ^{14}C among different subcellular fractions from a batch culture are compared to equilibrium labeled distributions (i.e. distributions in a culture which had been grown for at least 4 generations in the presence of inorganic ^{14}C). The operationally defined fractions included alcohol soluble material (intracellular free amino acids and low molecular weight metabolites), ether soluble material (lipophilic material), hot trichloroacetic acid soluble material (carbohydrate and nucleic acids), and an insoluble proteinaceous residue. The dotted line enveloped by the two solid lines indicates the mean with 95% confidence limits for the equilibrium label distribution.

Short term label distributions were initially very different from saturated label distributions. A majority of the label initially accumulates in the alcohol soluble material, and subsequently redistributes. This is an indication that the alcohol soluble material includes compounds which equilibrate more rapidly than other cell material. Discrepancies substantially diminished within the three hour incubation period, although significant differences persisted for the protein and alcohol soluble fractions. This is consistent with the observations of Cuhel and Waterbury (1984), whose results indicated that short term inorganic ^{14}C distributions underestimated equilibrium distributions in the protein fraction of the cyanobacterium, Synechococcus sp. even after 6 hours. The equilibrium proportions of label associated with the protein residue agreed well with estimates of

the proportion of total cellular carbon in protein based on chemical analyses (Table 3-2), suggesting that the equilibrium proportion of ^{14}C in the residue reflected primarily protein carbon.

Comparisons Between the Composition of Free Amino Acids and the Distribution of ^{14}C

In Figure 3-19, the time course distribution of ^{14}C -label in the intracellular free amino acids is compared to other metabolites in the alcohol soluble material. As can be seen, the time dependent variation in distribution is due primarily to the labelling patterns of the intracellular free amino acids. A similar pattern of free amino acid incorporation was observed at both high and low relative growth rates (Figure 3-20).

Although the specific activities of free amino acids were generally less than that of dissolved inorganic carbon, their proportion of total ^{14}C uptake exceeded their proportion of total cellular carbon. This is because the free amino acid pools, or portions thereof, saturated more rapidly than other cell material such as protein. The tendency for proportions of total ^{14}C uptake associated with free amino acids to exceed their proportion of total cellular carbon was particularly evident during the initial period of incubation with isotope. The proportions of total ^{14}C uptake associated with individual free amino acids are compared to proportions of cellular carbon for the high and low relative growth rates in Tables 3-6 and 3-7 respectively. Because free amino acid carbon was a small proportion of total carbon, the incorporation of ^{14}C into other cell material resulted in a decrease in the proportions of total ^{14}C uptake in free amino acids during the incubation period.

Figure 3-18. Short term time course of distribution of incorporated inorganic ^{14}C relative to equilibrium label distributions among subcellular fractions in a batch culture of Nannochloris sp. Dotted line indicates mean of equilibrium labeled culture (4 generations) with 95% confidence limits designated by solid lines.

Figure 3-19. Time course of the distribution of incorporated inorganic ^{14}C in alcohol soluble material from batch cultures of Nannochloris sp.

Figure 3-20. Time course of the distribution of incorporated inorganic ^{14}C in intracellular free amino acids of NH_4^+ -limited steady state cultures of Nannochloris sp. at high and low relative growth rate. Symbols (μ/μ_{max}): square, 0.933; cross, 0.229.

Table 3-6. Distributions of Total Cellular Carbon and Total ^{14}C Uptake Among Free Amino Acids in *Nannochloris* sp. at High Relative Growth Rate^a

Amino Acid	% Cell Carbon	Incubation Time (hrs):	% ^{14}C -Carbon				
			0.1	0.25	1.0	1.0	3.0
ASP	0.047 (0.010) ^b		4.6	2.8	1.2	1.2	0.28
GLU	0.41 (0.085)		8.3	6.1	3.8	4.2	1.6
GLN	0.51 (0.14)		2.0	2.8	2.4	2.2	1.0
THR	0.009 (0.001)		0.89	0.65	0.56	0.28	0.10
SER	0.017 (0.017)		2.5	1.3	1.0	n.d. ^c	n.d.
GLY + ALA	0.057 (0.036)		6.8	3.0	0.94	1.0	0.29
PRO	3.4 (0.58)		1.1	0.85	1.6	1.4	1.8
VAL	0.074 (0.023)		1.7	0.80	0.23	0.67	0.21
Total	4.5 (0.9)		27.9	18.3	11.7	11.0	5.3

^a $\mu/\mu_{\text{max}} = 0.933$.

^bValues in parentheses represent 95% confidence intervals.

^cn.d. = no data.

Table 3-7. Distributions of Cellular Carbon and Total ^{14}C Uptake
Among Free Amino Acids in Nannochloris sp. at Low Relative Growth Rate^a

Amino Acid	% Cell Carbon	Incubation Time (hrs):	% ^{14}C -Carbon						
			0.0	0.07	0.25	0.25	1.1	2.07	2.87
ASP	0.004 (0.005) ^b		3.1	1.0	0.40	0.48	0.28	0.29	0.008
GLU	0.16 (0.05)		10.2	6.3	3.0	3.0	2.8	0.69	0.64
GLN	0.01 (0.005)		ND ^c	0.26	0.08	0.11	0.14	n.d. ^d	0.03
SER	0.003 (0.003)		ND	1.1	0.30	0.37	0.17	n.d.	n.d.
THR	0.002 (0.001)		ND	n.d.	0.17	0.10	0.13	0.03	n.d.
GLY + ALA	0.03 (0.01)		14.2	4.6	1.5	1.6	0.54	0.27	0.19
PRO	0.06 (0.03)		ND	0.38	0.08	0.07	0.27	0.18	0.09
VAL	0.01 (0.004)		ND	0.76	0.22	0.26	0.09	0.04	0.05
Total	0.31 (0.07)		27.5	14.4	5.8	6.0	4.4	1.5	1.0

^a $\mu/\mu_{\text{max}} = 0.229$.

^bValues in parentheses represent 95% confidence intervals.

^cND = not detectable.

^dn.d. = no data.

Their proportion of total ^{14}C uptake approached the corresponding proportions of cellular carbon. An exception to this at the high relative growth rate was proline. Its proportion of ^{14}C uptake underestimated its proportion of cellular carbon and gradually increased rather than decreased. This is consistent with its slow saturation rate and large relative abundance of the intracellular proline pool at high relative growth rate (Table 3-3; Figure 3-1).

Examination of the ^{14}C distributions within the free amino acid pool revealed that most of the label was initially associated with glutamate, glycine + alanine, and aspartate (Tables 3-8 and 3-9). Other free amino acids which subsequently accounted for substantial proportions of label included glutamine, serine, proline and valine. Pathways for the biosynthesis of glutamate, glutamine, aspartate, alanine, glycine and serine are shown in Fig. 3-10. The initial accumulation of label in these amino acids is an indication of the close relationship between the photosynthetic carbon reduction cycle, and the production of carbon skeletons for amino acid biosynthesis (e.g. Miflin and Lea, 1976).

In Figure 3-21, distributions of ^{14}C are compared to distributions of cellular carbon within the intracellular free amino acid pool. Although there were quantitative differences, particularly in the case of proline, the label distribution did provide a qualitative description of the growth rate dependence of free amino acid composition. Qualitative changes in the amounts of label associated with glutamate and glutamine tended to reflect their relative abundance. Even, in the case of proline, although the proportion of ^{14}C consistently underestimated its proportion of carbon, qualitative relationships between high and low relative growth rates reflected the trends in actual abundance. Thus the

Table 3-8. Proportions of Intracellular Free Amino Acid Carbon and ^{14}C Associated With Individual Amino Acids of *Nannochloris* sp. at High Relative Growth Rate^a

Amino Acid	% Free Amino Acid Carbon	Incubation Time (hrs):	% ^{14}C -label				
			0.1	0.25	1.0	1.0	3.0
ASP	1.05 (0.25) ^b		16.5	15.3	9.9	10.9	4.6
GLU	9.2 (2.3)		29.7	33.2	31.3	39.4	27.0
GLN	11.5 (3.6)		7.3	14.9	19.8	20.5	17.2
SER	0.38 (0.38)		8.8	7.0	8.8	n.d. ^c	n.d.
THR	0.21 (0.04)		3.2	3.5	4.6	2.6	1.6
GLY + ALA	1.3 (0.9)		24.2	16.0	7.8	9.6	4.9
PRO	75.6 (16.5)		4.1	4.6	13.7	13.5	29.8
VAL	1.6 (0.5)		6.0	4.3	1.9	6.2	3.5

^a $u/u_{\text{max}} = 0.933$.

^bValues in parentheses represent 95% confidence intervals.

^cn.d. indicates no data were obtained.

Table 3-9. Proportions of Intracellular Free Amino Acid Carbon and ^{14}C Associated With Individual Amino Acids of *Nannochloris* sp. at Low Relative Growth Rate^a

Amino Acid	% Free Amino Acid Carbon	Incubation Time (hrs):	% ^{14}C -label					
			0.0	0.07	0.25	0.25	1.1	2.87
ASP	1.3 (1.6) ^b		10.4	5.6	6.5	7.7	6.0	7.2
GLU	53.4 (14.9)		34.3	34.3	50.0	47.4	61.1	59.4
GLN	3.7 (1.6)		ND ^c	1.5	1.4	1.2	3.0	2.8
SER	0.92 (0.69)		n.d.	5.9	5.0	5.9	3.8	n.d. ^d
THR	0.71 (0.31)		ND	n.d.	2.9	1.6	2.8	n.d.
GLY + ALA	8.8 (3.1)		48.0	25.3	25.2	25.6	11.6	17.6
PRO	21.4 (8.6)		n.d.	2.1	1.4	1.2	5.8	8.3
VAL	3.7 (1.3)		n.d.	4.1	3.5	4.2	2.0	4.4

^a $u/u_{\text{max}} = 0.229$.

^bValues in parentheses represent 95% confidence intervals.

^cND = not detectable.

^dn.d. = no data.

metabolism of inorganic ^{14}C is a useful probe of this sensitive level of cellular chemical composition, which is a reflection of population nitrogen status.

Time Course Distributions of ^{14}C Among Subcellular Fractions as a Function of Relative Growth Rate

It is clear from the previous discussion that the extent to which the distribution of ^{14}C reflects amino acid composition is time dependent, and influenced by growth rate. In this section, I discuss the time course distribution of ^{14}C among other cell materials as it is influenced by relative growth rate. In Figure 3-22, the time course label distributions for high ($u/u_{\text{max}} = 0.933$) and low ($u/u_{\text{max}} = 0.229$) relative growth rates are compared. At low relative growth rate, a higher proportion of label is associated with the ether soluble nonpolar material, a substantial proportion of which probably consists of lipid. Furthermore, a consistently lower proportion of label was associated with the protein residue at low relative growth rate. This is in agreement with the trend in the proportion of total particulate carbon associated with protein based on chemical analyses (Table 3-2). Initially, a slightly higher proportion of label is associated with the hot trichloroacetic acid soluble material at the low relative growth rate, but this subsequently decreased. Finally, the proportion of label associated with the alcohol soluble low molecular weight material is consistently lower at low relative growth rate than at high relative growth rate. This is due to the labelling characteristics of the intracellular free amino acids (Figure 3-20).

Figure 3-21. Proportions of intracellular free amino acid carbon and ^{14}C associated with individual amino acids of NH_4^+ -limited steady state cultures of Nannochloris sp. at high ($u/u_{\text{max}} = 0.933$) and low ($u/u_{\text{max}} = 0.229$) relative growth rates.

HIGH RELATIVE GROWTH RATE

LOW RELATIVE GROWTH RATE

Figure 3-22. Time course of the distribution of incorporated inorganic ^{14}C among subcellular fractions in NH_4^+ -limited steady state cultures of Nannochloris sp. at high and low relative growth rate. Symbols (μ/μ_{max}): square, 0.933; cross, 0.229.

Time (hours)

%

Figure 3-23. Distribution of total cellular carbon and total ^{14}C uptake among intracellular free amino acids and protein in NH_4^+ -limited steady state cultures of Nannochloris sp. at high ($\mu/\mu_{\text{max}} = 0.933$) and low ($\mu/\mu_{\text{max}} = 0.229$) relative growth rates.

The growth rate dependent differences in the distribution of ^{14}C among free amino acids and protein reflected actual trends in chemical composition (Table 3-1 and 3-2; Fig. 3-23). These data demonstrate the utility of using the dynamics of ^{14}C metabolism to probe actual compositional variations related to physiological state.

A higher proportion of label associated with ether soluble material at low relative growth rate was an indication of greater emphasis on energy storage rather than growth related metabolism. This is consistent with the large number of investigations which have reported increased C:N ratios (e.g. Conover, 1975; Goldman and Peavey, 1979; Goldman et al., 1979; Goldman, 1980), and increased abundance of storage materials relative to nitrogen containing biogenic material, and increased carbohydrate to protein ratios (e.g. Fogg, 1959; Handa, 1969; Myklestad and Haug, 1972; Edge and Ricketts, 1977; Myklestad, 1977; Rhee, 1978, 1980; Morris, 1981). In addition, my results are consistent with studies involving regulatory characteristics of enzymes controlling tradeoffs between energy storage and production of precursors for growth and protein synthesis (e.g. Hipkin and Syrett, 1977; Mohamed and Gnanan, 1979). Fogg (1956) similarly reported enhanced incorporation of ^{14}C -label into nonpolar cellular material in nitrogen starved cultures. Konopka and Schnur (1981) reported enhanced assimilation of ^{14}C into hot trichloroacetic acid extractable material (polysaccharide and nucleic acid) in nitrogen limited cultures of the cyanobacterium, Merismopedia tenuissima. In contrast, Morris et al. (1974) presented evidence for enhanced protein incorporation in nitrogen limited cultures of Phaeodactylum tricornutum, which he attributed to conservation of protein synthesis as a result of nitrogen limitation. Konopka and Schnur

(1981) suggested that the results of Morris et al. may have been due to the fact that nitrate limited cultures were resuspended in fresh medium before incubating with inorganic ^{14}C . This would have been equivalent to enriching the cultures with inorganic nitrogen. In the following sections, I examine the metabolic responses of an NH_4^+ -limited culture to enrichment with NH_4^+ .

II. EFFECTS OF NH_4^+ ENRICHMENT ON AMINO ACID AND TOTAL CARBON METABOLISM IN AN NH_4^+ -LIMITED STEADY STATE CULTURE OF NANNOCHLORIS SP.

In the preceding section, I presented results of experiments examining the relationship of amino acid and protein composition to incorporation of inorganic ^{14}C in steady state NH_4^+ -limited cultures. Because of the dynamic physicochemical environment in which they exist, natural phytoplankton populations are unlikely to be in a state of balanced growth. In this section, I discuss the relationship between free amino acid composition and incorporation of inorganic ^{14}C by NH_4^+ -limited cultures exposed to a saturating enrichment of NH_4^+ . The results illustrate the utility of using ^{14}C for tracing cellular composition and metabolism in non-steady state conditions. As will be demonstrated, intracellular free amino acid metabolism is more sensitive to increases in nitrogen supply than total ^{14}C uptake, supporting the view that probing responses at this level of metabolism may be useful in diagnosing the nitrogen status of phytoplankton populations.

METHODS

Nannochloris sp. was grown in continuous culture at a relative growth rate of .216 (.028) day⁻¹. The temperature was 24°C. NH₄Cl concentration in the media was 250 micromolar and all other nutrients were present at "h/2" concentrations (without silicate and nitrate, Guillard, 1975) in an artificial seawater base as described in chapter 2. The culture vessel was a liter Erlenmeyer flask, equipped with a butyl rubber stopper through which glass tubing was run for air sparging, sampling, media addition and effluent removal. Media was delivered through a latex rubber tube using a Harvard peristaltic pump. Efforts were made to maintain axenic conditions, however, they were not checked other than by microscopic inspection (1000X, oil immersion). Other culturing conditions were as described in chapter 2.

After the culture cell counts had stabilized for 2 consecutive days (approximate cell density of 10⁷ per ml), two 100ml aliquots were removed and placed in 250ml Erlenmeyer flasks under the same conditions of light and temperature. Inorganic ¹⁴C was added to both flasks and subsequently, to one of the flasks, NH₄Cl was added to a concentration of 200 micromolar. Samples were filtered onto Whatman GF/F filters, fumed with 6N HCl for 10 minutes, sealed in Nalgene filmware polypropylene envelopes, and stored at -20°C until analysis. 2ml samples were also collected for determination of acid non-volatile ¹⁴C. These samples were acidified in a 20ml glass scintillation vial to a concentration of 0.316N H₂SO₄, and left in a fume hood overnight. Subsequently, 15 ml Aquasol was added, and they were radioassayed as described previously.

Samples were fractionated according to the procedure described previously, within one week of the harvest. Radioactivity in the ether, hot trichloroacetic acid, and protein residue fractions was assayed as described in the general methods. The alcohol extract was stored at -20°C until amino acids were quantified and radioassayed as previously described.

All other methods are described in chapter 2.

RESULTS AND DISCUSSION

Increases in Intracellular Free Amino Acids in Response to Nitrogen Enrichment

Increases in concentrations of selected free amino acids occurred as a result of the saturating (200 micromolar) NH_4^+ enrichment. The most dramatic increase occurred in glutamine concentrations (Figure 3-24). Glutamine increased to approximately 70% of the low molecular weight amino acid carbon after 1.0 hour of enrichment and remained at this level through 1.5 hours (Table 3-10). Glutamate continuously increased after an initial lag (Figure 3-25), although its proportion of low molecular weight amino acid carbon declined after enrichment from 36% to roughly 6-9% (Table 3-10). Proline showed a shorter lag before increasing in concentration (Figure 3-26), and its proportion of free amino acid carbon declined as did that of glutamate (Table 3-10). Aspartate displayed an initial time dependent increase in concentration, and subsequently stabilized (Figure 3-27). Its proportion of the total intracellular free amino acid carbon remained relatively constant throughout 1.5 hours (Table 3-10). Other intracellular amino acids which increased in concentration over controls included glycine, alanine, and

Figure 3-24. Increase in intracellular free glutamine concentrations in response to NH_4Cl enrichment (200 micromolar) of an NH_4^+ -limited steady state culture of Nannochloris sp. Dotted line represents mean of glutamine concentration in control culture. Solid lines indicate 95% confidence limits. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 3-25. Increase in intracellular free glutamate concentrations in response to NH_4Cl enrichment (200 micromolar) of an NH_4^+ -limited steady state culture of Nannochloris sp. Dotted line represents mean of glutamate concentration in control culture. Solid lines indicate 95% confidence limits. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 3-26. Increase in intracellular free proline concentrations in response to NH_4Cl enrichment (200 micromolar) of an NH_4^+ -limited steady state culture of Nannochloris sp. Dotted line represents mean of proline concentration in control culture. Solid lines indicate 95% confidence limits. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 3-27. Increase in intracellular free aspartate concentrations in response to NH_4Cl enrichment (200 micromolar) of an NH_4^+ -limited steady state culture of Nannochloris sp. Dotted line represents mean of aspartate concentration in control culture. Solid lines indicate 95% confidence limits. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Table 3-10. Proportions of the Intracellular Free Amino Acid Carbon Pool Associated with Individual Amino Acids in Control and NH_4^+ Enriched Cultures of *Nannochloris* sp.

Amino Acid	Control	% Intracellular Amino Acid Carbon NH_4^+ Enriched		
		0.5 hr	1.0 hr	1.5 hr
ASP	2.2 (0.65) ^a	1.2 (0.36) ^b	1.4 (0.42)	1.3 (0.39)
GLU	36.1 (11.7)	9.3 (2.8)	5.5 (1.7)	6.4 (2.0)
GLN (+SER)	7.5 (8.1)	68.8 (25.9)	76.3 (29.6)	74.9 (28.9)
THR	0.86 (0.54)	0.35 (0.09)	0.12 (0.03)	0.03 (0.01)
GLY	0.94 (0.47)	1.02 (0.30)	0.55 (0.17)	0.36 (0.11)
ALA	4.4 (1.0)	3.5 (1.0)	1.6 (0.48)	0.88 (0.27)
BABA	7.5 (2.7)	n.d. ^c	0.82 (0.44)	0.72 (0.39)
PRO	23.6 (14)	4.8 (1.9)	7.5 (3.1)	7.9 (3.2)
ARG	10.5 (3.5)	6.2 (2.8)	n.d.	1.9 (0.89)
VAL	1.8 (0.41)	1.0 (0.35)	0.66 (0.24)	0.72 (0.18)
ILE	1.3 (0.56)	0.12 (0.06)	0.20 (0.11)	0.058 (0.031)
LEU	1.1 (0.99)	0.10 (0.03)	0.18 (0.055)	0.058 (0.018)
PHE	2.2 (1.1)	0.58 (0.26)	0.13 (0.06)	0.061 (0.028)
LYS	n.d.	2.92 (1.5)	3.9 (2.0)	3.6 (1.9)
HIS	ND ^d	n.d.	0.532 (0.18)	0.49 (0.17)
ORN	n.d.	n.d.	0.65 (0.28)	0.60 (0.26)
Total Free Amino Acid Carbon (micrograms/liter):	193 (36)	1190 (260)	1880 (450)	3000 (700)

^aErrors for control concentrations (in parentheses) represent 95% confidence intervals.

^bErrors for enriched concentrations (in parentheses) represent propagated estimates of analytical error based on 95% confidence intervals.

^cn.d. = no data.

^dND = not detectable.

Table 3-11. Comparisons of Intracellular Free Amino Acid Concentrations in Control and NH_4^+ Enriched Cultures of Nannochloris sp.

Amino Acid	Concentration (μM) ^a	
	Control	Enriched
GLY	0.076 (0.035) ^b	0.461 (0.100)
ALA	0.176 (0.023)	0.847 (0.322)
VAL	0.059 (0.007)	0.264 (0.117)
THR	0.0344 (0.021)	0.071 (0.080)
BABA	0.302 (0.091)	0.446 (0.301)
ARG	0.281 (0.077)	0.670 (1.10)
ILE	0.034 (0.014)	0.032 (0.044)
LEU	0.028 (0.026)	0.029 (0.037)
PHE	0.040 (0.018)	0.034 (0.065)

^aValues represent the mean over a 2.5 hour incubation period.

^bValues in parentheses represent 95% confidence intervals.

valine. Their mean concentrations over a 2.5 hour incubation period are compared in Table 3-11. The change in alanine was unique in that it was highest at 0.5 hours after enrichment, and declined asymptotically to a relatively constant value (Figure 3-28). No significant changes could be demonstrated for threonine, isoleucine, leucine, and phenylalanine (Table 3-11). All the intracellular free amino acids declined as a proportion of total low molecular weight free amino acid carbon except for glutamine, aspartate, glycine, lysine, and ornithine (Table 3-10).

The distribution of IFAA carbon among individual free amino acids in the control culture (Table 3-10) resembled the steady state values for low relative growth rate (c.f. Table 3-3). The distribution of the IFAA carbon in the enriched culture (Table 3-10) was similar to the high steady state relative growth rate distributions (c.f. Table 3-3) with the exception that glutamine was the dominant free amino acid in the enriched culture rather than proline as in the steady state case.

The increases in intracellular free amino acid concentrations as a result of nitrogen enrichment are a demonstration of an associated increase in the rate of assimilation of inorganic nitrogen. The accumulation of intracellular free amino acids in response to NH_4^+ enrichment is a general response of nitrogen depleted algal populations (Reisner et al., 1960; Turpin and Harrison, 1978; DeManche et al., 1979; DeManche, 1980; Syrett, 1981; Dortch, 1982; Wheeler, 1983; Wheeler et al., 1983). The increase in the free amino acid pool concentration is likely an indication that subsequent metabolic steps involving their utilization in biosynthesis of macromolecular material are rate limiting. However, the increases were not uniform among the different amino acids indicating intermediate rate limiting steps were also involved.

The dramatic accumulation of glutamine as a result of enrichment (approximately 70% free amino acid carbon after 1.0 hour) accompanied by an initial lag in the increase in glutamate concentrations would suggest that biosynthetic nitrogen flux became increasingly less limited by a step subsequent to the glutamine synthetase reaction. In light of the observed differences in glutamine amide and glutamate nitrogen turnover rates for high and low relative steady state growth (Table 3-4), it is likely that as a result of the enrichment, glutamine amide nitrogen turnover probably decreased, while glutamate nitrogen turnover increased. Thus, even though glutamate levels did not increase in association with glutamine, flux of nitrogen increased through the GS/GOGAT system.

Accumulation of glutamate and proline after an initial lag indicated that ultimately their utilization was also limited by subsequent biosynthetic steps. A decline in concentrations such as that observed for alanine would indicate a relative increase in the utilization/production ratio for that substrate. No significant changes could be demonstrated for threonine, isoleucine, leucine, and phenylalanine (Table 3-11). This is the result of either a lag in their pool size increases which extended beyond the incubation period, or a tight coupling of production and utilization of these amino acids.

These results are clear evidence for changes in mechanisms of processing intracellular nitrogen, which precede an acceleration of growth and protein synthesis. In the next section, I examine the relationship between these changes and patterns of incorporation of inorganic ^{14}C .

Figure 3-28. Increase in intracellular free alanine concentrations in response to NH_4Cl enrichment (200 micromolar) of an NH_4^+ -limited steady state culture of Nannochloris sp. Dotted line represents mean of alanine concentration in control culture. Solid lines indicate 95% confidence limits. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 3-29. Time course of the relative specific activity of total intracellular free amino acid carbon in control and enriched NH_4^+ -limited steady state cultures of Nannochloris sp. Relative specific activity is the specific activity of the amino acid normalized to that of the dissolved inorganic carbon pool; see text for further discussion. Symbols: square, enriched; cross, control. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Saturation of Free Amino Acids with ^{14}C in Control and Enriched Cultures

Saturation rates of total intracellular free amino acid carbon in the control and enriched cultures were similar throughout the first hour of incubation with isotope (Figure 3-29). The specific activity of the enriched culture increased slightly above the control by 1.5 hours. This indicated either enhanced turnover in the enriched culture, or a suppression of turnover in the control culture as a result of nitrogen depletion.

NH_4^+ enrichment effects on the saturation rates of selected IFAA's are shown in Table 3-12. There were no significant differences between controls and enriched cultures in the saturation rates of glutamate carbon, despite the increased production of glutamine. This increase presumably occurred via the glutamine synthetase reaction which uses glutamate as a substrate. That the source of glutamine was the glutamate pool was supported by the fact that the glutamine specific activity was similar to that of the glutamate pool in the enriched culture. This was also the case at the steady state low relative growth rate. The uniformity in glutamate saturation rates with and without enrichment indicates that the proportion of photosynthetically fixed carbon used in the production of alpha-ketoglutarate, the carbon skeleton of glutamate, was not significantly affected by enrichment (c.f. Fig. 3-10). Nevertheless, the increase in the concentration of glutamine would necessitate enhanced carbon flux through glutamate. This might be a consequence of a very large metabolic pool providing carbon skeletons for glutamate synthesis, such as respiratory intermediates derived from the tricarboxylic acid cycle whose labelling characteristics might change slowly despite an increased flux through glutamate.

Table 3-12. Relative Specific Activities of Intracellular Free Amino Acids in Control and Enriched Cultures of *Nannochloris* sp.

Amino Acid	Incubation Time (hrs)	Relative Specific Activity ^a	
		Control	Enriched
GLU	0.0	0.110 (0.024) ^b	n.d. ^c
	0.5	0.238 (0.054)	0.163 (0.036)
	1.0	0.328 (0.071)	0.329 (0.073)
	1.5	0.342 (0.074)	0.270 (0.060)
	2.5	n.d.	0.348 (0.077)
GLN	0.5	n.d.	0.246 (0.078)
	1.0	0.111 (0.037)	0.252 (0.082)
	1.5	0.082 (0.029)	0.320 (0.102)
ASP	0.5	n.d.	0.538 (0.121)
	1.0	0.814 (0.189)	0.203 (0.046)
	1.5	0.596 (0.154)	0.249 (0.054)
	2.5	n.d.	0.341 (0.073)
PRO	0.5	0.144 (0.065)	n.d.
	1.0	0.079 (0.028)	n.d.
	1.5	n.d.	0.366 (0.125)
	2.5	0.540 (0.186)	0.548 (0.188)

^aRelative specific activity = dpms/amino acid carbon normalized to specific activity of the dissolved inorganic carbon pool.

^bValues in parentheses represent propagated estimates of analytical error based on 95% confidence limits.

^cn.d. = no data.

Enrichment apparently did not enhance the saturation rate of proline (Table 3-12). The time course of proline specific activity resembled that of glutamate, its precursor. Glutamine saturation was enhanced. In contrast, the time course specific activity change of aspartate decreased as a result of enrichment, a possible indication that its carbon skeleton (oxaloacetate) was increasingly derived from unlabeled cell material such as lipid. The biosynthesis of ether soluble material was suppressed as a result of enrichment (see following section on effects of enrichment on carbon assimilation into subcellular fractions). It is possible that unlabeled lipid was used to increase the supply of carbon skeletons for protein synthesis.

As a result of the similarity in saturation rates, relative differences in amounts of ^{14}C associated with free amino acids in control and enriched cultures will reflect real differences in concentrations. Patterns of isotope incorporation are compared in the next section.

Effects of NH_4^+ Enrichment on Time Course Distributions of ^{14}C in Cellular Material

Significant effects of NH_4^+ enrichment on total inorganic ^{14}C fixation rates could not be demonstrated given the resolution of the time course sampling scheme used (Figure 3-30). In contrast, dramatic differences were observed in patterns of isotope incorporation into different cell material. Comparisons of isotope derived carbon incorporation into subcellular fractions for control and enriched cultures are shown in Figure 3-31. Enrichment led to a dramatic increase in the incorporation of isotopic carbon into the low molecular weight

metabolites, primarily at the expense of incorporation into the ether soluble fraction. There was initially no significant increase in protein incorporation and may have even been a slight depression relative to the control. However, after an initial lag, an increase in protein incorporation occurred. The lag coincided in approximate timing with the lags seen to precede the increases in glutamate concentrations (Figure 3-26), stabilization of aspartate (Figure 3-27) and alanine (Figure 3-28) concentrations, and increase in the total IFFA carbon specific activity over controls (Figure 3-29). The increase in incorporation associated with the low molecular weight metabolites was largely associated with an increase in ^{14}C in IFAA, particularly glutamine and glutamate (Table 3-13). Other amino acids whose incorporation contributed significantly included aspartate, glycine, alanine, proline, and valine, all of which also significantly increased in concentration over controls. Arginine and beta-aminobutyric acid (or a compound with similar elution characteristics) were also responsible for a small proportion of free amino acid ^{14}C incorporation. However, variability in concentration determinations precluded the demonstration of any consistent relationship to changes in their proportions of total ^{14}C uptake (c.f. Healey, 1979).

The accumulation of intracellular free amino acids under nitrogen replete conditions (DeManche et al., 1979; DeManche, 1980; Dortch, 1982; Wheeler, 1983; Wheeler et al., 1983) has led to the suggestion that protein synthesis ultimately limits the assimilation of nitrogen. This contention is supported by my data, in that the rate of labelling of the free amino acids increased within the first 0.5 hour of enrichment, while a subsequent increase in protein labelling rate over the control did not

Figure 3-30. Time course of total inorganic ^{14}C uptake by control and enriched NH_4^+ -limited steady state cultures of Nannochloris sp.

NH_4Cl enrichment of 200 micromolar was added at zero time. Culture temperature was 24°C .

Figure 3-31. Time course of the incorporation of inorganic ^{14}C into subcellular fractions in control and enriched NH_4^+ -limited steady state cultures of Nannochloris sp. growing at 24°C . Symbols: square, enriched; cross, control.

Table 3-13. Distributions of Total ^{14}C Uptake Among Intracellular Free Amino Acids in Control and Enriched Cultures of *Nannochloris* sp.

Amino Acid	Incubation Time (hrs):	% Total ^{14}C Uptake				Enriched		
		Control						
		0.0	0.5	1.0	1.5	0.5	1.0	1.5
GLU		1.3	1.9	1.4	0.89	3.3	2.6	2.4
GLN (+SER)		n.d. ^a	n.d.	0.17	0.07	36.3	27.3	33.8
ASP		0.12	n.d.	0.23	0.08	1.4	0.39	0.45
GLY		n.d.	n.d.	n.d.	n.d.	1.5	0.29	0.34
ALA		n.d.	1.26	0.10	n.d.	2.3	n.d.	0.71
PRO		n.d.	1.2	0.19	0.45	n.d.	n.d.	4.1
VAL		n.d.	n.d.	0.16	n.d.	n.d.	n.d.	0.96
ARG		n.d.	n.d.	0.21	n.d.	n.d.	n.d.	1.3
BABA ^b		n.d.	n.d.	0.13	n.d.	n.d.	0.37	0.31
Total		1.4	5.4	2.5	1.5	44.8	30.7	44.4

^an.d. = no data.

^bbeta-aminobutyric acid or a coelutant.

occur until after 1.5 hours (Figure 3-31). Reasons for such a lag in metabolism may be that the necessary protein synthesizing machinery (e.g. ribosomal RNA) had to be constructed, and amino acid pool sizes had to accumulate in order for nitrogen and carbon flux into protein to increase. It may be that this is only an apparent suppression resulting from dilution of photosynthetically derived carbon skeletons caused by an increased utilization of unlabeled storage material. This might also explain the relatively unaffected specific activity of free glutamate, despite increased carbon flux, and the decrease in aspartate specific activity.

Goldman and Dennett (1984) noted a slight suppression in photosynthesis after enrichment of NH_4^+ limited continuous cultures of Nannochloris sp. with 16 micromolar NH_4Cl . I did not observe significant differences between total ^{14}C uptake by control and enriched cultures (Figure 3-30). However, the effects referred to by Goldman and Dennett were of a duration and magnitude which would not be detectable at the level of precision of my sampling.

Although there were quantitative differences, the distribution of ^{14}C -label among IFAA's (Table 3-14) was a qualitative reflection of the actual carbon distribution (Table 3-10). The changes in patterns of ^{14}C incorporation as a result of enrichment were representative of changes in the actual concentrations and relative abundance of the nitrogen transport mediators. The increase in specific activity of IFAA carbon in the enriched culture over the control, coinciding with enhancement of protein incorporation, increasing glutamate concentration, and stabilization of aspartate and alanine concentrations, were indications of a major shift in metabolism towards accelerated growth.

Table 3-14. Distributions of ^{14}C Within the Intracellular
Free Amino Acid Pools of Control and Enriched
Cultures of Nannochloris sp.

Amino Acid	Incubation Time (hrs):	% Total Intracellular Free Amino Acid ^{14}C						
		Control				Enriched		
		0.0	0.5	1.0	1.5	0.5	1.0	1.5
GLU		91.6	36.0	56.8	59.8	7.3	8.4	5.4
GLN (+SER)		n.d. ^a	n.d.	6.9	5.0	81.0	89.0	76.1
ASP		8.4	n.d.	9.3	5.3	3.2	1.3	1.0
GLY		n.d.	n.d.	n.d.	n.d.	3.3	0.92	0.77
ALA		n.d.	3.4	0.63	n.d.	5.2	n.d.	1.6
PRO		n.d.	22.3	7.8	30.0	n.d.	n.d.	9.2
VAL		n.d.	n.d.	6.3	n.d.	n.d.	n.d.	2.2
ARG		n.d.	n.d.	8.7	n.d.	n.d.	n.d.	3.0
BABA		n.d.	n.d.	5.2	n.d.	n.d.	1.2	0.69

^an.d. = no data.

Attempts to probe the metabolism of natural communities which are intimately coupled to their physicochemical environment must recognize the need for more precise time course sampling in order to understand the biochemical events associated with transient physiological states.

III. PRELIMINARY OBSERVATIONS OF NH_4^+ ENRICHMENT EFFECTS ON CARBON METABOLISM IN TWO OTHER SPECIES

Thus far, I have discussed results with a single species, Nannochloris sp. In the following section, I examine interspecies variations in the characteristics of ^{14}C incorporation in response to NH_4^+ enrichment of NH_4^+ -limited cultures. The species studied were Dunaliella tertiolecta (clone Dun) and Thalassiosira weissflogii (clone Actin). In addition, the influence of variations in temperature on responses were assessed. Responses at three different temperatures were compared in D. tertiolecta (24°C , experiment 1; 16°C , experiment 2; 12°C , experiment 3), and two temperatures in T. weissflogii (16°C , experiment 2; 12°C experiment 3).

METHODS

Response of Intracellular Free Amino Acids of *Dunaliella tertiolecta* to Nutrient Enrichment

Enhanced production of IFAA's by D. tertiolecta as a result of enrichment was assessed using an early stationary phase batch culture of D. tertiolecta which had been grown in "h/2" media minus nitrate and silicate (Guillard and Ryther, 1962). Cell density was approximately 2.0×10^6 per ml. Replicate cultures of 10ml volume in 25 ml 100x18 mm borosilicate test tubes were grown at 21°C in a 12:12 light/dark cycle

to early stationary phase in media described in the general methods for stock cultures. Midway through the photoperiod, 5 ml fresh "h/2" media was added to one of the cultures and they were allowed to incubate for an additional 3 hours. Amino acids were extracted by filtering samples (volumes corrected for the dilution as a result of enrichment) onto a glass fiber filter, rinsing twice with 2ml artificial seawater, boiling the filters in deionized water for 1 min, and filtering the extract through a sterile 0.2 micron Millipore membrane filter. Cindy Lee analyzed the amino acids samples using fluorescence detection of o-phthaldialdehyde derivatives separated by reverse phase (5 micron, octyldecasilane) high pressure liquid chromatography (Lindroth and Mopper, 1979).

Effects of NH_4^+ Enrichment on Time Course Distributions of ^{14}C in Cellular Material

Experiment 1

Dunaliella tertiolecta was cultured under the same conditions as described for Nannochloris sp. in the previous section with the following exceptions. Media composition consisted of 100 micromolar NH_4Cl and 20 micromolar NaH_2PO_4 . All other nutrients were at "f/2" levels (without silicate and nitrate) in the artificial seawater base described in the General Methods section. The dilution rate was 0.380 ($.030$) day^{-1} and the temperature was 24°C . Aliquots were removed from the steady state culture as described in the previous Nannochloris sp. enrichment experiment and placed in 250ml Erlenmeyer flasks under similar conditions of light and temperature. Inorganic ^{14}C was added to both flasks, and NH_4Cl was added to a concentration of 200 micromolar in one

of the flasks. Samples were filtered on Whatman GF/F glass fiber filters at low vacuum (less than 200mm Hg). They were subsequently rinsed twice with 2ml artificial seawater, fumed for 10 minutes with 6N HCl, and sealed in Nalgene filmware polypropylene baggies. These were stored in liquid N₂ until analysis. The subcellular fractionation procedure differed from that described in the general methods section in that filters were initially extracted 2 times with 1.5ml room temperature 10% trichloroacetic acid instead of the methanol extraction. The procedure was described in the chapter 2 (see Table 2-4), and was analogous to that described by Roberts et al. (1963) as modified by Cuhel et al. (1981b).

Experiment 2

Joel Goldman kindly provided culture facilities and technical assistance in the incubation and harvesting experiments 2 and 3. D. tertiolecta and T. weissflogii were grown under NH₄⁺ limited steady state conditions using the methods and apparatus described in Goldman (1977) with the exception that concentrations of NH₄Cl and NaH₂PO₄ in the media were 100 micromolar and 20 micromolar respectively. Cultures were grown at 16°C at a dilution rate of 0.512 day⁻¹ for Dunaliella tertiolecta and 0.496 day⁻¹ for Thalassiosira weissflogii. Before addition of isotope, aliquots were withdrawn from the steady state cultures and placed in incubation vessels under similar conditions of light and temperature. Both aliquots were labeled with inorganic ¹⁴C and incubated for 50 minutes. After this period, one of the aliquots was enriched with NH₄Cl to 16 micromolar.

Samples were filtered onto Whatman GF/F glass fiber filters, rinsed twice with 2ml artificial seawater, sealed in Nalgene filmware baggies,

and stored at -20°C until analysis. Samples were fractionated as described in experiment 1.

Experiment 3

Chemostat cultures in this experiment were grown at 12°C . Dilution rates were 0.138 day^{-1} for D. tertiolecta and 0.360 day^{-1} for Thalassiosira weissflogii. Enrichment with NH_4Cl was at zero time rather than 50 minutes. Culture, incubation, and fractionation procedures were otherwise identical to those in Experiment 2.

RESULTS AND DISCUSSION

Dunaliella tertiolecta

The pattern of increase in IFAA levels in an early stationary phase culture of D. tertiolecta (Table 3-15) to some extent resembled those seen for Nannochloris sp. after NH_4Cl enrichment of a NH_4^+ -limited continuous culture (Table 3-11). The increases were primarily associated with a few free amino acids. Most of the increase could be attributed to glutamate, beta-glutamate, glutamine, glycine + threonine, and alanine + gamma-aminobutyric acid. A number of other amino acids increased relative to control levels, particularly the basic amino acids, ornithine and lysine. Proline was not assayable by the o-phthaldialdehyde method, and my separation did not distinguish beta-glutamate from glutamate. Hence, comparisons of these amino acids are not possible. It is important to recognize that the stationary phase cultures had been grown in a rich media where there was no clear limiting factor. The increase in IFAA's upon replenishment of the media reflects a general response of a culture which is shifting out of stationary phase.

Table 3-15. Intracellular Free Amino Acid Concentrations
in Control and Enriched Cultures of
D. tertiolecta

Amino Acid	Concentration (micrograms per liter)	
	Control	Enriched
ASP	4.82	7.78
GLU	7.28	18.8
BGLU	4.91	62.4
SER	2.02	11.8
GLN	0.0	24.4
GLY+THR	7.05	45.1
ALA+GABA	2.49	24.4
BABA	2.83	3.62
AABA	6.13	13.1
MET	5.44	6.92
VAL	6.26	10.9
PHE	5.64	11.5
ILE	6.94	12.8
LEU	7.59	12.1
ORN	0.0	12.5
LYS	0.0	4.16
TOTAL	69.4	282.5

In experiment 1, effects of NH_4^+ enrichment on patterns of carbon incorporation in an NH_4^+ limited steady state culture of Dunaliella tertiolecta resembled those reported for Nannochloris sp. in that there was little effect on total inorganic ^{14}C fixation, while isotope incorporation into the low molecular weight metabolites was enhanced relative to controls (Figure 3-32). However, the increased incorporation was at the expense of incorporation into the hot trichloroacetic acid soluble material, rather than the ether soluble nonpolar material as in the case of Nannochloris sp. In addition, enhancement of protein incorporation occurred immediately, without the lag which was observed for Nannochloris sp. (c.f. Figure 3-31).

In experiment 2, addition of NH_4^+ after one hour resulted in an immediate change in incorporation patterns of D. tertiolecta (Figure 3-33) which were consistent with the responses discussed in Experiment 1. Again, there were relatively minor effects on total rate (Figure 3-34). Sharp increases in labelling of cold acid soluble low molecular weight metabolites occurred at the expense of the hot trichloroacetic acid material as reflected in the relative distribution of label. Similarly, protein incorporation was immediately enhanced by enrichment (Figure 3-33).

The responses seen in experiment 3 were generally consistent with those of the other experiments, indicating the observed responses were largely temperature independent within the described range. D. tertiolecta again displayed large rapid increases in cold trichloroacetic acid soluble incorporation at the expense of incorporation into hot trichloroacetic acid (Figure 3-35), and protein residue incorporation was immediately enhanced. There was a slight reduction as a result of

NH₄Cl addition in total uptake (Figure 3-36) which was not apparent at the higher temperatures.

Thalassiosira weissflogii

Observations of the response of T. weissflogii to NH₄⁺ enrichment revealed intrinsic metabolic differences from the other species. There was a substantial reduction in total ¹⁴C uptake at 16°C (Experiment 2, Fig. 3-37) which was not apparent at 12°C (Experiment 3, Figure 3-38). As mentioned by Goldman and Dennett (1984), NH₃ at high levels can uncouple oxidative phosphorylation and be toxic. However, the levels of enrichment should not have been high enough to elicit such a response. Despite this result, enrichment appeared to have much less dramatic effects on the distribution of incorporated ¹⁴C (Fig. 3-39, exp. 2; Fig. 3-40, exp. 3) than observed for Nannochloris sp. and D. tertiolecta. There was apparently little effect of NH₄⁺ enrichment on the proportion of incorporated ¹⁴C associated with the cold trichloroacetic acid soluble material and, if anything, it resulted in a decreased proportion of label associated with the low molecular weight pools at 12°C (Fig. 3-40, exp. 3). Even before enrichment, a much larger proportion of total ¹⁴C-label was associated with low molecular weight metabolites in T. weissflogii than in the other species. There did appear to be some enhancement of protein incorporation, to some extent at the expense of the cold trichloroacetic acid and ether soluble fractions (Figure 3-39, experiment 2; Figure 3-40, experiment 3). This increase was small compared to the responses seen in D. tertiolecta and Nannochloris sp. Wheeler et al. (1983) found that T. weissflogii required a longer period to deplete an NH₄⁺ pulse from the medium,

Figure 3-32. Time course of the incorporation of inorganic ^{14}C into subcellular fractions in control and enriched NH_4^+ -limited steady state cultures of Dunaliella tertiolecta growing at 24°C . NH_4Cl enrichment of 200 micromolar was added at zero time. Symbols: open circles, enriched; closed circles, control.

Figure 3-33. Time course of the incorporation of inorganic ^{14}C into subcellular fractions in control and enriched NH_4^+ -limited steady state cultures of Dunaliella tertiolecta growing at 16°C . Dotted lines indicate point during incubation when NH_4Cl (16 micromolar) was added. Symbols: square, enriched; cross, control.

Time (hours)

%

Figure 3-34. Time course of total inorganic ^{14}C uptake by control and enriched NH_4^+ -limited steady state cultures of Dunaliella tertiolecta growing at 16°C . Dotted line indicates point during incubation when NH_4Cl enrichment (16 micromolar) was added. Symbols: square, enriched; cross, control.

Figure 3-35. Time course of the distribution of incorporated inorganic ^{14}C among subcellular fractions in control and enriched NH_4^+ -limited steady state cultures of Dunaliella tertiolecta growing at 12°C . Symbols: square, enriched; cross, control.

Time (hours)

%

Figure 3-36. Time course of total inorganic ^{14}C uptake by control and enriched NH_4^+ -limited steady state cultures of Dunaliella tertiolecta growing at 12°C . NH_4Cl enrichment of 16 micromolar was added at zero time. Symbols: square, enriched; cross, control.

Figure 3-37. Time course of total inorganic ^{14}C uptake by control and enriched NH_4^+ -limited steady state cultures of Thalassiosira weissflogii growing at 16°C . Dotted line indicates point during enrichment when NH_4Cl enrichment (16 micromolar) was added. Symbols: square, enriched; cross, control.

Figure 3-38. Time course of total inorganic ^{14}C uptake by control and enriched NH_4^+ -limited steady state cultures of Thalassiosira weissflogii growing at 12°C . NH_4Cl enrichment of 16 micromolar was added at zero time. Symbols: square, enriched; cross, control.

Figure 3-39. Time course of the distribution of incorporated inorganic ^{14}C among subcellular fractions in control and enriched NH_4^+ -limited steady state cultures of Thalassiosira weissflogii growing at 16°C . Dotted lines indicate point during incubation when NH_4Cl (16 micromolar) was added. Symbols: square, enriched; cross, control.

Figure 3-40. Time course of the distribution of incorporated inorganic ^{14}C among subcellular fractions in control and enriched NH_4^+ -limited steady state cultures of Thalassiosira weissflogii growing at 12°C . Symbols: square, enriched; cross, control.

Time (hours)

%

and intracellular nitrogen accumulated in the order NH_4^+ , IFAA's, and finally protein. It should be noted that the cultures used by Wheeler et al. were synchronous and growing in a light/dark cycle while my cultures were asynchronous growing in continuous light. As a result, some of the kinetics of the response may have been masked in my experiments. However, the results of Wheeler et al. (1983) demonstrated that initial uptake rates and overall depletion kinetics were very similar regardless of when the pulse was given in the light/dark cycle. Their demonstration of a slower rate of depletion of internal nitrogen pools is consistent with my observations of a lack of dramatic change in carbon metabolism as a result of enrichment. The expected increase in IFAA's based on their observations was not reflected in an increase in cold trichloroacetic acid soluble incorporation in my experiment. It is possible that NH_4^+ was assimilated into existing carbon skeletons. Another possibility is that the acid extraction included reserve polysaccharide, beta-1,3-glucan, which is present in diatoms and is acid soluble (Myklestad, 1977). The fractionation procedure used in this experiment would not distinguish this as carbohydrate, but rather include it as cold trichloroacetic acid soluble material.

In contrast to the relatively slow nitrogen metabolism, Goldman and Glibert (1982) found that specific NH_4^+ uptake was higher for T. weissflogii than D. tertiolecta over a wide range of nitrogen limited growth rates. The preliminary results from this study further emphasize the fact that metabolic responses to nitrogen limitation are highly species specific, as are patterns of carbon metabolism in general. Such differences may play a role in defining population structure in environments where nitrogen is a potential growth limiting substrate and

transient nitrogen uptake capabilities are important. More detailed comparisons of the behaviour of specific free amino acids (e.g. glutamate and glutamine) in different species may lead to the identification of metabolic responses which are not species specific, can be probed with inorganic ^{14}C , and would therefore serve as useful indicators of metabolic status of natural autotrophic populations.

CONCLUSIONS

This study characterized effects of variations in the NH_4^+ -limited steady state growth rate on compositional dynamics and ^{14}C incorporation of free and protein amino acids in the marine chlorophyte, Nannochloris sp. It was found that by characterizing the differential behaviour of intracellular free amino acids, considerable information about metabolism could be obtained. Low glutamine/glutamate ratios were associated with low relative growth rate, consistent with the increased importance of the GS reaction in limiting inorganic nitrogen assimilation.

The presence of proline as a large proportion of intracellular free amino acid pool of a microalga has not been previously reported to my knowledge. Decreases in cellular pools of free proline with increasing NH_4^+ limitation indicated that proline served as a nitrogen storage reservoir. Proline is structurally related to glutamate, and might also serve as a source of carbon skeletons during rapid assimilation of NH_4^+ . It may also play a role as an osmoregulator. Its slow saturation characteristics supported the view that it was not a central metabolic intermediate.

For glycine + alanine, aspartate, and valine at the high relative growth rate, saturation rates were rapid enough that specific activities could be assumed to be equivalent to that of the dissolved inorganic carbon pool. In general, specific activities of other free amino acids were less than the that of the dissolved inorganic carbon pool even after 3 hours. Saturation rates of individual free amino acids were generally lower at the low relative growth rate. However, the saturation rate of total IFAA carbon was similar for the high and low relative growth rates. This was largely the result of a decline in the proportion of IFAA carbon in proline at the low relative growth rate. Consequently, the abundance of IFAA carbon relative to cellular carbon provides a growth related index of carbon flux through the IFAA pool (i.e. higher relative abundance = higher relative growth rate). Nitrogen turnover rates were generally higher for the free amino acids than the saturation rates with inorganic ^{14}C , a consequence of the independence of carbon and nitrogen flux through the free amino acid pool. Trends related to growth rate in the turnover rates of carbon and nitrogen were similar.

The relationship between saturation characteristics of intracellular free amino acids and nascent protein amino acids was influenced by growth rate. At the low relative growth rate, the saturation rates of nascent protein glutamate, glycine + alanine, and valine were higher than corresponding free amino acid specific activities. This indicated metabolic segregation of protein amino acid precursor pools, which was not apparent at the high relative growth rate. Nascent protein glycine and alanine appeared to have rapidly saturating precursors at both high and low relative growth rates, providing a foundation for further study of their utility in accurately estimating protein synthesis. This approach is examined in Chapter 4.

Distributions of incorporated ^{14}C incorporation among individual free amino acids and protein provided qualitative descriptions of relative abundances of cellular carbon. Such an approach may be useful for assessing the physiological state of the metabolically active algal populations, without detrital or non-autotrophic interference.

Distributions of incorporated ^{14}C also qualitatively reflected increases in free amino acid concentrations caused by NH_4^+ enrichment. The responses of the free amino acids were highly sensitive to the increase in NH_4^+ concentration, in contrast to total ^{14}C uptake. Individual species differed in their responses to NH_4^+ enrichment with respect to the distribution of ^{14}C among different cell materials. It may be that responses at the level of the free amino acids are less subject to interspecies variations. If so, evaluating patterns of isotope incorporation into the free amino acids in response to NH_4^+ enrichment would be a useful means of diagnosing the nitrogen status of phytoplankton populations.

A more complete characterization of the manner in which other environmental conditions (such as light, temperature), diel variations, and species composition influence metabolism may eventually permit direct and rapid assessment of the physiological state of natural autotrophic populations, and rates and composition of the primary organic matter being produced. Such information will lead to a better understanding of the role of marine phytoplankton in influencing the dynamics of particulate organic matter, and a general understanding of this primary level in trophodynamic processes.

CHAPTER 4. RELATIONSHIPS BETWEEN NET SYNTHESIS RATES OF
PROTEIN AMINO ACIDS AND INCORPORATION OF
INORGANIC ¹⁴C IN MARINE PHYTOPLANKTON

INTRODUCTION

The production of protein is closely associated with the growth of all living organisms, and it is nutritionally essential to all heterotrophic organisms. Marine phytoplankton are the primary source of protein for the marine food chain. Accurate estimates of this primary input are required in order to understand marine ecosystem trophodynamics, and the role of phytoplankton in influencing the flux and compositional dynamics of particulate organic matter. Moreover, since the proportion of algal metabolism associated with protein synthesis varies in relationship with the growth rate of the population (e.g. Chapter 3), the ability to accurately trace autotrophic production of protein in relationship to total carbon fixation will be a useful tool for assessing population physiological state.

The ^{14}C technique for measuring rates of photosynthetic carbon fixation (Steeman-Nielsen, 1952) provided a sensitive and specific assay of phytoplankton activity in units which could be directly compared to biomass. Despite the controversy surrounding the methodology and interpretation of the results it provides, it remains one of the most powerful and widely applied methods for estimating photosynthetic activity. The ubiquitous nature of carbon in living biomass allows the use of ^{14}C to trace various levels of metabolism. This is the basis underlying fractionation procedures which involve the isolation and radioassay of operationally defined cellular material having general characteristics which can be related to different types of metabolism.

In chapter 3, I discussed methods for improving the utility of results obtained from fractionation procedures. These include: a) precise chemical definitions of cell material in order to clarify

relationships between observed isotope distributions and actual metabolic characteristics, and b) adequate descriptions of the kinetics of isotope distribution, and its relationship to chemical composition and biosynthetic rates. The application of these methods towards the isotopic tracing of protein synthesis are considered in this chapter. In addition, I have examined the potential impact of the interruption of steady state growth, such as might arise from sample confinement, on isotope incorporation into protein.

BACKGROUND:

Tracing Production of Particulate Protein Amino Acids

The essential role which amino acids play in the growth of all living organisms makes them an ideal class of biogenic compounds for investigating the relationship between the composition of primary organic matter produced by phytoplankton and the composition of total particulate organic material found in the environment. By tracing the production of this specific class of compounds with ^{14}C , I eliminate the ambiguities associated with undefined subcellular fractions. The second problem associated with fractionations, that is the dependence of isotopic distribution with time, will be evaluated in the laboratory in several representative phytoplankton species. The intracellular free amino acid pool specific activity may not necessarily reflect the specific activity of the protein amino acid precursor pool (e.g. Oaks and Bidwell, 1970; Bassham et al., 1964; Trewavas, 1972; Huffaker and Peterson, 1974; Wheeler and Stephens, 1977; chapter 3, this thesis). This results in an ambiguous relationship between isotope incorporation into protein and net

protein synthesis. In chapter 3, I noted that protein had the slowest isotope kinetics of the polymer fractions. Isotope kinetics were different for the individual protein amino acids, and varied with the nitrogen limited growth rate. Glycine and alanine contained in nascent protein consistently displayed rapid saturation kinetics. My approach in this chapter was to compare predicted net production of individual protein amino acids as defined by continuous culture dilution (growth) rate with observed production as determined by ^{14}C incorporation. The results provide information about the isotope kinetics of various protein amino acid precursor pools, and identify those protein amino acids whose precursors rapidly attain the specific activity of the exogenous dissolved inorganic carbon pool.

Li and Goldman (1981) compared carbon specific growth rates of several species with their continuous culture growth rates. In a number of instances, this rate did not agree with the growth rate defined by the culture dilution rate. Overestimates were probably due to turnover in excess of net production. Underestimates might have occurred as a result of nutrient depletion accompanying the interruption of steady state growth. Goldman et al. (1981a) found that unenriched samples which had been removed from steady state cultures of some species exhibited time dependent decreases in carbon incorporation within a few hours. This demonstrates the importance of time course sampling for distinguishing patterns of isotope kinetics from perturbations in culture metabolism as a result of the interruption of steady state.

Li and Harrison (1982) suggested that isotope incorporation into protein may be less likely to overestimate net synthesis than total isotopic carbon incorporation. During the eastern Canadian arctic summer

(24 hour photoperiod), Li and Harrison (1982) compared isotope incorporation patterns for samples from consecutive 2 hour incubations with those of a 32 hour incubation. From their results, they suggested that the short incubations tended to reflect gross isotope incorporation and as a result yielded higher estimates of incorporation than from samples taken in the latter portion of the extended incubation. The protein fraction seemed to show the least discrepancy between the long and consecutive short incubations. This might mean that protein is less subject to turnover in excess of net production. However, such turnover does occur in higher plants and algae, and is a function of physiological state (e.g. Trevawas, 1972; Huffaker and Peterson, 1974; Bates, 1981; Cuhel et al., 1984). I will present results which demonstrate that under certain conditions, protein turnover significantly affects isotope incorporation characteristics.

Radioisotopic Tracing of Microbial Activities

Radioisotopic tracers other than ^{14}C which can be associated with distinct types of cell material have been used to monitor microbial activities in natural systems. These include ^3H -adenine (Karl, 1981, Karl et al., 1981a,b; Karl, 1979; Winn and Karl, 1984) and $^{32}\text{P}\text{O}_4$ (Cuhel et al., 1983; Cuhel and Waterbury, 1984) to assess nucleic acid biosynthesis, and $^{35}\text{S}\text{O}_4$ to trace the production of protein (Bates, 1981; Cuhel et al., 1981a,b,c, 1982a,b, 1983). Unlike inorganic ^{14}C , these procedures do not provide an assay of metabolic activity specific to phytoplankton as bacterial activity will also be measured. Furthermore, sensitivity is limited in the case of $^{35}\text{S}\text{O}_4$ as a result of dilution by the large ambient SO_4 levels, and in the case of

^3H -adenine by the fact that the method traces only the salvage uptake of adenine, a minor contribution to total nucleic acid biosynthesis. Addition of ^3H -adenine and $^{32}\text{PO}_4$ may perturb existing uptake kinetics. In contrast, inorganic ^{14}C can be added in truly tracer amounts to very high specific activities. This investigation provides basic information required to optimize methods for ^{14}C tracing of protein amino acid production by natural phytoplankton populations.

* * * *

A major emphasis of this study will be to assess the impact of changes in various environmental conditions on the kinetics of isotope incorporation into protein. In order that these results can be useful to other investigators, descriptions of biomass parameters as a function of growth rate are provided. In particular, I described the compositional relationship of protein to other cellular materials. This information provides an index of the cultures' physiological states (e.g. Rhee, 1978; Goldman, 1980; Dortch, 1982; Maske, 1982). I initially described the compositional variations associated with steady state growth of Nannochloris sp. under two different light intensities and varying degrees of NH_4^+ limitation. Subsequently, intercomparisons were made of the chemical composition of three other species grown under NH_4^+ -limited steady state conditions at a single light intensity. The results provide a description of compositional differences, both within a single species as a result of environmentally induced changes in metabolism, and between species as a result of intrinsic metabolic differences. Such variations in metabolism influence observed kinetics of isotope incorporation. It is the main objective of this chapter to evaluate such variations in ^{14}C kinetics, and determine the optimal

conditions for accurate and precise estimation of protein synthesis on the basis of isotopic carbon incorporation. In chapter 5, I demonstrate the use of this information to assess the metabolic characteristics of a natural phytoplankton population, and the role of primary inputs of protein in influencing the compositional dynamics and flux of particulate organic matter.

METHODS

Comparison of Protein Assay Methodologies

Knowledge about the relationship of protein to other cellular materials is useful in providing an index of the growth and physiological state of the algal culture. Such information facilitates intercomparisons between results of other studies and the characteristics of protein synthesis and associated patterns of carbon incorporation which are presented here. In order to determine the extent to which results obtained by my method for protein analysis can be generalized to results of other studies, I compared different methodologies for assaying protein. Results are shown in Table 4-1 for log phase batch cultures of Nannochloris sp. adapted to a high light intensity ($176 \pm 70 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). The two methods, the Bradford assay (Bradford, 1976) using the Coomassie brilliant blue dye reagent provided commercially by Bio-Rad, and the analysis of individual protein amino acid residues, yield similar results. The estimate of protein nitrogen was slightly lower for the residue method. Differences between methods were within the limits of analytical error. A slight underestimate would be expected since the amide groups of glutamine and asparagine are lost and tryptophan is destroyed during acid hydrolysis (DeJong et al., 1981). Fowden (1954)

Table 4-1. Bulk Chemical Composition Parameters For High Light^a
Batch Cultures of Nannochloris sp.

% Total POC in Protein	% Total PON in Protein	<u>Chl a</u> Protein Carbon ($\mu\text{g}/\mu\text{g}$)x100	C:N	<u>Carbon</u> <u>Chl a</u> ($\mu\text{g}/\mu\text{g}$)
<u>Bradford protein assay:</u> ^b				
23.1 (6.2)	55 (15)	4.6 (1.0)	6.1 - 8.9	95.7 (6.2)
<u>Residue analysis of hydrolyzed protein:</u> ^c				
21.9 (3.9)	42.8 (9.5)	5.7 (0.8)	6.6	81 (12)

^a176+70 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

^bErrors (in parentheses) represent 95% confidence intervals.

^cErrors (in parentheses) represent propagated estimates of analytical error based on 95% confidence intervals.

also pointed out that the basic amino acids arginine, lysine and histidine may be partially destroyed during hydrolysis in the presence of carbohydrate. Finally, I did not assay tyrosine, cysteine, and methionine so they were not included in the results.

All other methods were described in chapter 2.

RESULTS AND DISCUSSION

Effects of Growth Conditions on Chemical Composition of *Nannochloris* sp.

The relationship of protein to other biomass parameters in high light adapted NH_4^+ -limited steady state cultures of *Nannochloris* sp. is shown in Table 4-2. The compositional parameters are representative of the steady state culture conditions. As noted in chapter 3 (Table 3-2), the percentage of particulate organic carbon associated with protein decreases with decreasing growth rate. In conjunction, the chlorophyll a/protein carbon and C:N ratios increase. No significant change in the POC/chlorophyll a ratio could be demonstrated. In NH_4^+ -limited cultures adapted to low light conditions ($28 \pm 9 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), no significant decrease in the percentage of POC in protein was seen with decreasing growth rate (Table 4-3), nor was there a substantial change in the C:N ratio. The chlorophyll a/protein carbon ratio decreased with increasing nitrogen limitation, in contrast to the increase seen under high light conditions. Thus, during growth under high light conditions, protein synthesis is more sensitive to nitrogen supply than chlorophyll a production, while under low light conditions, this relationship is reversed. The chlorophyll a/protein carbon ratio was generally higher and the POC/chlorophyll a ratio lower under low light relative to high light conditions. Chan (1978) found a similar pattern of chlorophyll

Table 4-2. Bulk Chemical Composition Parameters For High Light^a
 NH_4^+ -limited Steady State Cultures of *Nannochloris* sp.

Relative Growth Rate ^b	% Total POC in Protein ^c	% Total PON in Protein	Chl a Protein Carbon ($\mu\text{g}/\mu\text{g}$)x100	C:N	Carbon Chl a ($\mu\text{g}/\mu\text{g}$)
0.933 (0.031)	18.9 (4.9)	31.2 (21.0)	4.3 (1.1)	6.2	124 (42)
0.458 (0.014)	16.8 (3.0)	36.8 (8.4)	5.1 (0.5)	7.4	117 (28)
0.229 (0.016)	8.8 (2.0)	42.0 (7.3)	6.8 (1.0)	17.1	166 (38)

^a $176 \pm 70 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

^bGrowth rate normalized to maximum growth rate ($1.44 (0.04) \text{ day}^{-1}$) under similar conditions of light and temperature. Growth rate errors (in parentheses) represent 95% confidence intervals.

^cErrors for all compositional parameters (in parentheses) represent propagated estimates of analytical error based on 95% confidence intervals.

Table 4-3. Bulk Chemical Composition Parameters For Low Light^a
Batch and NH₄⁺-limited Steady State Cultures of Nannochloris sp.

Relative Growth Rate ^b	% Total POC in Protein ^c	% Total PON in Protein	Chl a Protein Carbon (ug/ug)x100	C:N	Carbon Chl a (ug/ug)
<u>Batch:</u>					
1.00 (0.09)	n.d.	n.d.	n.d.	7.5	52.3 (9.4)
<u>Steady State:</u>					
0.864 (0.092)	9.8 (1.9)	51 (35)	16.5 (1.8)	n.d.	n.d.
0.723 (0.063)	14.5 (2.8)	36.5 (7.9)	15.0 (1.6)	n.d.	n.d.
0.283 (0.060)	14.0 (2.6)	38.7 (8.6)	10.1 (1.6)	9.2	70.4 (14.7)

^a28+9 uE m⁻² s⁻¹.

^bGrowth rate normalized to maximum growth rate (0.58 (0.05) day⁻¹) under similar conditions of light and temperature. Growth rate errors (in parentheses) represent 95% confidence intervals.

^cErrors of all compositional parameters (in parentheses) represent propagated estimates of analytical error based on 95% confidence intervals.

a/protein ratios in response to light intensity in a survey of selected diatoms and dinoflagellates. This indicates that chlorophyll production was enhanced relative to total biomass in low light adapted cultures, a commonly observed response of algae (e.g. Beale and Appleman, 1971; Beardall and Morris, 1976; Prezelin, 1976; Falkowski and Owens, 1980; Perry et al., 1981; Richardson et al., 1983). The fact that the chlorophyll a/protein carbon ratio declined with decreasing growth rate under low light conditions indicates that nitrogen limitation suppressed the low light enhanced chlorophyll production. Reduction in steady state chlorophyll a content with increasing nitrogen limitation has been reported by other investigators (Caperon and Meyer, 1972; Thomas and Dodson, 1972). These results demonstrate that the regulation of chlorophyll synthesis in Nannochloris sp. is more sensitive to nitrogen supply under low light conditions. In contrast, the %POC in protein was lower at high relative growth rate under low light conditions, and did not decrease with increasing nitrogen limitation. This suggests that the proportion of carbon metabolism associated with protein synthesis was less sensitive to increasing nitrogen limitation at the low light intensity (c.f. Cook, 1963).

A possibility which must be recognized is that an unknown substrate other than NH_4^+ was limiting. For example, increased demand for some trace substrate might accompany growth under low light intensities. Further proof that inorganic nitrogen was limiting is required.

Interspecies Comparisons of Chemical Composition

Comparisons of compositional parameters among different species growing under high light NH_4^+ -limited steady state conditions reveal

differences (Table 4-4 and 4-5), but for the most part, these differences were within the range of environmentally induced variations seen for Nannochloris sp. The biomass parameters reported here strengthen earlier arguments by Parsons et al. (1961) that the chemical composition of different species is remarkably similar. Morris (1981) pointed out that environmentally induced variations can be more important than species changes in determining particulate organic matter composition. In Chattonella (Olisthodiscus) luteus, the percentage of PON in protein increased and the C:N ratio decreased with increasing growth rate as has been reported for other algae (Rhee, 1978; Dortch, 1982). The growth rate range may have been inadequate to detect changes in the other parameters.

Biomass parameters expressed on a per average cell basis are shown for high light (Table 4-6) and low light (Table 4-7) adapted continuous cultures of Nannochloris sp. at NH_4^+ -limited steady state. Cellular levels of both nitrogen and protein decreased with decreasing growth rate under high light conditions. In low light adapted cultures (Table 4-7), no decrease in nitrogen per cell was associated with decreasing growth rate. Carbon per cell remained relatively constant for both the light saturated and light limited cultures, an indication that carbon was a relatively conservative cellular material. Chlorophyll a per cell was generally higher at low light, consistent with the higher chlorophyll a/protein carbon and POC/chlorophyll a ratios (c.f. Table 4-3).

In contrast to the similarities among species in their bulk chemical composition, cellular abundances of different substances varied widely apparently in association with variations in cell size (e.g. Chan, 1978). C. luteus, which was the species of largest cell size, had the

Table 4-4. Bulk Chemical Composition Parameters For High Light^a
NH₄⁺-limited Steady State Cultures of Selected Marine Algae

Species	Relative Growth Rate ^b	% Total POC In Protein ^c	% Total PON In Protein	Chl a Protein Carbon (ug/ug)x100	C:N	Carbon Chl a (ug/ug)
<u>Chaetoceros simplex</u>	0.574 (0.064)	15.8 (3.0)	41.4 (9.3)	10.5 (1.3)	10.3	60.3 (9.5)
<u>Chroomonas salina</u>	0.804 (0.082)	20.0 (3.6)	34 (11)	9.5 (1.8)	6.5	53 (12)

^a176±70 μE · m⁻² · s⁻¹.

^bSteady state growth rate normalized to maximum under similar conditions of light and temperature. Maximum growth rate for C. simplex was 2.3 (0.23) day⁻¹. Maximum growth rate for C. salina was 0.52 (0.05) day⁻¹. All growth rate errors (in parentheses) represent 95% confidence intervals.

^cErrors of all compositional parameters (in parentheses) represent propagated estimates of analytical error based on 95% confidence intervals.

Table 4-5. Bulk Chemical Composition Parameters For High Light^a
Batch and NH₄⁺-limited Steady State Cultures of Chattonella luteus

Relative Growth Rate ^b	% Total POC in Protein ^c	% Total PON in Protein	Chl a Protein Carbon (μg/μg)x100	C:N	Carbon Chl a (μg/μg)
<u>Batch:</u>					
1.00 (0.11)	16.8 (4.0)	54 (15)	8.3 (1.8)	10.8	72 (13)
<u>Steady State:</u>					
0.920 (0.110)	9.8 (2.3)	55.1 (5.6)	11.2 (1.4)	21.3	92 (22)
0.540 (0.075)	15.9 (3.0)	85.0 (12.1)	6.7 (0.94)	17.7	94 (13)

^a176±70 μE · m⁻² · s⁻¹.

^bGrowth rate normalized to maximum growth rate (0.54 (0.06) day⁻¹) under similar conditions of light and temperature. Growth rate errors (in parentheses) represent 95% confidence intervals.

^cEstimates for μ/μ_{max} = 1.00 and 0.540 derived from Bradford assay of protein. Estimates for μ/μ_{max} = 0.920 derived from residue analysis of protein. Errors of all compositional parameters (in parentheses) represent propagated estimates of analytical error based on 95% confidence intervals.

Table 4-6. Cellular Chemical Composition Parameters of
High Light^a Batch and NH₄⁺-limited Steady State
Cultures of *Nannochloris* sp.

Relative Growth Rate ^b	Carbon per cell ^c (pg)	Nitrogen per cell (fg)	Chl a per cell (fg)	Protein Carbon per cell (pg)
<u>Batch:</u>				
1.0 (0.03)	4.2 (2.0)	550 (130)	50 (17)	0.74 (0.08)
<u>Steady State:</u>				
0.933 (0.030)	3.0 (0.7)	500 (330)	24.0 (6.0)	0.57 (0.06)
0.458 (0.014)	2.9 (0.8)	400 (130)	25.0 (3.0)	0.49 (0.08)
0.229 (0.016)	3.6 (0.7)	210 (28)	22.0 (2.0)	0.32 (0.04)

^a176±70 μE · m⁻² · s⁻¹.

^bGrowth rate normalized to maximum growth rate (1.44 (0.04) day⁻¹) under similar conditions of light and temperature. Growth rate errors (in parentheses) represent 95% confidence intervals.

^cErrors for all compositional parameters (in parentheses) represent propagated estimates of analytical error based on 95% confidence intervals.

Table 4-7. Cellular Chemical Composition Parameters of
Low Light^a Batch and NH₄⁺-limited Steady State
Cultures of Nannochloris sp.

Relative Growth Rate ^b	Carbon per cell ^c (pg)	Nitrogen per cell (fg)	Chl <u>a</u> per cell (fg)	Protein Carbon per cell (pg)
<u>Batch:</u>				
1.0 (0.08)	3.8 (0.9) ^b	490 (120)	74 (15)	n.d.
<u>Steady State:</u>				
0.864 (0.092)	n.d.	320 (180)	100 (16)	0.64 (0.12)
0.723 (0.063)	n.d.	510 (150)	100 (23)	0.69 (0.17)
0.283 (0.060)	5.0 (1.1)	540 (130)	71 (12)	0.70 (0.12)

^a29+9 uE m⁻² s⁻¹.

^bGrowth rate normalized to maximum growth rate (0.58 (0.05) day⁻¹) under similar conditions of light and temperature. Growth rate errors (in parentheses) represent 95% confidence intervals.

^cErrors of all compositional parameters (in parentheses) represent propagated estimates of analytical error based on 95% confidence intervals.

Table 4-8. Cellular Chemical Composition Parameters of High Light^a NH₄⁺-limited Steady State Cultures of Selected Marine Algae

Species	Relative Growth Rate ^b	Carbon per cell ^c (pg)	Nitrogen per cell (fg)	Chl <u>a</u> per cell (fg)	Protein Carbon per cell (pg)
<u>Chattonella luteus</u>					
	<u>Batch:</u>				
	1.0 (0.11)	610 (150) ^b	57000 (16000)	8500 (1900)	100 (30)
	<u>Steady State:</u>				
	0.921 (0.110)	311 (100)	14600 (4200)	3400 (860)	31 (8)
	0.540 (0.075)	340 (100)	19000 (5200)	3800 (990)	55 (16)
<u>Chaetoceros simplex</u>					
	0.574 (0.064)	10.9 (3.0)	1060 (720)	181 (42)	1.7 (.4)
<u>Chroomonas salina</u>					
	0.804 (0.082)	84 (25)	13000 (5200)	1600 (470)	17 (4)

^a170+70 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

^bSteady state growth rate normalized to maximum under similar conditions of light and temperature. Maximum growth rate for C. luteus was 0.54 (0.06) day⁻¹, 2.3 (0.23) day⁻¹ for C. simplex, and 0.52 (0.05) day⁻¹ for C. salina. All growth rate errors represent 95% confidence intervals.

^cErrors of all compositional parameters (in parentheses) represent propagated estimates of analytical error based on 95% confidence intervals.

largest values for carbon, nitrogen, chlorophyll a, and protein on a per average cell basis (Table 4-8). Values for Chroomonas salina and Chaetoceros simplex were intermediate between C. luteus and Nannochloris sp. (c.f. Tables 4-6 and 4-8).

Kinetics of Isotope Incorporation: Agreement Between Predicted and Observed Carbon Incorporation Rates

From the results presented in chapter 3 and in the preceding section, it is apparent that environmental conditions influence cellular composition, a reflection of their impact on metabolic processes. A prerequisite to estimating the rate of a specific biosynthetic process, in this case protein synthesis, using inorganic ^{14}C as a tracer, is to evaluate the potential impact of environmentally induced changes in metabolism on the isotope kinetics. I have compared predicted and observed carbon incorporation rates into total particulate organic carbon (POC), total particulate protein amino acids (PPAA), and individual protein amino acids in steady state cultures where the inorganic ^{14}C was added directly to the chemostat vessel. Assuming complete and instantaneous equilibration of precursors with the dissolved inorganic ^{14}C and negligible cell decay (c.f. Goldman, 1977), the rate of isotope incorporation into protein (dP^*/dt) in a steady state culture where influent flow is not interrupted will be the difference between the rate of protein synthesis and the dilution of labeled protein by inflowing media:

$$dP^*/dt = DP_0 - DP^*(t) \quad (4-1)$$

where D is the dilution rate of the chemostat, P_0 is the steady state protein carbon concentration, t is the incubation time, and $P^*(t)$ is the predicted quantity of isotope in protein. Equation (4-1) is a first order linear differential equation, with initial conditions $P^*(0) = 0$. Solving for $P^*(t)$:

$$P^*(t) = P_0 (1 - e^{-Dt}) \quad (4-2)$$

For most of the experiments, it was not possible to maintain influent flow throughout the incubation period. Under conditions where flow is interrupted, the predicted quantity of isotope in protein ($P^{*'}(t)$) was:

$$P^{*'}(t) = P_0 (e^{Dt} - 1) \quad (4-3)$$

The derivation of this equation is similar to that described by Roberts et al. (1963). It assumes no deviations from the steady state biosynthetic rates despite the interruption of flow, so that the chemostat is effectively viewed as a batch culture with growth rate D . As I will demonstrate, this assumption is not always appropriate.

Under conditions where Dt is small (i.e. less than 0.05), $P^*(t)$ approximates $P^{*'}(t)$. The initial rate of slope of observed incorporation was estimated by linear regression and compared to the predicted rate which was defined as:

$$R_p = P^*(\Delta t) / \Delta t \quad (4-4)$$

or:

$$R'_p = P^*(\Delta t)/\Delta t \quad (4-5)$$

where Δt was the period over which observed incorporation was regressed.

As previously pointed out, these equations are based on the assumption that precursors to protein synthesis are completely and instantaneously equilibrated with inorganic ^{14}C . If equilibration is not complete, than the rates predicted by equations 4-4 and 4-5 will overestimate observed incorporation. In contrast, if protein turnover in excess of net synthesis is significant, then the observed incorporation rate may be greater than the predicted rate. Examples of these cases are discussed in subsequent sections.

Predicted rates of POC production were obtained by substituting the steady state POC concentration for P_o in equations 4-1 through 4-5.

High Light Adapted Steady State Cultures of Nannochloris sp.: Isotope Kinetics at High and Low Relative Growth Rates

POC and PPAA

Comparisons of regressions of total inorganic ^{14}C uptake with predicted POC production rates under high light conditions indicated excellent agreement at all growth rates (Figure 4-1). In contrast, incorporation of isotope into isolated PPAA was less than the predicted rate of carbon incorporation (Figure 4-2). Higher predicted carbon incorporation into protein than observed isotope incorporation rates can

be caused by both incomplete saturation of precursors with inorganic ^{14}C as well as deviations from steady state in response to the interruption of steady state. In some cases, it is possible to distinguish these two sources of discrepancy by examining the time course patterns in incorporation.

The time course of isotopic carbon incorporation into total PPAA during the first 1.5 hours for the high and low relative growth rates are shown in Figure 4-3 in relationship to the predicted incorporation. The point at which the influent flow was stopped during the incubation is designated by an arrow. The predicted rate exceeded the observed rate before flow was stopped in both the high and low relative growth rate cases. This tendency was not an artifact due to perturbations of steady state, since it was also observed during the initial stages of a separate chemostat experiment where flow was not stopped and steady state was maintained throughout a 66 hour incubation period (Figure 4-4). Further evidence for the slow saturation characteristics of protein precursors was revealed in short term isotopic carbon distributions of batch cultures where nutrient depletion would not be a problem. Isotope incorporation initially underestimated the actual proportions of carbon associated with the protein fraction (Figure 3-18).

Nitrogen depletion effects did manifest themselves later in the NH_4^+ -limited cultures where flow was stopped. Examining the time course for the entire 3 hour incubation periods for total PPAA incorporation at high and low relative growth rate, it is evident that the observed incorporation rate decreases with time particularly at the low relative growth rate (Fig. 4-5). However, for the initial period where flow was not stopped, observed incorporation was less than the

predicted rates. A similar discrepancy was seen for the experiment where flow was not stopped (Figure 4-6). However, observed incorporation approached the predicted values as the incubation progressed. By 20 hours (Fig. 4-7), differences between predicted and observed values were negligible.

These results illustrate that the interruption of steady state can lead to the rapid onset of time dependent decreases in protein carbon incorporation rates. Therefore, inspection of time course incorporation patterns is necessary in order to distinguish non-steady state artifacts from kinetic phenomena as causes for discrepancies between observed and predicted carbon incorporation. As will be demonstrated, this is particularly important in understanding the characteristics of carbon incorporation into individual protein amino acids.

Carbon Incorporation into Individual Protein Amino Acids

In Figure 4-8, incorporation rates of selected protein amino acids are shown in relationship to the predicted rates for the high and low relative growth rates respectively. The biosynthetic pathways demonstrated in higher plants for these amino acids are diagrammed in Figure 4-9. Glycine and serine biosynthesis can be derived from 3-phosphoglycerate produced by the RuDP carboxylase reaction of the Calvin cycle (Miflin and Lea, 1977). Under these conditions, their production would be closely coupled to the supply of photosynthetically fixed carbon. This is consistent with the good agreement between predicted and observed incorporation rates of glycine (Figures 4-8 and 4-10), and the observations in chapter 3 that nascent protein glycine was immediately saturated with the inorganic carbon pool. Alanine also shows

close agreement between predicted and observed incorporation rates (Figures 4-8 and 4-10), in agreement with the rapid saturation of nascent protein alanine (chapter 3, Table 3-5). Alanine is a product of the reductive amination of pyruvate. A proposed route for the incorporation of photosynthetically derived ^{14}C into pyruvate is via the transport of triose phosphates from the chloroplast into the cytosol (Heber, 1974; Leech and Murphy, 1976). Once there, the action of the glycolytic enzymes could convert these molecules to pyruvate, which can subsequently diffuse (Schulze-Siebert et al., 1984) back into the chloroplast where it may undergo transamination with glutamate (e.g. Leech and Murphy, 1976). However, the close agreement between the observed and predicted incorporation into alanine indicates that its pyruvate carbon skeleton rapidly equilibrates with the dissolved inorganic carbon pool. If the above transport scheme is correct, this would require that either the entire cytosolic pyruvate pool was saturated or the source of pyruvate used for protein alanine biosynthesis was compartmentalized. The pool size of pyruvate was not measured in this study. The possibility that it was small and in a rapid state of turnover cannot be ruled out.

Recently, the presence of pathways for conversion of 3-phosphoglycerate (3PGA) to pyruvate have been demonstrated to exist within spinach chloroplasts (Fig. 4-11, taken from Schultze-Seiber et al., 1984). Such a mechanism would provide for a more direct means of production of alanine from photosynthetically fixed carbon. Furthermore, as I pointed out in chapter 3, at the low relative growth rate there were differences between the bulk intracellular free glycine + alanine pool specific activity and that of the nascent protein glycine and alanine. Such a phenomenon could be explained by the presence of an isolated

Figure 4-1. Comparisons of observed total ^{14}C uptake rates with theoretical particulate organic carbon production rates in high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Observed total ^{14}C uptake rates were determined by linear regression of the initial 1.5 hour time course of isotope incorporation. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 4-2. Comparisons of observed particulate protein amino acid isotope incorporation rates with theoretical rates in high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Observed isotope incorporation rates were determined by linear regression of a 3 hour time course. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 4-3. Observed and predicted 1.5 hour time courses of isotopic carbon incorporation into particulate protein amino acids where flow was interrupted during the incubation of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Upper solid line represents the predicted rate for the high relative growth rate culture ($\mu/\mu_{\text{max}} = 0.933$) and the dotted line the predicted rate for the low relative growth rate culture ($\mu/\mu_{\text{max}} = 0.229$). Lines connecting symbols drawn by eye. Arrows indicate points during the incubations when influent flow was stopped. Symbols: squares, sum of carbon incorporation into individual protein amino acid residues at the high relative growth rate; triangles, incorporation into hot trichloroacetic acid insoluble at the high relative growth rate; +, sum of carbon incorporation into individual protein amino acid residues at the low relative growth rate; X, incorporation into hot trichloroacetic acid insoluble at the low relative growth rate.

Figure 4-4. Observed and predicted 1.0 hour time courses of isotope incorporation into particulate protein amino acids of a high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state culture of Nannochloris sp. ($\mu/\mu_{\text{max}} = 0.458$). Flow was not interrupted during the incubation. Solid line represents the predicted rate.

Figure 4-5. Observed and predicted 3 hour time courses of isotope incorporation into particulate protein amino acids where flow was interrupted during the incubation of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Upper solid line represents the predicted rate for the high relative growth rate culture ($\mu/\mu_{\text{max}} = 0.933$) and the dotted line the predicted rate for the low relative growth rate culture ($\mu/\mu_{\text{max}} = 0.229$). Lines connecting symbols drawn by eye. Arrows indicate points during the incubations when influent flow was stopped. Symbols: squares, sum of carbon incorporation into individual protein amino acid residues at the high relative growth rate; triangles, incorporation into hot trichloroacetic acid insoluble at the high relative growth rate; +, sum of carbon incorporation into individual protein amino acid residues at the low relative growth rate; X, incorporation into hot trichloroacetic acid insoluble at the low relative growth rate.

Figure 4-6. Observed and predicted 6 hour time courses of isotope incorporation into particulate protein amino acids of a high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state culture of Nannochloris sp. ($\mu/\mu_{\text{max}} = 0.458$). Flow was not interrupted during the incubation. Solid line represents the predicted rate.

Total PPAA Carbon Incorporation

Figure 4-7. Observed and predicted 66 hour time courses of isotope incorporation into particulate protein amino acids of a high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state culture of Nannochloris sp. ($\mu/\mu_{\text{max}} = 0.458$). Flow was not interrupted during the incubation. Solid line represents the predicted rate. Area of overlap with Fig. 4-6 indicated by dotted lines.

Figure 4-8. Theoretical and observed rates of isotopic carbon incorporation into alanine (ALA), glycine (GLY), glutamate (GLU), and aspartate (ASP) in protein of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Results from high ($\mu/\mu_{\text{max}} = 0.933$) and low ($\mu/\mu_{\text{max}} = 0.229$) relative growth rate cultures are shown. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 4-9. Simplified diagram showing the major pathways associated with the biosynthesis of glycine (GLY), serine (SER), glutamine (GLN), glutamate (GLU), alanine (ALA), and aspartate (ASP). After Miflin and Lea (1977) and Miflin et al. (1980). PGA = phosphoglycerate, PEP = phosphoenolpyruvate, PYR = pyruvate, OAA = oxaloacetate, TCA = tricarboxylic acid cycle, KGA = alpha-ketoglutarate, RuDP = ribulose-1,5-diphosphate, FDP = fructose-1,6-diphosphate; G6P = glucose-6-phosphate. Formation of glycine and serine from glycolate may involve participation of peroxisomes and mitochondria (not indicated, see Keys et al., 1978, in Miflin et al., 1980). The proposed direct formation of phosphoenolpyruvate and pyruvate within the chloroplast (Schulze-Siebert et al., 1984) is also not shown.

Figure 4-10. Observed and predicted time courses of isotope incorporation into glycine, alanine, glutamate, and aspartate in protein of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Solid lines represent predicted rates for the high relative growth rate culture (0.933) and dotted lines the predicted rates for the low relative growth rate culture (0.229). Flow was stopped at 1 hour for $\mu/\mu_{\text{max}} = 0.229$ and 0.33 hours for $\mu/\mu_{\text{max}} = 0.933$ (indicated by arrows). Symbols: squares, carbon incorporation into the individual protein amino acid at the high relative growth rate; +, carbon incorporation into the individual protein amino acid at the low relative growth rate.

Figure 4-11. Diagram showing proposed routes for intrachloroplast production and utilization of PEP and pyruvate. From Schulze-Siebert et al. (1984).

rapidly turning over precursor pool (e.g. the chloroplast pool) which serves as the preferred source for protein synthesis. However, the observed rates of intrachloroplast carbon flow into pyruvate observed by Schulze-Siebert et al. were low, and addition of exogenous pyruvate to chloroplasts suspensions led to enhanced synthesis of alanine, lysine, and the branched chain amino acids (valine, isoleucine, and leucine). Thus further investigation is required in order to determine the relative importance of production of pyruvate directly from 3PGA within the chloroplast.

Predicted and observed incorporation rates of glutamate and aspartate agreed within analytical error at the high growth rate (Fig. 4-8). In the case of glutamate, this was at least partially due to the large regression error resulting from the low number of data points (4). At the lower growth rate, observed protein glutamate incorporation underestimated the predicted rate (Fig. 4-8). Examining the time course of incorporation for these protein amino acids (Fig. 4-10), it is clear that they both initially underestimate their predicted incorporation rates. However, the rate of incorporation approaches the predicted slope during the incubation reflecting the gradual equilibration of their precursor pools. Referring to Fig. 4-9, the carbon skeleton for aspartate is oxaloacetate (OAA) and that of glutamate is α -ketoglutarate. Both are involved in the tricarboxylic acid cycle (e.g. Miflin and Lea, 1977; Miflin et al., 1980; Schulze-Siebert et al., 1984). The presence of chloroplast enzymes for their synthesis has not been demonstrated. The cytosol carbon skeletons are able to exchange with the chloroplast pools. Consequently, the nonlinear patterns of incorporation into glutamate and aspartate probably reflect the

saturation characteristics of the "respiratory" intermediates from which they are derived. There was some indication of nutrient depletion effects at the low growth rate.

Phenylalanine is synthesized from erythrose-4-phosphate, an intermediate of the photosynthetic carbon reduction cycle, and phosphoenolpyruvate (PEP) (Fig. 4-12). Agreement of its observed labelling rate with the predicted rate (Fig. 4-13) suggests its carbon skeleton precursors saturated rapidly with inorganic ^{14}C .

Erythrose-4-phosphate is derived from the photosynthetic carbon reduction cycle, and would be expected to rapidly saturate with inorganic ^{14}C .

PEP is the precursor to pyruvate and oxaloacetate (OAA). As previously mentioned, the intrachloroplastic conversion of 3PGA to PEP and on to pyruvate has been demonstrated in spinach chloroplasts (Schulze-Siebert et al., 1984). This could explain the rapid equilibration of protein phenylalanine (see also chapter 3, nascent protein specific activity of phenylalanine, Table 3-5). The fact that protein aspartate did not show as rapid equilibration as phenylalanine could be explained if the OAA pool was large relative to the PEP pool, or if the OAA used to produce aspartate was derived from a separate more slowly saturating pool of PEP.

Biosynthetic pathways demonstrated in higher plants for the amino acids threonine, valine, isoleucine, leucine and lysine utilize aspartate and pyruvate as precursors (Fig. 4-14, taken from Mifflin and Lea, 1977). The characteristics of carbon incorporation into these amino acids (Fig. 4-15 and 4-16) were generally consistent with the biosynthetic pathways which have been described for higher plants. Protein threonine observed carbon incorporation agrees quite closely with the predicted rate at high

Figure 4-12. Pathways associated with the biosynthesis of tryptophan, tyrosine, and phenylalanine. From Mifflin and Lea (1977).

Figure 4-13. Observed and predicted time courses of isotope incorporation into phenylalanine in protein of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Solid line represents predicted rate for the high relative growth rate culture ($\mu/\mu_{\text{max}} = 0.933$) and dotted line the predicted rate for the low relative growth rate culture ($\mu/\mu_{\text{max}} = 0.229$). Flow was stopped at 1 hour for $\mu/\mu_{\text{max}} = 0.229$ and 0.33 hours for $\mu/\mu_{\text{max}} = 0.933$ (indicated by arrows). Symbols: squares, carbon incorporation into the protein phenylalanine at the high relative growth rate; +, carbon incorporation into protein phenylalanine at the low relative growth rate.

Figure 4-14. Pathways associated with the biosynthesis of methionine, threonine, isoleucine, lysine, valine, and leucine. From Mifflin and Lea (1977).

Figure 4-15. Theoretical and observed rates of isotopic carbon incorporation into isoleucine (ILE) + leucine (LEU), proline (PRO) + arginine (ARG), valine (VAL), phenyalanine (PHE), lysine (LYS), and threonine (THR) in protein of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. at high ($\mu/\mu_{\text{max}} = 0.933$) and low ($\mu/\mu_{\text{max}} = 0.229$) relative growth rates. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 4-16. Observed and predicted time courses of isotope incorporation into threonine, valine, isoleucine + leucine, and lysine in protein of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Solid lines represent predicted rates for the high relative growth rate culture ($\mu/\mu_{\text{max}} = 0.933$) and dotted lines the predicted rates for the low relative growth rate culture ($\mu/\mu_{\text{max}} = 0.229$). Flow was stopped at 1 hour for $\mu/\mu_{\text{max}} = 0.229$ and 0.33 hours for $\mu/\mu_{\text{max}} = 0.933$ (indicated by arrows). Symbols: squares, carbon incorporation into the individual protein amino acid at the high relative growth rate; +, carbon incorporation into the individual protein amino acid at the low relative growth rate.

Time (hours)

relative growth rate, but less so at low relative growth rate which is consistent with the saturation characteristics of its precursor, aspartate (Figure 4-10). Although there was no clear evidence for depletion effects at the high relative growth rate, this did appear to be a factor at the low relative growth rate.

The observed incorporation into lysine at the high relative growth rate was difficult to explain. Initially, the observed incorporation rate approached the predicted rate, but then continuously decreased (Fig. 4-16). This seems inconsistent with its presumed precursors aspartate and pyruvate, but may reflect a non-steady state response. Observed incorporation was consistently low at the low relative growth rate.

Observed carbon incorporation into valine and isoleucine+leucine consistently underestimated the predicted incorporation. At the high relative growth rate, the observed rate increasingly approached the predicted slope. At the low relative growth rate, decreasing incorporation in the latter part of the incubation period indicated that there was a depletion effect after the interruption of influent flow. As will be discussed in the next section, the biosynthetic characteristics of valine, and the analytical accessibility of both free and protein valine make it an ideal model amino acid for a more detailed kinetic analysis which clearly distinguishes isotope equilibration effects from those due to nitrogen depletion.

Elucidation of Nitrogen Depletion Effects Using Valine as a Model Protein Amino Acid

Unlike some of the other free amino acids such as aspartate and glycine, free valine has only one clearly defined sink, protein. The

ability to quantify both free and protein valine concentrations provide a unique opportunity to examine the kinetics of isotope incorporation into protein valine in more detail. This exercise provides a means for distinguishing isotope equilibration effects from non-steady state nitrogen depletion responses. The following assumptions were made in calculating predicted incorporation into protein valine:

- 1) assume that the intracellular free valine pool is not compartmentalized, and
- 2) the flux of carbon through the intracellular valine pool is equal to the net protein valine synthesis rate. Furthermore,
- 3) all carbon entering the free valine pool is in complete equilibrium with inorganic ^{14}C . Finally,
- 4) assume a steady state (i.e. no net increase in biomass, this is a reasonable assumption given the short incubation period). Given these assumptions, let C be the dissolved inorganic carbon pool, F be the intracellular free valine carbon concentration, and P the protein valine carbon formed from F:

where k is a production rate constant (c.f. Roberts et al., 1963). At time 0, the radioactive form of C (C^*) is added to the medium. The quantity of isotope in the intracellular free valine pool can be described as:

$$\text{F}^*(t) = \text{F}_0 (1 - e^{-kt}) \quad (4-6)$$

where $F^*(t)$ = the amount of label in the pool at time t , and F_0 = the initial free valine carbon concentration which is measured directly. The rate of isotope incorporation into protein valine (dP^*/dt) will be:

$$dP^*/dt = kF^*(t) \quad (4-7)$$

Integration yields the predicted quantity of isotope in protein valine at time t :

$$P^*(t) = kF_0 \left(t + (e^{-kt}/k) - (1/k) \right) \quad (4-8)$$

The term kF_0 is equal to the steady state rate of carbon flux into protein valine. This can be approximated for short incubation times as the product of the protein valine carbon concentration, P_0 , and the dilution rate of the chemostat, D , both of which can be determined directly. Substituting:

$$P^*(t) = DP_0 \left(t + (e^{-kt}/k) - (1/k) \right) \quad (4-9)$$

where $k = (DP_0/F_0)$ in units of hours^{-1} . Fig. 4-17 shows the relationship between the predicted incorporation and the observed value for protein valine carbon incorporation at high and low relative growth rates. There is excellent agreement initially, an indication that the assumptions were reasonable. Subsequently the observed values begin to fall below the predicted isotope incorporation, a likely nutrient depletion response. This is particularly evident at the low relative growth rate.

The fact that the kinetics of protein valine labelling can be described solely on the basis of the intracellular free valine pool concentration and the predicted rate of carbon flux into protein valine suggests that the specific activity of carbon entering the intracellular free valine pool, pyruvate, is similar to that of the dissolved inorganic carbon pool. This is also consistent with the rapid labelling of alanine, for which pyruvate is the carbon skeleton (Fig. 4-10).

* * * *

The results of the patterns of carbon incorporation under high light conditions have demonstrated that the majority of the discrepancy between observed and predicted carbon incorporation into protein is due to the incorporation characteristics of the branched chain amino acids valine, isoleucine, and leucine, as well as glutamate, and to some extent lysine. Initially, the kinetics of isotope distribution are the predominant cause of discrepancies. However, non-steady state effects are potentially important later in the incubation. Non-steady state effects were immediately evident at the low relative growth rate once the flow of media was stopped.

In the next section, the kinetics of protein incorporation of photosynthetically fixed inorganic ^{14}C in low light adapted ($28 \pm 9 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited cultures are described. As shall be demonstrated, incorporation patterns were substantially different in the situation where growth was limited by the energy supply as well as by the nutrient supply.

Figure 4-17. Comparisons of observed isotope incorporation into protein valine with that predicted on the assumption that all carbon flux through the free intracellular valine pool was associated with synthesis of protein valine. Curve represents results of model.

Comparisons of Carbon Incorporation and Net Protein Synthesis Rates in
Nannochloris sp. under Combined Conditions of NH₄⁺-limitation and Low
Light

POC Predicted versus Observed Production

As for the case of high light adapted cultures, predicted and observed POC production agreed within analytical error at the high relative growth rates (Fig. 4-18). There did appear to be a tendency for observed incorporation to exceed the predicted rate at the low relative growth rate, but the discrepancy was marginally significant.

Total PPAA Carbon Predicted versus Observed Production

Observed total PPAA carbon production was generally within analytical error of the predicted rates (Fig. 4-19), in contrast to the light saturated case (Fig. 4-2). Because of small sample sizes ($n = 4$), errors were large for the middle and low relative growth rates. Examination of the time course of carbon incorporation did reveal a tendency for the observed PPAA carbon incorporation to exceed the predicted value at the low relative growth rate (Fig. 4-20). Further evidence of observed carbon incorporation exceeding the predicted values was found by examining the individual protein amino acids.

Individual Protein Amino Acid Observed versus Predicted Carbon
Incorporation

As for the light saturated case, different amino acids behaved differently in their characteristics of isotope incorporation and their relationship to predicted rates. At all growth rates, both alanine and

glycine observed rates exceeded the predicted (Fig. 4-21). The differences were significant for both alanine and glycine at the middle growth rate ($\mu/\mu_{\max} = 0.723$). However, at the highest and lowest growth rate, only in the case of glycine was the difference significantly beyond the limits of analytical error. Examining the time course patterns of carbon incorporation into glycine and alanine, it can be seen that observed carbon incorporation consistently exceeds the predicted rate (Fig. 4-22). This is also the case for aspartate at the low relative growth rate. The regressions for alanine and aspartate at the low relative growth rate were not significantly different from zero due to the small number of points ($n=4$). Nevertheless, the observed data was consistently greater than the predicted incorporation. Glutamate observed carbon incorporation was low initially for all relative growth rates (Fig. 4-22), consistent with patterns seen in the light saturated cultures (Fig. 4-10). However, during the latter portion of the incubation at the low relative growth rate, observed production exceeded the predicted rate.

The agreement between observed and predicted carbon incorporation rates was generally well within the limits of analytical error for the other amino acids (Fig. 4-23), with the exception of proline for which observed incorporation was consistently below the predicted rates as in the high light cultures. Data obtained during the latter periods of incubation for isoleucine, leucine, threonine, phenylalanine also tended to be above the predicted incorporation at the low relative growth rate, and even for the middle relative growth rate in the case of phenylalanine and threonine (Fig. 4-24). Lysine observed incorporation was variable at the high relative growth rate, and was generally in agreement with

Figure 4-18. Observed total ^{14}C uptake rates and theoretical organic carbon production rates of low light ($28 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Observed total ^{14}C uptake rates were determined by linear regression of a 3 hour time course. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 4-19. Theoretical and observed rates of isotopic carbon incorporation into particulate protein amino acids (PPAA) of low light ($28 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Observed isotope incorporation rates were determined by linear regression of a 3 hour time course. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 4-20. Observed and predicted time courses of isotope incorporation into particulate protein amino acids of low light ($28 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Solid line represents the predicted rate for the high relative growth rate culture ($\mu/\mu_{\text{max}} = 0.864$). The long dashed line is the predicted rate for the middle relative growth rate culture ($\mu/\mu_{\text{max}} = 0.723$), and the short dashed line, the predicted rate for the low relative growth rate culture ($\mu/\mu_{\text{max}} = 0.324$). Flow was interrupted at zero time. Symbols: squares, sum of carbon incorporation into individual protein amino acid residues at the high relative growth rate; circles, sum of carbon incorporation into individual protein amino acid residues at the middle relative growth rate; +, sum of carbon incorporation into individual protein amino acid residues at the low relative growth rate.

Figure 4-21. Theoretical and observed rates of isotopic carbon incorporation into alanine (ALA), glycine (GLY), glutamate (GLU), and aspartate (ASP) in protein of low light ($28 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Observed isotope incorporation rates were determined by linear regression of a 3 hour time course. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 4-22. Observed and predicted time courses of isotope incorporation into alanine, glycine, glutamate, and aspartate in protein of low light ($28 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Solid line represents the predicted rate for the high relative growth rate culture ($\mu/\mu_{\text{max}} = 0.864$). The long dashed line is the predicted rate for the middle relative growth rate culture ($\mu/\mu_{\text{max}} = 0.723$), and the short dashed line, the predicted rate for the low relative growth rate culture ($\mu/\mu_{\text{max}} = 0.324$). Flow was interrupted at zero time. Symbols: squares, sum of carbon incorporation into individual protein amino acid residues at the high relative growth rate; circles, sum of carbon incorporation into individual protein amino acid residues at the middle relative growth rate; +, sum of carbon incorporation into individual protein amino acid residues at the low relative growth rate.

Time (hours)

micrograms carbon/liter

Figure 4-23. Theoretical and observed rates of isotopic carbon incorporation into isoleucine (ILE) + leucine (LEU), proline (PRO), phenylalanine (PHE), lysine (LYS), and threonine (THR) in protein of low light ($28 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Observed isotope incorporation rates were determined by linear regression of a 3 hour time course. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 4-24. Observed and predicted time courses of isotope incorporation into threonine, isoleucine + leucine, phenylalanine, and lysine in protein of low light ($28 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Nannochloris sp. Solid line represents the predicted rate for the high relative growth rate culture ($\mu/\mu_{\text{max}} = 0.864$). The long dashed line, the predicted rate for the middle relative growth rate culture ($\mu/\mu_{\text{max}} = 0.723$), and the short dashed line, the predicted rate for the low relative growth rate culture ($\mu/\mu_{\text{max}} = 0.324$). Flow was interrupted at zero time. Symbols: squares, sum of carbon incorporation into individual protein amino acid residues at the high relative growth rate; circles, sum of carbon incorporation into individual protein amino acid residues at the middle relative growth rate; +, sum of carbon incorporation into individual protein amino acid residues at the low relative growth rate.

Time (hours)

predicted incorporation at the low and middle growth rates.

Protein Turnover

The substantial discrepancies between predicted carbon incorporation and the observed isotope incorporation rate is in marked contrast to the results observed for the high light adapted cultures. The tendency for isotope incorporation to exceed the predicted rate was particularly evident in the case of the amino acids which exhibited rapid saturation kinetics under high light conditions (alanine, glycine, threonine, and phenylalanine). The discrepancy was most pronounced at the low relative growth rate.

The simplest explanation for these results would be that the gross rate of protein synthesis significantly exceeds the net protein synthesis rate. That is, the total flux of amino acids into protein exceeds the net increase in protein amino acids. Turnover of protein in excess of net synthesis has been recognized as a common phenomenon in higher plants (Huffaker and Peterson, 1974). However, due to limitations in methodology, little is known about the factors which regulate the breakdown of protein. One of the factors which makes the determination of protein degradation rates difficult is the potential compartmentalization of the precursor amino acid pools from the total intracellular free amino acid pool. The intracellular isolation of the precursor pools means that their specific activities may be quite different from the total intracellular free amino acid pool. Results from chapter 3 demonstrated that compartmentalization was a factor in the isotope kinetics in Nannochloris sp., and its relative importance varied in response to relative growth rate.

Trewavas (1972) solved the precursor pool problem by isolating and determining the specific activity of methionine bound to transfer RNA, which is unambiguously the immediate precursor source for incorporation of methionine into protein. He found that degradation was enhanced when cultures were grown at low levels of MgSO_4 .

Although it is not possible to estimate the true degradation rate of protein from the results I've presented, it is possible to derive a minimum value for protein degradation necessary to explain the overestimates of predicted incorporation rates by the observed carbon incorporation. This is a minimum estimate because protein precursor specific activities may be less than that of the dissolved inorganic carbon. However, from results obtained with the high light cultures, we know that precursor pools of glycine and alanine tend to equilibrate rapidly. Consequently, in basing my estimates of carbon incorporation on the characteristics of these two amino acids, it should be reasonable to assume that the precursor pool specific activity was equal to that of the inorganic carbon. Another reason this approach yields is only a minimum estimate is that degraded unlabeled protein may be reutilized in protein synthesis, thereby further diluting the precursor pool. Similarly, degradation of labeled protein would lead to a reduction in the incorporation rate and an underestimate of the total amino acid flux into protein. However, this latter constraint should not be substantial for the short incubation periods used here.

The calculation of protein turnover is based on the following equation adapted from Trewavas (1972):

$$dP/dt = P_0 (k_s - k_d) \quad (4-10)$$

where dP/dt = the net protein synthesis rate, P_o = the steady state carbon concentration of the protein amino acid, and k_s and k_d are the synthesis and degradation rate constants respectively. dP/dt can be approximated for short incubation times as the product of P_o and the dilution rate of the chemostat, D . The term $k_s P_o$, the total carbon flux through the protein amino acid, can be estimated from the rate of isotope incorporation (this assumes complete saturation of precursors with inorganic ^{14}C). Substituting into equation 4-10 and solving for k_d :

$$k_d = (k_s P_o - DP_o)/P_o \quad (4-11)$$

This can be further simplified as follows:

$$k_d = (k_s P_o)/P_o - D \quad (4-12)$$

Estimates for k_s and k_d are shown in Table 4-9 for the three experiments conducted under low light conditions. The results demonstrate the importance of protein degradation relative to total protein synthesis. The degradation constants are relatively unaffected by changes in the relative growth rate. It appears then that the increased discrepancy between observed and predicted carbon incorporation rates with decreasing relative growth rate is a consequence of the decline in the synthesis rate, as opposed to an increase in the degradation rate. The impact of protein turnover on incorporation patterns is substantial. At the low relative growth rate, the degradation rate constant was more than half the synthesis rate

constant. These results, in conjunction with observations of the characteristics of high light cultures, indicate that turnover becomes important under light limited situations.

Results with high light cultures did not indicate important turnover of protein in excess of net synthesis, as can be concluded from the close agreement between observed and predicted incorporation rates of glycine and alanine. The magnitude of protein turnover seen here was sufficiently large so as to have been apparent in the high light cultures if it was occurring. It may be concluded that either increased light intensity inhibited protein turnover, or that evidence for turnover was obscured at the higher light intensity for one or more of the reasons given previously. Li and Goldman (1981) noted that for some species, the observed incorporation of inorganic ^{14}C overestimated the predicted POC production rate. However, my results with Nannochloris sp. do not appear to represent an overall increase in the turnover of carbon biomass. Although there did appear to be an increasing trend in the degradation rate constant for total carbon, it was not significantly different from zero for any of the experiments (Table 4-9). This suggests that the turnover rate of protein exceeded that of the remaining carbon biomass.

The turnover of protein provides a way of altering the general protein constituents and enzymatic complement of the cell (Huffaker and Peterson, 1974). It is an important source of amino acids for protein synthesis when nutrients are limiting. Turnover of existing protein may be less energetically demanding than de novo synthesis which could explain why this process was enhanced under low light conditions in Nannochloris sp.

Table 4-9. Turnover Rate Constants for Light^a and
NH₄⁺-Limited Steady State Cultures of *Nannochloris* sp.

Relative Growth Rate ^b	Glycine ^c		Alanine		POC	
	k _s	k _d	k _s (day ⁻¹)	k _d	k _s	k _d
0.864	0.82 (0.26)	0.31 (0.26)	0.70 (0.23)	0.19 (.23)	0.43 (0.11)	-0.07 (0.11)
0.723	0.74 (0.24)	0.34 (0.24)	0.72 (0.19)	0.29 (0.19)	0.38 (0.10)	-0.05 (0.10)
0.324	0.50 (0.15)	0.34 (0.16)	0.43 (0.48)	0.26 (0.48)	0.22 (0.05)	0.05 (0.06)

^a28±9 μE · m⁻² · s⁻¹.

^bGrowth rate normalized to maximum growth rate (0.58 (0.05) day⁻¹) under similar conditions of light and temperature.

^cErrors of all rate constants (in parentheses) represent propagated estimates of analytical error based on 95% confidence intervals.

Morris et al. (1974) investigated the incorporation patterns of Phaeodactylum tricornutum under light limited conditions. They found that the ratio of carbon incorporation into the hot trichloroacetic acid insoluble fraction relative to total incorporation was higher under low light conditions. Konopka and Schnur (1980) also observed a relative increase in isotope incorporation into protein at lower light intensities. My results illustrate that protein turnover may have been a factor in their experiments, perhaps indicating that the patterns they observed were not entirely due to a relative increase in net protein synthesis, but rather, an increase in the importance of protein turnover in relationship to net protein synthesis.

Another consideration is the extent to which turnover of protein rather than net synthesis is responsible for observed night incorporation of isotope into hot trichloroacetic acid insoluble material of phytoplankton populations (e.g. Smith and Morris, 1980a,b; Morris and Skea, 1978; Morris et al., 1981; Cuhel et al., 1984). Cuhel et al. (1984) observed significant increases in equilibrium ^{14}C and ^{35}S labeled protein during the dark period in laboratory cultures of Dunaliella tertiolecta grown on a light/dark cycle. They concluded that night synthesis of protein was significant. On the basis of laboratory observations that exchange of $^{35}\text{SO}_4$ with protein sulfur in stationary phase cultures was substantially less than that of inorganic ^{14}C , they argued that short term incorporation of $^{35}\text{SO}_4$ more closely reflected net synthesis under conditions where protein turnover was significant. However, their results did not exclude the possibility of exchange of $^{35}\text{SO}_4$ in the absence of net protein synthesis.

Comparisons of Observed and Predicted Carbon Incorporation in Other Species of Marine Algae

Observed and Predicted Carbon Incorporation Into Particulate Organic Carbon

Observed and predicted POC production rates agreed well for all species examined under high light conditions, and moderate to high relative growth rates (Fig. 4-25). One exception to this was Chroomonas salina, in which observed incorporation exceeded the predicted rate. This indicates that the total flux of carbon exceeded the net synthesis rate of carbon biomass in this species under the specified growth conditions. The values of the degradation rate constants ($k_g = 0.674$ (0.111) day^{-1} , $k_d = 0.264$ (0.111) day^{-1}) show that degradation or respiratory breakdown accounts for a substantial proportion of total carbon fixation in this species.

PPAA Observed versus Predicted Carbon Incorporation

Observed PPAA carbon incorporation rates were, in all cases, below the predicted carbon incorporation rates (Fig. 4-26). In some cases the discrepancy was more than a factor of 6. There are two possible causes for such discrepancies. As pointed out for Nannochloris sp., the kinetics of saturation of protein precursors may result in the incorporation of amino acids of specific activity lower than that of the exogenous inorganic carbon pool. A second explanation for low observed rates of isotope incorporation relative to predicted rates was that interrupting the steady state growth conditions may have led to nonlinear decreases in the protein carbon incorporation rate. In the experiments

conducted with Chattonella luteus, Chroomonas salina, and Chaetoceros simplex, influent flow was interrupted at time zero (i.e. the time of isotope addition). For Nannochloris sp., flow was interrupted after .33 hours beyond isotope addition, so that these experiments are comparable. Time courses of PPAA observed carbon incorporation revealed a pronounced time dependent decrease in the incorporation rate for cultures of C. simplex (Fig. 4-27). Such a decrease is strong evidence for the manifestation of non-steady state responses. Although the observed slopes of incorporation for the other species were below that predicted, non-linearity was less apparent. As will be demonstrated, different amino acids respond differently to the interruption of steady state.

Observed versus Predicted Carbon Incorporation into Individual Protein Amino Acids

Chattonella luteus

Observed carbon incorporation rates into individual protein amino acids in C. luteus are compared to the predicted rates in Fig. 4-28. They resembled those for Nannochloris sp. in that the observed and predicted carbon incorporation rates into protein glycine and alanine agreed well. Agreement was also good for protein serine. The time course incorporation patterns for these protein amino acids indicates rapid saturation of their precursor pools (Figure 4-29). For glutamate and aspartate, the observed rates were below the predicted rates (Fig. 4-28). However, the time course of carbon incorporation revealed that the observed slope of incorporation approached the predicted rate by 3 hours (Fig. 4-29).

Figure 4-25. Observed total ^{14}C uptake rates and theoretical particulate organic carbon production rates of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of four species of marine phytoplankton. Nanno = Nannochloris sp. (μ/μ_{max} , 0.933); Olith = Chattonella luteus (0.921), Chaet = Chaetoceros simplex (0.574); Chroom = Chroomonas salina (0.804). Observed total ^{14}C uptake rates were determined by linear regression of a 3 hour time course. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 4-26. Theoretical and observed rates of isotopic carbon incorporation into particulate protein amino acids of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of four species of marine phytoplankton. Nanno = Nannochloris sp. (μ/μ_{max} , 0.933); Olsth = Chattonella luteus (0.921), Chaet = Chaetoceros simplex (0.574); Chroom = Chroomonas salina (0.804). Observed isotope incorporation rates were determined by linear regression of a 3 hour time course. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 4-27. Observed and predicted time courses of isotopic carbon incorporation into particulate protein amino acids of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Chattonella luteus (μ/μ_{max} , 0.921), Chroomonas salina (0.804), and Chaetoceros simplex (0.574). Flow was stopped at zero time. Solid line represents the predicted rate.

Figure 4-28. Theoretical and observed rates of isotopic carbon incorporation into alanine (ALA), glycine (GLY), glutamate (GLU), aspartate (ASP), and serine (SER) in protein of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Chattonella luteus (μ/μ_{max} , 0.921), Chroomonas salina (0.804), and Chaetoceros simplex (0.574). Observed isotope incorporation rates were determined by linear regression of a 3 hour time course. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

micrograms carbon/liter/hour

Figure 4-29. Observed and predicted time courses of observed isotope incorporation into glycine, alanine, glutamate and aspartate in protein of a high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Chattonella luteus ($\mu/\mu_{\text{max}} = 0.921$). Flow was stopped at zero time. Solid line represents the predicted rate.

Time (hours)

For the remaining protein amino acids, the observed incorporation substantially underestimated the predicted rate (Fig. 4-30). This was also the case for Nannochloris sp. at the low relative growth rate. Threonine was low, a consequence of the saturation characteristics of its likely precursor, aspartate. However, both in the case of threonine and the other protein amino acids, there was evidence for non-steady state responses. The observed incorporation into phenylalanine, isoleucine+leucine, valine+arginine, lysine, and proline exhibited time dependent decreases in rates (Fig. 4-31). This indicates that the supply of newly fixed inorganic carbon to the production of the precursors to these protein amino acids was suppressed, presumably as a result of the interruption of influent flow into the chemostat. The fact that observed incorporation into alanine and glycine continues to reflect their predicted net synthesis rates, and that observed slopes of incorporation into glutamate and aspartate continuously approach their predicted carbon incorporation rates (Fig. 4-29) is an indication that protein synthesis continued despite the reduction in flux of photosynthetically fixed inorganic carbon. This suggests a selective regulation of the metabolic flux of photosynthetically fixed carbon into specific amino acids in response to the reduced NH_4^+ supply.

C. luteus is a large celled algae. It is possible that continued synthesis of the other amino acids became more important at an intracellular location other than the photosynthetic site. A reduced supply of NH_4^+ could lead to a reduction in the supply of photosynthetically derived carbon skeletons for amino acid biosynthesis as has been demonstrated in higher plants (e.g. Platt et al., 1977; Paul et al., 1978; Mohamed and Gnaman, 1979). Such a regulatory effect might

result in an increased utilization of unlabeled PEP and pyruvate from the cytosol. PEP is a substrate in the biosynthesis of threonine, isoleucine, and lysine, and pyruvate in the biosynthesis of leucine and valine. So an unlabeled supply of pyruvate to the chloroplast could generate the decline in the observed label incorporation rates. However, pyruvate is also the carbon skeleton of alanine. Thus this argument is inconsistent with the rapid labelling characteristics of alanine. PEP is a substrate in the biosynthesis of phenylalanine. However, one would expect the inputs of unlabeled PEP to be reflected as decreased labelling rate of aspartate which was not observed. These results suggest that synthesis of threonine, valine + arginine, isoleucine + leucine, lysine, phenylalanine, and proline was metabolically segregated from glycine, alanine, glutamate, and aspartate.

The high initial rates of incorporation suggest that early in the incubation period, a greater proportion of photosynthetically derived carbon supplies the production of these protein amino acids. If indeed protein synthesis continues at a relatively constant rate as suggested by the incorporation characteristics of glycine and alanine, then the simplest explanation for the rapid initial rate of label incorporation followed by a time dependent decrease in the rate of isotope incorporation into the other amino acids is that their precursors must be supplied with carbon from a source other than the chloroplast.

Chroomonas salina

The labelling characteristics of individual protein amino acids of C. salina were similar in many respects to the observations of the other species, supporting the view that basic biosynthetic pathways associated

Figure 4-30. Theoretical and observed rates of isotopic carbon incorporation into isoleucine (ILE) + leucine (LEU), valine (VAL) + arginine (ARG), proline (PRO), phenyalanine (PHE), lysine (LYS), and threonine (THR) in protein of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Chattonella luteus (μ/μ_{max} , 0.921), Chroomonas salina (0.804), and Chaetoceros simplex (0.574).

Observed isotope incorporation rates were determined by linear regression of a 3 hour time course. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Figure 4-31. Observed and predicted time courses of isotope incorporation into threonine, valine + arginine, isoleucine + leucine, lysine, phenylalanine, and proline in protein of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Chattonella luteus ($\mu/\mu_{\text{max}} = 0.921$). Flow was stopped at zero time. Solid line represents the predicted rate.

with algal protein metabolism are similar among different taxonomic groups. Alanine and glycine again show good agreement between observed and predicted carbon incorporation rates (Fig. 4-28 and Fig. 4-32). The observed isotope incorporation into glycine did slightly exceed the predicted rate, similar to the situation observed for low light cultures of Nannochloris sp. This was also the case for serine, whose synthesis is closely linked to that of glycine by way of the photorespiratory cycle. However, regression analysis did not reveal differences outside the limits of analytical error (Fig. 4-28).

The observed incorporation rate into protein glutamate continuously increased during the incubation (Fig. 4-32). This pattern resembled that seen for Nannochloris sp. and C. luteus, and could be explained by the presence of a relatively large slowly saturating precursor pool. Aspartate incorporation was variable, but tended to be less than the predicted rate (Fig. 4-32).

Incorporation into other amino acids also substantially underestimated the predicted rate, as was seen for C. luteus (Fig. 4-30). Threonine observed incorporation was substantially below the predicted value but tended to increase (Fig. 4-33). Time courses of incorporation into valine + arginine, isoleucine + leucine, lysine, and phenylalanine resembled the patterns observed for C. luteus in that they displayed an initial rapid rate of incorporation of labelling followed by a lower relatively steady rate. However, unlike C. luteus, incorporation rates of phenylalanine and isoleucine + leucine did not approach zero (c.f. Figs. 4-31 and 4-33). Both appear to display an initial rapid rate of incorporation of label followed by a lower relatively steady rate. It is unlikely that these represent linear rates of incorporation, since the

resulting intercept would be far in excess of the blank. This nonlinear decrease in carbon incorporation again suggests a decreased flux of photosynthetically derived carbon into the carbon skeletons of these amino acids. The observed incorporation into proline reflected the labelling characteristics of glutamate, the precursor to proline.

Chaetoceros simplex

C. simplex was particularly sensitive to the interruption of steady state. All observed rates underestimated the predicted incorporation rates (Figs. 4-28 and 4-34), even in the case of glycine and alanine where predicted rates were underestimated by more than a factor of 2. Underestimates were even more dramatic for threonine, isoleucine + leucine, phenylalanine, lysine, and proline (Figs. 4-32 and 4-35). For glycine, alanine, phenylalanine, threonine, and isoleucine + leucine the observed incorporation continuously decreased from an initially higher rate. In the case of glycine and alanine, this initial rate was close to the predicted rate. Non-linear decreases were less evident for glutamate, proline, and aspartate, despite the fact that observed incorporation rates were less than predicted rates. This was likely due to a compensating effect of the saturation of precursor pools, which would counter the depletion induced decrease.

Protein metabolism of C. simplex appears to be extremely sensitive to the interruption of steady state. This was not a generalized metabolic response since non-steady state decreases were not observed in total ¹⁴C uptake rate (c.f. Goldman et al., 1981b). This illustrates how considerably more information about population metabolism can be obtained through the tracing of specific biosynthetic processes. It appears that

Figure 4-32. Observed and predicted time courses of isotope incorporation into glycine, alanine, glutamate and aspartate in protein of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Chroomonas salina ($\mu/\mu_{\text{max}} = 0.804$). Flow was stopped at zero time. Solid line represents the predicted rate.

Figure 4-33. Observed and predicted time courses of isotope incorporation into threonine, valine + arginine, isoleucine + leucine, lysine, phenyalanine, and proline in protein of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Chroomonas salina ($\mu/\mu_{\text{max}} = 0.804$). Flow was stopped at zero time. Solid line represents the predicted rate.

Figure 4-34. Observed and predicted time courses of isotope incorporation into glycine, alanine, glutamate and aspartate in protein of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Chaetoceros simplex ($\mu/\mu_{\text{max}} = 0.574$). Flow was stopped at zero time. Solid line represents the predicted rate.

Figure 4-35. Observed and predicted time courses of isotope incorporation into threonine, valine + arginine, isoleucine + leucine, lysine, phenylalanine, and proline in protein of high light ($176 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) NH_4^+ -limited steady state cultures of Chaetoceros simplex ($\mu/\mu_{\text{max}} = 0.574$). Flow was stopped at zero time. Solid line represents the predicted rate.

under nitrogen limited conditions, C. simplex is unable to accumulate an internal supply of nitrogen sufficient to maintain growth for an extended period. C. simplex had the highest growth rate of all the species examined in this study. It may be that this higher rate of metabolism led to a more rapid manifestation of non-steady state responses.

CONCLUSIONS

This investigation has contributed to existing information about two potentially important sources of variation in the protein content of primary organic matter being introduced into the marine environment. These include 1) environmental conditions affecting metabolism within a single organism, and 2) intrinsic metabolic differences among species. The results demonstrate that environmentally induced variations within a single species may encompass the range of differences between species growing under similar conditions. For the species examined in this study grown under similar conditions, protein is a relatively constant proportion of total carbon. This is consistent with the original conclusions of Parsons et al. (1961), who surveyed the chemical composition of a variety of marine algae and found them to be quite similar. As Morris (1981) noted, it appears that environmental factors are more important than variations in species composition in influencing the relative abundance of protein in the primary organic matter. This conclusion is supported by the variety of studies which have illustrated the substantial variations in algal protein relative to other cell material in response to changing growth conditions (e.g. Cook, 1963; Handa, 1969; Myklestad and Haug, 1972; Darley et al., 1976; Rhee, 1978; Hitchcock, 1980; Dortch, 1982).

The main objective of this investigation was to evaluate the characteristics of isotopic incorporation into protein in order to optimize methods for quantifying the production of particulate protein amino acids by autotrophs. The variations in composition referred to above reflect variations in the metabolism. Such metabolic changes also influence the isotope kinetics, and must be recognized as potentially important factors affecting relationship of isotope incorporation into protein and net protein synthesis. The environmental condition which had the most dramatic effect on the isotope kinetics in Nannochloris sp. was light intensity. Precursors saturated much more rapidly under low light conditions, and as demonstrated in Table 4-9, the low synthesis rates associated with low light intensity and low relative growth rate make the impact of protein degradation on total isotope incorporation significant. Consequently, attempts to estimate protein synthesis using isotope incorporation under dark or low light and low nutrient conditions, must recognize that some of the observed isotope incorporation may be the result of turnover, rather than net protein synthesis.

In addition to the characteristics of precursor saturation, the effects of nutrient depletion must be recognized, and incorporation must be evaluated in a time course in order to know the appropriate time scales over which to determine protein synthesis rates (Goldman et al., 1981). Results with high light NH_4^+ -limited cultures of Nannochloris sp. indicated that nutrient depletion effects were a function of the nutrient prehistory, being more substantial in more nutrient limited cultures. This may reflect the presence of variable internal pools of nitrogen which can be utilized after the exogenous nitrogen supply is

interrupted. Such pools may be present in higher levels in the less nutrient limited cultures. This is certainly true for the intracellular free amino acids (chapter 3, Table 3-1). However, time dependent reductions in the IFAA pools were not found to be associated with the observed decrease of carbon incorporation into protein (Table 4-10). I did not measure the intracellular NH_4^+ pool. It may be that this pool was more important in regulating the flux of photosynthetic carbon into protein amino acids. This would be consistent with the observations of regulatory effects of NH_4^+ on photosynthetic enzymes in a higher plant (Mohamed and Gnaman, 1979). The potential impact of nutrient depletion may be substantial, and yet not necessarily reflected in an obviously nonlinear pattern of incorporation into total PPAA carbon due to the mixed kinetics of the different individual amino acids. Efforts have been made to measure protein synthesis based on ^{14}C incorporation in nitrogen limited populations (DiTullio and Laws, 1983; Laws et al., 1985). Of course, the environment of a chemostat is very different from a natural system which contains more than just phytoplankton. However, it is important to recognize that long incubation periods without detailed time course descriptions of carbon incorporation may yield rates which underestimate the actual protein synthesis rate.

The other species also showed evidence for nutrient depletion effects. In the case of C. simplex, there was marked nonlinearity in incorporation patterns even in the amino acids which were least sensitive to this effect (i.e. glycine and alanine). The comparisons between the different species did illustrate, however, fundamental similarities in the basic pathways associated with the supply of photosynthetically fixed carbon to protein production. Precursors to glycine and alanine

Table 4-10. Intracellular Free Amino Acid
Carbon Concentrations in High Light^a
NH₄⁺-limited Steady State Cultures of Nannochloris sp.

Incubation Time (hrs)	Relative Growth Rate ^b	IFAA Micrograms Carbon per Liter
0.005	0.229	116
0.07		120
0.25		138
1.1		150
2.1		100
2.9		127
0.1	0.933	450
0.25		455
1.0		450
1.0		611
3.0		355

^a176±70 μE · m⁻² · s⁻¹.

^bGrowth rate normalized to maximum growth rate (1.44 (0.04) day⁻¹)
under similar conditions of light and temperature.

consistently saturated rapidly, so that agreement between predicted and observed carbon incorporation was good. Although the observed incorporation into protein glycine and alanine tended to be less than the predicted rate in C. simplex, the initial incorporation rates did approximate the predicted rates before the onset of depletion effects.

The characteristics of protein glycine and alanine carbon incorporation make them ideal model protein amino acids for tracing protein synthesis in mixed autotrophic populations. Their relative abundance in protein was found to be highly consistent, irrespective of growth conditions or species (Table 4-11). The proportions I found were similar to those found by other investigators (Table 4-12). My estimates did tend to exceed the relative abundances determined by sequencing protein. This is likely a consequence of the preferential recovery of glycine and alanine from hydrolysis leading to an apparent increase in their relative abundance (see Methods, Table 2-4; see also Roberts et al., 1963). Despite the discrepancy, the studies do suggest that glycine and alanine are a highly consistent proportion of protein from a wide range of organisms. An optimal approach towards estimating protein synthesis will be to characterize the synthesis rates of glycine and alanine, and use their known average abundance in protein to determine the total protein synthesis rate. In Table 4-13, I extrapolated glycine and alanine incorporation rates to total protein carbon incorporation based on their relative abundance in average protein. These estimates are compared to observed and predicted carbon incorporation rates. Observed rates were consistently the lowest, a consequence of slow kinetics of labelling of other amino acids, and non-steady state decreases in protein synthesis. Predicted rates were below the

Table 4-11. Relative Abundance of Glycine and Alanine in Protein of Different Algal Sources

Source	Alanine	Mole % ^a	Glycine
<u>Nannochloris</u> sp.:			
High Light: ^b			
$\mu/\mu_{\max} = 0.933$	12.5 (3.2)		13.8 (3.1)
0.229	12.4 (7.0)		14.5 (3.4)
Low Light: ^c			
$\mu/\mu_{\max} = 0.864$	11.2 (2.4)		14.8 (3.4)
0.324	13.9 (3.6)		11.8 (2.7)
<u>Chaetoceros simplex</u>	8.4 (1.8)		12.4 (2.8)
<u>Chroomonas salina</u>	12.2 (2.6)		12.9 (2.9)
<u>Chattonella luteus</u>	12.3 (2.6)		13.0 (2.9)

^aErrors (in parentheses) represent propagated estimates of analytical error based on 95% confidence intervals.

^b $176 \pm 70 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

^c $28 \pm 9 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

Table 4-12. Relative Abundance of Glycine and Alanine in Protein

Source	Alanine	Mole %	Glycine
<u>SEQUENCED PROTEIN:</u>			
Jukes et al. (1975)	8.7		7.9
Doolittle (1981):			
Newat	7.6		7.9
Atlas	8.2		8.8
<u>ACID HYDROLYSIS:</u>			
Fowden (1954)			
<u>Chlorella vulgaris</u>	9.5		7.6
<u>Anabaena cylindrica</u>	7.0		6.5
<u>Navicula pelliculosa</u>	7.6		7.1
<u>Tribonema aequale</u>	10.4		7.6
Cowey and Corner (1963)			
<u>Skeletonema</u>	9.2		11.6
suspended particulate	8.4 (6.7-11.4)		16.6 (12.5-21.7)
Degens (1970)			
Sargasso Sea (surface)	5.8-6.5		7.8-10.0
Buzzards Bay "	8.8		12.1
Siezen and Mague (1978)			
(suspended particulate)	10.0		13.4
Maïta and Yanada (1978)			
(suspended particulate)	9.0		15.4

Table 4-13. Comparison of Observed, Predicted and Extrapolated Protein Carbon Incorporation Rates

Relative Growth Rate ^b	Protein Carbon Incorporation Rates ^a (micrograms carbon/liter/hour)				
	Observed	Predicted	Extrapolated Gly	Ala	Combined Mean
0.933	85 (34)	111 (12)	203 (44)	148 (30)	165 (24)
0.229	18 (11)	37 (5)	72 (18)	56 (15)	62 (11)

^aErrors (in parentheses) represent propagated estimates of analytical error based on 95% confidence intervals.

^bGrowth rate normalized to maximum growth rate (1.44 (0.04) day⁻¹) under similar conditions of light and temperature.

extrapolated rates. This is expected since the predicted rate is based on the protein carbon which is recovered from hydrolysis. Some of the amino acids are destroyed, while alanine and glycine are preferentially recovered.

Because glycine and alanine are preferentially recovered, and generally show good agreement between predicted and observed incorporation rates, they are ideal model protein amino acids for tracing the primary production of particulate protein. In the next chapter, I describe a study where this approach was used to estimate the proportion of primary production associated with protein production in a natural phytoplankton community and compare this to the protein content of particulate organic matter.

CHAPTER 5. PRIMARY PRODUCTION OF PARTICULATE ORGANIC CARBON
AND PARTICULATE PROTEIN AMINO ACIDS IN SALT POND, MA:
THE RELATIONSHIP TO THE COMPOSITION OF
PARTICULATE ORGANIC MATTER

INTRODUCTION

Particulate organic matter (POM) in euphotic systems is a complex matrix of chemical substances which originate largely as a result of biological activity. Photoautotrophs account for the bulk of POM production in euphotic systems of the world's oceans. The production and metabolism of biogenic compounds by photoautotrophs will depend on environmentally induced and intrinsic metabolic characteristics of the species making up the phytoplankton population (e.g. Morris, 1981). However, the relationship of the chemical composition of POM to the biochemical composition of living algal biomass may be obscured by the presence and activities of other organisms and detritus (e.g. Zeitzchel, 1970; Banse, 1974, 1977; Beers et al., 1975; Eppley et al., 1977; Barlow, 1982a). Hence, the abundance and composition of POM cannot be exclusively attributed to the activities of primary producers.

The composition of POM is a function of the rates of production of individual organic components, and their rates of removal from POM (Figure 5-1). The inputs of POM to euphotic systems consist of gross primary production by photoautotrophs, chemosynthetic production, secondary production at the expense of dissolved organic matter, and allocthonous sources such as aeolian transport, resuspension, groundwater intrusion, runoff and advection and diffusion from other water masses. Processes which remove POM include autotrophic and heterotrophic respiration, autolysis, excretion and exudation, macroheterotrophic grazing and assimilation, sedimentation, and advection and diffusion. The unique chemical characteristics of individual biogenic molecules make them differentially susceptible to heterotrophic degradation and transformation (e.g. Wakeham et al., 1980; Lee et al., 1983; Gagosian et

Figure 5-1. Processes involved in production and removal of organic matter in aquatic environments.

al., 1982; Karl, 1982). As a result, chemically distinct types of particulate organic matter will, under some circumstances, have different turnover rates in euphotic systems. Precise descriptions of the dynamic relationship between distinct components making up POM will provide insight as to the kinds of processes which control POM composition and concentration, as well as the characteristics of organisms which mediate these processes (e.g. Lee and Cronin, 1984). Information about time dependent concentration changes is not by itself adequate to describe the dynamics of individual materials, since it reveals nothing about the actual rate of flux in a system where there are inputs as well as outputs throughout the water column. Through assessment of differential changes in the production and concentrations of individual biogenic materials, it is possible to distinguish the impact of primary production from the effects of secondary transformations on organic matter composition and concentration.

Previous investigations have compared the relationship of carbon and nitrogen assimilation by natural phytoplankton populations to the relative abundance of particulate organic carbon and particulate organic nitrogen (e.g. Eppley et al., 1977; Slawyk et al., 1978; Eppley et al., 1979; Sharp et al., 1980; Harrison et al., 1983). Although positively correlated, C:N assimilation ratios were generally quantitatively different from the particulate ratios. Both larger and smaller assimilation ratios were observed, and the range was much larger than for particulate ratios. Some of the discrepancies may be attributable to the fact that carbon and nitrogen uptake may be uncoupled, and consequently not reflect the algal elemental composition (Collos and Slawyk, 1979; Goldman et al., 1981a).

Because of the nutritional importance of particulate protein, factors which control its relative abundance in particulate organic matter ultimately influence the flux of organic matter to higher trophic levels. In Chapter 4, I described the optimal approach to estimating the production of particulate amino acids on the basis of radioisotopic carbon incorporation. The ability to measure both concentrations and primary production of particulate protein amino acids (PPAA) in conjunction with particulate organic carbon (POC) make it possible to determine whether the relative abundance of POC and PPAA reflects the metabolism of the active autotrophic population.

To date, there has not been an investigation which has independently estimated primary production of POC and PPAA, and compared these measurements to the relative abundance of POC and PPAA in the particulate organic matter. A major objective of this study was to estimate rates of POC and PPAA primary production in natural populations growing under various environmental conditions. This provided an indication of the proportion of photosynthetic metabolism associated with protein synthesis. The relative magnitude of these primary inputs was compared to the relative abundance of POC and PPAA in order to determine the extent to which POM composition reflects population metabolism (Figure 5-2).

The study was conducted in a small marine pond, Salt Pond (Kim and Emery, 1971). The semi-enclosed nature of the pond increased the probability that processes occurring within the pond ecosystem were primarily responsible for POM composition and abundance. Under conditions where POM composition did not reflect the biosynthetic activities of the photoautotrophic population, I sought to determine

Figure 5-2 . Simplified diagram illustrating the influence of primary inputs and net removal of organic matter on particulate organic matter composition.

whether such discrepancies were the result of differential changes in rates at which POC and PPAA are introduced by primary production, or a consequence of differential removal of POC and PPAA by secondary transformational processes. Net rates of removal of POC and PPAA from the mixed layer were estimated by quantifying differences between estimated production and observed changes in concentration.

RESULTS AND DISCUSSION

Compositional Dynamics of Particulate Organic Matter in a Well Mixed Euphotic System

The following assumptions regarding Salt Pond are implicit in subsequent discussion of field observations:

1) Salt Pond has a shallow well mixed euphotic layer, so that POM is generally homogeneously distributed throughout a mixed euphotic layer. This assumption states that POM composition at one meter, where sampling took place, reflects composition throughout the mixed layer. This assumption is supported by several observations. On the days where incubations were conducted, chlorophyll a concentrations were uniform throughout the upper 1.0 - 1.5 meters (Figure 5-3). The mixed layer depth extended to between 1.0 and 3.0 meters, as indicated by a distinct pycnocline in this depth range (Figure 5-4). Langmuir circulation is commonly observed in Salt Pond as evidenced by distinct slicks (convergence zones) interspersed between turbulent areas (divergence zones). Dispersion of dye added at the surface was rapid during such a period.

2) Non-in situ inputs of particulate organic carbon (POC) (e.g. mixing from other water masses (Vineyard Sound), run-off and ground water intrusion, bottom resuspension, and atmospheric sources) are negligible for the upper mixed layer of Salt Pond. This assumption states that the bulk of organic matter in the euphotic layer is produced by organisms residing there, and is supported by the semi-enclosed nature of Salt Pond. However, the potential for allochthonous inputs of organic matter must be recognized, particularly in view of the presence of a large bird population and the observed accumulation of eelgrass (Zostera marina) along the leeward shore.

Surface salinities in Salt Pond were of the order of 10 o/oo lower than Vineyard Sound, the adjoining water mass. This indicated substantial freshwater inputs, and the potential for exogenous inputs of DOC, which through utilization by microheterotrophs might ultimately influence POM composition. In order to assess the importance of non-in situ inputs of DOC, concentrations of DOC were determined in ground water 1 meter below ground at a site adjacent to the pond where inputs were most likely to be significant. These concentrations were compared to rain water and surface Salt Pond water in Table 5-1 along with corresponding dates of sampling. Although ground water and rain water concentrations were of the same order of magnitude, Salt Pond surface water had the highest concentration. If the transformation of exogenous DOC into POC was significant relative to total primary production, then a decrease in the surface water DOC concentrations relative to exogenous sources would be expected. This was apparently not the case.

Figure 5-3. Depth profiles of chlorophyll a on days during which incubations were conducted in Salt Pond. Symbols: square, chlorophyll a; cross, total pigments (phaeopigments + chlorophyll a). A, 20 July 83; B, 5 Aug 83; C, 10 Aug 83; D 26 Aug 83; E, 1 Sept 83; F, 3 Oct 83.

Figure 5-4. Depth profiles of specific gravity during study period in Salt Pond. $\text{Sigma}_t = [(\text{density of seawater at } t^\circ\text{C}/\text{density of pure water at } 4^\circ\text{C}) - 1]1000$, after Cox et al. (1970). 19 July 83 and 26 July 83 data were provided courtesy of Brian Howes and Stuart Wakeham.

Table 5-1. Dissolved Organic Carbon Concentrations (DOC) From
Ground Water, Rain Water, and Salt Pond Surface Water

Date	Source/ Location	DOC (mg carbon/liter)		
		mean	standard error	n
25 Sept 84	Ground Water (1m) ^a	3.52	0.05	2
25 Sept 84	Surface Runoff ^b	2.00	-	-
4 Sept 84	Rainwater	1.70	-	-
12 Sept 84	"	1.70	-	-
	Salt Pond			
24 Aug 84	Surface (1m)	6.38	0.08	2
27 Aug 84	"	5.30	0.05	2
13 Sept 84	"	6.59	0.29	2
20 Sept 84	"	5.17	0.08	2

^aTaken from site one meter below the ground adjacent to pond where
ground water intrusion was likely to be significant.

^bTaken from drainage ditch flowing into pond.

3) Heterotrophic transformational processes differentially affect the chemically distinct constituents of POM over time, resulting in selective changes in composition. This assumption is amply supported by observations of depth dependent transformations in the composition of POM (Wakeham et al., 1980; Lee et al., 1983; Gagosian et al., 1982), selective assimilation of biochemical components in the food source (Scott, 1980), and release of fecal material depleted in nitrogen (Small et al., 1983). Processes such as advection and diffusion, sedimentation, and grazing by macroheterotrophs may or may not be selective with respect to chemical composition. The above ideas provide a basis for determining which processes are controlling POM flux in the mixed layer.

Given the above assumptions, it is possible to define a POM mass balance at the sampling site. The changes in the concentration C_1 of a given component of POM at this point can be expressed as a difference between in situ rates of production and removal:

$$dC_1/dt = (P_1 + P_2 + P_{a/d} + P_c) - (R_r + R_{DOC} + R_s + R_{a/d} + R_g) \quad (5-1)$$

The term dC_1/dt is the rate of change in concentration of a given component. The terms on the right hand side of the equation are grouped into production (P) terms and removal (R) terms. Production terms include autotrophic or primary production (P_1), secondary production through microheterotrophy (P_2), chemosynthetic production (P_c), and allochthonous inputs through advection or diffusion ($P_{a/d}$). Removal terms include losses due to respiratory degradation (R_r), losses due to

conversion to DOC (R_{DOC}), losses by physical processes such as sedimentation (R_s) or advective or diffusive losses ($R_{a/d}$), and finally, macroheterotrophic grazing (R_g). Microheterotrophs would be included in filtered samples, and were considered part of the POM.

In this study, measurements were made of POC and PPAA production and concentration. Thus dC_1/dt and P_1 are known for POC and PPAA. The other production and removal terms can be combined into a composite rate term, R_N , which represents the rate of net outward flux of POM from the sampling point. Thus:

$$dC/dt = P_1 - R_N$$

where:

$$\begin{aligned} R_N &= P_1 - dC/dt \\ &= (R_r + R_{DOC} + R_s + R_{a/d} + R_g) - (P_2 + P_{a/d} + P_c) \end{aligned} \quad (5-2)$$

This relationship will be used as a guideline for the discussion of results comparing concentrations and production characteristics of POC and PPAA.

Incubation Procedures

Production rates were determined using in situ incubation techniques (sampler incubation device, SID; Taylor et al., 1983) at the 1 meter depth. All estimates were derived from relatively short term (2-4 hours) time course sampling methods. In situ incubations were conducted on six separate days throughout the two and one-half month sampling period (20

July - 3 October 83). The use of in situ incubation methods was an essential aspect of this investigation since the main focus was to examine the relationship of fluxes of chemically distinct organic substances which may be differentially susceptible to sampling artifacts (e.g. Chapter 4). The fact that incubations were initiated and sampled in situ minimizes the potential problem that production characteristics might be a result of artifacts due to sampling methodology.

An additional and fundamental consideration of any study examining the distribution of radioisotopic carbon among different organic substances is the degree to which this distribution is a function of the time of incubation, whether it be the result of isotopic kinetics within the photoautotrophs, or containment effects which would cause deviations from the actual in situ rates as a result of physiological changes. I have attempted to minimize the latter artifact by keeping incubations relatively short (2-3 hours) and conducting time course sampling so that the onset of containment effects would be signaled by characteristic deviations from linearity (Taylor et al., 1983; Goldman et al., 1981a; see also Chapter 4). Furthermore, assessments of PPAA production will include estimates based on isotope incorporation characteristics of protein glycine and alanine, which have been shown to have rapid precursor saturation and relatively close agreement between theoretical and isotopically derived production rates for several algal species (Chapter 4).

A further consideration is the effect of transformation of labeled primary organic matter by microzooplankton grazers included in the incubation vessel (e.g. Smith et al., 1984). For the short incubation periods used in this study, this should not have a significant impact on

incorporation characteristics unless the grazing rate was considerably in excess of the algal growth rate. Under these conditions, a significant decrease in algal standing biomass would have lead to a non-linear decrease in carbon incorporation rates. For the majority of incubations conducted, short term uptake of C^{14} -inorganic carbon was effectively linear (e.g. Figure 5-5), and consequently should have been a reflection of the steady state in situ incorporation rates.

In Table 5-2, results show little or no discrepancies between total fixed carbon (acid non-volatile) and particulate carbon (retained on GF/F filter) for incubations throughout the summer. This rules out the possibility that significant accumulation of labeled DOC was occurring either as a result of algal excretion or exudation, or as a result of "sloppy feeding". Significant DOC production could still have occurred if it was derived from an unlabeled source (c.f. Mague et al., 1980), or was taken up as rapidly as it was produced (e.g. Ammerman et al., 1984; Hagstrom et al., 1984). However, it is likely that linear incorporation of label into individual materials for relatively short incubation times was primarily a reflection of algal metabolism.

Primary Production of Particulate Organic Carbon

Rates of POC primary production are shown for the study period along with concentrations of chlorophyll a and POC (Figure 5-6). In the first three experiments (20 July 83 - 10 August 83), 2 or more replicate incubations were made in order to assess the analytical variation in POC production rate determinations. In Table 5-3, the coefficient of variation in production rates for the replicates is compared to that for the overall mean for that period (mean = 47 micrograms carbon/liter/hour,

95% conf. int. = 11, $n = 7$). The proportion of variability associated with replicate measurements was 33.5%, while that associated with between day variation was 66.5%. Differences between replicate incubations were not significant, while between day differences were (ANOVA, P less than 0.05).

During the latter portion of the summer, consecutive morning and midday incubations were conducted. Coefficients of variation were slightly higher than for the replicate incubations, although there was no consistent trend in the differences between the morning and midday incubations (Table 5-4).

Between day differences during the period from 20 July to 3 October 83 could not be explained by correlations of either assimilation numbers (i.e. micrograms carbon fixed per microgram chlorophyll a per hour) or total POC production (micrograms carbon fixed per liter per hour) with either temperature or phosphate concentration (Table 5-5). However, there was a significant positive correlation between POC production and total inorganic nitrogen concentrations. As will be discussed, nitrogen may be an important factor influencing the population metabolism and POM compositional dynamics.

Integrated POC Production Estimates

In comparing POC production (P_1) to observed changes in POC concentration (dC/dt), mean production rates were used. Daily production of POC was estimated by extrapolating the mean production rate for the whole summer over an 11 hour photoperiod. This corresponded to the period when sunlight was at or greater than 40% or more of maximum (Figure 5-7). This represents 90% or more of the integrated light energy

Figure 5-5. Representative incubation (5 Aug 83) showing POC production in conjunction with surface light regime.

Figure 5-6. POC production during sampling period shown in relationship to dynamics of chlorophyll a and particulate POC. Solid line represents the overall mean for the summer (dotted lines indicate 95% confidence limits). Units for chlorophyll a are multiplied by ten to bring them up to scale.

Figure 5-7. Diel light regime from 1982 during the same time of year as the period of study in Salt Pond. The interval of daylight over which midday production rates were extrapolated is indicated by the dotted lines. Data provided courtesy of Dr. Pat Glibert.

June 23, 1982

Table 5-2. Comparison of Inorganic ^{14}C Fixation as Determined by Acidification/Sparging and Filtration

Date	Incubation Period (hours)	Production Rate ($\mu\text{gC} \cdot \text{l}^{-1} \cdot \text{hr}^{-1}$)	n	Method
20 July 83	1.8	38 (10) ^a	14	filtration acid
	1.7	36 (6)	10	
5 Aug 83	2.8	55 (13)	6	filtration acid
	2.8	55 (6)	23	
10 Aug 83	2.6	45 (6)	4	filtration acid
	2.5	43 (8)	14	
1 Sept 83	3.2	21 (2)	12	filtration acid
	1.98	24 (2)	16	
3 Oct 83	2.6	87 (12)	12	filtration acid
	2.7	89 (9)	16	

^aErrors in parentheses represent 95% confidence intervals.

Table 5-3. Coefficients of Variation for Replicate Production Estimates

Date	Mean Production Rate ($\mu\text{gC} \cdot \text{l}^{-1} \cdot \text{hr}^{-1}$)	n	Coefficient of Variation (%)
20 July 83	39	2	19
5 Aug 83	63	2	9
10 Aug 83	41	3	10
Overall Mean	47	7	23

Table 5-4. Particulate Organic Carbon Production Rates for Consecutive Incubations in Salt Pond, Summer 1983

Date	Time of Day	Production ^a	Mean	Coefficient of Variation
26 Aug 83	0930 - 1250	25 (12)	29	23
	1130 - 1415	34 (12)		
1 Sept 83	0900 - 1200	47 (6)	34	54
	1050 - 1400	21 (2)		
3 Oct 83	0815 - 1050	59 (6)	73	27
	1010 - 1230	87 (11)		

^aμg carbon fixed/liter/hour. Errors represent 95% confidence intervals.

Table 5-5. Product-Moment Correlation Coefficients for the Relationships Between Selected Environmental Parameters and Assimilation Number and Production Rate

Parameter	Assimilation number ^a		Production ^b	
	r	n	r	n
PO ₄	-0.644	9	-0.283	9
Temperature	0.228	9	-0.366	9
Dissolved Inorganic N	0.269	9	0.728 ^c	9

^aμg carbon fixed/μg chl_a/hour

^bμg carbon fixed/liter/hour

^cSignificant at the P = 0.05 level.

during the photoperiod. I assumed that diel fluctuations in production around the midday rate will not be significantly greater than between-day variation in rates, and hence adequately described by the error associated with the overall production mean for the summer. However, in view of observations of substantial diel variations in photosynthetic ability of natural phytoplankton populations (Lorenzen, 1963; Harding et al., 1982a,b) the potential for such variations must be recognized. In general, these studies indicated that maximal photosynthetic ability occurred before or near the middle of the light period. Extrapolation of midday rates in this study may have resulted in a slight overestimate of production. However, my observations showed that midday rates were neither consistently higher or lower than morning rates (Table 5-4, Figure 5-6).

In Figure 5-6, the overall mean for the summer (46 micrograms carbon/liter/hour, 95% confidence interval = 11, n = 13) is compared to the individual determinations of midday production at one meter. Although it provided an upper estimate of production during the bloom, this mean rate was still not adequate to explain POC increases associated with the bloom peak, indicating a transient increase in primary production in excess of measured rates during that period. This will be discussed further in a later section.

An additional consideration in estimating production is the validity of extrapolating rates measured at one meter throughout the mixed layer. I assumed that the mean production rate represents a light saturated photosynthetic rate, and that this rate is uniform throughout the mixed layer. Simulated in situ photosynthesis-irradiance experiments during summer of 1982 revealed that the production was generally saturated at or

above 30% surface incident light (Figure 5-8, Table 5-6). Surface incident light regimes were comparable for the 1982 and 1983 incubations (not shown). For the period from 20 July to 10 August 83, extinction coefficients determined for the water column indicated that one meter in situ light intensities were between 20 - 40% I_0 (Figure 5-9, Table 5-7). Based on the 1982 field observations and published values of photosynthesis-irradiance relations for pure cultures (Falkowski and Owens, 1980), I argue that at the light intensities observed, the potential reduction in production rates at one meter due to light limitation was within the limits of analytical variation. Light limitation was certainly a factor lower in the mixed layer.

Light limitation may have also been important later in the summer. The correlation between the natural logarithm of the assimilation number for all incubations conducted in summer 1983 and quantum flux (microeinsteins per m^2 per hour, integrated over the incubation period and corrected for extinction by the water column) was significant at the 99% level (correlation coefficient = 0.828, $n = 9$). This is probably indicative of light limitation of production rates during the bloom period when extinction was high (Table 5-7) and incident solar radiation was relatively low (Table 5-8).

Particulate Protein Amino Acid Production

PPAA production rates based on observed incorporation into total PPAA, as well as incorporation in hot trichloroacetic acid insoluble material, and protein glycine and alanine for the period from 20 July to 3 October 83 are shown in Table 5-9. I also included rates of total PPAA

Figure 5-8. Time course of carbon fixation by surface samples from Salt Pond incubated during midday under natural sunlight attenuated to different intensities. Upper panel is an experiment conducted on 9 September 1982, and lower panel is an experiment conducted on 17 August 1982. Symbols: squares, 80% incident light; circles, 30%; triangles, 10%; X, 1%.

MICROGRAMS CARBON/LITER

Figure 5-9. Proportion of surface light reaching 1 meter during study period in Salt Pond.

Table 5-6. Simulated In Situ Production Rates for Salt Pond, 1982

%I ₀	Production Rate ^a	
	17 Aug 82	9 Sept 82
80	204 (19)	44 (4)
30	157 (300)	43 (13)
10	90 (30)	19 (4)
1	n.d.	7 (2)

^aμg carbon/liter/hour. Errors represent 95% confidence intervals.

Table 5-7. Light Extinction in the Mixed Layer of Salt Pond,
Summer 1983

Date	Extinction Coefficient ^a	%I ₀ at 1 meter
20 July 83	-1.18 (0.19) ^b	23 - 37
5 Aug 83	-1.24 (0.28)	23 - 37
10 Aug 83	-1.36 (.18)	21 - 31
26 Aug 83	-1.89 (.38)	10 - 22
1 Sept 83	-2.66 (.34)	5 - 10
3 Oct 83	-1.22 (0.06)	28 - 31

^aExtinction coefficient = $\ln(I_z/I_0) \times (1/z)$ where I_z/I_0 is the proportion of incident light reaching depth z in meters.

^bErrors represent 95% confidence intervals.

Table 5-8. Surface Incident and Atmosphere Corrected
Solar Radiation: 10 Aug - 1 Sept 83, Salt Pond^a

Date	Surface Incident	Atmosphere Corrected	A/B
	(A) (10 ³ watt-hours/m ²)	(B)	
10 Aug 83	7.1	10.3	0.7
11	2.1	10.2	0.2
12	1.2	10.2	0.1
13	2.8	10.1	0.3
14	5.0	10.1	0.5
15	4.3	10.0	0.4
16	6.9	10.0	0.7
17	6.8	9.9	0.7
18	0.8	9.9	0.1
19	5.2	9.8	.5
20	6.0	9.8	.6
21	7.1	9.7	0.7
22	5.4	9.6	0.6
23	6.6	9.6	0.7
24	6.7	9.5	0.7
25	6.5	9.5	.7
26	5.9	9.4	0.6
27	5.3	9.4	0.6
28	3.7	9.3	0.4
29	1.6	9.2	0.2
30	1.8	9.2	0.2
31	1.6	9.1	0.2
1 Sept 83	5.5	9.1	0.6

^aData provided courtesy of Dr. Richard Payne.

Table 5-9. Particulate Protein Amino Acid Production Rates

Date	Total PPAA	TCA Insoluble	Production Rate ^a		Extrapolated	
			glycine	alanine	hydrolyzed	sequenced
20 July 83	3.3 (2.3)	2.9 (0.6)	0.26 (0.18)	0.26 (0.19)	3.9 (2.0)	5.8 (3.1)
5 Aug 83	3.1 (2.2)	5.5 (3.1)	0.25 (0.15)	0.52 (0.48)	4.8 (2.5)	7.7 (4.1)
10 Aug 83	3.3 (1.6)	3.6 (1.6)	0.31 (0.14)	0.28 (0.18)	4.6 (1.8)	6.8 (2.8)
26 Aug 83	2.8 (2.4)	4.4 (2.1)	0.16 (0.27)	0.18 (0.34)	2.6 (3.3)	3.9 (5.1)
1 Sept 83						
Morning	6.0 (0.9)	6.4 (2.7)	0.68 (0.10)	0.78 (0.13)	11.3 (1.5)	16.8 (2.9)
Midday	3.1 (1.2)	3.9 (1.2)	0.24 (0.09)	0.20 (0.31)	4.2 (1.6)	6.5 (2.7)
3 Oct 83	7.4 (2.7)	7.6 (2.8)	0.38 (0.09)	0.52 (0.13)	6.9 (1.3)	10.6 (2.3)
Midday Means:						
20 July - 10 Aug	3.2 (1.1)		0.27 (0.07)	0.35 (0.13)		
20 July - 1 Sept	3.1 (0.8)		0.23 (0.05)	0.27 (0.09)		

^aμg carbon/liter/hour. Errors represent 95% confidence intervals.

production extrapolated from the incorporation characteristics of protein glycine and alanine. Extrapolated rates were determined by two methods: a) from the average proportions of glycine and alanine in protein recovered by my method, and b) from average proportions of glycine and alanine reported for sequenced proteins (Jukes et al., 1975; Dolittle, 1981). These proportions are compared to those observed for Salt Pond protein in Table 5-10. The proportions from hydrolyzed protein were quite similar for laboratory and field experiments, while those from sequenced proteins were lower, consistent with observations discussed in Chapter 4 regarding the preferential recovery of glycine and alanine residues from hydrolysis. Comparisons of extrapolated rates to observed incorporation into total PPAA (Table 5-9) indicated the extrapolated rates were generally higher, consistent with observations of rapid saturation of glycine and alanine precursor pools (Chapters 3 and 4). However, for a given incubation, rates generally agreed within analytical error. An exception to this occurred for the morning incubation on 1 September 83, where extrapolated rates were consistently higher than both the observed incorporation into total PPAA, and the incorporation into hot acid insoluble material. Rates of incorporation into the hot trichloroacetic acid insoluble fraction consistently agreed with the incorporation rates into total PPAA, suggesting that protein is the predominant substance responsible for incorporation into the hot acid insoluble fraction. However, this relationship cannot be generalized to all systems, particularly in populations containing significant proportions of dinoflagellates (Hitchcock, 1981). Dinoflagellates were not a major species in the populations in this study.

Table 5-10. Proportion of Glycine and Alanine Carbon in Protein Recovered from Hydrolysis

Source	% Protein Carbon	
	Glycine	Alanine
Salt Pond:		
20 July 83	6.4	7.2
5 Aug 83	6.8	9.4
10 Aug 83	6.3	6.8
1 Sept 83	6.0	7.6
3 Oct 83	5.7	8.4
Mean from laboratory experiments:	5.6 (0.4)	7.4 (1.0)
Mean based on sequenced protein: ^a	3.4 (0.7)	5.1 (0.8)

^aJukes et al., 1975; Dolittle, 1981

In general, all rates were remarkably uniform from day to day, which suggested a relative stable rate of population growth over a wide range of environmental conditions. Consistency in production rates was assumed and mean protein production rates were used to estimate flux of PPAA through the mixed layer. The observed total PPAA incorporation rates (Table 5-9) were used as an estimate of total PPAA production, and regarded as a conservative minimum estimate of the actual net protein synthesis rate. Calculations were also made individually for glycine and alanine for comparative purposes. For flux calculations, midday production rates were pooled and the mean rate used (Table 5-9). The rates were assumed to represent an integrated rate of production over the mixed layer as for POC production. Daily production estimates were made by assuming an 11 hour photoperiod. The 95% confidence intervals were propagated throughout all calculations.

Relationship Between Production and Concentration

Total PPAA concentration is illustrated in Figure 5-10 along with chlorophyll a and POC concentrations. During the latter portion of August, increases in concentrations occurred which were associated with a bloom of Olisthodiscus magnus. Projected concentration changes of POC and PPAA based on mean production rates were compared to observed concentrations (Figure 5-11). It is obvious that production generally exceeded observed changes in concentrations, which was evidence for substantial net removal of organic matter from the mixed layer. One exception to this occurred for POC during the interval between 26 - 30 Aug 83 immediately before the bloom. During this interval, projected POC concentrations were substantially lower than observed concentrations.

The estimated minimum POC production rate necessary to account for the increase in POC during this interval given an 11 hour photoperiod was 229 micrograms carbon per liter per hour. This rate exceeded the mean rate by a factor of 4.

The otherwise relatively high projected concentrations suggest substantial losses of particulate organic matter were occurring. The observed rates of change in concentration (dc/dt) and the estimated daily production rates (P_1) appear in Table 5-11. Differences are expressed as a daily net removal rate (R_N). In order to facilitate comparisons between PPAA and POC, the net daily removal rates were expressed as percentages of the mean concentrations of POC and PPAA for the corresponding period. If net loss occurred preferentially from PPAA carbon, this would result in a discrepancy between the ratio of PPAA production/POC production and the PPAA carbon concentration/POC concentration ratio. In the remainder of the discussion, the characteristics of POC compositional dynamics for the periods before and during the bloom are compared.

Prebloom Comparisons of POC and PPAA Dynamics

In Figure 5-12, the PPAA carbon production/POC production ratios are compared to the PPAA/POC concentration ratios. Before 1 September 83, the PPAA/POC production ratios were similar to the concentration ratios. This was also the case for glycine. Net daily removal rates of PPAA, glycine, and alanine were virtually the same as those of POC, in the range of approximately 10 - 15% per day. These observations suggest that during the period before the bloom, the composition of bulk POM reflected that being produced by the primary photoautotrophs, and that substantial

Figure 5-10. Particulate organic carbon (POC), chlorophyll a, and particulate protein amino acid (PPAA) concentrations in the Salt Pond mixed layer during the study period. Arrows indicate days during which production incubations were conducted.

Figure 5-11. Comparison of observed concentrations of particulate organic carbon (POC) and particulate protein amino acids (PPAA) to the projected concentrations based on mean production rates.

Figure 5-12. Comparison of production and concentration ratios of particulate protein amino acids (PPAA) and protein glycine relative to particulate organic carbon (POC). Percentages represent either the proportion of total POC in PPAA carbon, or the proportion of total observed ^{14}C uptake (POC production) associated with incorporation into PPAA. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

Table 5-11. Production and Net Removal of POC and PPAA^a

Dates	Material	dC/dt	P ₁ (μg carbon/liter/day)	R _N (P ₁ - dC/dt)	x ^b (day ⁻¹)
20 July - 5 Aug	POC	100 (40)	520 (120)	420 (160)	16 (5)
	Total				
	PPAA	1.2 (3.6)	35 (14)	33 (14)	11 (4)
	Glycine	0.14 (0.36)	2.9 (0.9)	2.8 (1.0)	14 (4)
	Alanine	0.52 (0.78)	3.9 (1.6)	3.4 (1.7)	14 (6)
5 - 10 Aug	POC	-70 (150)	520 (120)	590 (210)	17 (5)
	Total				
	PPAA	11 (14)	35 (14)	25 (20)	7 (4)
	Glycine	0.4 (1.3)	3.0 (1.0)	2.6 (1.6)	12 (5)
	Alanine	-1.0 (2.4)	3.8 (1.6)	4.8 (2.8)	18 (8)
10 - 26 Aug	POC	150 (62)	500 (150)	350 (170)	8 (3)
	Total				
	PPAA	3.0 (4.4)	34 (11)	32 (12)	8 (3)
	Glycine	0.12 (0.50)	2.6 (0.7)	2.4 (0.7)	12 (3)
	Alanine	-0.06 (1.1)	3.0 (1.1)	3.0 (1.5)	12 (6)
26 Aug - 1 Sept	POC	670 (140)	500 (150)	-150 (170)	-0.5 (2.6)
	Total				
	PPAA	8 (12)	34 (11)	26 (16)	6 (3)
	Glycine	0.4 (1.4)	3.2 (0.7)	2.8 (0.9)	11 (3)
	Alanine	2.0 (3.0)	3.0 (1.2)	1.1 (3.0)	-

^adC/dt is the change in concentration divided by the number of days in the period indicated. P₁ is the daily production rate based on midday production estimates multiplied by an 11 hour photoperiod. See text for further discussion. Errors represent propagated estimates of analytical error based on 95% confidence intervals.

^bRepresents R_N expressed as a percentage of the mean concentration for the corresponding time period.

amounts of POM were being removed from the euphotic system of Salt Pond by processes which did not differentiate between PPAA and POC.

Referring to equation (2), non-selective removal processes could include sedimentation (R_s), non-selective grazing (R_g), and advection and diffusion ($R_{a/d}$). The possibility that POM was being removed by sedimentation out of the euphotic mixed layer was considered by determining the relationship between the remineralization of organic matter below the mixed layer and POM lost from the layer. These calculations, based on integrated changes in dissolved inorganic carbon and POC concentrations are given in Table 5-12. Clearly, this a complicated system, and the assumption that changes in the concentrations of CO_2 are due solely to remineralization is subject to dispute. Many other factors influence this concentration including advective or diffusive exchange, reassimilation by chemosynthesis or sulfur photosynthesis, and remineralization of allochthonous inputs of organic matter such as eelgrass or groundwater intrusion. Nevertheless, the results indicated that CO_2 production, which could not be accounted for by in situ changes in POC, exceeded the loss rate of organic matter from the mixed layer. This indicates that degradation rates below 3 meters are adequate to account for the losses estimated from the euphotic layer if such losses are entirely due to sedimentation. Therefore, sedimentation may have been a significant source of removal.

Non-selective grazing (R_g) is another route of removal which would not differentiate between POC and PPAA. For example, macrozooplankton, which would be excluded from POM by our sampling methods (202 μ mesh), might graze and subsequently assimilate, respire, and excrete or release resulting waste products. Such waste products may be either dissolved

Table 5-12. Changes in Dissolved Inorganic and Particulate Organic Carbon in
Relationship to the Net Removal of Particulate Organic Carbon
From the Mixed Layer in Salt Pond, Summer 1983

Dates	CO ₂	<u>Rate of Change</u> <u>Below 3 meters</u>	<u>Estimated Net POC Removal</u> <u>From Mixed Layer</u>
		POC (moles carbon/m ² /day) ^a	
18 July - 24 Aug	0.116 (0.009)	-0.004 (0.003)	0.055 (0.013)
24 Aug - 1 Sept	-0.001 (0.040)	0.005 (0.007)	0.018 (0.022)

^aErrors in parentheses indicate propagated estimates of analytical error based on 95% confidence limits.

organic or inorganic material, or particulate fecal matter. However, if the release of fecal material is significant, this could introduce particulate material into the POM of significantly altered composition (Small et al., 1983), which is not necessarily consistent with my observations. Alternatively, if the characteristics of fecal matter is such that it rapidly sinks, production of fecal matter might be a means of accelerating sedimentation of POM out of the mixed layer. This material would likely not be included in the incubation vessel.

Microzooplankton would be included in both the incubation vessel and filtered subsamples. Furthermore, grazing by microzooplankton may result in particle production (Stoecker, 1984). The presence and activities of the microzooplankton population did not lead to a measurable discrepancy between POM composition and primary organic matter produced by the algal population during the prebloom period (Fig. 5-12). Additional information is required about the composition, abundance, and activities of the microheterotrophs, and the fate of the organic matter on which they feed, in order to assess their importance in the compositional dynamics of POM in the mixed layer.

Advective losses ($R_{a/d}$) may have occurred via the outlet channel connecting to Vineyard Sound. Estimates of exchange through the channel indicated it may have been as much as 5% day⁻¹ of the total volume of Salt Pond, and 11% day⁻¹ of the mixed layer volume. Further information about the hydrodynamics of the pond and actual organic matter transport through the channel is required in order to quantitate such losses.

Contributions of POM not due to in situ autotrophic activity could occur from advection and diffusion ($P_{a/d}$), chemotrophic production (P_c), in situ secondary production (P_2), and adsorption of DOM to POM. If processes which result in input of POM other than primary production are not negligible relative to primary production during the prebloom period, then either they were introducing material of similar composition with respect to PPAA and POC, or were coupled to efficient removal so that accumulation of non-primary POM is negligible. Dark uptake of inorganic ^{14}C was generally low relative to total primary production (Table 5-13), which would indicate that chemosynthetic production (P_c) was small relative to autotrophic production. Secondary production (P_2) could contribute non-primary organic matter. The potential importance of such production is currently the subject of considerable dispute (e.g. Sorokin, 1981; Williams, 1981). Cuhel (1981) reported ranges in protein/POC composition of two bacterial species depending on nutritional state, which span the ranges for POM reported here. Hence it is possible bacteria could be introducing POM of similar composition to that produced by photoautotrophs. On the other hand, some loss of organic matter would occur due to bacterial respiration (R_r). Other components of R_r include microzooplankton respiration and autotrophic respiration. Such activities would be expected to selectively enrich the PPAA content of POM according to reports of several investigators that protein is relatively less susceptible to respiratory degradation than other cell material (e.g. Van Liere et al., 1979; Handa, 1969; Lorenzen and Kaushik, 1976; Darley et al., 1976; Hitchcock, 1980; Scott, 1980; Fukami et al., 1985). My observations for the prebloom period are not consistent with the hypothesis that these

Table 5-13. Dark Uptake Rates of Inorganic ^{14}C :
Salt Pond, Summer 1983

Date	Uptake Rate ^a
20 July 83	10 (11)
5 Aug	1.4 (0.4)
10 Aug	-0.6 (1.4)
26 Aug	-0.3 (2.8)
1 Sept	0.7 (1.2)
3 Oct	-13 (7)

^a $\mu\text{g carbon/liter/hour}$. Errors represent 95% confidence intervals.

selective processes are the predominant routes of removal of POM from the euphotic zone.

Advective inputs ($P_{a/d}$) would include those from Vineyard Sound through the channel, and other sources within the pond. Chlorophyll concentrations were generally higher in Salt Pond than in Vineyard Sound (data not shown). It seems likely that in situ production would have been more important in influencing the composition of POM in the pond mixed layer. In view of the dynamic mixing regime of Salt Pond, it seems likely that advective and diffusive inputs and outputs of POM within the pond were occurring. If this is the case, then the composition of incoming material must have been similar to that of the POM produced in situ at the sampling site during the pre-bloom period. This would suggest that primary organic matter of relatively similar composition is being produced throughout the mixed layer (i.e. no significant vertical or horizontal gradients in POC production relative to PPAA production).

The results indicate that within the limits of analytical precision, the protein composition of primary produced organic matter was similar to the existing POM composition. Furthermore, estimated rates of primary production were more than adequate to account for observed POM concentration increases. This is consistent with the view that algal biosynthetic activity was the predominant factor influencing the protein content of POM, and ultimately, controlled its flux to higher trophic levels during the period preceding the bloom.

POC and PPAA Dynamics During Bloom

During the decline of the bloom, the PPAA/POC production ratio was significantly higher than the PPAA/POC concentration ratio (Figure 5-12). Glycine showed a similar trend. This pattern was observed for both the morning and midday incubations (Table 5-14), although for the morning incubation the difference between production ratios and concentration ratios were significantly outside the limit of analytical error only in the case of glycine and alanine for the morning incubation. This was primarily because PPAA/POC concentration ratios were significantly higher than for the midday incubation. In view of the short interval between the initiation of the incubations (2 hours), it is difficult to envision that a real change in POM composition of the magnitude indicated actually occurred. PPAA concentration ratios were based on estimates from samples taken directly from the incubation chamber. In contrast, POC concentrations were estimated from samples taken independently. This leads to speculation that observed compositional differences between incubations may have been due to spatial and/or temporal heterogeneity in the distribution of organic matter. However, as previously mentioned, the depth profiles of chlorophyll a concentrations did indicate a relatively uniform distribution around the one meter depth where sampling and incubations were conducted (Fig. 5-3). Therefore, it is also possible that the difference was related to population metabolism. Despite the differences, both incubations did show a tendency for the production ratios to exceed the concentration ratios. In the next section, I will discuss how changes in environmental conditions may have generated metabolic responses leading to the observed discrepancies.

Table 5-14. Comparisons of Morning and Midday Production and Composition Ratios, Salt Pond, 1 Sept 83

Time of Day	Material	%	%
		Total POC Production	Total POC
0900 - 1200	total PPAA	12.6 (2.5) ^a	9.1 (1.8)
	Glycine	1.4 (0.3)	0.54 (0.13)
	Alanine	1.6 (0.3)	0.71 (0.16)
1050 - 1400	total PPAA	14.6 (5.8)	4.7 (0.9)
	Glycine	1.2 (0.4)	0.28 (0.07)
	Alanine	0.9 (1.5)	0.36 (0.07)

^aErrors in parentheses represent 95% confidence intervals.

Production Characteristics and Population Metabolism

During the bloom, significant changes in physical parameters occurred which may have resulted in changes in population metabolism. Inorganic nitrogen concentrations (particularly NH_4^+) were high immediately before the bloom, and subsequently decreased rapidly (Figure 5-13). Light intensities at the one meter depth also decreased during the bloom (Figure 5-9, Table 5-7). As previously discussed, significant correlations were observed for the relationships between total POC production rate versus inorganic nitrogen concentrations (Table 5-5), and assimilation number versus quantum flux at one meter (correlation coefficient = 0.828, P less than 0.05). These observations indicate that light limitation, nitrogen limitation, or both led to the bloom decline. Additional evidence in support of light limitation of the bloom population was the pronounced decrease in the POC/chlorophyll a ratios (Figure 5-14).

In Chapter 4, it was noted that under conditions of light and nitrogen limitation, protein turnover could account for a significant portion of total protein label incorporation in Nannochloris sp., leading to an overestimate of net protein synthesis. Such a phenomenon could explain the observed discrepancies between the high proportions of primary production associated with PPAA, as compared to the PPAA/POC concentration ratios. This would mean the discrepancy was due to a kinetic artifact of isotope incorporation, rather than a real difference between the biosynthetic patterns of the population and the composition of POM. This view is supported by the fact that discrepancies were larger in the case of glycine and alanine (Table 5-14) than for total protein, an observation similar to that seen for light and nitrogen

Figure 5-13. Orthophosphate, ammonium, nitrate, and nitrite concentrations at the one meter sampling depth in relationship to particulate organic carbon (POC) and chlorophyll a concentrations during the period of study in Salt Pond.

Figure 5-14. Particulate organic carbon to chlorophyll a ratios during the study period in Salt Pond.

limited cultures of Nannochloris sp. Obviously, the metabolic characteristics of Olisthodiscus magnus may be different from Nannochloris sp. In addition, metabolic and compositional characteristics of populations growing under diel light cycles are likely to be different from laboratory continuous cultures.

Variations between day and night metabolism were considered as a possible explanation for the fact that the midday PPAA/POC production ratio was higher than the midday PPAA/POC concentration ratio (Figure 5-12, Table 5-14). This would require either night production of total particulate carbon at a higher relative rate than particulate amino acids, or preferential catabolism or respiratory degradation of protein relative to other cell material. Neither of these possibilities is consistent with previous observations of preferential conservation of protein at the expense of storage material during the dark period in synchronous laboratory cultures grown in light/dark cycles (Handa, 1969; Darley et al., 1976; Lorenzen and Kaushik, 1976; VanLiere et al., 1979; Hitchcock, 1980). However, day/night variations in protein metabolism of Olisthodiscus species has not been investigated, so generalizations should be considered tentative.

In view of the low capacity of Olisthodiscus luteus for nitrogen uptake (K_s approximately 2 micromolar, Tomas, 1979), it is reasonable to assume that the bloom population was nitrogen limited. Although nutrient concentrations during the bloom were below the saturation uptake determined for O. luteus (Figure 5-13), it is likely that they could have been utilized by other algae. The pattern of biosynthesis during the decline of the bloom may have reflected the metabolic activity of other species in the mixed algal population. Changes in patterns of

incorporation of labelled carbon into subcellular material provided additional evidence that changes in population physiological state occurred during the bloom (Figure 5-15). One interpretation of these observations is that as a result of the accumulation of senescent biomass, the bulk composition of POM did not reflect the metabolism of an active algal population which was distinct from the declining bloom population. This would result in a real input of PPAA in excess of its proportion in POC. In the following section, I propose several mechanisms which could lead to enhanced removal of protein and the resulting discrepancy between production and composition.

Net Removal of PPAA Relative to POC During the Bloom

The calculations of net removal shown in Table 5-11 indicate more rapid removal of PPAA relative to POC during the bloom. Referring to equation (2), selective grazing (R_g) of protein enriched organic matter would be a potential mechanism for enhanced PPAA removal. Investigations with another Olisthodiscus species, Olisthodiscus luteus, have found it to be relatively unpalatable to zooplankton (Verity and Stoecker, 1982; Tomas and Deason, 1981). Thus it is likely that algae able to utilize existing nitrogen concentrations would be selectively grazed (R_g). Furthermore, grazers may preferentially assimilate protein (Scott, 1980), and release fecal material depleted in nitrogen (Small et al., 1983). Such activities would suppress the influence of the active autotrophs on POM composition, despite their potential importance as a source of protein for higher trophic levels.

Differential susceptibilities of POC and PPAA to decomposition and transformation into DOC (R_{DOC}) could also lead to selective removal of

Figure 5-15. Proportion of total ^{14}C incorporation associated with subcellular fractions of natural phytoplankton populations during the study period in Salt Pond. Open bars, hot trichloroacetic acid insoluble (including protein); thinly hatched, hot trichloroacetic acid soluble (polysacharride and nucleic acid); densely hatched, lipophylic; solid bars, alcohol soluble.

protein. As the bloom neared its peak, the bloom species population likely became increasingly senescent. Hence, both autolytic activities and decomposition of organic matter would have become relatively more important in affecting POM composition (c.f. Jassby and Goldman, 1974). Lee and Cronin (1984) reported that depth dependent losses of particulate amino acids from suspended particulate matter were enhanced in more productive areas. Significant extracellular proteolytic action on soluble proteins has been demonstrated (Hollibaugh and Azam, 1983; Somville and Billen, 1983) and could also serve as a mechanism for the accelerated breakdown of protein in inactive algal biomass. Release of DOC accompanying blooms has been observed previously, including the release of amino acids (Ittekkot, 1982). From results of decomposition studies (e.g. Harrison, 1980), phosphorus is known to be one of the most rapidly remineralized elements. PO_4^{3-} concentrations varied directly with POC and chlorophyll a (Figure 5-13), and the observed increase may have reflected accelerated decomposition of organic matter. Further evidence in support of enhanced decomposition was provided by the work of Lee and Hicke (mans. sub.). They determined that production of putrescine, a polyamine indicative of protein degradation, was accelerated during the bloom period.

Although it is likely that significant chemotrophic production (P_c) can be ruled out on the basis that dark uptake was not important relative to total productivity (Table 5-12), secondary production (P_2) could introduce POM relatively low in PPAA/POC. This will be a function of the physiological state of the bacterial population as well as the composition of their growth substrate. Fukami et al. (1985) found that decomposing algal material was initially enriched in protein due to

colonization by bacteria. However, the extent to which this type of laboratory study can be generalized to natural systems is questionable.

As previously mentioned, because of the potential for spatial and temporal heterogeneity in sampling, advection or diffusion could have selectively influenced the PPAA/POC ratio. Therefore, for future investigations, I recommend that direct comparisons of materials within the incubation chamber be made.

Physical removal (R_p) such as sedimentation could also be selective with respect to PPAA/POC content if more rapidly sedimenting particles were enriched in protein. Lee and Hicke (mans. sub.) observed an increase in the concentration of putrescine and ornithine with depth, which would be consistent with decomposition of sinking phytoplankton protein. However, diffusive flux from the sediment or decomposition of allocthonous organic matter such as eel grass could also contribute to the levels of these compounds.

The fact that the bloom consisted of a large-celled flagellated organism (Olisthodiscus magnus) may have lead to a proportionate decrease in sedimentation rates. CO_2 production below three meters, (as determined by changes in concentration) apparently decreased during the bloom (Table 5-13), but such circumstantial evidence cannot be used to infer changes in actual flux from the mixed layer. It is possible that the characteristics of Olisthodiscus magnus led to an increased residence time of senescent biomass in the mixed layer, thereby masking the impact of the biosynthetic activities of other algae on POM composition.

CONCLUSIONS

This study is a first attempt to determine the importance of primary inputs of POC and PPAA in controlling their relative abundances in the POM, and to assess the degree to which the relative abundances of these biochemicals were indicative of the metabolic state of the phytoplankton population. In addition, production of PPAA and POC was compared to observed changes in their concentrations, in order to estimate and compare net removal rates and evaluate the potential impact of autotrophic inputs on POM composition.

During the period before the bloom, the composition of bulk POM suggested production by the photoautotrophs. Primary inputs were significant relative to observed concentrations, consistent with the view that algal biosynthetic activity was an important factor controlling the composition of particulate organic matter. Production exceeded observed changes in concentration of POC and PPAA, indicating that substantial amounts of POM were being removed from the euphotic system of Salt Pond by processes which did not differentiate between PPAA and POC. Non-selective removal processes which could have been responsible for these observations included sedimentation, non-selective grazing, and advection and diffusion.

During the bloom period, the proportion of total POC production associated with particulate protein amino acid production was significantly higher than the PPAA/POC ratio in the particulate organic matter. In view of low inorganic nitrogen concentrations and low light intensities, it was suggested that the decline of the Olisthodiscus magnus bloom may have resulted from both light and nitrogen limitation. The fact that the PPAA/POC production ratio was higher than the PPAA/POC

concentration ratio could be explained by turnover of protein in excess of net synthesis. Other mechanisms were proposed as possible explanations of the discrepancy including selective grazing and enhanced decomposition of accumulated senescent biomass, which would have suppressed the influence of the active photoautotrophs on POM composition.

Discrepancies between primary production and POM composition were observed only in a situation of unusually high particulate organic matter concentrations and only during a single sampling period. In general, within the limits of analytical precision, the composition of particulate organic matter reflected the biosynthetic activities of the algal population. These results are consistent with the view that, during the period of study, algal biosynthetic activity was the predominant factor controlling the relative abundance of protein in the particulate organic matter, and the corresponding flux of protein to higher trophic levels in the mixed layer of Salt Pond.

A major limitation in interpreting these results was the need for greater precision and higher frequency in sampling. Nevertheless, this study illustrates an approach which can lead to an increased understanding of the compositional dynamics of individual biogenic compounds, and the characteristics of organisms which control them. It is my opinion that through careful assessments of the behaviour of compounds which are markers of biochemical activities, it will be possible to advance our understanding of the role of microbial metabolism in trophodynamic and biogeochemical activity in aquatic ecosystems.

REFERENCES

- Allredge, A. L., 1979. The chemical composition of macroscopic aggregates in two neretic seas. *Limnol. Oceanogr.* 24, 855-866.
- Ammerman, J. W., J. A. Fuhrman, A. Hagstrom, and F. Azam, 1984. Bacterioplankton growth in seawater: I. Growth kinetics and cellular characteristics in seawater cultures. *Mar. Ecol. Prog. Ser.* 18, 31-39.
- Andersson, A., C. Lee, F. Azam, and A. Hagstrom, 1985. The release of free amino acids by heterotrophic marine microflagellates. *Mar. Ecol. Prog. Ser.* in press.
- Andrews, P., and P. J. leB. Williams, 1971. Heterotrophic utilization of dissolved organic compounds in the sea. III. Measurement of the oxidation rates and concentrations of glucose and amino acids in sea water. *J. Mar. Biol. Assoc., U.K.* 51, 111-125.
- Anikouchine, W. A., and R. W. Sternberg, 1973. The World Ocean: An Introduction to Oceanography. Prentice Hall, Inc. New Jersey. 338 pp.
- Anitia, N. J., B. R. Berland, and D. J. Bonin, 1980. Proposal for an abridged nitrogen turnover cycle in certain marine planktonic systems involving hypoxanthine-guanine excretion by ciliates and their re-utilization by phytoplankton. *Mar. Ecol. Prog. Ser.* 2, 97-103.
- Azam, F., and J. W. Ammerman, 1984. Cycling of organic matter by bacterioplankton in pelagic marine ecosystems: microenvironmental considerations. In: Flows of Energy and Materials in Marine Ecosystems (M. J. R. Fasham, ed.) Plenum Publishing Corp., New York.
- Azam, F., T. Fenchel, J. G. Field, J. S. Gray, L. A. Meyer-Reid, and F. Thingstad, 1983. The ecological role of water-column microbes in the sea. *Mar. Ecol. Prog. Ser.* 10, 257-263.
- Azam, F., and O. Holm-Hansen, 1973. Use of tritiated substrates in the study of heterotrophy in seawater. *Mar. Biol.* 23, 191-196.
- Banase, K., 1974. On the role of bacterioplankton in the Tropical Ocean. *Mar. Biol.* 24, 1-5.
- Banase, K., 1977. Determining the carbon-to-chlorophyll ratio of natural phytoplankton. *Mar. Biol.* 41, 199-212.
- Banase, K., 1982. Cell volumes, maximal growth rates of unicellular algae and ciliates, and the role of ciliates in the marine pelagial. *Limnol. Oceanogr.* 27, 1059-1071.
- Barlow, R. G., 1980. The biochemical composition of phytoplankton in an upwelling region off South Africa. *J. exp. mar. Biol. Ecol.* 45, 83-93.
- Barlow, R. G., 1982a. Phytoplankton ecology in the southern Benguela current. I. Biochemical composition. *J. exp. Mar. Biol. Ecol.* 63, 209-227.
- Barlow, R. G., 1982b. Phytoplankton ecology in the Southern Benguela current. II. Carbon assimilation patterns. *J. Exp. Mar. Biol. Ecol.* 63, 229-237.
- Barlow, R. G., and J. L. Henry, 1982. Patterns of carbon assimilation in phytoplankton from the Southern Ocean. *Fish. Bull. S. Afr.* 16, 25-29.
- Bassham, J. A. and M. Kirk, 1964. Photosynthesis of amino acids. *Biochim. Biophys. Acta* 90, 553-562.

- Bassham, J. A., A. A. Benson, L. D. Kay, A. Z. Harris, A. T. Wilson, and M. Calvin, 1954. The path of carbon in photosynthesis. XXI. The cyclic regeneration of carbon dioxide acceptor. *J. Amer. Chem. Soc.* 76, 1760-1770.
- Bassham, J. A., B. Morawiecka, and M. Kirk, 1964. Protein synthesis during photosynthesis. *Biochim Biophys. Acta* 90, 542-552.
- Bates, S. S., 1981. Determination of the physiological state of marine phytoplankton by use of radiosulfate incorporation. *J. exp. mar. Biol. Ecol.* 51, 219-239.
- Bayer, E., E. Gram, B. Kaltenegger, and R. Uhmman, 1976. Separation of amino acids by high performance liquid chromatography. *Anal. Chem.* 48, 1106-1109.
- Baylor, E. R., and W. H. Sutcliffe, Jr., 1963. Dissolved organic matter in seawater as a source of particulate food. *Limnol. Oceanogr.* 4, 369-371.
- Beale, S. I., and D. Appleman, 1971. Chlorophyll synthesis in *Chlorella*. Regulation by degree of light limitation of growth. *Plant. Phys.* 47, 230-235.
- Beardall, J., and I. Morris, 1976. The concept of light intensity adaptation in marine phytoplankton: some experiments with *Phaeodactylum tricornutum*. *Mar. Biol.* 37, 377-387.
- Beers, J. R., F. M. H. Reid, and G. L. Stewart, 1975. Microplankton of the North Pacific Central Gyre. Population structure and abundances, June 1973. *Int. Revue. ges. Hydrobiol.* 60, 607-638.
- Bell, W. H., 1980. Bacterial utilization of algal extracellular products. 1. The kinetic approach. *Limnol. Oceanogr.* 25, 1007-1020.
- Bell, W. H., 1983. Bacterial utilization of algal extracellular products. 3. The specificity of the algal-bacterial interaction. *Limnol. Oceanogr.* 28, 1131-1143.
- Bell, W. H., and E. Sakshaug, 1980. Bacterial utilization of algal extracellular products. 2. A kinetic study of natural populations. *Limnol. Oceanogr.* 25, 1021-1033.
- Bell, W. H., and R. Mitchell, 1972. Chemotactic and growth responses of marine bacteria to algal extracellular products. *Biol. Bull.* 143, 265-277.
- Beudeker, R. F., 1984. Glycolate metabolism is under nitrogen control in *Chlorella*. *Plant Phys.* 75, 516-520.
- Bidwell, R. G. S., R. A. Barr, and F. C. Steward, 1964. Protein synthesis and turn-over in cultured plant tissue: sources of carbon synthesis and the fate of the protein breakdown products.
- Bishop, J. K. B., D. R. Ketten, M. P. Bacon, and W. B. Silker, 1977. The chemistry, biology, and vertical flux of particulate matter from the upper 400m of the equatorial Atlantic Ocean. *Deep-Sea Res.* 24, 511-548.
- Bishop, J. K. B., D. R. Ketten, and J. M. Edmond, 1978. The chemistry, biology, and vertical flux of particulate matter from the upper 400m of the Cape Basin in the southeast Atlantic Ocean. *Deep-Sea Res.* 25, 1121-1161.
- Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-55.
- Calvin, M. and P. Massini, 1952. The path of carbon in photosynthesis. XX. The steady state. *Experientia* 8, 445-484.

- Caperon, J., and J. Meyer, 1972. Nitrogen limited growth of marine phytoplankton. I. changes in population characteristics with steady state growth rate. *Deep Sea Res.* 19, 601-618.
- Caperon, J., D. Schell, J. Hirota, and E. Laws, 1979. Ammonium excretion rates in Kaneohe Bay, Hawaii measured by an ^{15}N isotope dilution technique. *Mar. Biol.* 54, 33-7.
- Carpenter, E. J., and J. J. McCarthy, 1975. Nitrogen fixation and uptake of combined nitrogenous nutrients by *Oscillatoria* (*Trichodesmium*) *thiebautii* in the western Sargasso Sea. *Limnol. Oceanogr.* 20, 389-401.
- Carpenter, E. J., and J. S. Lively, 1980. Review of estimates of algal growth using ^{14}C tracer techniques. In: *Primary Productivity in the Sea* (P. G. Falkowski, ed.) Plenum Press, New York. pp. 161-178.
- Cattolico, R. A., J. C. Boothroyd, and S. P. Gibbs, 1976. Synchronous growth and plastid replication in the naturally wall-less algal *Olisthodiscus luteus*. *Plant Physiol.* 57, 497-503.
- Cauwet, G., 1978. Organic chemistry of sea water particulates: concepts and developments. *Oceanol. Acta* 1, 99-105.
- Cauwet, G., 1981. Non-living particulate matter. In: *Marine Organic Chemistry. Evolution, Composition, Interactions and Chemistry of Organic Matter in Seawater* (E. K. Duursma and R. Dawson, eds.) Elsevier Scientific Publishing Co., New York. pp. 71-89.
- Chan, A. T., 1978. Comparative physiological study of marine diatoms and dinoflagellates in relation to irradiance and cell size. I. Growth under continuous light. *J. Phycol.* 14, 396-402.
- Chan, A. T., 1980. Comparative physiological study of marine diatoms and dinoflagellates in relation to irradiance and cell size. II. Relationship between photosynthesis, growth, and carbon/chlorophyll *a* ratio. *J. Phycol.* 16, 428-432.
- Chau, Y. K., L. Chuecas, and J. P. Riley, 1967. The component combined amino acids of some marine phytoplankton species. *J. mar. biol. Ass. U.K.* 47, 543-554.
- Chisholm, S. W., 1981. Temporal patterns of cell division in unicellular algae. In: *Physiological Bases of Phytoplankton Ecology* (T. Platt, ed.) Can. Bull. Fish. Aquat. Sci. 210, 150-181.
- Christie, W. W., 1973. *Lipid Analysis. Isolation, Separation, Identification, and Structural Analysis of Lipids.* Pergamon Press, New York.
- Cole, J. J., G. E. Likens, and D. L. Strayer, 1982. Photosynthetically produced dissolved organic carbon: an important carbon source for planktonic bacteria. *Limnol. Oceanogr.* 27, 1080-1090.
- Collos, Y. and G. Slawyk, 1979. ^{13}C and ^{15}N uptake by marine phytoplankton. I. Influence of nitrogen source and concentration in laboratory cultures of diatoms. *J. Phycol.* 15, 186-190.
- Conover, R. J., 1980. General rules of grazing in pelagic ecosystems. In: *Primary Production in the Sea* (P. G. Falkowski, ed.) Plenum Press, New York. pp. 461-485.
- Conover, S. A. M., 1975. Partitioning of nitrogen and carbon in cultures of the marine diatom *Thalassiosira fluviatilis* supplied with nitrate, ammonium, or urea.
- Cook, J. R., 1963. Adaptations in growth and division in *Euglena* effected by energy supply. *J. Protozool.* 10, 436-444.

- Cowey, C. B., and E. D. S. Corner, 1963. On the nutrition and metabolism of zooplankton. II. Relationship between the marine copepod Calanus helgolandidis and particulate material in Plymouth sea water, in terms of amino acid composition. J. mar. biol. Ass. U.K. 43, 495-511.
- Cox, R. A., M. J. McCartney, and F. Culkin, 1970. The specific gravity/salinity/temperature relationship in natural sea water. Deep-Sea Res. 17, 679-689.
- Crawford, C. C., J. E. Hobbie, and K. L. Webb, 1974. The utilization of dissolved free amino acids by estuarine microorganisms. Ecology 55, 551-563.
- Cuhel, R. L., 1981. Assimilatory sulfur metabolism in marine microorganisms. Ph.D. thesis, Woods Hole Oceanogr. Inst. Also Woods Hole Oceanogr. Inst. Publ. 81-29. 418pp.
- Cuhel, R. L., C. D. Taylor, and H. W. Jannasch, 1981a. Assimilatory sulfur metabolism in marine microorganisms: characteristics and regulation of sulfate transport in Pseudomonas halodurans. J. Bacteriol. 147, 340-349.
- Cuhel, R. L., C. D. Taylor, and H. W. Jannasch, 1981b. Assimilatory sulfur metabolism in marine microorganisms: sulfur metabolism, growth, and protein synthesis of Pseudomonas halodurans and Alteromonas luteo-violaceus during sulfate limitation. Arch. Microbiol. 130, 1-7.
- Cuhel, R. L., C. D. Taylor, and H. W. Jannasch, 1981c. Assimilatory sulfur metabolism in marine microorganisms: sulfur metabolism, protein synthesis, and growth of Pseudomonas halodurans and Alteromonas luteo-violaceus during unperturbed batch growth. Arch. Microbiol. 130, 8-13.
- Cuhel, R. L., C. D. Taylor, and H. W. Jannasch, 1982a. Assimilatory sulfur metabolism in marine microorganisms: sulfur metabolism, protein synthesis, and growth of Pseudomonas halodurans and Alteromonas luteo-violaceus during unperturbed batch growth. Appl. Environ. Microbiol. 43, 151-159.
- Cuhel, R. L., C. D. Taylor, and H. W. Jannasch, 1982b. Assimilatory sulfur metabolism in marine microorganisms: considerations for the application of sulfate incorporation into protein as a measure of natural marine microbial protein synthesis. Appl. Environ. Microbiol. 43, 160-168.
- Cuhel, R. L., H. W. Jannasch, C. D. Taylor, and D. R. S. Lean, 1983. Microbial growth and macromolecular synthesis in the northwestern Atlantic Ocean. Limnol. Oceanogr. 28, 1-18.
- Cuhel, R. L., P. B. Ortner, and D. R. S. Lean, 1984. Night synthesis of protein by algae. Limnol. Oceanogr. 29, 731-744.
- Cuhel, R. L., and J. B. Waterbury, 1984. Biochemical composition and short term nutrient incorporation patterns in a unicellular marine cyanobacterium, Synechococcus (WH7803). Limnol. Oceanogr. 29, 370-374.
- Darley, W. M., C. W. Sullivan, and B. E. Volcani, 1976. Studies on the biochemistry and fine structure of silica shell formation in diatoms. Division cycle and chemical composition of Navicula pelliculosa during light-dark synchronized growth. Planta (Berl.) 130, 159-167.

- Davenport, J. B., and B. Maguire, Jr., 1984. Quantitative grain density autoradiography and the intraspecific distribution of primary productivity in phytoplankton. *Limnol. Oceanogr.* 29, 410-416.
- Dawson, R., and K. Gocke, 1978. Heterotrophic activity in comparison to the free amino acid concentration in Baltic sea water samples. *Oceanol. Acta* 1, 45-54.
- Dawson, R., and E. K. Duursma, 1981. State of the art. In: *Marine Organic Chemistry* (E. K. Duursma and R. Dawson, eds.) Elsevier Scientific Publishing Co., New York. pp. 497-512.
- DeJong, C., G. J. Hughes, E. VanWieringen, and K. J. Wilson, 1982. Amino acid analyses by high-performance liquid chromatography. An evaluation of the usefulness of pre-column Dns derivatization. *J. Chromatog.* 241, 345-359.
- DeManche, J. M., 1980. Variations in phytoplankton physiological parameters during transient nitrogen environments. Ph.D. thesis, Oregon State University, Corvallis.
- DeManche, J. M., H. C. Curl, Jr., D. W. Lundy, and P. L. Donaghay, 1979. The rapid response of the marine diatom Skeletonema costatum to changes in external and internal nutrient concentration. *Mar. Biol.* 53, 323-333.
- Degens, E. T., 1970. Molecular nature of nitrogenous compounds in sea water and recent marine sediments. *Occ. Publ. Inst. Mar. Sci., Univ. Alaska* 1, 77-106.
- Derenbach, J. B., and P. J. leB. Williams, 1974. Autotrophic and bacterial production: fractionation of plankton populations by differential filtration of samples from the English Channel. *Mar. Biol.* 25, 263-269.
- DiTullio, G. R., E. A. Laws, 1983. Estimates of phytoplankton N uptake based on $^{14}\text{CO}_2$ incorporation into protein. *Limnol. Oceanogr.* 28, 177-185.
- Dolittle, R. F., 1981. Similar amino acid sequences: chance or common ancestry? *Science* 214, 149-159.
- Donaghay, P. L., J. M. DeManche, and L. F. Small, 1978. On predicting phytoplankton growth rates from carbon:nitrogen ratios. *Limnol. Oceanogr.* 23, 359-362.
- Dortch, Q., 1982. Effect of growth conditions on accumulation of internal nitrate, ammonium, amino acids, and protein in three marine diatoms. *J. Exp. Mar. Ecol. Biol.* 61, 243-264.
- Dring, M. J., and D. H. Jewson, 1982. What does ^{14}C uptake by phytoplankton really measure? A theoretical modelling approach. *Proc. R. Soc. Lond. B* 214, 351-368.
- Dugdale, R. C., and J. J. Goering, 1967. Uptake of new and regenerated forms of nitrogen in primary productivity. *Limnol. Oceanogr.* 12, 196-206.
- Edge, P. A., and T. R. Ricketts, 1977. The effect of nitrogen refeeding on the carbohydrate content of nitrogen starved cells of Platymonas striata Butcher. *Planta* 136, 159-162.
- Eppley, R. W., W. G. Harrison, S. W. Chisholm, and E. Stewart, 1977. Particulate organic matter in surface waters off southern California and its relationship to phytoplankton. *J. Mar. Res.* 35, 671-696.
- Eppley, R. W., and B. J. Peterson, 1979. Particulate organic matter flux and planktonic new production in the deep ocean. *Nature* 282, 677-680.

- Falkowski, P. G., and T. G. Owens, 1978. Effects of light intensity on photosynthesis and dark respiration in six species of marine phytoplankton. *Mar. Biol.* 45, 289-295.
- Falkowski, P. G., and T. G. Owens, 1980. Light-shade adaptation: two strategies in marine phytoplankton. *Plant Phys.* 66, 592-595.
- Fallon, R. D., and T. D. Brock, 1979. Decomposition of blue-green algal (cyanobacterial) blooms in Lake Mendota, Wisconsin. *Appl. Environ. Microbiol.* 37, 820-830.
- Ferguson, R. L., E. N. Buckley, and A. V. Palumbo, 1984. Response of marine bacterioplankton to differential filtration and confinement. *Appl. Environ. Microbiol.* 47, 49-55.
- Ferguson, R. L., and W. G. Sunda, 1984. Utilization of amino acids by planktonic marine bacteria: importance of clean technique and low substrate additions. *Limnol. Oceanogr.* 29, 258-274.
- Fisher, T. R., P. R. Carlson, and R. T. Barber, 1981. Some problems in the interpretation of ammonium uptake kinetics. *Mar. Biol. Letters* 2, 33-44.
- Fitzwater, S. E., G. A. Knauer, and J. H. Martin, 1982. Metal contamination and its effect on primary production measurements. *Limnol. Oceanogr.* 27, 544-551.
- Fogg, G. E., 1956. Photosynthesis and formation of fats in a diatom. *Annls. Bot., N. S.* 20, 265-285.
- Fogg, G. E., 1959. Nitrogen nutrition and metabolic patterns in algae. *Symp. Soc. Exp. Biol.* 13, 106-125.
- Fogg, G. E., 1962. Extracellular products. In: Physiology and Biochemistry of Algae (R. A. Lewin, ed.). Academic Press, New York. pp. 475-489.
- Fogg, G. E., 1977. Excretion of organic matter by phytoplankton. *Limnol. Oceanogr.* 22, 576-577.
- Fowden, L., 1954. A comparison of the composition of some algal proteins. *Annls. Bot.* 18, 258-266.
- Fukami, K., U. Simidu, and N. Taga, 1985. Microbial decomposition of phyto- and zooplankton in seawater. I. Changes in organic matter. *Mar. Ecol. Prog. Ser.* 21, 1-5.
- Gagosian, R. B., S. O. Smith, and G. E. Nigrelli, 1982. Vertical transport of steroid alcohols and ketones measured in a sediment trap experiment in the equatorial Atlantic Ocean. *Geochim. Cosmochim. Acta* 46, 1163-1172.
- Gagosian, R. B., and C. Lee, 1981. Processes controlling the distribution of biogenic organic compounds in seawater. In: Marine Organic Chemistry. Evolution, Composition, Interactions and Chemistry of Organic Matter in Seawater (E. K. Duursma and R. Dawson, eds.) Elsevier Scientific Publishing Co., New York. pp. 91-123.
- Garfield, P. C., T. T. Packard, and L. A. Codispoti, 1979. Particulate protein in the Peru upwelling system. *Deep Sea Res.* 26 (6A), 623-639.
- Garside, C., 1984. Apparent ¹⁵N uptake kinetics resulting from remineralization. *Limnol. Oceanogr.* 29, 204-210.
- Garside, C., and P. M. Glibert, 1984. Computer modeling of ¹⁵N uptake and remineralization experiments. *Limnol. Oceanogr.* 29, 199-204.
- Gast, V., and U. Horstmann, 1983. N-remineralization of phyto- and bacterioplankton by the marine ciliate, Euplotes vannus. *Mar. Ecol. Prog. Ser.* 13, 55-60.

- Gieskes, W. W. C., G. W. Kroes, and M. A. Baars, 1979. Current ^{14}C methods for measuring primary production: gross underestimates in oceanic waters. *Netherlands J. Sea Res.* 13, 58-78.
- Glibert, P. M. and J. C. Goldman, 1981. Rapid ammonium uptake by marine phytoplankton. *Mar. Biol. Letters* 2, 25-31.
- Glibert, P. M., F. Lipschultz, J. J. McCarthy, and M. A. Altabet, 1982. Isotope dilution models of uptake and remineralization of ammonium by marine plankton. *Limnol. Oceanogr.* 27, 639-650.
- Goldman, J. C., 1977. Steady state growth of phytoplankton in continuous culture: comparison of internal and external nutrient equations. *J. Phycol.* 13, 251-258.
- Goldman, J. C., 1980. Physiological processes, nutrient availability, and the concept of relative growth rate in marine phytoplankton ecology. In: *Primary Productivity in the Sea* (P. G. Falkowski, ed.) Plenum Press, New York. pp. 179-194.
- Goldman, J. C., and J. J. McCarthy, 1978. Steady-state growth and ammonium uptake of a fast-growing marine diatom. *Limnol. Oceanogr.* 23, 695-703.
- Goldman, J. C., and D. G. Peavey, 1979. Steady-state growth and chemical composition of the marine chlorophyte *Dunaliella tertiolecta* in nitrogen-limited cultures. *Appl. Environ. Microbiol.* 38, 894-901.
- Goldman, J. C., J. J. McCarthy, and D. W. Peavey, 1979. Growth rate influence on the chemical composition of phytoplankton in oceanic waters. *Nature* 279, 210-215.
- Goldman, J. C., C. D. Taylor, and P. M. Glibert, 1981a. Nonlinear time-course uptake of carbon and ammonium by marine phytoplankton. *Mar. Ecol. Prog. Ser.* 6, 137-148.
- Goldman, J. C., M. R. Dennett, and C. B. Riley, 1981b. Marine phytoplankton photosynthesis and transient ammonium availability. *Mar. Biol. Letters* 2, 323-331.
- Goldman, J. C., and P. M. Glibert, 1982. Comparative rapid ammonium uptake by four species of marine phytoplankton. *Limnol. Oceanogr.* 27, 814-827.
- Goldman, J. C., and P. M. Glibert, 1983. Kinetics of inorganic nitrogen uptake by phytoplankton. In: *Nitrogen in the Marine Environment* (E. J. Carpenter and D. G. Capone, eds.) Academic Press, New York. pp. 233-274.
- Goldman, J. C., and M. R. Dennett, 1984. Photosynthetic responses of 15 phytoplankton species to ammonium pulsing. *Marine Ecology Progress Series*.
- Goltermann, H. L., 1964. Mineralization of algae under sterile conditions or by bacterial breakdown. *Verh. Internat. Verein. limnol.* 15, 544-548.
- Guillard, R. R. L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: *Culture of Marine Invertebrate Animals* (W. L. Smith and M. H. Chanley, eds.) Plenum Publishing Corp., New York. pp. 29-60.
- Guillard, R. R. L., and J. H. Ryther, 1962. Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.* 8, 229-239.
- Gutel'makher, B. L., 1973. Autoradiography as a method of determining the relative contribution of individual algal species to the primary production of plankton. *Hydrobiol. J.* 9, 61-64.

- Hagström, A., J. W. Ammerman, S. Henrichs, and F. Azam, 1984. Bacterioplankton growth in seawater: II. Organic matter utilization during steady state growth in seawater cultures. *Mar. Ecol. Prog. Ser.* 18, 41-48.
- Hammer, K. D., and U. H. Brockman, 1983. Rhythmic release of dissolved free amino acids from partly synchronized *Thalassiosira rotula* under nearly natural conditions. *Mar. Biol.* 74, 305-312.
- Handa, N., 1969. Carbohydrate metabolism in the marine diatom, *Skeletonema costatum*. *Mar. Biol.* 4, 208-214.
- Handa, H., 1970. Dissolved and particulate carbohydrates. *Occ. Publ. Inst. Mar. Sci., Univ. Alaska*, 1, 129-152.
- Harding, L. W., Jr., B. B. Prezelin, B. M. Sweeney, and J. L. Cox, 1982a. Diel oscillations of the photosynthesis-irradiance (P-I) relationship in natural assemblages of phytoplankton. *Mar. Biol.* 67, 167-178.
- Harding, L. W., Jr., B. B. Prezelin, B. M. Sweeney, and J. L. Cox, 1982b. Primary production as influenced by diel periodicity of phytoplankton photosynthesis. *Mar. Biol.* 67, 179-186.
- Harris, G. P., S. I. Heaney, and J. F. Talling, 1979. Physiological and environmental constraints in the ecology of the planktonic dinoflagellate *Ceratium hirundinella*. *Freshwater Biol.* 9, 413-428.
- Harris, G. P., and B. B. Piccinin, 1977. Photosynthesis by natural phytoplankton populations. *Arch. Hydrobiol.* 80, 405-457.
- Harrison, W., 1978. Experimental measurements of nitrogen remineralization in coastal waters. *Limnol. Oceanogr.* 23, 684-694.
- Harrison, P. J., H. L. Conway, and R. C. Dugdale, 1976. Marine diatoms grown in chemostat under silicate or ammonium limitation. 1. Cellular chemical composition and steady state growth kinetics of *Skeletonema costatum*. *Mar. Biol.* 35, 177-186.
- Harrison, W. G., 1980. Nutrient regeneration and primary production in the sea. In: *Primary production in the sea* (P. G. Falkowski, ed.) Plenum Press, New York, pp. 433-460.
- Harrison, W. G., D. Douglas, P. Falkowski, G. Rowe, and J. Vidal, 1983. Summer nutrient dynamics of the Middle Atlantic Bight: nitrogen uptake and regeneration. *J. Plankton Res.* 5, 539-556.
- Harvey, G. R., 1983. Dissolved carbohydrates in the New York bight and the variability of marine organic matter. *Mar. Chem.* 12, 333-339.
- Haug, A., S. Mykkestad, and 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.
- Healey, F. P., 1979. Short-term responses of nutrient deficient algae to nutrient addition. *J. Phycol.* 15, 289-299.
- Heber, U., 1974. Metabolite exchange between chloroplasts and cytoplasm. *Ann. Rev. Plant Physiol.* 25, 393-421.
- Hellebust, J. A., 1965. Excretion of some organic compounds by marine phytoplankton. *Limnol. Oceanogr.* 10, 192-206.
- Hellebust, J. A., and R. G. S. Bidwell, 1964. Protein turnover in attached wheat and tobacco leaves. *Can. J. Bot.* 42, 1-12.
- Hendrickson, P., K. G. Sellner, B. Rojas de Mendiola, N. Ochoa, and R. Zimmermann, 1982. The composition of particulate organic matter and biomass in the Peruvian upwelling region during ICANE 1977 (Nov. 14-Dec. 2). *J. Plank. Res.* 4, 163-186.

- Hipkin, C. R., and P. J. Syrett, 1977. Some effects of nitrogen-starvation and carbohydrate metabolism in Ankistrodesmus braunii. Planta 133, 209-214.
- Hitchcock, G. L., 1978. Labelling patterns of carbon-14 in net plankton during a winter-spring bloom. J. exp. mar. Biol. Ecol. 31, 141-153.
- Hitchcock, G. L., 1980. Diel variation in chlorophyll a, carbohydrate and protein content of the marine diatom Skeletonema costatum. Mar. Biol. 57, 271-278.
- Hitchcock, G. L., 1981. Photosynthate partitioning in cultured marine phytoplankton. I. Dinoflagellates. J. exp. mar. Biol. Ecol. 69, 21-36.
- Hobbie, J. E., C. C. Crawford, and K. L. Webb, 1968. Amino acid flux in an estuary. Science 159, 1463-1464.
- Hobson, L. A., 1971. Relationships between particulate organic carbon and micro-organisms in upwelling areas off Southwest Africa. ??
- Hobson, L. A., D. W. Menzel, and R. T. Barber, 1973. Primary productivity and sizes of pools of organic carbon in the mixed layer of the oceans. Mar. Biol. 19, 298-306.
- Hollibaugh, J. T., A. B. Carruthers, J. A. Fuhrman, and F. Azam, 1980. Cycling of organic nitrogen in marine plankton communities studied in enclosed water columns. Mar. Biol. 59, 15-21.
- Hollibaugh, J. T., and F. Azam, 1983. Microbial degradation of dissolved proteins in seawater. Limnol. Oceanogr. 28, 1104-1116.
- Holligan, P. M., R. P. Harris, R. C. Newell, D. S. Harbour, R. N. Head, E. A. S. Linley, M. I. Lucas, P. R. G. Tranter, and C. M. Weekley, 1984. Vertical distribution and partitioning of organic carbon in mixed, frontal, and stratified water of the English Channel. Mar. Ecol. Prog. Ser. 14, 111-127.
- Holm-Hansen, O., K. Nishida, V. Moses, and M. Calvin, 1959. Effects of mineral salts on short term incorporation of carbon dioxide in Chlorella. J. Exptl. Bot. 10, 109-124.
- Huffaker, R. C., and L. W. Peterson, 1974. Protein turnover in plants and possible means of its regulation. Ann. Rev. Plant Physiol. 25, 363-392.
- Ittekkot, V., 1982. Variations of dissolved organic matter during a plankton bloom: qualitative aspects based on sugar and amino acid analyses. Mar. Chem. 11, 143-158.
- Ittekkot, V., U. Brockman, W. Michaelis, and E. T. Degens, 1981. Dissolved free and combined carbohydrates during a phytoplankton bloom in the northern North Sea. Mar. Ecol. Prog. Ser. 4, 299-305.
- Ittekkot, V., E. T. Degens, and U. Brockmann, 1982. Monosaccharide composition of acid-hydrolyzable carbohydrates in particulate matter during a plankton bloom. Limnol. Oceanogr. 27, 770-776.
- Jannasch, H. W., 1974. Steady state and the chemostat in ecology. Limnol. Oceanogr. 19, 716-720.
- Jassby, A. D., and C. R. Goldman, 1974. Loss rates from a lake phytoplankton community. Limnol. Oceanogr. 19, 618-627.
- Jeffries, H. P., 1969. Seasonal composition of temperate plankton communities: free amino acids. Limnol. Oceanogr. 14, 41-52.
- Jeffries, H. P., 1970. Seasonal composition of temperate plankton communities: fatty acids. Limnol. Oceanogr. 15, 419-426.

- Jensen, L. M., 1984. Antimicrobial action of antibiotics on bacterial and algal carbon metabolism: on the use of antibiotics to estimate bacterial uptake of algal extracellular products. *Arch. Hydrobiol.* 99, 423-432.
- Johannes, R. E., 1964. Phosphorus excretion and body size in marine animals: microzooplankton and nutrient regeneration. *Science* 146, 923-924.
- Johannes, R. E., 1965. Influence of marine protozoa on nutrient regeneration. *Limnol. Oceanogr.* 10, 434-442.
- Johnson, B. D., 1976. Non-living particle formation from bubble dissolution. *Limnol. Oceanogr.* 21, 444-446.
- Joiris, C., 1977. On the role of heterotrophic bacteria in marine ecosystems: some problems. *Helgoländer wiss. Meeresunters.* 30, 611-621.
- Jukes, T. H., R. Holmquist, and H. Moise, 1975. Amino acid composition of proteins: selection against the genetic code. *Science* 189, 50-51.
- Kanazawa, T., K. Kanazawa, M. R. Kirk, and J. A. Bassham, 1972. Regulatory effects of ammonia on carbon metabolism in Chlorella pyrenoidosa during photosynthesis and respiration. *Biochim. Biophys. Acta* 256, 656-669.
- Kanazawa, T., M. R. Kirk, and J. A. Bassham, 1970. Regulatory effects of ammonia on carbon metabolism in photosynthesizing Chlorella pyrenoidosa. *Biochim. Biophys. Acta* 205, 401-408.
- Karl, D. M., 1980. Cellular nucleotide measurements and applications in microbial ecology. *Microbial. Rev.* 44, 739-796.
- Karl, D. M., 1981. Simultaneous rates of ribonucleic acid and deoxyribonucleic acid synthesis for estimating growth and cell division of aquatic microbial communities. *Appl. Environ. Microbiol.* 42, 802-810.
- Karl, D. M., 1982. Microbial transformations of organic matter at oceanic interfaces: a review and prospectus. *EOS* 63, 138-140.
- Karl, D. M., C. D. Winn, and D. C. L. Wong, 1981a. RNA synthesis as a measure of microbial growth in aquatic environments. I. Evaluation, verification and optimization of method. *Mar. Biol.* 64, 1-12.
- Karl, D. M., C. D. Winn, and D. C. L. Wong, 1981b. RNA synthesis as a measure of microbial growth in aquatic environments. II. Field Applications. *Mar. Biol.* 64, 13-21.
- Kattner, G., G. Gercken, and K. D. Hammer, 1983. Development of lipids during a spring plankton bloom in the northern North Sea. II. Dissolved lipids and fatty acids. *Mar. Chem.* 14, 163-173.
- Kim, C. M., and K. O. Emery, 1971. Salt Pond: topography, sediments, and water. Salt Pond Bird Sanctuaries, Inc.--Annual Report 1971.
- Kirchman, D., 1983. The production of bacteria attached to particles suspended in a freshwater pond. *Limnol. Oceanogr.* 28, 858-872.
- Kirk, P. R., and R. M. Leech, 1972. Amino acid biosynthesis by isolated chloroplasts during photosynthesis.
- Konopka, A., 1982. Physiological ecology of a metalimnetic Oscillatoria rubescens population. *Limnol. Oceanogr.* 27, 1154-1161.
- Konopka, A., and M. Schnur, 1981. Biochemical composition and photosynthetic carbon metabolism of nutrient limited cultures of Merismopedia tenuissima (Cyanophyceae). *J. Phycol.* 17, 118-122.
- Konopka, A., and M. Schnur, 1980. Effect of light intensity on macromolecular synthesis in cyanobacteria. *Microb. Ecol.* 6, 291-301.
- Koybayashi, Y., and D. V. Maudsley, 1974. Biological applications of liquid scintillation counting. Academic Press, New York. 196 pp.

- Landry, M. R., 1977. A review of important concepts in the trophic organization of pelagic ecosystems. *Helgoländer wiss. Meeresunters.* 30, 8-17.
- Larsen, P. O., K. L. Cornwell, S. L. Gee, and J. A. Bassham, 1981. Amino acid synthesis in photosynthesizing spinach and rates of labeling from $^{14}\text{CO}_2$. *Plant Physiol.* 68, 292-299.
- Larsson, U., and A. Hagstrom, 1979. Phytoplankton exudate release as an energy source for the growth of pelagic bacteria. *Mar. Biol.* 52, 199-206.
- Laws, E. A., and D. C. L. Wong, 1978. Studies of carbon and nitrogen metabolism by three marine phytoplankton species in nitrate-limited continuous culture. *J. Phycol.* 14, 406-416.
- Laws, E. A., and T. T. Bannister, 1980. Nutrient- and light-limited growth of *Thalassiosira fluviatilis* in continuous culture with implications for phytoplankton growth in the oceans. *Limnol. Oceanogr.* 25, 457-473.
- Laws, E. A., W. G. Harrison, and G. R. DiTullio, 1985. A comparison of nitrogen assimilation rates based on ^{15}N uptake and autotrophic protein synthesis. *Deep-Sea Res.* 32, 85-95.
- Lean, D. R. S., and B. K. Burnison, 1979. An evaluation of errors in the ^{14}C method of primary production measurement. *Limnol. Oceanogr.* 24, 917-928.
- Lee, C., and C. Cronin, 1982. The vertical flux of particulate organic nitrogen in the sea: decomposition of amino acids in the Peru upwelling area and the equatorial Atlantic. *J. Mar. Res.* 40, 227-251.
- Lee, C., S. G. Wakeham, and J. W. Farrington, 1983. Variations in the composition of particulate organic matter in a time series sediment trap. *Mar. Chem.* 13, 181-194.
- Lee, C., and C. Cronin, 1984. Particulate amino acids in the sea: effects of primary production and decomposition. *J. Mar. Res.* 42, 1075-1097.
- Leech, R. M., and D. J. Murphy, 1976. The cooperative function of chloroplasts in the biosynthesis of small molecules. In: *The Intact Chloroplast* (J. Barber, ed.) pp. 365-401.
- Li, W. K. W., and J. C. Goldman, 1981. Problems in estimating growth rates of marine phytoplankton from short-term ^{14}C assays. *Microbial Ecol.* 7, 113-121.
- Li, W. K. W., H. E. Glover, and I. Morris, 1980. Physiology of carbon photoassimilation by *Oscillatoria thiebautii* in the Caribbean Sea. *Limnol. Oceanogr.* 25, 447-456.
- Li, W. K. W., and T. Platt, 1982. Distribution of carbon among photosynthetic end-products of phytoplankton of the eastern Canadian arctic. *J. Phycol.* 18.
- Li, W. K. W., and W. G. Harrison, 1982. Carbon flow into the end-products of photosynthesis in short and long incubations of a natural phytoplankton population. *Mar. Biol.* 72, 175-182.
- Little, J. E., R. E. Sjogren, and G. E. Carson, 1979. Measurement of proteolytic activity in natural waters. *Applied Environ. Microbiol.* 37, 900-908.
- Lorenzen, C. J., 1963. Diurnal variation in photosynthetic activity of phytoplankton populations. *Limnol. Oceanogr.* 8, 56-62.
- Lorenzen, H., and B. D. Kaushik, 1976. Experiments with synchronous *Anacystis nidulans* Ber. Deutsch. Bot. Ges. Bd. 89, 491-498.

- MacNicol, P. K., 1978. Determination of specific radioactivity of plant amino acids using dansylation. *Anal. Biochem.* 85, 71-78.
- Mague, T. H., E. Friberg, D. J. Hughes, and I. Morris, 1980. Extracellular release of carbon by marine phytoplankton; a physiological approach. *Limnol. Oceanogr.* 25, 262-279.
- Maguire, B. Jr., and W. E. Neill, 1971. Species and individual productivity in phytoplankton communities. *Ecology* 52, 903-907.
- Maity, Y., and M. Yanada, 1978. Particulate protein in coastal waters, with special reference to seasonal variation. *Mar. Biol.* 44, 329-336.
- Marra, J., and K. Heinemann, 1984. A comparison between noncontaminating and conventional incubation procedures in primary production measurements. *Limnol. Oceanogr.* 29, 389-392.
- Maske, H., 1982. Ammonium-limited continuous cultures of Skeletonema costatum in steady and transitional state: experimental results and model simulations. *J. mar. biol. Ass. U. K.* 62, 919-943.
- McCarthy, J. J. and J. C. Goldman, 1979. Nitrogenous nutrition of marine phytoplankton in nutrient depleted waters. *Science* 203, 670-672.
- McCarthy, J. J., 1972. The uptake of urea by natural populations of marine phytoplankton. *Limnol. Oceanogr.* 17, 738-748.
- Menzel, D. W., and J. H. Ryther, 1970. Distribution and cycling of organic matter in the oceans. *Occ. Publ. Inst. Mar. Sci., Univ. Alaska*, 1, 31-54.
- Menzel, D. W., and R. F. Vaccaro, 1964. The measurement of dissolved organic and particulate carbon in seawater. *Limnol. Oceanogr.* 9, 138-142.
- Mifflin, B. J., P. J. Lea, and R. M. Wallsgrove, 1980. The role of glutamine in ammonia assimilation and reassimilation in plants. In: Glutamine: Metabolism, Enzymology, and Regulation (ed.) Academic Press, New York. pp. 213-234.
- Mifflin, B. J., and P. J. Lea, 1977. Amino acid metabolism. *Ann. Rev. Plant Physiol.* 28, 299-329.
- Mills, W. R., P. J. Lea, and B. J. Mifflin, 1980. Photosynthetic formation of the aspartate family of amino acids in isolated chloroplasts. *Plant Physiol.* 65, 1166-1172.
- Mohamed, A. H., and A. Gnanam, 1979. A possible mechanism of ammonium ion regulation of photosynthetic carbon flow in higher plants. *Plant Physiol.* 64, 263-268.
- Mopper, K., and P. Lindroth, 1982. Diel and depth variations in dissolved free amino acids and ammonium in the Baltic Sea determined by shipboard HPLC analysis. *Limnol. Oceanogr.* 27, 336-347.
- Morris, I., 1981. Photosynthetic products, physiological state, and phytoplankton growth. In: Physiological Bases of Phytoplankton Ecology. (T. Platt, ed.) *Can. Bull. Fish. Aquat. Sci.* 210, 83-102.
- Morris, I., A. E. Smith, and H. E. Glover, 1981. Products of photosynthesis in phytoplankton off the Orinoco river and in the Caribbean Sea. *Limnol. Oceanogr.* ?
- Morris, I., H. E. Glover, and C. S. Yentsch, 1974. Products of photosynthesis by marine phytoplankton: the effect of environmental factors on the relative rates of protein synthesis. *Mar. Biol.* 27, 1-9.
- Morris, I., and W. Skea, 1978. Products of photosynthesis in natural populations of marine phytoplankton from the Gulf of Maine. *Mar. Biol.* 47, 303-312.

- Myklestad, S. and 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, 125-136.
- 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 carbohydrates by Chaetoceros affinis var. willei (Gran) Hustedt and Skeletonema costatum (Grev.) Cleve. J. exp. mar. Biol. Ecol. 29, 161-179.
- Neess, J. C., R. C. Dugdale, V. A. Dugdale, and J. J. Goering, 1962. Nitrogen metabolism in lakes. II Measurement of nitrogen fixation with ¹⁵N. Limnol. Oceanogr. 7, 163-169.
- Nienhaus, P. H., 1981. Distribution of organic matter in living organisms. In: Marine Organic Chemistry. Evolution, Composition, Interactions and Chemistry of Organic Matter in Seawater (E. K. Duursma and R. Dawson, eds.) Elsevier Scientific Publishing Co., New York. pp. 31-69.
- Oaks, A., and R. G. S. Bidwell, 1970. Compartmentation of intermediary metabolites. Ann. Rev. Plant Physiol. 21, 43-66.
- Olive, J. H., and J. H. Morrison, 1967. Variations in distribution of ¹⁴C in cell extracts of phytoplankton living under natural conditions. Limnol. Oceanogr. 12, 383-391.
- Olive, J. H., and D. M. Benton, and J. Kishler, 1969. Distribution of C-14 in products of photosynthesis and its relationship to phytoplankton composition and rate of photosynthesis. Ecology 50, 380-386.
- Packard, T. T., and Q. Dortch, 1975. Particulate protein-nitrogen in North Atlantic Surface waters. Mar. Biol. 33, 347-354.
- Paerl, H. W., 1974. Bacterial uptake of dissolved organic matter in relation to detrital aggregation in marine and freshwater systems. Limnol. Oceanogr. 19, 966-972.
- Paerl, H. W., 1984. An evaluation of freeze fixation as a phytoplankton preservation method for microautoradiography. Limnol. Oceanogr. 29, 417-426.
- Parris, N., and D. Galleli, 1984. Dansylation of amino acids and byproduct formation. J. Liquid Chrom. 7, 917-924.
- Parsons, T. R., K. Stephens, and J. D. H. Strickland, 1961. On the chemical composition of eleven species of marine phytoplankters. J. Fish. Res. Bd. Can. 18, 1001-1016.
- Parsons, T. R., M. Takahashi, and B. Hargrave, 1977. Biological Oceanographic Processes. Pergamon Press, New York. 332 pp.
- Paul, J. S., K. L. Cornwell, and J. A. Bassham, 1978. Effects of ammonia on carbon metabolism in photosynthesizing isolated mesophyll cell from Papaver somniferum L. Planta 142, 49-54.
- Perry, M. J., M. C. Talbot, and R. S. Alberte, 1981. Photoadaptation in marine phytoplankton: response of the photosynthetic unit. Mar. Biol. 62, 91-101.
- Peterson, B. J., 1980. Aquatic primary productivity and the ¹⁴C-CO₂ method: a history of the productivity problem. Ann. Rev. Ecol. Syst. 11, 359-385.
- Pine, M. J., 1972. Turnover of intracellular proteins. Ann. Rev. Microbiol. 26, 103-126.

- Platt, S. G., Z. Plaut, and J. A. Bassham, 1977. Ammonia regulation of carbon metabolism in photosynthesizing leaf discs. *Plant Physiol.* 60, 739-742.
- Pomeroy, L. R., 1974. The ocean's food web, a changing paradigm. *Bioscience* 24, 499-504.
- Porter, K., M. L. Pace, and J. F. Battery, 1979. Ciliate protozoans as links in freshwater planktonic food chains. *Nature* 277, 563-565.
- Poulet, S. A., and V. Martin-Jezequel, 1983. Relationships between dissolved free amino acids, chemical composition and growth of the marine diatom *Chaetoceros debile*. *Mar. Biol.* 77, 93-100.
- Prezelin, B. B., 1976. The role of peridinin-chlorophyll *a*-proteins in the photosynthetic light adaptation of marine dinoflagellates, *Glenodinium* sp. *Planta (Berl.)* 130, 225-233.
- Prisco, J. C., and C. R. Goldman, 1983. Carboxylating enzyme activity and photosynthetic endproducts of phytoplankton in the shallow and deep chlorophyll layers of Castle Lake. *Limnol. Oceanogr.* 28, 1168-1181.
- Rausch, T., 1981. The estimation of microalgal protein content and its meaning to the evaluation of algal biomass. I. Comparison of methods for extracting protein. *Hydrobiologia* 78, 237-251.
- Redalje, D. G., 1983. Phytoplankton carbon biomass and specific growth rates determined with the labeled chlorophyll *a* technique. *Mar. Ecol. Prog. Ser.* 11, 217-225.
- Redalje, D. G., and E. A. Laws, 1983. The effects of environmental factors on growth and the chemical and biochemical composition of marine diatoms. I. Light and temperature effects. *J. Exp. Mar. Biol. Ecol.* 68, 59-79.
- Redalje, D. G., and E. A. Laws, 1981. A new method for estimating phytoplankton growth rates and carbon biomass. *Mar. Biol.* 62, 73-79.
- Reisner, G. S., R. K. Gering, and J. F. Thompson, 1960. The metabolism of nitrate and ammonia by *Chlorella*. *Plant Physiol.* 35, 48-52.
- Remsen, C. C., 1971. The distribution of urea in coastal and oceanic waters. *Limnol. Oceanogr.* 16, 732-740.
- Reynolds, C. S., H. R. Morison, and C. Butterwick, 1982. The sedimentary flux of phytoplankton in the south basin of Windermere. *Limnol. Oceanogr.* 27, 1162-1175.
- Rhee, G-Yull, 1973. A continuous culture study of phosphate uptake, growth rate, and polyphosphate in *Scenedesmus* sp. *J. Phycol.* 9, 495-506.
- Rhee, G. Y., 1978. Effects of N:P atomic ratio and nitrate limitation on algal growth, cell composition, and nitrate uptake. *Limnol. Oceanogr.* 23, 10-25.
- Rhee, G., 1980. Continuous culture in phytoplankton ecology. *Adv. Aquat. Microbiol.* 2, 151-203.
- Richardson, K., J. Beardall, and J. A. Raven, 1983. Adaptation of unicellular algae to irradiance: an analysis of strategies. *New Phytol.* 93, 157-191.
- Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Botton, and R. J. Britten, 1963. Studies of biosynthesis in *Escherichia coli*. *Carnegie Inst. Wash. Publ.* 607. 521pp.
- Schell, D. M., 1974. Uptake and regeneration of free amino acids in marine waters of Southeast Alaska. *Limnol. Oceanogr.* 19, 260-270.

- Schiffrin, N. S., and S. W. Chisholm, 1981. Phytoplankton lipids: interspecific differences and effects of nitrate, silicate, and light-dark cycles. *J. Phycol.* 17, 374-384.
- Schindler, D. W., and S. K. Holmgren, 1971. Primary production and phytoplankton in the experimental lakes area, Northwestern Ontario, and other low carbonate waters, and a liquid scintillation method for determining ^{14}C activity in photosynthesis. *J. Fish. Res. Board Can.* 28, 189-201.
- Schulze-Siebert, D., D. Heineke, H. Scharf, and G. Schultz, 1984. Pyruvate derived amino acids in spinach chloroplasts. Synthesis and regulation during photosynthetic carbon metabolism. *Plant Physiol.* 76, 465-471.
- Scott, J. M., 1980. Effect of growth rate of the food alga on the growth/ingestion efficiency of a marine herbivore. *J. mar. biol. Ass. U.K.* 60, 681-702.
- Sharp, J. H., 1977. Excretion of organic matter by phytoplankton: Do healthy cells do it? *Limnol. Oceanogr.* 22, 381-399.
- Sharp, J. H., M. J. Perry, E. H. Renger, and R. W. Eppley, 1980. Phytoplankton rate processes in the oligotrophic waters of the central North Pacific Ocean. *J. Plank. Res.* 2, 335-353.
- Sieburth, J. McN., K. M. Johnson, C. M. Burney, and D. M. Lavoie, 1977. Estimation of *in situ* heterotrophy using diurnal changes in dissolved organic matter and growth rates of picoplankton in diffusion culture. *Helgoländer wiss. Meeresunters.* 30, 565-574.
- Sieburth, J. McN., V. Smetacek, and J. Lenz, 1978. Pelagic ecosystem structure: Heterotrophic compartments of the plankton and their relationship to plankton size fractions. *Limnol. Oceanogr.* 23, 1256-1263.
- Siezen, R. J., and T. H. Mague, 1978. Amino acids in suspended particulate matter from oceanic and coastal waters of the Pacific. *Mar. Chem.* 6, 215-231.
- Slawyk, G., Y. Collos, and J. Auclair, 1977. The use of ^{13}C and ^{15}N isotopes for the simultaneous measurement of carbon and nitrogen turnover rates in marine phytoplankton. *Limnol. Oceanogr.* 22, 925-932.
- Small, L. F., S. W. Fowler, S. A. Moore, and J. LaRosa, 1983. Dissolved and fecal pellet carbon and nitrogen release by zooplankton in tropical waters. *Deep-Sea Res.* 30, 1199-1220.
- Smayda, T. J., 1970. The suspension and sinking of phytoplankton in the sea. *Oceanogr. Mar. Biol. Ann. Rev.* 8, 353-414.
- Smith, A. E., and I. Morris, 1980a. Synthesis of lipid during photosynthesis by phytoplankton of the Southern Ocean. *Science* 207, 197-199.
- Smith, A. E., and I. Morris, 1980b. Pathways of carbon assimilation in phytoplankton from the Antarctic Ocean. *Limnol. Oceanogr.* 25, 865-872.
- Smith, D. C., J. A. Bassham, and M. Kirk, 1961. Dynamics of the photosynthesis of carbon compounds. II. Amino acid synthesis. *Biochim. Biophys. Acta* 48, 299-313.
- Smith, D. F., and S. M. J. Horner, 1981. Tracer kinetic analysis applied to problems in marine biology. In: *Physiological Bases of Phytoplankton Ecology* (T. Platt, ed.) *Can. Bull. Fish. Aquat. Sci.* 210, 113-125.

- Smith, R. C., K. S. Baker, and P. Dustan, 1981. Fluorometer techniques for the measurement of ocean chlorophyll in the support of remote sensing. Ref. Rep. Scripps Instn. Oceanogr., 81-17, 1-14. (Scripps Institution of Oceanography, La Jolla, California, U.S.A. 92093).
- Smith, R. E. H., and J. Kalff, 1983. Sample preparation for quantitative autoradiography of phytoplankton. *Limnol. Oceanogr.* 28, 383-389.
- Somville, M., and G. Billen, 1983. A method for determining exoproteolytic activity in natural waters. *Limnol. Oceanogr.* 28, 190-193.
- Sorokin, Y. I., 1981. Microheterotrophic organisms in marine ecosystems. In: Analysis of marine ecosystems (A. R. Longhurst, ed.) Academic Press, London. pp. 293-342.
- Steele, J. H., 1974. The structure of marine ecosystems. Harvard University Press, Cambridge, MA. 128 pp.
- Steele, J. H., and I. E. Baird, 1962. Carbon-chlorophyll relations in cultures. *Limnol. Oceanogr.* 7, 101-102.
- Steeman-Nielsen, E., 1952. The use of radioactive carbon (^{14}C) for measuring organic production in the sea. *J. Cons., Cons. Int. Explor. Mer.* 18, 117-140.
- Stewart, C. R., S. F. Boggess, D. Aspinall, and L. G. Paleg, 1977. Inhibition of proline oxidation by water stress. *Plant Physiol.* 59, 930-932.
- Stoecker, D. K., 1984. Particle production by planktonic ciliates. *Limnol. Oceanogr.* 29, 930-940.
- Stout, J. D., 1980. The role of protozoa in nutrient cycling and energy flow. *Adv. Microb. Ecol.* 4, 1-50.
- Strathmann, R. R., 1967. Estimating organic carbon content of phytoplankton from cell volume or plasma volume. *Limnol. Oceanogr.* 12, 411-418.
- Syrett, P. J., 1953. The assimilation of ammonia by nitrogen-starved cells of *Chlorella vulgaris*. II. The assimilation of ammonium to other compounds. *Annls. Bot. (London)* 17, 20-36.
- Syrett, P. J., 1981. Nitrogen metabolism of microalgae. In: Physiological bases of phytoplankton ecology (T. Platt, ed.) Can. Bull. Fish. Aquat. Sci. 210, 182-210.
- Tapuhi, Y., D. E. Schmidt, W. Lindner, and B. L. Karger, 1981. Dansilation of amino acids for high performance liquid chromatography analysis. *Anal. Biochem.* 115, 123-129.
- Taylor, C. D., J. J. Molongoski, S. E. Lohrenz, 1983. Instrumentation for the measurement of phytoplankton production. *Limnol. Oceanogr.* 28, 781-787.
- Thomas, W. H., and A. N. Dodson, 1972. On nitrogen deficiency in tropical pacific oceanic phytoplankton. II. Photosynthetic and cellular characteristics of a chemostat-grown diatom. *Limnol. Oceanogr.* 17, 515-523.
- Thomas, W. H., and R. W. Krauss, 1955. Nitrogen metabolism in *Scenedesmus* as affected by environmental changes. *Plant Physiol.* 30, 113-122.
- Tomas, C. R., 1978. *Olisthodiscus luteus* (Chrysophyceae). I. Effects of salinity and temperature on growth, motility, and survival. *J. Phycol.* 14, 309-313.
- Tomas, C. R., 1978. *Olisthodiscus luteus* (Chrysophyceae). II. Formation and survival of a benthic stage. *J. Phycol.* 14, 314-319.

- Tomas, C. R., 1979. Olisthodiscus luteus (Chrysophyceae). III. Uptake and utilization of nitrogen and phosphorus. J. Phycol. 15, 5-12.
- Tomas, C. R., 1980. Olisthodiscus luteus (Chrysophyceae). IV. Effects of light intensity and temperature on photosynthesis and cellular composition. J. Phycol. 16, 149-156.
- Tomas, C. R., 1980. Olisthodiscus luteus (Chrysophyceae). V. Its occurrence, abundance, and dynamics in Narragansett Bay, Rhode Island. J. Phycol. 16, 157-166.
- Tomas, C. R., and E. E. Deason, 1981. The influence of grazing by two Acartia species on Olisthodiscus luteus Carter. P. S. Z. N. I: Marine Ecology 2, 215-223.
- Trewavas, A., 1972. Determination of the rates of protein synthesis and degradation in Lemna minor. Plant Physiol. 49, 40-46.
- Turpin, D. H., and P. J. Harrison, 1978. Fluctuations in free amino acid pools of Gymnodinium simplex (Dinophyceae) in response to ammonia perturbation: evidence for glutamine synthetase pathway. J. Phycol. 14, 461-464.
- Van Liere, L., L. R. Mur, and C. E. Gibson, and M. Herdman, 1979. Growth and physiology of Oscillatoria agardhii Gomont cultivated in continuous culture with a light-dark cycle. Arch Microbiol. 123, 315-318.
- Venrick, E. L., J. R. Beers, and J. F. Heinbokel, 1977. Possible consequences of containing microplankton for physiological rate measurements. J. exp. mar. Biol. Ecol. 26, 55-76.
- Verity, P. G., and D. Stoecker, 1982. Effects of Olisthodiscus luteus on the growth and abundance of tintinnids. Mar. Biol. 72, 79-87.
- Vollenweider, R. A., and A. Nauwerck, 1961. Some observations on the ¹⁴C method for measuring primary production. Verh. Internat. Verein. Limnol. 14, 134-139.
- Wakeham, S. G., J. W. Farrington, R. B. Gagosian, C. Lee, H. DeBarr, G. E. Nigrelli, B. W. Tripp, S. O. Smith, and N. M. Frew, 1980. Organic matter fluxes from sediment traps in the equatorial Atlantic Ocean. Nature 286, 798-800.
- Wangersky, P. J., 1977. The role of particulate matter in the productivity of surface water. Helgoländer wiss. Meeresunters 30, 546-564.
- Watt, W. D., 1971. Measuring the primary production rates of individual phytoplankton species in natural mixed populations., Deep Sea Res. 18, 329-339.
- Webb, K. L., and R. E. Johannes, 1967. Studies of the release of dissolved free amino acids by marine zooplankton. Limnol. Oceanogr. 12, 376-382.
- Wheeler, P. A., 1983. Phytoplankton nitrogen metabolism. In: Nitrogen in the Marine Environment (E. J. Carpenter and D. G. Capone, eds.) Academic Press, New York. pp. 309-346.
- Wheeler, P. A., B. B. North, and G. C. Stephens, 1974. Amino acid uptake by marine phytoplankters. Limnol. Oceanogr. 19, 249-259.
- Wheeler, P. A., and G. C. Stephens, 1977. Metabolic segregation of intracellular free amino acids in Platymonas (Chlorophyta). J. Phycol. 13, 193-197.

- Wheeler, P. A., R. J. Olson, and S. W. Chisholm, 1983. Effects of photocycles and periodic ammonium supply on three marine phytoplankton species. II. Ammonium uptake and assimilation. *J. Phycol.* 19, 528-533.
- Whittle, K. J., 1977. Marine organisms and their contribution to organic matter in the ocean. *Mar. Chem.* 5, 381-411.
- Wiebe, W. J., and D. F. Smith, 1977. Direct measurement of dissolved organic carbon release by phytoplankton and incorporation by microheterotrophs. *Mar. Biol.* 42, 213-223.
- Wiedmeier, V. T., S. P. Porterfield, and C. E. Hendrich, 1982. Quantitation of Dns-amino acids from body tissues and fluids using high-performance liquid chromatography. *J. Chromatog.* 231, 410-417.
- Williams, P. J. leB., 1981. Incorporation of microheterotrophic processes into the classical paradigm of the planktonic food web. *Kieler Meeresforsch., Sonderh.* 5, 1-28.
- Williams, P. J. leB., T. Berman, and O. Holm-Hansen, 1976. Amino acid uptake and respiration by marine heterotrophs. *Mar. Biol.* 35, 41-47.
- Winn, C. D., and D. M. Karl, 1984. Laboratory calibrations of the [³H]adenine technique for measuring rates of RNA and DNA synthesis in marine microorganisms. *Appl. Environ. Microbiol.* 47, 835-842.
- Woodwell, G. M., R. H. Whittaker, W. A. Reiners, G. E. Likens, C. C. Delwiche, and D. B. Batkin, 1978. The biota and the world carbon budget. *Science* 199, 141-146.
- Yentsch, C. S., 1974. Some aspects of the environmental physiology of marine phytoplankton: a second look. *Oceanogr. Mar. Biol.* 12, 41-75.
- Yentsch, C. S., and D. W. Menzel, 1963. A method for the determination of phytoplankton chlorophyll and pheophytin by fluorescence. *Deep Sea Res.* 10, 221-231.
- Zeitzschel, B., 1970. The quantity, composition and distribution of particulate matter in the Gulf of California. *Mar. Biol.* 7, 305-318.

BIOGRAPHY

I was born in Newton, Kansas on the 20th of December, 1955. Newton is a town in the heart of the Midwest, about as far from any large body of water as you can get while still being in the United States. I began my gradual migration towards aquatic environments in 1959 when my family moved to Marshfield, Wisconsin, where my father, an M.D. in Internal Medicine, joined the Marshfield Clinic. Although Marshfield is not exactly coastal, it was much closer to the Great Lakes and other bodies of water than Kansas. The beauty of northern Wisconsin and Michigan lakes is unique and wonderful. My first glimpse of an ocean was in 1965 when my family visited Atlantic City (then a pretty resort city) on our way to the New York world's fair. I do not remember specifically how I felt about a career choice at that point, but I do know it was the beginning of a lasting love for the ocean.

Around the seventh and eighth grades, after some vacillation between the choices of being an M.D. or an oceanographer, I decided to make oceanography my career. My father supported my choice, and helped me research educational opportunities in the field. It was at this point that I began to realize being an oceanographer entailed more than just learning how to scuba dive. Fortunately, I had an excellent chemistry teacher in high school, T. K., who made the subject an enjoyable challenge rather than an unpleasant requirement.

I majored in chemistry during my freshman and sophomore years at the University of Wisconsin in Madison. After a semester doing undergraduate research on the synthesis of cis, trans-dicyclopropenes, I realized a need for more biological flavor in my studies. Oceanography, it seemed, was the ideal vehicle for combining both biological and chemical research. I made the decision to transfer to the University of Oregon in Eugene in hopes that its relative proximity to the ocean might provide more opportunity for marine related studies.

The summer of 1976 marked a major transition in my life. My father's death led to second thoughts about leaving my family and friends for the West Coast. Yet their support of my decision, and my father's earlier encouragement helped me through this difficult period. Once at the University of Oregon, I knew I had made the right decision. A summer at the Oregon Institute of Marine Biology strengthened my desire to study oceanography. A course in invertebrate zoology qualified me for a job with Beak Consultants of Portland, OR, conducting a brief survey of intertidal community gradients for Mobil Oil in Puget Sound. During the following term at the U. of O., I continued research along these lines working under Greg Daly, a graduate student studying tidal gradient effects on competition among rocky intertidal invertebrates.

Although I enjoyed these experiences, I felt they lacked the balance between biology and chemistry I was seeking. I took advantage of U. of O.'s extensive biochemistry curriculum, and became involved in a project with Dr. Robert Terwilliger using spectropolarimetric techniques to compare structures of polychaete hemoglobins. The summer of 1978, I returned to Marshfield to work for Dr. Mary Treuhaft at the Marshfield Medical Foundation on a project involving the immunochemical characterization of thermophilic actinomycetes. I gained experience in microbiological techniques which later paid off during my thesis research.

BIOGRAPHY (continued)

Returning to the U. of O., I continued my undergraduate research using spectropolarimetry to study temperature dependent premelting phenomena in calf thymus DNA. My varied interests resulted in my graduating with a B.A. in biology and chemistry in december of 1978. For the next few months, I waited for word on my applications to graduate programs in oceanography and paid the bills by working for my friend and advisor, Dr. William Bradshaw, studying the developmental physiology of larval mosquitos.

I was lucky enough to be accepted by the MIT/WHOI Joint Program, and traversed the country to the East Coast. The unique nature of the program fosters independence and allowed me to choose thesis research which drew on my past experiences, and introduced me to new and challenging ones.