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#### DOCTOR OF PHILOSOPHY

#### Interaction between the bacterial and phytoplanktonic inorganic nitrogenous nutrition

de Vasconcelos Rodrigues, Rubina Maria Neves

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## Interaction Between the Bacterial and Phytoplanktonic Inorganic Nitrogenous Nutrition

Thesis submitted in accordance with the requirements of the University of Wales, Bangor for the degree of Doctor of Philosophy



Rubina Maria Neves de Vasconcelos Rodrigues



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School of Ocean Sciences Menai Bridge, Anglesey University of Wales, Bangor United Kingdom



### Summary

The present work investigates the inorganic nitrogenous nutrition of the phytoplanktonic community and its association with the bacterial role in the remineralisation and/or utilisation of inorganic nitrogen, in the waters of the Menai Strait. The field study lead to the recognition of four distinct phases in the seasonal cycle, based on measurements of primary plankton production and metabolism of nitrate and ammonium by the algae and bacteria. These were first the net autotrophic phase (April and May), followed by the net heterotrophic phase (June), then the secondary net autotrophic phase (July and August) and finally, the regenerative phase (remainder of the year).

The phytoplankton seasonal pattern was characterised by nitrate-based production in the spring followed by ammonium-based production in summer and autumn, associated with the ambient availability of these nutrients. Bacteria accounted for up to 70% of the total inorganic nitrogen taken up with nitrate contributing heavily to bacterial production (up to 80%) early in the year. Whilst the maximum contribution of the heterotrophs to the total uptake was observed during the net heterotrophic phase, the lowest (<25%) was during the net autotrophic periods of the seasonal cycle. Calculations suggest that a maximum of 67% of algal ammonium uptake came from bacterial recycling. Only in June was there evidence of competition between algae and bacteria for nitrogen which is believed to have contributed to the sharp decline of the phytoplankton, at this time of the year. There was no evidence for nutrient limitation of the algae at other times of the season. There was also evidence for uptake of nitrate and ammonium in conditions of total darkness by both the bacteria and the algae, with bacteria accounting for a maximum of ~75% the total nitrate and ammonium uptake. Ammonium was preferred over nitrate by the autotrophs and the heterotrophs. However, early in the year, when both primary and bacterial production were nitrate based, nitrate was utilised equitably with its environmental availability. For increasing concentrations of ammonium, bacteria clearly rejected the more oxidised form of inorganic nitrogen.

The seasonal variation of <sup>13</sup>C (as NaH<sup>13</sup>CO<sub>3</sub>) uptake by the populations studied was also investigated and there was strong evidence for heterotrophic uptake. Over the diel cycle, the contribution of bacteria to the total <sup>13</sup>C uptake amounted to a maximum of 44% during the net heterotrophic phase. In dark conditions, this percentage increased to a value of 60%, measured on the same occasion. The ambient availability of inorganic nitrogen seemed to influence the proportion of carbon fixed by the autotrophs that was inferred to be transferred to the bacteria. The assumed carbon transference from the autotrophs to the heterotrophs was highest in the spring, following a period of sufficiency of inorganic nitrogen, and lowest during summer (despite peaks of primary production), associated with conditions of ambient nitrogen depletion.

It is widely hypothesised that bacteria take up inorganic nitrogen in order to be able to utilise carbon-rich, nitrogen-deficient compounds and so maintain a constant C:N cell quota. From a series of laboratory experiments, it was observed that the C:N ratio of the dissolved organic matter (DOM) used by the bacteria set the boundary on the role of these organisms as inorganic N net consumers or remineralisers. The laboratory work was interpreted as showing that when DOM was the major nitrogen source for growth, bacteria excreted the excess nitrogen as ammonium whereas at high C:N ratios, bacteria took up and conserved nitrogen. In the latter circumstances, when nitrogen was limiting, bacteria appeared to increase respiratory losses in order to dispose of the excess carbon. By contrast at low C:N ratios, carbon conservation occurred. It was concluded that if the release of DOM by the algae is the prime source of organic material for the bacteria, then this switching of the inorganic nitrogen metabolism of the bacteria, will be ultimately driven by the algae. These results were used to interpret the seasonal dynamics of the phytoplanktonic and bacterial inorganic nitrogen metabolism in the Menai Strait.

## Contents

#### **CHAPTER I**

INTRODUCTION	1
1.1. Sources and Distribution of Inorganic Nitrogen in Sea Water	2
1.2. Phytoplanktonic Utilisation of Inorganic Nitrogen	5
1.2.1. The Kinetics of Nutrient Uptake	5
1.2.2. The Enzymes of Nutrient Uptake and Assimilation	8
1.2.3. Evaluation of Preference for a Given Form of Nitrogen: the Relative Preference Index	9
1.2.4. "New" and "Regenerated" Production	11
1.3. The Role of Bacteria in the Pelagic Ecosystem	13
1.3.1. 'Microbial Loop' and Microbial Food Web Versus the Classical Food Chain	13
1.3.2. Bacterial Inorganic Nitrogen Uptake	16
1.3.2.1. Physiological Mechanisms of Bacterial Nitrogen Uptake and Assimilation	17
1.3.2.2. Competition between Bacteria and Phytoplankton for Inorganic Nitrogen- an Apparent Paradox?	18
1.3.2.3. Controls of the Bacterial Inorganic Nitrogen Uptake: the Effect of Organic Substrate	20
1.4. Aims and Objectives of the Present Work	24
CHAPTER II Characterisation of the Studied Marine Environment	26
2.1 Hydrography	26
2.2. Chemical, Physical and Biological Characteristics of the Menai Strait Waters	29
2.2.1 Drysical and Chemical Parameters	30
2.2.1. r hysical and Chemical r drameters	30
2.2.1.1. Temperature	31
2.2.1.2. Samily	33
• •	

#### **CHAPTER III**

General Methodology		
3.1. Sampling	50	
3.2. Sample Size Fractionation	51	
3.2.1. Background	51	
3.2.2. Limitations of the Size Fractionation Procedure	52	
3.2.3. Experimental Procedure	52	
3.3. Chemical Determinations: Inorganic Nitrogenous Nutrients	53	
3.4. Biological Parameters	55	
3.4.1. Clorophyll	55	
3.4.2. Total Bacteria Numbers	56	
3.4.2.1. Background	56	
3.4.2.2. Sources of Error in Bacteria Counts	58	
3.4.2.3. Experimental Procedure	59	
3.4.3. Oxygen Flux Measurements: Respiration, Gross and Net Community Production	60	
3.4.3.1. Background	60	
3.4.3.2. Sources of Error in the Oxygen Technique	61	
3.4.3.3. Experimental Procedure	62	
3.4.4. Inorganic Nitrogen Uptake: The <sup>15</sup> N Technique	63	
3.4.4.1. Background	63	
3.4.4.2. Assumptions Made in <sup>15</sup> N Experiments	64	
3.4.4.3. Limitations of the <sup>15</sup> N Tracer Technique and Practical		
Considerations on its Application	65	
3.4.4.4. Experimental Procedure in the Field	70	
3.4.4.5. Calculation of the Uptake Rates	71	
3.4.5. Nitrogen (and Carbon) Content and Isotopic Composition of the Particulate		
Organic Matter	74	
3.4.5.1. Background	74	
3.4.5.2. Sample Preparation	76	
3.4.5.3. Instrument Performation and Calibration	76	
3.4.6. The <sup>13</sup> C-Technique	80	
3.4.6.1. Background	80	
3.4.6.2. Interpretation of the <sup>13</sup> C-Technique	80	
3.4.6.3. Errors Inherent to the Technique	82	
3.4.6.4. Experimental Procedure	84	
3.4.6.5. Calculation of Primary Production Rates	84	

#### **CHAPTER IV**

MENAI STRAIT SEASONAL STUDY		
4.1. Measured Physical and Chemical Features of the Studied Environment	88	
4.1.1. Temperature	88	
4.1.2. Dissolved Inorganic Nitrogen ,	89	
4.2. Whole Community Related Characteristics and Metabolic Processes	90	
4.2.1. Chlorophyll	90	
4.2.2. Particulate Organic Matter	91	
4.2.2.1. Measurements in 1995	91	

	4.2.2.2. Measurements in 1994
	4.2.3. Oxygen Flux Measurements: Gross and Net Community Production and
	Dark Respiration
	4.2.4. Uptake of "N-Nitrate and "N-Ammonium
	4.2.4.1. Determinations in 1995
	4.2.4.2. Determinations in 1994
	4.2.5. "f Ratio"
	4.2.6. <sup>13</sup> C Uptake
	4.2.6.1. Determinations in 1995
	4.2.6.2. Determinations in 1994
	4.2.7. C:N Uptake Ratio
4.3.	Picoplanktonic (<2um) Characteristics and Metabolic Processes
	4 3 1 Bacteria Abundance
	4.3.2 Chlorophyll Content of the Picoplanktonic Fraction
	4.3.3 Oxygen Gross and Net Production and Dark Respiration
	4.3.4. Destiguilate Organic Carbon and Nitrogen Contents of the Sum Fraction
	4.5.4. Faithculate Organic Carbon and Mitrogen Contents of the \2µm Flaction
	4.5.4.1. Measurements in 1995
	4.5.4.2. Measurements in 1994
	4.3.5. Uptake of "N-Nitrate and "N-Ammonium by the Picoplankton
	4.3.5.1. Uptake Over the Diel Cycle
	4.3.5.2. Uptake in Dark Only Conditions
	4.3.6. <sup>13</sup> C Uptake by the Picoplankton
	4.3.6.1. Uptake Over the Diel Cycle
4.4.	4.3.6.2. Uptake in Dark Only Conditions
4.4.	4.3.6.2. Uptake in Dark Only Conditions 4.3.7. C:N Uptake Ratio Characterisation of the Four Phases of the Menai Strait Seasonal Cycle: a Summary
4.4. 4.5.	4.3.6.2. Uptake in Dark Only Conditions 4.3.7. C:N Uptake Ratio Characterisation of the Four Phases of the Menai Strait Seasonal Cycle: a Summary Discussion
4.4. 4.5.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li></ul>
4.4. 4.5.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li></ul>
4.4. 4.5.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li></ul>
4.4. 4.5.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li></ul>
4.4. 4.5.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li> <li>4.3.7. C:N Uptake Ratio</li> <li>Characterisation of the Four Phases of the Menai Strait Seasonal Cycle: a Summary</li> <li>Discussion</li> <li>4.5.1. Seasonal Variation of the Nitrogenous Nutrition of Menai Strait's Phytoplanktonic Community</li> <li>4.5.1.1. Relative Uptake of Nitrate and Ammonium: the Calculation of "f Ratios"</li> <li>4.5.1.2. Relationship Between Regenerated Production and Bacterial Respiration</li> </ul>
4.4. 4.5.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li></ul>
4.4. 4.5.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li> <li>4.3.7. C:N Uptake Ratio</li> <li>Characterisation of the Four Phases of the Menai Strait Seasonal Cycle: a Summary</li> <li>Discussion</li> <li>4.5.1. Seasonal Variation of the Nitrogenous Nutrition of Menai Strait's Phytoplanktonic Community</li> <li>4.5.1.1. Relative Uptake of Nitrate and Ammonium: the Calculation of "f Ratios"</li> <li>4.5.1.2. Relationship Between Regenerated Production and Bacterial Respiration</li> <li>4.5.2. Dark Uptake of Nitrate and Ammonium</li> <li>4.5.3. Seasonal Variation of the Picoplankton Nitrogenous Nutrition</li> </ul>
4.4. 4.5.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li> <li>4.3.7. C:N Uptake Ratio</li> <li>Characterisation of the Four Phases of the Menai Strait Seasonal Cycle: a Summary</li> <li>Discussion</li> <li>4.5.1. Seasonal Variation of the Nitrogenous Nutrition of Menai Strait's Phytoplanktonic Community</li> <li>4.5.1.1. Relative Uptake of Nitrate and Ammonium: the Calculation of "f Ratios"</li> <li>4.5.1.2. Relationship Between Regenerated Production and Bacterial Respiration</li> <li>4.5.3. Seasonal Variation of the Picoplankton Nitrogenous Nutrition</li> <li>4.5.3.1. Characterisation of the Sum Fraction</li> </ul>
4.4. 4.5.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li> <li>4.3.7. C:N Uptake Ratio</li> <li>Characterisation of the Four Phases of the Menai Strait Seasonal Cycle: a Summary</li> <li>Discussion</li> <li>4.5.1. Seasonal Variation of the Nitrogenous Nutrition of Menai Strait's Phytoplanktonic Community</li> <li>4.5.1.1. Relative Uptake of Nitrate and Ammonium: the Calculation of "f Ratios"</li> <li>4.5.1.2. Relationship Between Regenerated Production and Bacterial Respiration</li> <li>4.5.3. Seasonal Variation of the Picoplankton Nitrogenous Nutrition</li> <li>4.5.3.1. Characterisation of the Contibution of the Heterotrophic</li> </ul>
4.4.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li> <li>4.3.7. C:N Uptake Ratio</li> <li>Characterisation of the Four Phases of the Menai Strait Seasonal Cycle: a Summary</li> <li>Discussion</li> <li>4.5.1. Seasonal Variation of the Nitrogenous Nutrition of Menai Strait's Phytoplanktonic Community</li> <li>4.5.1.1. Relative Uptake of Nitrate and Ammonium: the Calculation of "f Ratios"</li> <li>4.5.1.2. Relationship Between Regenerated Production and Bacterial Respiration</li> <li>4.5.3. Seasonal Variation of the Picoplankton Nitrogenous Nutrition</li> <li>4.5.3.1. Characterisation of the Contibution of the Heterotrophic Component of the &lt;2µm Fraction (Bacteria) to the Total Uptake</li> </ul>
4.4. 4.5.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li> <li>4.3.7. C:N Uptake Ratio</li> <li>Characterisation of the Four Phases of the Menai Strait Seasonal Cycle: a Summary</li> <li>Discussion</li> <li>4.5.1. Seasonal Variation of the Nitrogenous Nutrition of Menai Strait's Phytoplanktonic Community</li> <li>4.5.1.1. Relative Uptake of Nitrate and Ammonium: the Calculation of "f Ratios"</li> <li>4.5.1.2. Relationship Between Regenerated Production and Bacterial Respiration</li> <li>4.5.3. Seasonal Variation of the Picoplankton Nitrogenous Nutrition</li> <li>4.5.3.1. Characterisation of the &lt;2μm Fraction</li> <li>4.5.3.2. Seasonal Variation of the &lt;2μm Fraction</li> </ul>
4.4.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li> <li>4.3.7. C:N Uptake Ratio</li> <li>Characterisation of the Four Phases of the Menai Strait Seasonal Cycle: a Summary</li> <li>Discussion</li> <li>4.5.1. Seasonal Variation of the Nitrogenous Nutrition of Menai Strait's Phytoplanktonic Community</li> <li>4.5.1.1. Relative Uptake of Nitrate and Ammonium: the Calculation of "f Ratios"</li> <li>4.5.1.2. Relationship Between Regenerated Production and Bacterial Respiration</li> <li>4.5.3. Seasonal Variation of the Picoplankton Nitrogenous Nutrition</li> <li>4.5.3.1. Characterisation of the &lt;2µm Fraction</li> <li>4.5.3.2. Seasonal Variation of the &lt;2µm Fraction (Bacteria) to the Total Uptake of Inorganic Nitrogen</li> <li>4.5.3.3. Inorganic Nitrogen Uptake by Bacteria in Relation to that by the Phytoplankton</li> </ul>
4.4.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li></ul>
4.4.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li></ul>
4.4.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li> <li>4.3.7. C:N Uptake Ratio</li> <li>Characterisation of the Four Phases of the Menai Strait Seasonal Cycle: a Summary</li> <li>Discussion</li> <li>4.5.1. Seasonal Variation of the Nitrogenous Nutrition of Menai Strait's Phytoplanktonic Community</li> <li>4.5.1.1. Relative Uptake of Nitrate and Ammonium: the Calculation of "f Ratios"</li> <li>4.5.1.2. Relationship Between Regenerated Production and Bacterial Respiration</li> <li>4.5.3.1. Characterisation of the Picoplankton Nitrogenous Nutrition</li> <li>4.5.3.2. Seasonal Variation of the Contibution of the Heterotrophic Component of the &lt;2µm Fraction</li> <li>4.5.3.3. Inorganic Nitrogen</li> <li>4.5.3.4. Bacterial Inorganic Nitrogen Preference</li> <li>4.5.4. Relationship Between Carbon and Nitrogen uptake by the Phytoplankton and Bacterial Respiration</li> </ul>
4.4.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li> <li>4.3.7. C:N Uptake Ratio</li> <li>Characterisation of the Four Phases of the Menai Strait Seasonal Cycle: a Summary</li> <li>Discussion</li> <li>4.5.1. Seasonal Variation of the Nitrogenous Nutrition of Menai Strait's Phytoplanktonic Community</li> <li>4.5.1.1. Relative Uptake of Nitrate and Ammonium: the Calculation of "f Ratios"</li> <li>4.5.1.2. Relationship Between Regenerated Production and Bacterial Respiration</li> <li>4.5.3. Seasonal Variation of the Picoplankton Nitrogenous Nutrition</li> <li>4.5.3.1. Characterisation of the Contibution of the Heterotrophic Component of the &lt;2μm Fraction (Bacteria) to the Total Uptake of Inorganic Nitrogen</li> <li>4.5.3.4. Bacterial Inorganic Nitrogen Preference</li> <li>4.5.4.1. Algel Uptake</li> </ul>
4.4.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li> <li>4.3.7. C:N Uptake Ratio</li> <li>Characterisation of the Four Phases of the Menai Strait Seasonal Cycle: a Summary</li> <li>Discussion</li> <li>4.5.1. Seasonal Variation of the Nitrogenous Nutrition of Menai Strait's Phytoplanktonic Community</li> <li>4.5.1.1. Relative Uptake of Nitrate and Ammonium: the Calculation of "f Ratios"</li> <li>4.5.1.2. Relationship Between Regenerated Production and Bacterial Respiration</li> <li>4.5.3. Seasonal Variation of the Picoplankton Nitrogenous Nutrition</li> <li>4.5.3.1. Characterisation of the Contibution of the Heterotrophic Component of the &lt;2μm Fraction</li> <li>4.5.3.2. Seasonal Variation of the &lt;2μm Fraction (Bacteria) to the Total Uptake of Inorganic Nitrogen</li> <li>4.5.3.4. Bacterial Inorganic Nitrogen Preference</li> <li>4.5.4. Relationship Between Carbon and Nitrogen uptake by the Phytoplankton and Bacteria</li> </ul>
4.4.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li></ul>
4.4.	<ul> <li>4.3.6.2. Uptake in Dark Only Conditions</li></ul>

4.6.	Conclusions		203
------	-------------	--	-----

#### **CHAPTER V**

LABORATORY EXPERIMENTS	
5.1. Introduction	206
5.2. Experimental Strategy	209
5.2.1. Experiments in 1995	209
5.2.2. Experiments in 1996	212
5.3. Results and Discussion	215
5.3.1. Mineralisation of Organic Carbon	215
5.3.2. Bacteria as Users of Inorganic Nitrogen	221
5.3.3. Evidence for Competition	236
5.3.4. Variation of the Bacterial C:N ratio with the Composition of the Substrate	240
5.3.5. Summary and Conclusions	242

#### **CHAPTER VI**

GENERAL DISCUSSION AND CONCLUSIONS	244
6.1. Dynamics of the Bacterial and Algal Inorganic Nitrogen Metabolism in the Menai Strait	245
6.2. Environmental and Physiological Controls of Bacterial Uptake and Remineralisation of Nitrogen	249
6.3. Ecological Consequences of the Control of the Bacterial Roles in the Planktonic Community	253

#### APPENDIX

Experimental Study of the Retentiveness of Glass Fibre Filters ("Whatman" GF/F)

#### REFERENCES

#### **ACCOMPANYING MATERIAL**

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## **List of Figures**

#### CHAPTER I

- 1.1. Schematic diagram of inorganic nitrogen uptake across the phytoplankton cell membrane from aqueous pool into functional and storage components within the cell, which are then used in growth processes leading to the synthesis of cellular structural components. Adapted from Goldman & Glibert (1983).
- 1.2. Pathways of phytoplankton uptake and assimilation of different forms of nitrogen.
- 1.3. Simplified pelagic food web structure with I. classical food chain; II. 'microbial loop' and III. the microbial food web which includes the 'microbial loop' and autotrophic picoplankton and nanoplankton >5µm. Adapted from Lenz (1992).
- 1.4. Nitrogen cycling through the microbial food web. Adapted from Tupas & Koike (1991).

#### **CHAPTER II**

- 2.1. Location of the sampling area: the Menai Strait.
- 2.2. Annual variation of Menai Strait water temperatures. Monthly means and ranges were estimated from discontinuous measurements undertaken between 1969 and 1993, assembled in "The Menai Database".
- 2.3. Annual variation of Menai Strait water salinity. Monthly means and ranges estimated from discontinuous measurements assembled in "The Menai Database".
- 2.4. Secchi Disc readings from the Menai Strait. After Jones & Spencer, 1970.
- 2.5. Annual variation of inorganic nutrients in Menai Strait waters. Monthly means calculated from discontinuous measurements undertaken between 1975 and 1977, collected in "The Menai Database".
- 2.6. Annual variation of dissolved organic carbon in the waters of the Menai Strait. Monthly means and ranges estimated from Morris & Foster (1971).
- 2.7. Annual variation of chlorophyll a concentrations in Menai Strait waters. Monthly means calculated from discontinuous measurements undertaken between 1969 and 1993, collected in "The Menai Database".

#### **CHAPTER III**

- 3.1. Uptake of <sup>15</sup>N-ammonium and -nitrate by the whole community (WC) and "bacterioplankton" (<2µm size fraction), over a diel cycle.
- 3.2. Schematic representation of the experimental procedure applied for the measurement of <sup>15</sup>N uptake by the whole community (WC) and  $<2\mu$ m fraction. 3.3. Schematic diagram illustrating <sup>15</sup>N and <sup>13</sup>C analysis by an ANCA-MS. (Europa Scientific Ltd,
- Cheschire, United Kingdom).

#### **CHAPTER IV**

- 4.1. Menai Strait surface water temperature during 1994 and 1995. Comparison with historical data from 'The Menai Database'.
- 4.2. Seasonal variation of dissolved inorganic nitrogen in Menai Strait waters between 1993 and 1995.

- **4.3.** Seasonal variation of chlorophyll concentration for the years of 1994 (<200μm size fraction from Blight *et al.*, 1995 and author's own determinations) and 1995 (whole community).
- 4.4. Seasonal variation of the a) particulate organic carbon; b) particulate organic nitrogen and c) C:N ratio of the particulate matter of the Menai Strait waters, during 1995. Comparison with 1993 data from Blight *et al.*, (1995).
- **4.5.** Seasonal variation of **a**) gross and **b**) net community production and **c**) dark community oxygen respiration, during 1995. Comparison with 1994 data from Blight *et al.*, (1995) and author's own determinations.
- 4.6. Seasonal variation of the <sup>15</sup>N-nitrate uptake by the whole community a) over the diel cycle and b) in dark only conditions. Results from the 6 and 24 hour incubation experiments.
- 4.7. Seasonal variation of the <sup>15</sup>N-ammonium uptake by the whole community a) over the diel cycle and b) in dark only conditions. Results from the 6 and 24 hour incubation experiments.
- 4.8. a) Seasonal variation of the "f-ratio" during 1995. Estimations derived from the 6 and 24 hour incubation experiments. b) Analogy between the phases defined for the "f-ratio" and those for the net community production.
- **4.9.** a) Seasonal variation of <sup>13</sup>C uptake by the whole community over the diel cycle. b) Total, "new" and "regenerated" C production, with the "new" and "regenerated" C production calculated with the basis on estimated "*f*-ratios". c) Seasonal variation of <sup>13</sup>C uptake by the whole community in dark only conditions: absolute and relative (as percentage of C uptake over the diel cycle) estimates.
- **4.10.** a) Seasonal variation of the whole community uptake and compositional C:N ratio; b) detail of the lower part of a).
- **4.11.** Seasonal variation of bacteria abundance in the Menai Strait during 1995. Comparison with 1994 data from Blight *et al.*, (1995).
- 4.12. Seasonal variation of the chlorophyll absolute and relative contents (as a percentage of the whole community) in the <2μm size fraction.
- 4.13. Seasonal variation of the oxygen fluxes by the  $<2\mu$ m size fraction during 1995.
- 4.14. Seasonal variation of the <2μm a) absolute b) relative (percentage of the unfractionated matter) particulate organic carbon and nitrogen and c) of the corresponding C:N ratio. Comparison with data from 1994.</p>
- 4.15. a) Seasonal variation of the <sup>15</sup>N-nitrate uptake by the picoplankton over the diel cycle, in 1994 (24 hour incubations) and 1995 (6 and 24 hour incubation experiments); b) picoplanktonic uptake as a percentage of that by whole community.
- **4.15.c)** Relationship between the chlorophyll content and the uptake of nitrate by the  $<2\mu m$  size fraction.
- **4.15.** Seasonal variation of the picoplanktonic autotrophic and heterotrophic nitrate uptake over the diel cycle, in 1995. Results from the d) 6 and the e) 24 hour incubation experiment.
- **4.15.** Proportion of autotrophic and heterotrophic picoplanktonic nitrate uptake over the diel cycle during 1995. Results from the **f**) 6 and **g**) 24 hour incubation experiments.
- **4.15.** h) Seasonal variation of the contribution of the heterotrophic picoplanktonic (bacterial) nitrate uptake to the unfractionated uptake over the diel cycle, in 1995.
- 4.16. a) Seasonal variation of the <sup>15</sup>N-ammonium uptake by the picoplankton over the diel cycle, in 1994 (24 hour incubation experiments) and 1995 (6 and 24 hour incubation experiments); b) picoplankton uptake as a percentage of that by the whole community.
- **4.16.** Seasonal variation of the picoplankton autotrophic and heterotrophic ammonium uptake over the diel cycle, during 1995. Results from the c) 6 and d) 24 hour incubation experiments.
- **4.16.** Proportion of autotrophic and heterotrophic picoplanktonic ammonium uptake over the diel cycle during 1995. Results from the e) 6 and the f) 24 hour incubation experiments.
- **4.16.** g) Seasonal variation of the contribution of the heterotrophic picoplanktonic (bacterial) ammonium uptake to the unfractionated uptake over the diel cycle, during 1995.
- **4.17.** Seasonal variation of the relative heterotrophic uptake of nitrate in relation to the total heterotrophic inorganic nitrogen uptake (nitrate + ammonium), over the diel cycle, in 1995. Results from the 6 and 24 hour incubation experiments.

- **4.18.** Seasonal variation of the contribution of the heterotrophic uptake (bacterial) to the total unfractionated nitrogen uptake over the diel cycle, in 1995. Results from the 6 and 24 hour incubation experiments.
- 4.19. a) Seasonal variation of the <sup>15</sup>N-nitrate uptake by the picoplankton in dark only conditions, in 1994 (24 hour incubation experiments) and 1995 (6 and 24 hour incubations): b) picoplankton uptake as a percentage of that by the whole community.
- **4.19.** Seasonal variation of the picoplanktonic autotrophic and heterotrophic nitrate uptake in the dark, during 1995. Results from the c) 6 and d) 24 hour incubation experiments.
- **4.19.** Proportion of autotrophic and heterotrophic picoplanktonic nitrate uptake in dark only conditions during 1995. Results from the e) 6 and f) 24 hour incubation experiments.
- **4.19.** g) Seasonal variation of the contribution of the heterotrophic picoplanktonic (bacterial) nitrate uptake to the unfractionated uptake in dark only conditions, during 1995. Results from the 6 and 24 hour incubation experiments.
- 4.20. a) Seasonal variation of the <sup>15</sup>N-ammonium uptake by the picoplankton in the dark, during 1994 (24 hour incubations) and 1995 (6 and 24 hour incubations);: b) picoplankton uptake as a percentage of that by the whole community.
- **4.20.** Seasonal variation of the picoplanktonic autotrophic and heterotrophic ammonium uptake in the dark, during 1995. Results from the c) 6 and d) 24 hour incubation experiments.
- **4.20.** Proportion of autotrophic and heterotrophic picoplanktonic ammonium uptake in dark only conditions during 1995. Results from the **e**) 6 and **f**) 24 hour incubation experiments.
- **4.20.** g) Seasonal variation of the contribution of the heterotrophic picoplanktonic (bacterial) ammonium uptake to the unfractionated uptake in dark only conditions, during 1995.
- **4.21. a)** Seasonal variation of <sup>13</sup>C uptake by the total and the heterotrophic picoplankton over the diel cycle during 1995; contribution of the latter to the unfractionated uptake.
- **4.21.** b) Contribution of the heterotrophic (bacterial) and autotrophic picoplanktonic inorganic carbon uptake to the total uptake by the <2μm fraction, over the diel cycle, in 1995.
- **4.22.** a) Seasonal variation of <sup>13</sup>C uptake by the total and the heterotrophic picoplankton in the dark, during 1995; contribution of the latter to the unfractionated uptake.
- **4.22.** b) Contribution of the heterotrophic (bacterial) and autotrophic picoplanktonic uptake to the total uptake by the  $<2\mu m$  fraction, in the dark, during 1995.
- **4.23. a)** Seasonal variation of the <2μm fraction C:N uptake ratio over the diel cycle, during 1995. Comparison with <2μm POM compositional C:N ratio.
- 4.23. b) Seasonal variation of the bacterial C:N uptake ratio during 1995.
- 4.24. Relationship between percent "f-ratio" and ambient nitrate concentration in the Menai Strait.
- **4.25.** Relationship between nitrate uptake by the whole community and the ambient concentration of ammonium.
- **4.26.** a) Seasonal variation of ammonium and nitrate RPI of Menai Strait phytoplankton. b) Relationship between nitrate RPI and ambient ammonium concentration.
- 4.27. Seasonal variation of the chlorophyll-specific nitrogen uptake rates.
- **4.28.** a) Relationship between ammonium based production and bacterial respiration. b) Detail of graph a) showing linear relationship between these two parameters for the points outside the ellipse.
- 4.29. Proportion of the bacterial and algal dark uptake of a) ammonium and b) nitrate.
- 4.30. Seasonal variation of the proportion of ammonium and nitrate assimilated by a) bacteria and b) algae, in the dark.
- **4.31.** Seasonal variation of the contribution of the bacterial uptake to the total unfractionated inorganic N utilised.
- **4.32.** Seasonal variation of the algal and bacterial ammonium and nitrate uptake in the Menai Strait, during 1995.
- **4.33.** a) Seasonal variation of ammonium and nitrate RPI for Menai Strait bacteria. b) Relationship between bacterial nitrate RPI and ambient ammonium.
- **4.34.** Comparison between C:N composition and uptake ratio for the Menai Strait phytoplankton in 1995.

- **4.35.** Relationship between **a**) POC and **b**) PON and chlorophyll concentrations in the Menai Strait during the 1995 seasonal cycle.
- **4.36.** Seasonal variation of the algal and bacterial **a**) diel and **b**) dark uptake of inorganic C and **c**) of the inferred percent "transference" of algal assimilated carbon to the bacteria.
- **4.37.** a) Seasonal variation of the total N and C uptake by the bacteria and resulting C:N uptake ratios, over the diel cycle and b) in the dark.
- **4.38.** Relationship between the observed carbon assimilation by Menai Strait bacteria and that predicted by Romanenko's (1963) and Bryan's (1979) ratios of CO<sub>2</sub>:O<sub>2</sub> uptake.

#### **CHAPTER V**

- 5.1. a) *Hypothesis I.* Control of inorganic nitrogen uptake by bacteria by the C:N ratio of the DOM and cell C:N quota. Model of conservation of a constant proportion of carbon and competition for nitrogen.
- 5.1. b) Hypothesis II. Bacteria inorganic nitrogen metabolism is not controlled by the C:N ratio of the DOM substrate and cell C:N quota. Model of disposal of excess carbon determining bacteria growth yield.
- 5.2. Variation with incubation time of the net uptake of <sup>13</sup>C by the "bacterial" fractions in 1995 (a to c) and 1996 (d & e) laboratory experiments.
- **5.3.** a) to d) Variation of the bacterial abundance in high and low C:N ratio substrates during a 72 hour incubation.
- **5.4. a)** to **c)** Temporal variation of the fractionated and unfractionated particulate organic carbon in 1995 and 1996 Experiments.
- 5.5. Uptake of inorganic nitrogen by the whole community and "bacterial" fractions in substrates of high, low and "balanced" C:N ratio.
- 5.6. Uptake of ammonium and nitrate by the whole community (wc) and the <2μm fraction in 1995 "control" Experiment. Temporal variation of the <sup>15</sup>N-atom% in the organic particulate nitrogen.
- 5.7. a) Production and b) uptake of ammonium in substrates of low and high C:N ratio.
- **5.8.** Temporal variation of the unfractionated chlorophyll concentrations in high and low C:N ratio substrates and in "control" incubations carried out during the 1996 Experiments.
- 5.9. Temporal variation of the C:N ratio of the "bacterial" particulate matter in 1995 and 1996 Experiments, in substrates of high (a to d), low (a to d) and "balanced" (e) C:N ratio.
- 5.10. Variation of the bacteria C:N ratio with the C:N composition of the available substrate.

#### CHAPTER VI

- 6.1. Seasonal variation of the algal and bacterial uptake of inorganic N.
- 6.2. Seasonal variation of ammonium and nitrate turnover rates.
- 6.3. Uptake and inferred production of ammonium by bacteria, from May until July.
- 6.4. Schematic diagram to illustrate relationship among nitrate uptake by heterotrophic bacteria, new production and N export. From Kircham *et al.*, (1992).

## List of Tables

#### **CHAPTER I**

- 1.1. Distribution of inorganic nitrogen species in sea water.
- 1.2. Postulated transport mechanisms for uptake of nitrogen by the phytoplankton.

#### **CHAPTER II**

2.1. Variation of the mean tidal range in the Menai Strait, using Holyhead as port of reference.

#### **CHAPTER III**

- **3.1.** Addition of <sup>15</sup>N-ammonium and -nitrate according to the ambient concentration of these nutrients in the seawater.
- 3.2. Reproducibility of <sup>15</sup>N-atom % (0.3663) in reference material of varying nitrogen content.
- 3.3. Reproducibility of <sup>13</sup>C-atom % (1.108) in reference material of different carbon content.

#### **CHAPTER IV**

- **4.1.** Particulate organic carbon and nitrogen measurements undertaken during 1994 and C:N ratio of the particulate matter.
- **4.2.** <sup>15</sup>N uptake rates ( $\mu$ M d<sup>-1</sup>) by the whole community, over the diel cycle and in dark only conditions in 1994.
- **4.3.** <sup>13</sup>C uptake rates ( $\mu$ M d<sup>-1</sup>) by the whole community, over the diel cycle and in dark only conditions in 1994.
- **4.4.** Contribution of the  $<2\mu$ m fraction inorganic nitrogen uptake to that by the whole community and of the bacterial to the total picoplanktonic and unfractionated uptake over the diel cycle, during the PNAPh.
- **4.5.** Contributions of the  $<2\mu$ m fraction inorganic nitrogen uptake to that by the whole community and of the bacterial to the picoplanktonic and unfractionated uptake in dark only conditions, during the PNAPh.
- **4.6.** C:N uptake ratios by the whole community, total picoplankton and heterotrophic component of the  $<2\mu$ m size fraction during the PNAPh.
- **4.7.** Distribution of the uptake of ammonium and nitrate between the whole community, total picoplankton and bacteria over the diel cycle, during the NHPh.
- **4.8.** Distribution of the uptake of ammonium and nitrate between the whole community, total picoplankton and heterotrophic picoplankton in dark only conditions, during the NHPh.
- **4.9.** Contributions of the  $<2\mu$ m fraction <sup>13</sup>C uptake to that by the whole community and of the heterotrophic to the picoplanktonic and unfractionated uptake over the diel cycle and in the dark only conditions, during the NHPh.
- **4.10.** C:N uptake ratios by the whole community, total picoplankton and heterotrophic component of the  $<2\mu$ m size fraction (bacteria) during the NHPh.
- **4.11.** Contributions of the  $<2\mu$ m fraction inorganic N uptake to that by the whole community and of the bacteria to the total picoplanktonic and unfractionated uptake over the diel cycle, during the SNAPh.
- **4.12.** Contributions of the  $<2\mu$ m fraction inorganic N uptake to that by the whole community and of the bacterial to the picoplanktonic and unfractionated in dark only conditions, during the SNAPh.

- **4.13.** Contributions of the  $<2\mu$ m fraction <sup>13</sup>C uptake to that by the whole community and of the heterotrophic uptake to the picoplanktonic and unfractionated uptake over the diel cycle and in the dark only conditions, during the SNAPh
- **4.14.** Contributions of the  $<2\mu$ m fraction inorganic N uptake to that by the whole community and of the bacterial uptake to the picoplanktonic and unfractionated uptake over the diel cycle, during the RPh.
- **4.15.** Contributions of the  $<2\mu$ m fraction inorganic N uptake to that by the whole community and of the bacterial to the picoplanktonic and unfractionated in dark only conditions, during the RPh.
- **4.16.** Contributions of the  $<2\mu$ m fraction <sup>13</sup>C uptake to that by the whole community and of the heterotrophic uptake to the picoplanktonic and unfractionated uptake over the diel cycle and in the dark only conditions, during the RPh.
- **4.17.** C:N uptake ratios by the whole community, total picoplankton and heterotrophic component of the  $<2\mu$ m size fraction, during the RPh.
- **4.18.** Comparison of the values of  $f_{max}$  and m for the asymptotic curves relating "new production" and ambient concentrations of nitrate, obtained in the present study and those reported in the literature.
- **4.19.** Calculation of the predicted heterotrophic C fixation in the dark, from bacterial O<sub>2</sub> consumption, with basis on Romanenko's (1964) and Bryan's (1979) ratios.

#### **CHAPTER V**

- 5.1. Summary of the predicted metabolic and/or ecological consequences of the two proposed hypotheses.
- 5.2. Chemical experimental conditions at the start of the 1995 Experiments.
- 5.3. Chemical experimental conditions at the start of the 1996 Experiments.
- 5.4. Uptake rates (mean  $\pm$  se) of total inorganic nitrogen by the whole community and "bacterial" fractions during the surge uptake phase.
- 5.5. "Bacterial" uptake of organic C in high C:N ratio substrates and corresponding inorganic N demand, actual N availability and utilisation.
- 5.6. Percentage of <sup>15</sup>N recovered in the PON at the end of the rapid uptake phase, in relation to that added (mean  $\pm$  se).
- 5.7. Percent recovery of <sup>15</sup>N in the particulate matter in relation to that added, after one hour incubation (mean  $\pm$  se).
- 5.8. Average C:N composition of the "bacterial" particulate matter at each Experiment carried out.

#### **CHAPTER VI**

- 6.1. Bacterial ammonium and oxygen metabolism in the Menai Strait from May until July.
- **6.6.** Mean composition of the primary metabolites of marine phytoplankton estimated by Anderson (1995).

#### APPENDIX

I. Effectiveness of GF/F filters to retain bacteria from unfractionated (whole community) and fractionated seawater subsamples of varying volumes, given as the percentage of cells trapped in the filter in relation to the number of cells in the pre-filtered seawater.

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### "There are more things in heaven and earth (...), Than are dreamt of in your philosophy."

(from "Hamlet" by W. Shakespeare)

"Science must begin with myths, and with the criticism of myths." (Karl Popper)

## CHAPTER I INTRODUCTION

Nitrogen is a vital element. As a central component of two essential classes of biochemicals, proteins and nucleic acids, nitrogen is a logical choice to measure the growth of planktonic organisms such as phytoplankton and bacteria. Measurements of population growth using nitrogen may, in fact, show less scatter than using carbon or phosphorus because the latter two elements are also associated with non-growth processes such as storage.

Nitrogen is essential to marine life. As it is more closely associated with the vital processes in the cell, its transfer through the food chain is arguably of greater fundamental significance than the more commonly studied element - carbon. The spatial and temporal distribution of the biologically reactive, inorganic forms of this element (principally that of nitrate) is believed to determine to a large extent the global distribution of oceanic and coastal productivity, giving rise to the notions of oligotrophy and eutrophy, the former being associated with areas of low concentrations of inorganic nitrogen and primary productivity and the latter with the opposite situation. There are, one recognises, seemingly legitimate claims for chemical factors limiting production, such as iron (Martin *et al.*, 1990a, 1990b; Cullen, 1991), and a resurgence of the view of phosphorus as a limiting nutrient (Thingstad, 1987; Thingstad & Rassouzadegan, 1995; Thingstad *et al.*, 1998).

This thesis deals specifically with the dynamics of inorganic nitrogen (in particular nitrate and ammonium) in coastal waters. In the following sections of this Introduction and subsequent experimental parts, less attention will be given to the distribution of dinitrogen and the organic forms of nitrogen or to systems other than coastal.

### **1.1. SOURCES AND DISTRIBUTION OF INORGANIC NITROGEN IN SEA WATER**

Nitrogen is introduced to the marine environment by waste water disposal, river transport, microbiological fixation and by direct deposition from the atmosphere onto the sea surface. It is removed as gases (both dinitrogen and ammonia) by diffusion across the sea surface into the atmosphere and is permanently trapped in various organic forms in the sediments. Nitrogen occurs in the sea primarily in five oxidation states:  $-3(NH_4^+; NH_2)$ ,  $0(N_2)$ ,  $+2(NO_2)$ ,  $+3(NO_2)$ , and  $+5(NO_3)$ . The most abundant species in the sea is dinitrogen but has low biological reactivity and as a consequence is found throughout most oceans and coastal waters at very near saturation values (Kester, 1975). Neglecting the organic forms, the next most abundant nitrogen species is the biologically reactive molecule, nitrate. The other bioactive inorganic forms; nitrite and ammonium, are generally less abundant but can be of local significance. Table 1.1 summarises the distribution of the major inorganic nitrogen species in different marine systems.

**Table 1.1.** Distribution of inorganic nitrogen species in sea water. Adapted from Sharp (1983). Concentrations in  $\mu$ M.

N species	Ocean Surface (0-100m)	Deep Ocean (>100m)	Coastal Waters	Estuarine waters
N <sub>2</sub>	800	1150	700-1100	700-1100
NO3	0.2	35	0-30	0-350
$NO_2$	0.1	<0.1	0-2	0-30
$\mathrm{NH_4}^+$	<0.5	<0.1	0-25	0-600

Biological processes are dominant in the interconversion of the various nitrogen species in the sea and, in turn, all marine organisms are dependent, directly or indirectly upon the stocks of inorganic nitrogen in the sea for the synthesis of many of their components. Thus, ambient concentrations represent the balance between rates of supply and consumption. Low concentrations can mask a rapid flux of nitrogen through the primary producers and high turnover rates of the dissolved inorganic nutrients pools.

Nitrate is the thermodynamically stable form of combined inorganic nitrogen in well oxygenated waters and the final oxidation product of nitrogen compounds in the sea water. The concentration of this nutrient in the euphotic zone is governed by the advective transport of nitrate into surface layers, the microbial oxidation and the uptake of primary producers. If light penetration into the water is sufficient and temperature and plankton biomass adequate, uptake rates are generally faster than the processes transporting nitrate into the surface layers; as a consequence, nitrate concentrations on occasions may be close to the measurable zero, with the biology controlling small scale variations. In coastal areas nitrate varies from undetectable during bloom situations to >100 $\mu$ M in estuaries of rivers draining agricultural and urbanised areas (Spencer, 1975; Sharp, 1983). Of all inorganic nitrogen forms the most investigated is nitrate as it is a limiting nutrient for the growth of marine phytoplankton.

Studies since the early work of Cooper (1933, 1938) and Atkins *et al.* (1954), have shown that in temperate coastal areas, the distribution of this ion usually shows a predictable seasonal pattern characterised by accumulation during winter due to the upward flux of nitrate from deep-ocean reserves and absence or much reduced uptake by the autotrophs. With the onset of spring, density stratification markedly reduces the vertical diffusion of nitrate and the increased utilisation of this nutrient in surface waters by the vernal blooming of phytoplankton results in a summer nitrate minimum. The breakage of the stratification leading to the upward mixing of nitrate from deeper waters to the surface may result in a late summer/autumn phytoplankton bloom, of smaller magnitude than that in the spring.

Nitrite is the intermediate oxidation state between ammonium and nitrate, therefore it can appear as transient in both the oxidation of ammonium and the reduction of nitrate. Both of these processes are predominantly biologically mediated although some photochemical reduction can also occur (Hamilton, 1964; Spencer, 1975 and references cited therein). In addition, nitrite may be excreted by the phytoplankton especially during periods of luxury feeding. Natural levels of nitrite are usually very low in comparison with nitrate ( $\leq 5\%$  of the nitrate level). In some shallow estuaries (McCarthy *et al.*, 1984), in upwelling areas and in the vicinity of sewage outfalls nitrite concentrations can increase substantially (Grasshoff, 1983b; Sharp, 1983 and references cited therein). Near the base of the euphotic zone, a nitrite maximum is often registered in both the Pacific and Atlantic Oceans (McCarthy, 1980). With a few exceptions (McCarthy *et al.*, 1977), the importance of this nutrient in phytoplankton nitrogenous nutrition has been generally ignored.

The lack of a simple method to measure accurately the concentrations of ammonium at the often low concentrations in the environment, has greatly impeded research on the dynamics of this nutrient in the marine environment. Despite this, it has been observed that for oceanic, neritic, unpolluted estuarine and freshwater environments, ammonium concentrations are normally present in lower concentrations than other nitrogenous nutrients, varying considerably both temporally and spatially (McCarthy, 1980). With terrigenous input, often from sewage, ammonium can reach 20-25 µmol dm<sup>-3</sup> in open coastal waters (Sharp, 1983 and references cited therein). Soluble and particulate organic nitrogen compounds resulting from decaying organisms, together with those excreted by plants and animals, are rapidly decomposed to ammonium by proteolytic bacteria; ammonia is also excreted by animals together with urea and peptides. Additionally, bacterial denitrification of nitrate in anoxic conditions leads to the production of ammonium in addition to dinitrogen (Grasshoff, 1983a). Subsequent to a period of high productivity, ammonium can be the most abundant form of inorganic nitrogen in surface waters, after phytoplankton have removed the more oxidised species such as nitrate. As a consequence, for phytoplankton, ammonium is often the most important source of inorganic nitrogen sustaining their growth during summer, when nitrate is depleted.

# **1.2. PHYTOPLANKTONIC UTILISATION OF INORGANIC NITROGEN.**

It was the German chemist Liebig (1840) who probably first recognised the nitrogen requirement for plant growth, but the study of the nitrogen metabolism of algae may be considered to have started with the work of Warburg and Negelein (1920), who showed that light stimulated nitrate reduction by the alga *Chlorella*. Since then, studies of algal metabolism have been published at an ever-increasing rate. Landmarks in this field of research have been the proposal that nitrogen is probably the most important element limiting biological production in large portions of the world's ocean (Ryther & Dunstan, 1971) and the introduction of the concepts of "new" and "regenerated" production (Dugdale & Goering, 1967). The most important innovation that has contributed to our present knowledge about the utilisation and recycling of nitrogen compounds has been the introduction of stable isotope techniques in the study of marine nitrogen dynamics, namely the <sup>15</sup>N, introduced by Dugdale and co-workers (Dugdale & Goering, 1967; Dugdale, 1967). This technique will be considered at length in CHAPTER III.

#### **1.2.1.** The Kinetics of Nutrient Uptake

Contemporary ideas on phytoplankton nitrogen kinetics for the most part stem from the work of Droop (1968, 1970, 1974 and other work by this author reviewed in Droop, 1983) and Dugdale (1967) in which it was proposed that rates of phytoplankton nutrient uptake could be described by a rectangular hyperbolic equation. This concept had far-reaching implications towards a more complete understanding of how phytoplankton compete for nutrients, which nutrients may be limiting, and how nutrient availability, either through physical transport processes or biologically regeneration processes, controls the growth and primary production rates of marine populations.

Dugdale (1967; Dugdale & Goering, 1967), with natural populations of marine phytoplankton and Eppley et al., (1969), with pure cultures, showed that the relationship

between rate of nitrate (or ammonium) uptake and the concentration of the nutrient was a hyperbolic curve and could be described by an equation of the following type

$$V = V_{max} [N / K_s + N]$$
 (1.1)

where  $V_{max}$  is the nutrient-specific uptake rate V at infinite substrate concentration; N is the concentration of the limiting nutrient;  $K_s$  is the half-saturation constant, the concentration of limiting nutrient at which  $V = V_{max}/2$ .

Equation (1.1), which has origins in gas and enzyme kinetics, was originally used by Monod (1942) to describe the relationship between bacterial growth rates and limiting concentration of organic substrate. The original Monod equation was defined as

$$\mu = \mu_{\max}[S/K_u + S] \tag{1.2}$$

in which  $\mu$  is the specific growth rate;  $\mu_{max}$  is the maximum specific growth rate; S is the concentration of the limiting substrate and  $K_u$  is the concentration of S for which  $\mu$ = 0.5 $\mu_{max}$ . Although there is no arithmetic difference between equations (1.2) and (1.1) they describe dissimilar physiological processes, the latter defining only the rate by which a particular nutrient is incorporated into the cell from the surrounding aqueous medium (**uptake**) and the former defining the net sum of all physiological processes, including the incorporation of the material taken up into the cell's components (**assimilation**), cell division and biomass increase (**growth**) (Figure 1.1).

In the present work the terms 'uptake' and 'assimilation' will be used interchangeably even though the author is aware that the uptake of nutrients is not always accompanied by their assimilation into amino acids leading to growth; rather, the inorganic nitrogen taken up can be stored into reserve components (Figure 1.1).

Our understanding of the manner in which nutrient uptake and assimilation and growth are coupled and regulated are still too incomplete for the distinction to be made with certainty in field studies (Eppley, 1981). Only under steady-state conditions is  $\mu = V$ ,  $K_u = K_s$  and  $\mu_{max} = V_{max}$ . However, in a natural system, the unique conditions of balanced uptake and growth are rarely met, *i.e.*, the external nutrient concentration is rarely in equilibrium with the sum of all storage, functional and structural components comprising the cellular

nutrient mass, even more if it is considered that the nutritional status of phytoplankton in most of the marine systems is that of deficiency. Thus, uncoupling between nutrient uptake and population growth may be observed (Eppley & Renger, 1974; McCarthy & Goldman, 1979; Goldman, 1980; McCarthy, 1981; Eppley, 1981).



Figure 1.1. Schematic diagram of inorganic nitrogen uptake across the phytoplankton cell membrane from aqueous pool into functional and storage components within the cell, which are then used in growth processes leading to the synthesis of cellular structural components. Adapted from Goldman & Glibert (1983).

Despite assuming steady-state conditions, Dugdale's model has been used widely by phytoplankton ecologists to describe the affinity of a phytoplankton species for a given nutrient using only the parameters  $K_s$  and  $V_{max}$ : for example, a combination of low  $K_s$  and high  $V_{max}$  conceptually indicates high nutrient affinity whereas low nutrient affinity would be represented by high  $K_s$  and low  $V_{max}$ , in the classical ecological terms respectively r-and K- strategies (MacArthur & Wilson, 1967; Pianka, 1970; Hairston *et al.*, 1970; Kilham & Kilham, 1980)<sup>1.1.</sup>

<sup>&</sup>lt;sup>1.1.</sup> r stands for the intrinsic growth rate and K, the upper asymptote of the hyperbolic growth curve of a species (*i.e.* the point at which a resource becomes absolutely limiting). r - selected species have a high growth rate and can respond quickly to the availability of environmental resources but do not and cannot maintain their maximum density for long; on the other hand, K - selected species grow slower and have an ability to tolerate or accomodate to periods of resource-stress. The former may be recognised as colonising, oportunistic species whilst the latter will be autochthonous, in a constant equilibrium.

### 1.2.2. The Enzymes of Nutrient Uptake and Assimilation

In their nitrogenous nutrition, phytoplankton can use a number of nitrogen forms, namely nitrate, ammonium, nitrite and urea, with the first two being those predominantly taken up in the great majority of marine environments.

In view of the very low concentrations of available nitrogen and the common occurrence of saturation kinetics, it has been presumed that phytoplankton possess efficient membrane transport for nitrogenous nutrients; in Table 1.2, the transport systems for inorganic and organic nitrogen, in phytoplankton cells are summarised.

In general, there is a preference for the most reduced forms and the preference order ammonium > urea > nitrite > nitrate has been reported from several offshore, coastal and freshwater environments (Eppley *et al.*, 1971, 1973, 1977; Kanda *et al.*, 1985; McCarthy *et al.*, 1977, 1982; Paasche & Kristiansen, 1982; Glibert *et al.*, 1982a,b; Furnas, 1983 among many others).

**Table 1.2.** Postulated transport mechanisms for uptake of nitrogen by the phytoplankton. Adapted from Wheeler, (1983).

N Source	Transport Mechanisms
ammonium	diffusion; mediated diffusion or secondary active transport
urea	diffusion and secondary active transport
amino acids	secondary active transport
nitrate	primary active transport

Diffusion is the non-specific passage of a solute down a chemical gradient and is usually characterised by linear uptake kinetics; mediated diffusion is also down a chemical gradient but is substrate-specific and characterised by saturable uptake kinetics; active transport is both substrate-specific and against a chemical gradient and is also characterised by saturable kinetics; the primary active transport system requires a direct input of energy whereas secondary active transport is driven by ion gradient established by a primary system (Wheeler, 1983).

Such preference can be anticipated on energetic grounds: in a very simplistic way, amino acid synthesis arising from the uptake of either nitrate or nitrite involve reducing transformations yielding ammonium as an end product (Figure 1.2) which is then converted into organic compounds. Two enzymes appear to be involved in the steps: nitrate reductase (NR) which affects the reduction of nitrate to nitrite and nitrite reductase (N<sub>i</sub> R) which





transferase. Pathways I and II are seen as alternative routes for the assimilation of ammonium into organic components. Pathway I is more direct, involving only the Figure 1.2. Pathways in phytoplankton uptake and assimilation of different forms of nitrogen. NR = Nitrate Reductase; NiR = nitrite reductase; GDH = Glutamate Dehydrogenase; GS = Glutamine Synthetase; U = Urease; UC = Urea Carboxylase; AH = Allophnate Hydrolase; GOGAT = Glutamate Synthetase; t-ase = aminoof ammonium into amino acids because GS has much higher affinity for ammonium than does GDH. However, the physiological and biochemical controls of these enzyme GDH and not requiring ATP. Pathway II involves enzymes GS and GOGAT, requiring ATP. It appears that the latter is the major route for the incorporation processes are not well understood (Syrett, 1981). Figure adapted from McCarthy (1980), with modifications. catalyses the reduction of nitrite to ammonium (McCarthy, 1980; Syrett, 1981). These reducing reactions are energetically expensive, the first step requiring two electrons and the second, six:

$$NO_3^- \xrightarrow{2e^-} NO_2^- \xrightarrow{6e^-} NH_4^+$$
 (1.3)

Therefore, it is not surprising that algae will take up preferentially those forms of nitrogen present in the environment already in a more reduced state which do not require energy expenditure to be assimilated.

Urea has been shown to be an excellent nitrogen source for natural populations of phytoplankton (McCarthy, 1972a,b; McCarthy *et al.*, 1977; Glibert *et al.*, 1991; Metzler *et al.*, 1997) needing to be metabolised into  $CO_2$  and ammonium before incorporation into organic materials, a reaction that is mediated by the enzyme urease:

$$CO(NH_2)_2 + H_2O \longrightarrow CO_2 + 2NH_3$$
(1.4)  
urease

In algae that do not possess this enzyme, urea conversion to  $CO_2$  and ammonium is catalysed by the enzymes urea carboxylase and allophnate hydrolase. The result is the same as that achieved by the urease reaction but at the expense of a molecule of ATP per molecule of urea (Syrett, 1981).

In Figure 1.2. the pathways of urea, nitrate, nitrite and ammonium uptake and of ammonium assimilation into amino acids is shown, as well as the enzymes responsible for catalysing those processes.

## **1.2.3.** Evaluation of Preference For a Given Form of Nitrogen: the Relative Preference Index

The above order of preference of nutrient utilisation by the phytoplankton has been evaluated based either on direct comparisons of uptake rates of different nitrogen sources measured with the <sup>15</sup>N technique or on the Relative Preference Index (RPI) introduced by

McCarthy *et al.*, (1977). This ratio is a way of describing nitrogen utilisation relative to its availability and is defined by the following expression:

$$RPI_{N1} = \frac{\rho_{N1} / \Sigma \rho_i}{[N_1] / \Sigma [N_i]}$$
(1.5)

where  $\rho N_1$  and  $\Sigma \rho_i$  are, respectively, the uptake rate of nutrient 1 and the sum of all the measured uptake rates; and  $[N_1]$  and  $\Sigma [N_i]$ , respectively the ambient concentrations of substrate 1 and the sum of the concentrations of all substrates considered. A value of unity for a particular nitrogen form indicates utilisation equitable with availability, a value in excess of unity reflects preference whereas a value lower than 1 means rejection. McCarthy *et al.*, (1977) calculated RPI values for four substrates: ammonium, nitrate, urea and nitrite. In the present work however, only ammonium and nitrate ambient concentrations and rates of uptake were determined, hence,

$$\sum \rho_i = \rho_{NO3} + \rho_{NH4}$$
, and

$$\sum[N_i] = [NO_3] + [NH_4].$$
(1.6)

The analysis of *in situ* uptake measurements and concentrations is, however, hampered by a number of factors:

- analytical difficulties due to the often extremely low nutrient concentrations in surface waters (Eppley et al., 1977);
- (2)- the fact that <sup>15</sup>N uptake measurements often require substrate additions which significantly affect ambient nutrient concentrations (Eppley *et al.*, 1977; Harrison, 1983a), thus producing results which represent something between natural uptake rates and the uptake potential of the community;
- (3)- the use of different pool sizes of nitrogenous nutrients in the RPI equation.

Points (1) and (2) will be further commented upon at a later stage (CHAPTER III); reason (3) is obviously problematic because the availability of a given nutrient during periods when primary production is based on regenerated forms of nitrogen such as ammonium, is more a function of supply of the nutrient through mineralisation than of concentration as such. During summer, when ambient levels of nutrients are depleted, in particular of nitrate, and the rapid regeneration of ammonium sustains primary production, the RPI will

tend to exaggerate preference for the nitrogen source which is rapidly supplied by regeneration processes. Consequently, if different nitrogen sources are not equally available hence precluding free selection from, true preference may be difficult to ascertain in natural conditions. It could, therefore, be argued that preference in a strict sense can be evaluated only experimentally by manipulating nutrient availability. This conceptual drawback has not hindered the application of the RPI to *in situ* studies and reports in the literature are numerous (Eppley *et al.*, 1977; Glibert *et al.*, 1982a,b; Paasche & Kristiansen, 1982; Goldman & Glibert, 1982; Cochlan, 1986, to name but a few), with the interpretation of the ratios not always cautious.

#### 1.2.4. "New" and "Regenerated" Production

The concept of nitrogen limitation in the sea was formalised in the now classical work of Dugdale & Goering (1967) from which emerged the distinction between "new" and "regenerated" production, according to the nitrogen form predominantly used by the autotrophs. "New" production is fuelled by allochthonous nitrogen sources, primarily nitrate, mixed into surface from the deep ocean reserves, and secondarily, nitrogen fixation, riverine and rainfall inputs; "regenerated" production is fuelled by autochthonous nitrogen sources, principally ammonium, derived from biological processes (nutrient recycling) occurring in *situ*. Thus, the distinction between these two forms of production is largely a discrimination between physically and biologically mediated supplies of nutrients for primary producers.

Dugdale & Goering (1967) suggested that the amount of production associated with "new" nitrogen utilisation would be equivalent to that available for export (mainly in the form of particle sinking and organic nitrogen transfer to higher trophic levels) from the photic zone. Eppley & Peterson (1979) developed this concept even further by suggesting the need for a balance to be maintained between the upward flux of nitrate into the euphotic zone and the downward flux of organic nitrogen out of the euphotic zone, if biomass and productivity were to be kept at near steady-state. In order to quantify the contribution of "new" production to the total primary production measured at a given area, Eppley & Peterson (1979) introduced the use of the so called f-ratio, defined as:

$$f = \rho_{\rm NO3} / (\rho_{\rm NO3} + \rho_{\rm NH4} + \rho_{\rm urea})$$
(1.7)

where  $\rho_{NO3}$ ,  $\rho_{NH4}$  and  $\rho_{urea}$  are the uptake rates of nitrate, ammonium and urea, respectively.

The concept of the f- ratio as a measure of the amount of "exportable production" is now established firmly in biological oceanography and in recent years it has assumed even greater significance because it also provides a measure of the upper limit of production of a marine ecosystem. In terms of carbon it equates to the amount of CO<sub>2</sub> that can be sequestered from the atmosphere with implications for the potential regulation of global warming (Owens *et al.*, 1991).

Eppley & Peterson (1979) showed also that broad oceanographic regions could be distinguished from each other by the proportion of "new" and "regenerated" production occurring there, with f values increasing from a value of 0.05 in extremely oligotrophic waters offshore to a value >0.5 in highly productive upwelling areas having an annual primary production in excess of 200g C m<sup>-2</sup>. Based on <sup>15</sup>N uptake measurements, it appears therefore, that whilst regenerated nitrogen is the predominant nitrogen source sustaining production in oceanic and inshore waters, nitrate is a much more important nitrogen form for the primary producers on the continental shelf. Furthermore, values of f can vary spatially and seasonally within a region, the most striking feature of the latter being the marked decrease in f values from early spring through summer and early autumn, indicating a shift from "new" to "regenerated" production during the primary production seasonal cycle (McCarthy *et al.*, 1977; Eppley *et al.*, 1979a,b; Olson, 1980; Glibert *et al.*, 1982a,b; Paasche & Kristiansen, 1982; Harrison *et al.*, 1983; Wheeler & Kokkinakkis, 1990; Dauchez *et al.*, 1991; Smith, 1993; Tamminen, 1995, and others).

The major limitation of the *f*-ratio concept arises when it is applied to coastal areas of less than 200m depth or inshore waters (Eppley & Peterson, 1979). When the bottom sediments are in direct contact with the water harbouring the growing phytoplankton, the distinction between "new" and "regenerated" nitrogen in the sense of Dugdale & Goering (1967) and Eppley & Peterson (1979) is no longer tenable because any nitrate released from the sediments would have been regenerated *in situ* and thus represented regenerated nitrogen. Conversely, ammonium could have an allochthnous origin, for instance from sewer outfall and land drainage, constituting in these cases "new" nitrogen. Thus, in coastal and inshore waters indices such as f merely serve to indicate the contribution of nitrate and ammonium (1-f) to the total nitrogen production. In such circumstances, it is more correctly to express production as being nitrate- or ammonium-based since terms like "new" and "regenerated" would be misleading.

# **1.3.** THE ROLE OF BACTERIA IN THE PELAGIC ECOSYSTEM

## **1.3.1.** 'Microbial Loop' and Microbial Food Web Versus the Classical Food chain

Classically the role attributed to heterotrophic bacteria has been that of decomposers of organic matter, returning inorganic nutrients to the environment. With the development of epifluorescence techniques to estimate bacteria abundance (Porter & Feig, 1980; Hobbie *et al.*, 1977) and methods which allowed bacterial production to be measured (Furhman & Azam, 1980), it became possible to obtain realistic estimates of these two parameters, leading to a re-examination of the role of heterotrophic bacteria in the sea. The proposal of the significance of the microbial food web (Pomeroy, 1974; Williams, 1981) and the introduction of the 'microbial loop' term by Azam *et al.*, (1983)<sup>1.2</sup>, lead to extensive research on the role of microbial activity within the classical food web (*e.g.*, Ducklow *et al.*, 1986), from which a new paradigm started to emerge on the importance of bacteria in cycling not only C but also N, in the world's marine systems. It now appears that the once perceived supremacy of the classical food chain which was maintained over many decades is shaken (Steele, 1974; Williams, 1981) and that the hitherto largely neglected bacteria and protists have taken the leading role in recent models of pelagic systems.

The term classical, traditional or conventional food wed (or chain) has been well established for a long time (Odum, 1959), and it involves the transference of particulate organic matter from the primary producers to the higher trophic levels (nekton) of the marine ecosystem, via grazing by micro-, meso- and carnivorous zooplankton (Figure 1.3).

<sup>&</sup>lt;sup>1.2</sup> Although the designation 'microbial loop' was coined by Azam *et al.*, (1983), the concept itself has its roots in syntheses by Pomeroy (1974) and Williams (1981).

Within this food chain concept, harvestable yield plays a great role and the number of food levels from the primary producers to commercially exploitable fish products determines together with environmental factors (nutrients, light and temperature), the fishery potential of aquatic ecosystems (Ryther, 1969). The classical food chain predominates in shelf seas and cold-water ecosystems and low latitude upwelling systems (Ryther, 1969). Its base is formed by the larger nano- and microphytoplankton species building up a standing stock which often accumulates to originate a bloom, such as the spring bloom in higher latitudes. The development of the phytoplankton grazers lags behind with the effect that the grazers, mainly copepods and euphausids, take advantage of the accumulated biomass. The accumulation of biomass is thus an important feature in the transfer of energy and matter to higher trophic levels.

The so called 'microbial loop' described by Azam et al., (1983) has bacteria as the vehicles transferring dissolved organic matter of photosynthetic origin (which otherwise would be lost to the system) to the higher trophic levels of the classical food chain, via grazing by heterotrophic nanoflagelates and ciliates (Figure 1.3). The conversion of dissolved organic C to particulate organic carbon, as mediated by heterotrophic bacteria, therefore, represents a microbial dissolved organic carbon retention or recovery system, in all likelihood optimising trophic transfer of fixed organic C. It is now regarded that bacteria can process a large fraction of primary production in many marine environments (15-50%; Larsson & Hagstrom, 1982; Lancelot & Billen, 1984; Cole et al., 1988, among many others) therefore, it is not surprising that Sherr & Sherr (1988) consider that the 'microbial loop' should be considered as a component and integral part of a larger microbial food web (Figure 1.3) which includes all pro- and eukaryotic unicellular organisms, both autotrophic and heterotrophic. From this point of view, the entire microbial food web and not simply the primary producers supports the metazoan food web. In this resides the most evident difference between the classical food chain and the new conceptual microbial food web. Furthermore, contrary to the classical food chain which is mostly controlled by the availability of nutrients, the microbial food web appears to be controlled by grazing activity (Thingstad et al., 1997).

The role of the 'microbial loop' is particularly important in rapidly recycling nutrients but, contrary to the classical view of bacteria as remineralisers, in this new paradigm, when

bacteria act as nutrient sinks, it is the heterotrophic flagellates and microzooplankton that play a major role in returning inorganic nutrients to the medium (Azam *et al.*, 1983), a view that had been proposed many years ago by Johannes (1965) and that only in the 1980s started to be re-examined and confirmed.



Figure 1.3. Simplified pelagic food web structure with I. classical food chain; II. 'microbial loop' and III. the microbial food web which includes the 'microbial loop' and autotrophic picoplankton and nanoplankton  $>5\mu$ m. Arrows of varying thickness indicate qualitative magnitude of the materials transference. Adapted from Lenz (1992).

The domain of the microbial food web are oligotrophic warm-water systems where regenerated production predominates although the microbial loop and autotrophic

picoplankton are present in all types of pelagic ecosystems. Inter-ecosystem variability on the predominant food chain in operation may give rise to diverging opinions among investigators on whether bacteria act mainly as 'links' ('microbial loop') or 'sinks' (classical view of remineralisers) for carbon in the flow of organic matter in the pelagic environment (Ducklow *et al.*, 1986; Sherr & Sherr, 1988;). This role variability has implications for carbon and nitrogen flux pathways and for predictive ecosystem models. Therefore, it is of paramount importance to uncover the causes and mechanisms of this variability, in particular the regulation of C and nitrogen into the bacteria since these fluxes are derived from varied pools of organic and inorganic materials which are distributed heterogeneously in space and time, being further influenced by biogeochemical, trophic and physiological interactions in the environment.

Whichever role predominates in a given system at a particular time, heterotrophic bacteria will have a large impact on the cycling of nitrogen in the sea because the ratios of bacterial to primary production are often very high (Cole *et al.*, 1988; Furhman *et al.*, 1989; Cho & Azam, 1990; Ducklow & Carlson, 1992), the compositional C:N ratio of bacteria is low (~ 4-5; Nagata, 1986) and bacteria account for nearly all DON (Azam & Hodson, 1977) and sometimes a large part of DIN uptake (Kirchman, 1994).

#### **1.3.2.** Bacterial Inorganic Nitrogen Uptake

Although there are relatively few studies of inorganic nitrogen utilisation by pelagic heterotrophic bacteria, it is now beyond doubt that these organisms at times can account for a large proportion of the total ammonium and, to a lesser extent, nitrate in the marine environment. The work of Kirchman and collaborators in particular, has been pivotal to the quantification and current understanding of the heterotrophic inorganic nitrogen uptake dynamics (Wheeler & Kirchman, 1986; Kirchman *et al.*, 1989, 1990, 1991; 1992, 1994; Keil & Kirchman, 1991; Kirchman, 1990, 1994) but the bacterial use of ammonium has been discussed for some time (*e.g.* Eppley *et al.*, 1977).

Initially, bacteria were thought to be unimportant utilisers of nitrate (Wheeler & Kirchman, 1986), with ammonium utilisation more ubiquitous However, evidence suggests the

possibility of high nitrate uptake (Horrigan *et al.*, 1988; Kirchman *et al.*, 1991) specially in the presence of additional supplies of dissolved organic compounds such as sugars and carbohydrates (Parsons *et al.*, 1981; Kirchman *et al.*, 1992). Nevertheless, of the two forms of nitrogen, nitrate is the least favoured nitrogen source, with bacteria using preferentially dissolved free amino acids (DFAA) followed by ammonium (Kirchman *et al.*, 1989, 1992). This order of preference is consistent with the energetics of using each nitrogen source for macromolecular synthesis: based on this argument,  $NO_3^-$  would be the least preferred nitrogen source since it must be reduced to the oxidation state of R-NH<sub>2</sub>, involving a group of reactions that are energetically expensive, as already described.

Uptake of inorganic nitrogen by heterotrophic bacteria is embedded in the concept of the 'microbial loop', leading to the first suggestions by Azam *et al.*, (1983) that in the pelagic marine ecosystem, the basic links of the microbial food web (heterotrophic bacteria and phytoplankton) may be competing for growth limiting nutrients, a concept that will be discussed in the next sections.

## **1.3.2.1.** Physiological Mechanisms of Bacterial Nitrogen Uptake and Assimilation

From a review by Billen (1984), it appears that the physiological mechanisms of bacterial nitrogen uptake and assimilation are identical to those described for phytoplankton. Active transport systems for both mineral and organic nitrogen compounds have been demonstrated in heterotrophic micro-organisms as well as in algae, although studies on the former organisms are fewer.

The reduction reactions of nitrate and nitrite to ammonium by the NR and NiR enzymes appear also to be present in bacteria as well as the two assimilation pathways of ammonium into amino acids, via the GS + GOGAT and GDH routes (Figure 1.2), with the former being much more efficient than the latter (Billen, 1984). For energy economy reasons identical to those explained for the algae, bacteria uptake of inorganic nitrogen is biased towards the uptake of the more reduced forms.

The uptake kinetics of bacterial substrates appears also to be adequately described by a Michaelis-Menten type relationship between the rates of the uptake of a nutrient and its

ambient concentration, with the Ks values obtained for natural communities characterising the affinity towards the substrate of the dominant microbial populations. By comparison with phytoplankton, bacteria appear to be better able to take up nutrients at low concentrations, but not at high (Suttle *et al.*, 1990), having lower half-saturation constants (K<sub>s</sub>) than the algae (Cotner & Wetzel, 1992). The ability of bacteria to better phytoplankton at low concentrations of substrate is usually attributed to the small size of bacteria and their high surface area to volume ratios. The trade off is that phytoplankton have higher maximum uptake velocities (V<sub>max</sub>) than bacteria, consequently having the possibility of outcompeting bacteria in high substrate concentrations (Thingstad *et al.*, 1993).

## **1.3.2.2** Competition Between Bacteria and Phytoplankton for Inorganic Nitrogen - an Apparent Paradox?

Even before the importance of bacterial uptake of ammonium and nitrate had been fully ascertained, Bratbak & Thingstad (1985) called attention to an apparently paradoxical situation that seemed to occur between bacteria and phytoplankton in their identical need for inorganic nitrogenous nutrients. When the ambient concentrations of ammonium and (secondarily) nitrate are depleted, a situation of competition between the algae and bacteria can develop. As seen in the previous section of this introduction, bacteria are very effective at taking up mineral nutrients, being able to outcompete the algae in certain situations (Rhee, 1972; Parker *et al.*, 1975; Parsons *et al.*, 1981; Horstmann & Hoppe, 1981; Currie & Kalff, 1984a,b,c).

The paradoxical situation arises from the fact that it appears that phytoplankton excretes additional organic compounds when deficient in inorganic nutrients (Ignitiates & Fogg, 1973; Lancelot, 1983, 1984; Joiris *et al.*, 1982). If so, phytoplankton responds to a stressed situation of nutrient depletion in a manner that they (apparently) stimulate their competitors and worsen the situation. However, Wood & Van Valen (1990) argue that as long as the algae nutrient supply is temporarilly or spatially uncoupled from the conditions favouring the exudation of photosynthetic products, the algae are released from the apparent paradox<sup>1.3.</sup>, and Kirchman *et al.*, (1994) argue that any negative impact on the primary

<sup>&</sup>lt;sup>1.3.</sup> According to the conditions postulated by Wood & Van Valen (1990), photosynthate release occurs during periods when light energy is superabundant relative to nutrient availability and /or to the cell's capacity for growth and energy storage, whereas nutrient uptake occurs at a different time and, in a different nutrient environment (e.g. the thermocline where nutrient levels can be high enough that bacteria may not

producers by the heterotrophic uptake of ammonium would only be transient because bacteria ultimately depend on phytoplankton as a source of carbon and energy substrates.

In nature the paradox may be solved by the presence of higher trophic levels such as bacteria predators which would relieve the pressure of bacteria on phytoplankton and would also provide nutrient regeneration (Bratbak & Thingstad, 1985). However, it is not clear whether or not this nutrient regeneration by the bacterial grazers would be sufficient to sustain the primary producers, as the predator chain would tie up mineral nutrients in their biomass as well. Flagellates have C:N ratios similar to that of bacteria, so, the mineralisation of bacterial nitrogen after grazing is exactly parallel to the carbon dioxide produced by respiration of the diet. According to Fenchel (1982), respiration by flagellates represents 25% of the ingested bacterial C. Assuming this to be the case, consequently if bacteria are actively being grazed by microflagellates, a maximum of a quarter of the nitrogen immobilised in their biomass may be released as ammonium, with a few days lag, the rest being incorporated in the flagellate biomass or egested as faeces.

Physiological as well as environmental factors may also influence this competitive relationship between bacteria and phytoplankton, resulting in a coupling between autotrophs and heterotrophs that approximates more the effects of mutualism than the detrimental ones of competition (Currie, 1990).

The controls and regulations of bacterial utilisation of both organic and inorganic substrates are still to be fully understood. This results on the present lack of complete understanding of the relationships between bacteria and phytoplankton in the sea, and in the attribution of the term 'paradox' to those situations when a significant discrepancy between predictions and the real behaviour of natural populations exist and that has so far resisted full elucidation.

It has become increasingly clear that the basis for our full understanding of the biogeochemical cycling of the main bioelements nitrogen and carbon, will stem from the uncovering of the fluxes of organic and inorganic substrates through bacteria, their

effectively out compete phytoplankton for nutrients. Because of the temporal and spatial separation of carbohydrate release and nutrient uptake by phytoplankton under these conditions, stimulation of bacterial growth at the point of carbohydrate release will have relatively little effect on the long term algal growth dynamics.
dynamics and controls. Since the microbial food web is a highly interactive and dynamic system, mathematical models will be instrumental for a deeper insight into the composite functioning of this microbial food web, conjugating the many factors which interact to regulate it (*e.g.* Thingstad & Pengerud, 1985).

# **1.3.2.3** Controls of the Bacterial Inorganic Nitrogen Uptake: the Effect of the Organic Substrate

The factors controlling the switching role of bacteria between nutrient remineralisers and consumers are not yet fully established, as well as the consequences that heterotrophic nutrient uptake could have on the planktonic community and biogeochemical processes such as "new production". Some variability in the degree of nutrient uptake by bacteria is expected because of variation in several parameters related to bacterial growth such as the bacterial biomass itself, temperature, ambient concentration of the inorganic nutrient or, more relevant for the present work, the chemical nature of the available dissolved organic matter (Kirchman, 1994), as will be discussed.

Based on simple stoichiometric considerations of cell quota, Billen (1984) argued that whether bacteria assimilated or regenerated ammonium depended on the relationship between the C:N composition of the organic substrate and that of the bacterial biomass. Indeed, Goldman *et al.*, (1987) found that ammonium regeneration by enriched bacterial assemblages did depend on C:N of the added DOM, as predicted. Goldman *et al.*, (1987) argued further that since it was apparent that natural assemblages of bacteria utilised inorganic nitrogen, then, it was quite likely, albeit still unknown, that the C:N of the DOM used by natural bacteria assemblages is high and that these micro-organisms were not important producers of ammonium after all. However, this was not the findings of Anderson & Williams (1998) when using a stoichiometric basal model to describe the seasonal cycle of DOC and DON in the English Channel.

Lancelot & Billen (1985) proposed a model to examine the question of organic nitrogen immobilisation versus mineralisation. According to these authors, the mass balance of nitrogen during organic matter utilisation can be written as

organic N taken up = ammonification + N immobilisation , 
$$(1.8)$$

where ammonification, in this case can be positive or negative, referring to release or uptake of inorganic nitrogen into or from the surrounding medium. Taking into account the C:N ratio of the organic substrates used  $(C:N)_s$  and of bacterial biomass formed  $(C:N)_b$  this balance may be written as follows:

$$\frac{\text{organic C taken up}}{(\text{C:N})_{\text{s}}} = \text{ammonification} + Y \frac{\text{organic C taken up}}{(\text{C:N})_{\text{b}}}$$
(1.9)

which can be rearranged as

$$\underline{\text{ammonification}}_{\text{organic C taken up}} = \underline{1}_{(C:N)_s} - \underline{Y}_{(C:N)_b}, \qquad (1.10)$$

where Y is the fractional growth yield, the ratio of carbon incorporated into biomass to total carbon utilised. Experimentally (Billen, unpublished; Hollibaugh, 1978), a good fit to the above equation was obtained with the relationship

Lancelot & Billen (1985) estimated Y to be 0.33 - 0.48 given a C:N ratio of bacterial biomass in the range of 3.3-4.8 and concluded that during degradation of organic matter, net uptake instead of release of mineral nitrogen occurs for substrates of  $(C:N)_s$  higher than 10. The only problem with this model is, of course, that the C:N composition of the substrates utilised by bacteria are still largely unknown, making it difficult to predict the predominant role a natural assemblage will have, in a certain environment. Kirchman (1994) suggest that bacteria use relatively few compounds on the daily time scale, with dissolved free amino acids accounting for a large fraction of the nitrogen supporting bacterial production and glucose being an important C source.

In fact, according to Kirchman *et al.*, (1986, 1989), it is the relative contribution of amino acids (specifically dissolved free amino acids, and not other dissolved nitrogenous compounds) to the nitrogen required for bacterial growth that controls whether bacteria assimilate or excrete ammonium. Their results suggest the following physiological model: amino acids are first used for biosynthesis of protein by the bacteria and are only catabolised after biosynthesis needs have been met. High amino acids concentrations repress those enzymes needed for ammonium utilisation and  $NH_4^+$  is excreted during

amino acid catabolism. When amino acid concentrations are low, enzymes for ammonium utilisation are de-repressed and net uptake of ammonium occurs.

Based on these results, Kirchman (1994) proposed a plausible hypothesis indicating the conditions that might lead to bacterial utilisation of dissolved inorganic nutrients along an environmental gradient from estuaries to the open ocean. He hypothesised that the organic compounds used as substrates for bacterial growth in highly oligotrophic environments (such as the open ocean) have predominantly high C:N ratios as a consequence of nutrient limitation of the algal community in these environments. This in turn results in the production of organic compounds with high C:N ratios. The utilisation of nitrogen-poor organic compounds by bacteria leads to an increased reliance on inorganic nitrogen for bacterial growth demands. By comparison, organic materials produced by algae in eutrophic coastal environments (with higher nutrient concentrations) would be expected to have C:N ratios that are relatively low, thereby meeting a larger percentage of the nitrogen growth demand of the bacteria utilising these compounds.

A similar scenario to the estuarine-oceanic gradient along a vertical profile in any vertically stratified water column can be easily envisaged, with nutrient-limited phytoplankton in surface waters producing predominantly high C:N organic compounds which, when eventually utilised by bacteria would result in competition between these two populations for the limiting inorganic nutrients. By contrast, light limitation (and nutrient sufficiency) in the deeper layers of the stratified water column could result in the production of dissolved organic materials of relatively low C:N ratios. Bacteria utilising these organic substrates may act as net nutrient remineralisers (Caron, 1994).

Figure 1.4 comprises the currently adopted view on the nitrogen cycling through the microbial food web. The classical function of heterotrophic marine bacteria as remineralisers (Figure 1.4, path 4) has been modified recently by studies showing that microbial growth rates reflect a high nitrogen demand which cannot be totally met by the utilisable portion of the available DON in sea water (Wheeler & Kirchman, 1986). In summary, ammonium and to a lesser extent nitrate, were found to supplement total bacterial nitrogen demands (path 2a), which has been shown to occur commonly in different marine environments (Wheeler & Kirchman, 1986; Goldman *et al.*, 1987; Kirchman *et al.*, 1989).



Figure 1.4. Nitrogen cycling through the microbial food web. Pathways involved are: (1) dissolved organic nitrogen (DON) assimilation; (2) ammonium assimilation; (3) nitrate assimilation; (4) DON mineralisation (ammonium regeneration); (5) grazing; (6) detrital formation; (7) excretion, sloppy feeding; (8) excretion, release and (9) detrital assimilation, degradation. Adapted from Tupas & Koike (1991).

The net uptake and regeneration of ammonium by bacteria have been attempted to be explained as a function of the carbon and nitrogen content of the bacterial biomass and substrates - C:N ratio model (Lancelot & Billen, 1985; Goldman *et al.*, 1987; Anderson, 1992). Interpretations from this model are based on the generally accepted idea that cells metabolise their substrates for maximum economy of growth. The idea that ammonium is utilised as a supplemental nitrogen source teleologically implies that ammonium regeneration will be minimal or not take place at all because of the high demand for nitrogen (Goldman *et al.*, 1987). Conversely, because of the preferential utilisation of amino acids over ammonium, the mineralisation of such compounds is held to imply nitrogen sufficiency; thus ammonium is not considered necessary for bacterial growth (Kirchman *et al.*, 1986, 1989).

This "either...or" theory of bacterial nitrogen metabolism, as inorganic nitrogen utilisers or remineralisers has been challenged by observations of concomitant uptake and remineralisation of ammonium by natural assemblages of bacteria (Tupas & Koike, 1991). Furthermore, bacterial regeneration of ammonium occurred to a large extent even when the C:N models predicted no net production of  $NH_4^+$ , with the dual function appearing to be regulated not by substrate specifications but rather by metabolic constraints. The work of Tupas & Koike (1991) is consistent with the traditional view that bacteria are important remineralisers of nitrogen in the marine environment, and at the same time supports the idea that marine bacteria subsist on ammonium for growth, in some cases to a large extent. Both roles are attributed equal weights in the flow of inorganic nitrogen through the bacterial component of the planktonic food web (paths 4 and 2a in Figure 1.4).

## **1.4.** AIMS AND OBJECTIVES OF THE PRESENT WORK

The coastal environment of the Menai Strait has been extensively researched (for a review see CHAPTER II) in particular the sequence of autotrophic and heterotrophic production over the seasonal cycle in this ecosystem (Blight *et al.*, 1995; Blight, 1996). Such studies of the phytoplanktonic and bacterial ecology have much to gain by the inclusion of information on the nitrogen sources for these organisms, allowing a more complete understanding of their dynamics in this particular environment and the way they may compare with other ecosystems.

The primary aim of the present work was first to study the inorganic nitrogenous nutrition of the Menai Strait phytoplanktonic community and its association with the bacterial nitrogen metabolism, and second to understand further the factors regulating the roles of bacteria as nitrogen remineralisers and/or consumers. To progress to these aims the following objectives were set:

 to investigate the temporal variation of the contribution of ammonium and nitrate to the nutrition of the phytoplankton from the Menai Strait, determined by uptake rates of <sup>15</sup>N labelled nutrients (CHAPTER IV);

- to estimate the seasonal variation of the contribution of bacteria to the total ammonium and nitrate uptake and its association (or not) with the primary production cycle in these waters (CHAPTER IV);
- to explore the relationships between the algal and bacterial inorganic nitrogenous nutrition and fluctuations in parameters such as ambient concentrations of nutrients, phytoplankton biomass, gross community oxygen production and respiration, bacterial abundance and bacterial respiration (CHAPTERS IV and VI);
- 4) to investigate the effects of bacteria organic substrates of varying C:N composition on the role of these heterotrophs as nutrient (ammonium and nitrate) utilisers or remineralisers and on the algal community by means of a series of manipulation experiments using Menai Strait's natural microbial assemblages and <sup>15</sup>N and <sup>13</sup>Clabelled compounds (CHAPTER V).
- 5) to attempt a synthesis of the results obtained from the laboratory with the ecology of phytoplankton and bacteria, in this particular coastal ecosystem (CHAPTER VI).

"Study the past, if you would divine the future." (Confucius)

# **CHAPTER II**

# CHARACTERISATION OF THE STUDIED MARINE ENVIRONMENT

The present work was carried out in a coastal marine environment - the Menai Strait. Coastal waters support complex biogeochemical processes which are, to a great extent, influenced by the intrinsic characteristics of each specific region. It is, therefore, allimportant to characterise the water mass under study, to lay the basis for future interpretation of the results obtained regarding particular aspects of the dynamics of the ecosystem in question. The following characterisation of the Menai Strait is based exclusively upon historical records, and it is not intended to be exhaustive.

# 2.1. HYDROGRAPHY

The Menai Strait separates the isle of Anglesey from the mainland of North Wales. The channel runs for approximately 25 Km from Puffin Island to Fort Belan at Abermenai, in a general NE-SW direction (Figure 2.1). It is a narrow stretch of water (700m mean width)



Figure 2.1. Location of the sampling area: the Menai Strait.

with a mean depth of 10m, at low tide. Although depths between 1 to 5m predominate along the Strait, at certain sites these increase substantially, namely at the northern entrance, by Trwyn Du (26m deep pool), at Gallows Point (19m deep gully) and at the south-west of the Swellies where the deepest soundings are of 21m (Admiralty Chart 1464, 1986).

The bed of the Menai Strait consists mainly of solid rock and stone, specially at the midchannel region where the high energy of the tidal currents prevents significant sedimentation. At the Swellies, the narrowest and shallowest region of the Strait, the tidal scour is at its strongest, sweeping clean the rocky seabed. At the low energy environment of the intertidal zone and at wider parts of the Strait, the weakened currents allow deposition of sediments which can vary from course to very fine particles. Examples of this sedimentation can be found at the north-east end at a vast inter-tidal sand flat uncovered at low tides called the Lafan Sands and at the region south of the Swellies (Fouéré, 1966; Boitier, 1982).

Most sounds and straits have complex tidal movements. The Menai Strait is no exception, with its tides governed by the water masses of Liverpool Bay and Caernarfon Bay. Very briefly, the tidal pattern is as follows: the inflowing tide enters Abermenai running in a north-east direction. However, before it reaches the other extremity of the Strait, the tidal wave has passed around Anglesey coming into the channel at Puffin Island. Consequently, the two counter-flowing masses of water meet, at a varying point between Bangor Pier and the Swellies, becoming thoroughly mixed. Two hours before high water in Liverpool, an equilibrium is reached and the flow ceases. After a short period of time (approximately 30 minutes) the ebb starts at Abermenai and the south-west going flow predominates, breaking this balance. At about three to four hours after high water in Liverpool, the tide begins to run eastwards while the Swellies ebb continues to drain westwards, as before.

The south-westerly flowing tidal current (ebb) was found to be stronger than the inflow to the north-east (flood). Based on the biological investigations of Jones & Haq (1963) and on evidence collected by himself from the movements of Woodhead Sea-Bed Drifters, direct current measurements and variations of temperature and salinity at both ends of the Menai Strait, Harvey (1965, 1968) confirmed the existence of a residual flow from north-east to south-west of a magnitude of approximately  $30 \times 10^6$  m<sup>3</sup> each semidiurnal spring tidal cycle.

He attributed this residual flow to the tidal range at the north-eastern end exceeding that at the south-western end. At the mid Strait region the mean tidal range is 3.5m at neap tides and 6.6m at spring tides (Table 2.1). The magnitude of this residual flow seemed also to be reduced by the phase of the tide being later at Puffin Island than at Abermenai (about 90 minutes).

The water flow through the Menai Strait is affected by weather conditions. Harvey (1967) reported changes in the water level along the channel which were closely (but inversely) correlated with a decrease in the atmospheric pressure. During severe weather, strong north-west winds can augment the flow whereas south-west winds can reduce or even reverse the residual flow from its usual direction. The wind speed likely to cause this reversal depends on the tidal period with stronger winds needed during spring (20 m/s) than during neap tides (12.5 m/s) (Simpson *et al.*, 1971).

The tidal flow is also strongly influenced by the topography: at the Swellies the rocky bottom produces resistance to the flow causing increased turbulence. This area offers a greater friction to the flow towards Puffin Island than it does to the flow to the south-west; as a result, it takes approximately 2 to 3 days for the complete flushing of this area to occur.

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Location	MHWS (m)	MHWN (m)	MLWN (m)	MLWS (m)	Mean Spring Tidal Range (m)	Mean Neap Tidal Range (m)
Beaumaris (north-eastern end)	7.6	6.0	2.5	0.8	6.8	3.5
Menai Bridge (mid strait region)	7.3	5.8	2.3	0.7	6.6	3.5
Fort Belan (south-western end)	4.6	3.5	1.8	0.6	4.0	1.7

Table 2.1. Variation of the mean tidal range in the Menai Strait, using Holyhead as port ofreference. From "Admiralty Tide Tables. European Waters including Mediterranean Sea". Vol.1.Admiralty Charts and Publications (1996).

M, H, W, S, N, L stand for Mean, High, Water, Spring, Neap and Low, respectively.

There are several freshwater inputs into the Menai Strait. The two major ones are the Ogowen River, which enters the Strait at the north-eastern part, and the River Anafon at the

opposite end. Nevertheless, their discharge is small compared with either the tidal streams or with the residual flow of seawater through the Strait.

# 2.2. CHEMICAL, PHYSICAL AND BIOLOGICAL CHARACTERISTICS OF THE MENAI STRAIT WATERS

The two water currents entering the Menai Strait have different physical and chemical properties, with the water inflowing from the south-west entering from Caernarfon Bay and that from the north-east originating from Liverpool Bay. The differences between these two water bodies arise from the fact that whilst Liverpool Bay water is markedly influenced by several freshwater outflows, in particular by the discharges from major river systems (Mersey and Dee Rivers), Caernarfon Bay water retains predominantly the characteristics of the Irish Sea. As a consequence, Liverpool Bay waters are less saline and present a larger seasonal fluctuation in nutrients and temperature that those of the Irish Sea. Furthermore, Liverpool Bay hydrology has been considered rather complex constituted by a "multi-component dilution system" where up to 6 different water masses have been distinguished with the individual characteristics of the overall system (Hunt & Foster, 1977; Hunt, 1978; Foster *et al.*, 1978a,b; 1982a,b; 1983; 1984; Voltolina *et al.*, 1986).

The influence of the particular local hydrography and meteorology on the flow of the two water masses in the Menai Strait results in a mixed water system whose dynamics are reflected on the general characteristics of this channel, as a whole.

Menai Strait's seawater has been extensively researched. Data regarding its biology, chemistry and physics are now extensive. The great majority of the various parameters studied have been determined at Menai Bridge area, namely from St George's Pier which was, in fact, the sampling location for the present study. Observations made during complete tidal cycles at this site and in the mid-stream by Harvey (1972), indicated that the water sampled at this pier is representative of that running in the mid-channel. Exception to this happens when periods of slack water between the north-east and the south-west going tidal currents are extended at the pier, and when back eddies become established in that

region in conditions of very strong flow in the mid-stream. From his observations, Harvey (1978) also concluded that the water present at Menai Bridge Pier during any part of the tidal cycle in normal weather conditions is provenient from Liverpool Bay. However, during periods of strong southerly or westerly winds, Caernarfon Bay water could enter the Strait at the northern end and thus, be present at this location during at least part of the tidal cycle. The water in the Menai Strait may also occasionally include a proportion of temporary incursions of water from the more central portions of Irish Sea (Ewins & Spencer, 1967).

## **2.2.1.** Physical and Chemical Parameters

#### 2.2.1.1. Temperature

Records of Menai Strait water temperature have been taken more or less regularly over the past 30 years. Early measurements by Harvey (1972) showed a seasonal trend characterised by highest temperatures during August (approximately 16°C) and lowest in February (approximately 5°C), (Figure 2.2 - Data compiled from 'The Menai Database'<sup>2.1</sup>). This seasonal trend has been confirmed in later determinations, between 1974 and 1983, by the *Coastal Surveillance Unit* (Fletcher & Jones, 1975, 1976; Fletcher *et al.*, 1978; Jones *et al.*, 1975 and 1979 to 1983) and it was also described by Lennox, (1979), El Hag & Fogg (1986), Fogg & Bajpai (1988) and Blight *et al.*, (1995), among many others.

In addition to the seasonal variations, short-period fluctuations have been identified by Harvey (1972) in his comprehensive study of the Menai Strait thermal features. Harvey reports not only semi-diurnal and diurnal oscillations but fortnightly as well, with temperatures 0.7°C higher at minimum neap tides than at maximum spring tides in summer (May to August) and 0.5°C higher at maximum spring tides than at minimum neap tides in winter (mid-November to late March). He considered these seasonal differences in fortnightly variations consistent with the hypothesis that during summer the increased mixing associated with spring tides reduces the temperatures whereas in winter temperatures are raised by such mixing.

<sup>&</sup>lt;sup>2.1</sup> - 'The Menai Database' is an archive of observations made on the Menai Strait in the relational database Access, which will be deposited in the British Oceanographic Data Center.



Figure 2..2. Annual variation of Menai Strait water temperatures. Monthly means and ranges were estimated from discontinuous measurements undertaken between 1969 and 1993, assembled in 'The Menai Database'.

Diurnal variations were attributed to the daily heating cycle and their amplitude was between 0.04 and 0.2°C. Semidiurnal oscillations were thought to be caused by the peculiar character of the Strait's tidal pattern. The water present at the head of the north-east going tide is the most recently arrived in the channel, while that at the beginning of the south-west going stream will have spent more time flowing forward and backwards in the Strait and adjacent shallow areas.

Variations in the amount of time the water has been in contact with these areas (where in winter it is cooled and in summer heated) could, therefore, be the cause for the semidiurnal oscillations in the water temperature, whose amplitude was between 0.07 and 1.15°C (Harvey, 1972). Both diurnal and semidiurnal fluctuations in the seawater temperature were shown to vary seasonally with the amplitude of the semidiurnal oscillations less variable than that of the diurnal. The largest amplitude in diurnal variations were recorded near the summer solstice; the smallest, near the winter solstice.

Being shallow and turbulent, the water of the Menai Strait does not stratify. This was also confirmed by Harvey (1972) who reports temperatures at the near surface and at the near bottom not varying more than 0.1°C, at depths between 2 and 9m.

#### 2.2.1.2. Salinity

The salinity of Menai Strait water lies between 32-34, falling below 31 and rising above 34 infrequently. Its temporal fluctuations follows a seasonal pattern characterised by slightly

slightly increased concentrations during the summer months and lower from September onwards (Figure 2.3.). This is possibly the result of variations in the fresh water inflow particularly during autumn and winter due to increased rain fall, having an overall diluting effect of the receiving water's salinity.

Short-term oscillations have also been recorded, associated with the diurnal tidal cycle and with the spring-neap tidal effect (Harvey & Spencer, 1962). These variations have been attributed to major changes in the pattern of the water movements into and within the Irish Sea rather than to changes in local determinants such as freshwater runoff and were of a smaller magnitude than those of longer-term.



Figure 2.3. Annual variation of Menai Strait water salinity. Monthly means and ranges estimated from discontinuous measurements assembled in 'The Menai Database'.

The range of salinity is greater at Abermenai than at Bangor region during semidiurnal periods (Harvey, 1968). This is in accordance with the tidal movements of seawater through the Menai Strait: whilst the water at Bangor is only provenient from Liverpool Bay, that flowing past Fort Belan at Abermenai comes, at least, from two different sources, namely Caernarfon Bay, where salinity is relatively high, and Liverpool Bay, after having run through the

channel and being diluted.

Abnormal hydrographical conditions can give rise to atypical salinities and temperatures in the Menai Strait: Harvey & Spencer (1962) registered unusually high values for both these parameters between February and August 1961. Furthermore, not only were they positively correlated with each other but also with the air temperature, with similar patterns of anomalies (Harvey, 1972). These observations were in agreement with the hypothesis defending an unusually strong incursion of Atlantic water into the Irish Sea as a result of a sudden change in strength or/and direction of the Gulf stream system (Harvey & Spencer, 1962). Ewins & Spencer's (1967) findings further supported this theory in reporting an inverse relationship between salinity and nitrate concentrations in the waters of the Menai Strait in the early months of 1964, consistent with the inflow of high-salinity, low-nitrate seawater into the Strait. This reduction in nitrate concentration was dissociated with the spring diatom bloom which only occurred later on and which normally results in a decline of nitrate concentrations in these waters.

#### 2.2.1.3. Optical Properties - Turbidity

Carrying a heavy load of particulate suspended matter, the waters of the Menai Strait are quite turbid. Figure 2.4 shows that the transparency of these waters increases towards the summer months (Jones & Spencer, 1970), associated with a decrease in seston concentrations at this time of the year.



Transmittance studies have shown a high correlation between turbidity and the mean wind, tidal range and rainfall. with the equations derived accounting for about 80% of the variation of the suspended load on a short term scale (Thomson, 1974). The tidal effect was highly

Figure 2.4. Secchi Disc readings from the Menai Strait. After Jones & Spencer (1970).

determinant of the short term fluctuations in the transmittance, in agreement with the seasonal studies of Buchan *et al.*, (1967, 1973), particularly when conjugated with the mean wind (tidal range x mean wind). Rainfall, although playing a smaller part was still

significant in dictating this kind of oscillations contrasting with the findings of Buchan *et al.*, (1967).

### 2.2.1.4. Suspended Particulate Matter

The quantification and characterisation of the suspended material in the waters of the Menai Strait were carried out during the 1960s by Buchan *et al.*, (1967, 1973) and Floodgate (1965). Although other measurements have been made since, very few data have been published; hence the present knowledge on the source and chemical nature of this material in these waters is rather limited.

**Inorganic fraction** - This component constitutes approximately 75% of the total suspended load, both at the surface and at (1m above) the sea bed (Buchan *et al.*, 1973), reflecting the character of the Irish Sea suspended load which is dominated by mineral particles, the organic fraction rarely exceeding 20% of the total weight (Simpson & Brown, 1987). Thus, the origin of the suspended matter in the Menai Strait waters appears to be allochthonous [though its exact source is not clear (D. Bowers, pers. comm.)] with changes at the surface representing real differences in the load carried by the whole water column rather than being caused by vertical resuspension (Buchan *et al.*, 1967).

The amount of the seston ash weight is strongly influenced by the tidal period with higher concentrations during spring than neap tides, what can be accounted for by the greater turbulence accompanying the former than the later. In relation to the tidal cycle in the Strait, no difference in the suspended load was found between the ebb and the flood periods (Buchan *et al.*, 1967).

After subtracting the effect of the spring and neap tides, a seasonal variation in the amount of suspended inorganic particles is still evident and characterised by minimum loads during summer and maximum in winter, over a range between 0.5 and 50 mg dm<sup>-3</sup> (Buchan *et al.*, 1967). Among the various factors thought to influence the load of suspended inorganic material in these waters only temperature was shown to correlate strongly (but negatively) with it. Easterly winds increased the suspended burden even though wind velocity was found to be unimportant in determining the quantity of inorganic seston in this waters. Neither rainfall, freshwater runoff or salinity appeared to have a marked effect, in the long

term (Buchan *et al.*, 1967). The seasonal variations in the inorganic suspended load appear to have a biological rather than a physical origin since changes in the thermal stability and viscosity of the seawater are unlikely to cause such fluctuations. Two processes explanatory of the observed seasonal trend could be partaking simultaneously: 1) filmforming micro-organisms could bind the surface of the fine particles, reducing their erosion and dispersion. This process would be particularly active during summer as the activity of these micro-organisms are highly conditioned by temperature; 2) Filter-feeding organisms, could remove the suspended particles, again more effectually during the warmer months. One single bed of mussels at the eastern end of the Strait was estimated to filter up to 33% of the residual flow through the channel on each tidal cycle. Given that along the Strait much larger areas are intensely populated by filter-feeding benthic and epibenthic fauna, this process could have an important impact on the levels of suspended inorganic material in these waters (Buchan *et al.*, 1967).

**Organic fraction** - Unlike the inorganic fraction, the organic load of the seston in the Menai Strait is believed to be autochthonous in origin. Although this water course receives a considerable input of organic matter from local sewage discharges, the source of the suspended organic particles appears to be other, namely the periodical blooming of phytoplankton in these waters (D. Bowers, pers. commun.). The highest concentrations have been measured in June (approximately 12 mg dm<sup>-3</sup>) associated with a large *Phaeocystis* bloom, but high levels of suspended organic particles were also registered during the winter months, suggesting that the seston's organic component is less temperature-dependent than the inorganic (Buchan *et al.*, 1973). This could be explained by the inorganic particles binding together by bacterial adhesive substances, particularly during the warmest months as discussed above, whereas organic particles would remain free, and so subject to resuspension and transportation by the tide, independently of the water temperature. The fact that the period of the tide is strongly correlated with the organic load of the Menai Strait waters, with a higher burden during spring than neap tides, supports this theory (Buchan *et al.*, 1973).

**Chemical Composition of the Suspended Matter -** Early studies by Floodgate (1965) revealed a total nitrogen contents of the suspended matter between 0.04 and 0.07 mg dm<sup>-3</sup>. It varied seasonally, with the highest concentrations in spring and the lowest in summer

and autumn, being largely determined by physical and meteorological conditions. The amino-nitrogen constituted 40 to 100% of this particulate nitrogen showing a seasonal pattern of variation significantly correlated with that of chlorophyll, with concentrations between a winter's maximum of 0.020 mg dm<sup>-3</sup> and a spring's maximum of 0.060 to 0.070 mg dm<sup>-3</sup>.

More recent measurements of the suspended particulate organic carbon and nitrogen have been carried out although their relation to the total suspended matter is not known as seston quantifications were not performed concomitantly. Both components of the particulate organic fraction varied seasonally exhibiting a trend very close to that of the chlorophyll *a*. The highest concentrations (up to 2.4 mg C dm<sup>-3</sup> and 0.3 mg N dm<sup>-3</sup>) were registered during spring, associated with the seasonal blooming of phytoplankton and the lowest (0.4 mg C dm<sup>-3</sup> and 0.07 mg N dm<sup>-3</sup>) in summer and autumn (Blight *et al.*, 1995), in agreement with the trend described by Floodgate (1965) but suggesting that either his measurements were underestimations or the organic suspended load has increased significantly over the past three decades.

#### 2.2.1.5. Dissolved Matter

**Inorganic nutrients** - Studies of the chemical composition of Menai Strait seawater regarding the main inorganic micronutrients, nitrate, silicate and phosphate, date from the mid 1960s by Ewins (1964) and Ewins & Spencer (1967) who carried out a comprehensive study of the seasonal variation in these waters. Discontinuous determinations of some of these compounds have been made since, (*e.g.* Hunt, 1978; Lennox, 1979; Hunt & Foster, 1985; Troëng, 1994, among others) with more recently measurements of the nitrogenous nutrients, nitrate, nitrite and ammonia having been regularly undertaken (Blight *et al.*, 1995). The seasonal variation of these nutrients show a particularly close similarity to each other, not only in relation to larger but also to smaller scale fluctuations, as will be discussed. These fluctuations of nitrate, silicate and phosphate ambient concentrations result from the interaction of several factors such as biological utilisation and regeneration, inflow of land runoff and of rivers and sewage discharges, water movement into and within the Irish Sea and other abiological processes. Ultimately, nutrient dynamics in the Menai Strait waters, particularly at Menai Bridge area where most of the aforementioned studies

were undertaken, will reflect the more general situation in the originating water mass from Liverpool Bay (Foster *et al.*, 1978a).

**Nitrate -** Ewins (1964) and Ewins & Spencer (1967) have described the seasonal cycle of nitrate variation in Menai Strait waters as being "typical of many temperate inshore waters", characterised by a decline in concentration during spring and summer and accumulation during autumn and winter (Figure 2.5).



Figure 2.5. Annual variation of inorganic nutrients in Menai Strait waters. Monthly means calculated from discontinuous measurements between 1975 and 1977, collected in 'The Menai Database'.

Concentrations between 0.05 and 23  $\mu$ mol dm<sup>-3</sup> have been reported (Ewins, 1964; Ewins & Spencer, 1967; Lennox, 1979; Blight *et al.*, 1995), with minimum values usually reached at the end of May, and maximum normally during March. The accumulation of nitrate during winter ceases with the outbreak of the early spring blooming of diatoms. This event does not completely exhaust the supply of this nutrient, what is only attained by the onset of the following phytoplankton bloom, dominated by the flagellate *Phaeocystis*, during May and June. Throughout summer, the concentration of nitrate remains very low (<1  $\mu$ mol dm<sup>-3</sup>),

starting to rise steadily around October, until the following spring. (Ewins & Spencer, 1967; Lennox, 1979; Blight et al., 1995).

The continual build up of nitrate throughout the winter appears to be the result of the slow processes of remineralization and nitrification, and also of land runoff and river discharges, which are expected to have a cumulative effect during this period of reduced biological utilisation. However, the river influence is all but clear since the discharge of the river Anafon was found uncorrelated with variations in nitrate concentrations (Hunt, 1978).

Other fluctuation, particularly those occurring at the end of March and that did not appear to be linked to phytoplanktonic utilisation, were attributed to the inflow of high-salinity low-nitrate into the Menai Strait which, as seen previously, can occur episodically in this area (Ewins & Spencer, 1967; Harvey & Spencer, 1962).

Silicate - The general pattern of silicate seasonal variation in the waters of the Menai Strait is very close to that of nitrate with maximum values (up to 14  $\mu$ mol dm<sup>-3</sup>) reached during autumn and winter and minimum (0.50 $\mu$ mol dm<sup>-3</sup>) during spring and summer (Ewins, 1964; Ewins & Spencer, 1967; Lennox, 1979) (Figure 2.5). However, in contrast to nitrate, silicate concentrations have already nearly reached their maximum values by November, remaining fairly stable thereafter, whereas nitrate levels increase continuously throughout the winter. Furthermore, initial silicate removal in the spring occurs before any significant decrease of nitrate or phosphate, reflecting the phytoplankton succession in these waters, with the diatoms dominating the early stages of the productive season (Ewins & Spencer, 1967; Lennox, 1979; Blight *et al.*, 1995).

The accumulation of silicate in these waters was considered to be a function of river discharge and land drainage rather than of regeneration processes (Ewins & Spencer, 1967). However this situation is compounded by the fact that whilst at times the riverine water concentrations of dissolved silicate are higher than those of the receiving waters, in other occasions the freshwater will dilute the seawater silicate contents instead of rising it (Hunt & Foster, 1985). This is because some rivers exhibit a negative correlation between silicate concentration and discharge, of which the river Anafon is an example (Hunt, 1978; Hunt & Foster, 1985), giving rise to complex salinity to silicate relationships in the receiving waters.

As with nitrate concentrations, many of the marked fluctuations in the silicate levels during the winter months show an inverse relationship with salinity, so it is likely that the same explanation applies in the form of an incursion of Atlantic Sea water into the Irish Sea. However, only 15% of the variance of silicate concentrations could be accounted for by regression on salinity, suggesting that other factors may be operative in causing these fluctuations (Ewins & Spencer, 1967).

**Phosphate** - The seasonal variation of this nutrient in Menai Strait waters appears to be characterised by highest concentrations from October until March, characteristically reaching a maximum of around 1.5  $\mu$ mol dm<sup>-3</sup> (although values up to 5  $\mu$ mol dm<sup>-3</sup> have been reported by Lennox, 1979), then attenuated during spring and summer as a result of phytoplankton uptake, to a minimum concentration of 0.1  $\mu$ mol dm<sup>-3</sup> (Lennox, 1979) (Figure 2.5). A departure to this pattern has been reported by Ewins & Spencer (1967) who describe little effect of the 1964 *Phaeocystis* bloom on the concentration of this nutrient, and unexpectedly low concentrations in winter and relatively high in summer. As with nitrate and silicate, the observed fluctuations in phosphate concentration in winter were associated with the influx of high salinity waters of a different origin.

The major fluctuations in nitrate, silicate and phosphate concentrations can be explained by the complex balance between biological uptake and regeneration and water advection processes. The annual cycle of the three main nutrients in consideration exhibit some anomalous features which are not easily explained solely in these terms, namely the continuous rise in nitrate concentrations during winter, the stability in silicate levels throughout the winter months despite continued addition of this nutrient by land drainage and finally the relatively low concentrations of phosphate during winter and unexpectedly high during summer. These anomalous trends have been attributed to the buffering action of sorption of some of these inorganic species to suspended matter in which the Menai Strait waters are particularly rich (Buchan, 1965; Spencer & Ewins, 1965; Spencer, 1968). The sorption processes involved appear to be relatively irreversible in the case of silicate but desorption of reactive phosphate is likely to occur into waters of low reactive phosphate concentration. This explanatory abiological control of nutrients concentrations is however refuted by Hunt (1978) who defends the cause for different patterns of variation of silicate and nitrate to be the changing characteristics of the land drainage. Apart from the long-term changes in the nutrients concentration, short-term variations have been recorded by Troëng (1994), with nitrogenous species and phosphate showing semidiurnal fluctuations. Minimum concentrations were found approximately two hours before low water while the highest levels were reached three hours after low tide. This semidiurnal cyclicity was interpreted in terms of the interactions between the two water masses meeting in the Menai Strait, with extreme concentrations occurring when the water at the sampling station (Menai Bridge pier) was dominated by a single water body. Minimum concentrations were associated with the dominance of Irish Sea water whilst maximum were characteristic of the presence of the Liverpool Bay waters. Silicate concentrations did not exhibit such oscillations appearing to be rather constant over the tidal cycle.

**Organic Compounds** - Despite its importance for the understanding of the ecology of any marine ecosystem, particularly at the microbial level (Williams, 1981; Azam *et al.*, 1983; Kirchman *et al.*, 1991; Blight *et al.*, 1995), the concentrations and composition of the dissolved organic matter are poorly documented in the great majority of marine environments. The Menai Strait waters are no exception with studies regarding organic matter carried out sporadically.

The seasonal variation of dissolved organic carbon (DOC) was found to follow a regular annual cycle characterised by minimum values during winter (ca. 1 mg C dm<sup>-3</sup>), followed by a regular build up throughout spring and summer reaching maximum concentrations in October (up to 7 mg C dm<sup>-3</sup>) succeeded by a sharp return to winter values (Morris & Foster, 1971), (Figure 2.6). This trend was somewhat unexpected since there was no pronounced increase of dissolved organic carbon during the phytoplankton blooms and the late peak arrived at a time of very low primary production. Earlier studies have shown, however, that the accumulation of DOC during spring and summer derive from autochthonous biological production and was not of terrestrial origin (Foster & Morris, 1971).

The relatively low levels of DOC in spring and summer in comparison with those in the autumn were explained as being the result of high *in situ* biological utilisation, with the continual summer build-up attributed to the rate of supply by excretion and dissolution

from dead and decaying organisms exceeding that of bacterial and chemical oxidative regeneration.



This large summer supply would be due to high primary production levels the low despite phytoplanktonic standing stock and low chlorophyll contents (Morris & 1971). The Foster, autumn peak resulted from the summer continual accumulation of DOC and finally of the decay of organisms at the end of the productive season.

Figure 2.6. Annual variation of dissolved organic carbon in the waters of the Menai Strait. Monthly means and ranges estimated after Morris & Foster (1971).

The less labile fraction of

the dissolved organic matter have also received some attention having been studied optically by UV absorption (Frost, 1973). The Gelbstoff absorption was found to be lowest in July after which it increased reaching a maximum in early November. By the end of November and early December this high absorption had already been eroded to lower levels.

The absorption of non-Gelbstoff material showed a slightly different temporal variation with similar values in September to those in July. These increased to a maximum in October, declining thereafter. This is in accordance with the observations of Morris & Foster (1971) who reported highest dissolved organic carbon concentrations at this time of the year, followed by a sharp fall. The peak of the more labile fraction of the organic matter occurred earlier than that of the less labile which supports the hypothesis that the humic material in coastal waters is mainly produced from the exudates and decay products of algae (Frost, 1973).

During September and October both fractions of the DOM were found to be inversely correlated which contrast with the positive correlations obtained for freshwater streams (Frost, 1973). This suggests that during this period, land drainage and river runoff had little control of the DOM in the waters of the Menai Strait. However, towards the end of the year the inverse correlation between Gelbstoff and non-Gelbstoff was less marked, possibly as a result of land drainage having a stronger influence from November onwards. The negative correlation between these two fractions could be the result of a varying degree of *in situ* formation of Gelbstoff from the more labile portion of the organic matter (Frost, 1973).

Contrary to the humic material, the non-Gelbstoff was inversely correlated with the tidal range. An explanation for this was based on the presence of dense beds of brown algae in the proximity of the studied area of the Menai Strait. These beds of brown algae are below the mean water level so it is possible that the assumed constant daily exudation of organic matter is more diluted by the greater turbulence and inflow of water into the Menai Strait at spring tides compared to neap tidal periods. Reflecting the mixed character of the waters of Menai Strait no differences were found between the absorption values of samples taken at low tide and those taken at high tide nor differences between the two water masses meeting in this channel were evident from this study (Frost, 1973).

The seasonal variation of glycollate, a phytoplankton extracellular product, was followed in the waters of the Menai Strait by Al-Hasan *et al.*, (1975). The range of variation of this compound was between 0-60  $\mu$ g dm<sup>-3</sup>, with minimum values occurring in August, February and May and maximum in the early winter and early summer. These fluctuations were correlated with those of chlorophyll *a* and total pigments but not with numbers of any one phytoplankton species suggesting that if glycollate is being produced by these organisms then more than one species is involved in this process. The concentrations of glycollate found in these waters were thought to represent a balance between algae release and heterotrophic uptake by bacteria. This would justify the low levels observed in August, when production rates were low and bacteria uptake was suggested to be high, associated with the high water temperature. However, this could not be confirmed since there was no information available on turnover rates (Al-Halsan *et al.*, 1975).

# 2.2.2. Biological Parameters

# **2.2.2.1.** Chlorophyll *a* and Phytoplanktonic Seasonal Dynamics in the Menai Strait.

The temporal variations of planktonic chlorophyll *a* in the Menai Strait follows a pattern characterised by high concentrations between April and June associated with the vernal blooming of phytoplankton and respective productive season, followed by an abrupt decline towards minimum levels reached over the winter months. Minor increases in the chlorophyll concentrations can also be detected between late September and late October (Figure 2.7).



Figure 2.7. Annual variation of chlorophyll *a* concentrations in Menai Strait waters. Monthly means calculated from discontinuous measurements undertaken between 1969 and 1994, collected in 'The Menai Database'. A - Diatom bloom; B - *Phaeocystis* bloom; C - mixed autumn bloom (dominance of diatoms).

This relatively simple cycle first was outlined by Spencer (1965), but has been confirmed further and complemented in later studies (Ewins Spencer, 1967; & Spencer, Jones & 1970; Morris 1974; Al-Hasan et al., 1975; Lennox, 1979; Blight et al., 1995, among many others). Minimum

concentrations are

normally down to  $\leq 1\mu g \text{ dm}^{-3}$ , between January and early March rising to a maximum of up to 16  $\mu g \text{ dm}^{-3}$  in May, although values between 6-10  $\mu g \text{ dm}^{-3}$  are more commonly reported (Spencer, 1965; Morris, 1974; Lennox, 1979; Newton, 1986; Spencer, 1987; Blight *et al.*, 1995). Interestingly, there has been an increase with time in the maximum chlorophyll concentrations reported in the literature, which could be due not only to more accurate analytic techniques but also to the intensification of the factors directly

influencing the magnitude of the phytoplankton crops, such as the nutrients levels or the particulate suspended matter load which provides a favourable environment for the generation of colonies of certain types of algae such as *Phaeocystis* (Spencer, 1987).

The phytoplankton productive season in this area is characterised by a major annual bloom occurring between April and June, with its timing and magnitude varying from year to year. The early part of the bloom is predominantly diatomaceous (Figure 2.7), composed primarily by various species of *Rhizosolenia*, such as *R. deliculata* (Jones & Spencer, 1970; Spencer, 1987; Blight *et al.*, 1995), *R. faeröense* (Jones & Spencer, 1970) and *R. shrubsolei* (Jones & Spencer, 1970; Al-Hasan *et al.*, 1975) and by the species *Skeletonema costatum* (Spencer, 1965; Jones & Spencer, 1970; Al-Hasan *et al.*, 1975) and by the species of *Biddulphia* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and also *Guinardia flaccida* (Spencer, 1965; Jones & Spencer, 1970) and Jones of chlorophyll *a* associated with the d

A mixed population of diatoms and flagellates immediately follows, the later becoming more dominant as the former declines. The standing crop of flagellates increases up to 95% of the total biomass by volume, of that of the total phytoplankton. The largest proportion of the population is dominated (by number) by organisms of 5 to 10  $\mu$ m in size, predominantly belonging to the genus *Phaeocystis* (Spencer, 1965; Jones & Spencer, 1970; Al-Hasan *et al.*, 1975; Lennox, 1979; Spencer, 1987).

To the bloom of *Phaeocystis* corresponds the highest seasonal chlorophyll peak (Figure 2.7), normally between late May and early June (Jones & Spencer, 1970; Al-Hasan *et al.*, 1975; Lennox, 1979; Blight *et al.*, 1995). Exceptions to this have been reported with *Phaeocystis* colonies being first encountered in the Menai Strait in late June (Spencer, 1987). The timing and magnitude of the *Phaeocystis* burst of growth is variable from year to year but either appear to be closely linked to the earlier diatomaceous bloom. The timing of the start of the flagellate dominance suggests that this alga may not be able to compete favourably with the diatoms species until their growth is limited by silicate depletion. It appears also, that the growth of *Phaeocystis* bladders is compromised by high ambient nutrient concentrations (Lennox, 1979). Thus, the appearance of this alga after the main

bloom of diatoms may be due to the *Phaeocystis* requiring the water to be pre-conditioned by the former organisms.

The magnitude of the *Phaeocystis* population is variable, with the highest number of cells per litre between  $4.6 \times 10^5$  and  $3.4 \times 10^7$  (Jones & Spencer, 1970; Lennox, 1979; Newton, 1986; Blight *et al.*, 1995) normally recorded towards the middle stages of the bloom (Lennox, 1979), which can last for 50 or more days (Lennox, 1979; Spencer, 1987). The maximum number of bladders could be up to  $6 \times 10^3$  per litre, decreasing sharply towards the end of the bloom when the colonies disintegrate (Lennox, 1979; Blight *et al.*, 1995).

As the main phytoplankton peak declines, a period of low standing crops succeeds through summer and early autumn, occasionally interrupted by small growth outbursts of a single species, represented to a lesser extent in the spring bloom (*e.g.*, *Thalassiosira* spp; Al-Hasan *et al.*, 1975) and which become more prominent at this time (Figure 2.7). The occurrence of these autumnal blooms is irregular being almost imperceptible in some years and small in relation to the main spring bloom (Jones & Spencer, 1970; Blight *et al.*, 1995). In contrast, in other years their effect on the general chlorophyll levels can be significant (Lennox, 1979) and of a magnitude comparable to that of the vernal phytoplankton bloom (Al-Hasan *et al.*, 1975).

The concentration of coccoid cyanobacteria which is normally low in these waters  $(4x10^3 \text{ cells cm}^{-3})$  can occasionally increase by up to two orders of magnitude with the highest counts found between May and October but more frequently in early July or August. The build up to a maximum concentration and decline is a short lived event strongly correlated with water temperature though apparently independent from the nutrients seasonal cycle: the peak of this algal group occurs at the time of nutrient depletion. This could be possible due to the small size of these cells, allowing high rates of diffusion of nutrients through the cell surface even at low nutrient concentrations (El Haq & Fogg, 1986).

Although the seasonal sequence of phytoplankton growth in the Menai Strait, described above, follows a regular inter-annual pattern, some exceptions to it have been recorded (Spencer, 1987), with diatoms dominating throughout the productive season and *Phaeocystis* exhibiting a less marked and short-lived presence. Among other factors, wind direction and intensity play an important role in determining the magnitude of the *Phaeocystis* bloom in the Menai Strait: under strong westerly winds, more water from Liverpool Bay ingresses in the Menai Strait bringing its own organisms. In this way, the size of the population in the Menai Strait is influenced by metereological conditions varying from year to year up to extreme cases which results in the anomalous situation described by Spencer (1987).

Despite the intense early to mid-summer productive season in the Menai Strait, these waters support a low phytoplankton standing crop throughout the year which is uncharacteristic of temperate inshore waters (Spencer, 1965; Jones & Spencer, 1970).

#### 2.2.2.2. Autotrophic and Heterotrophic Planktonic Processes

The annual productive period in the Menai Strait comprehended between early spring and late summer is characterised by a succession of autotrophy and heterotrophy dominated phases which are closely linked, with phytoplankters as the major primary producers and bacteria the main respirers (see attached publication - Blight *et al.*, 1995- for an in depth discussion of the sequencing of these processes in the Menai Strait).

The blooming of phytoplankton described above is accompanied by gross oxygen community production reaching up to 50  $\mu$ mol dm<sup>-3</sup> d<sup>-1</sup>, associated with the presence of high abundance of *Phaeocystis* in the seawater parallelled by high chlorophyll concentration. Whilst during the spring, meso- and microphytoplankters dominate the gross community production, towards summer nanophytoplankters become more prominent although of low biomass and activity (Blight *et al.*, 1995).

The consequential net community oxygen production at first follows very closely that of gross production. However, there are periods when it becomes negative as a result of the net autotrophic community being succeeded by a net heterotrophic one dominated by bacteria. The predominance of heterotrophic processes (dark community oxygen respiration - up to 17  $\mu$ mol O<sub>2</sub> dm<sup>-3</sup> d<sup>-1</sup> by organisms smaller than 0.8 $\mu$ m) is normally concurrent with the decline of the *Phaeocystis* bloom and with the seasonal highest bacterial abundance (4.5x10<sup>6</sup> cells cm<sup>-3</sup>) and spans a period of a month (Blight *et al.*, 1995).

This positive-negative temporal sequence of the net community production has its origins in the very close links between the phytoplankton and the bacterioplankton which are argued to be via high and low molecular weight organic material provenient from phytoplankton exudates and decay products (Blight et al., 1995). When these are of low molecular weight they are readily assimilable by bacteria and no or very short delay occurs between the peak of gross community production and the peak of dark community respiration which are then closely coupled. By contrast, high molecular weight organic materials must first be hydrolysed by ectoenzymes prior to bacterial assimilation. This is speculated to give rise to a delay of one or more weeks between the two events. The former situation is regarded to be more characteristic of early spring diatom blooms whilst the latter of the later stages of the Phaeocystis bloom. It can also be the case that a peak in gross oxygen production has no response in terms of dark oxygen respiration. This could be due to the passage of organic matter from the phyto- to the bacterioplankton being rather limited and only sufficient for the maintenance of the bacterial population, with other loss processes more predominant, such as phytoplankton sedimentation or direct grazing. It is thought therefore that the time scales of the different routes by which organic matter of an autotrophic origin becomes available to bacteria, are the basis of the mechanisms dictating the phasing of autotrophy and heterotrophy dominated periods during the productive season in the Menai Strait (Blight et al., 1995).

# 2.3. SEASONAL PHYTOPLANKTONIC DYNAMICS IN LIVERPOOL BAY

The changes in the phytoplankton populations and hydrochemistry of the Menai Strait, particularly at the area where this study has been conducted, are to a large extent the result of sequential chemical, hydrological and biological processes occurring at Liverpool Bay, with the succession of species in the Menai Strait mirroring that in the later environment.

The phytoplankton seasonal development in Liverpool Bay is essentially conditioned by hydrological factors. As mentioned before, this bay receives several freshwater outflows corresponding to water types of different "origin, history and physical and chemical characteristics" (Foster *et al.*, 1982a). This heterogeneity is reflected on the marked distinction between the offshore Irish sea and the inshore water masses along vertical and horizontal density discontinuities dictated by differences in salinity, nutrients concentrations and temperature.

During the phytoplanktonic productive season the particular composition of the various water sources entering the bay and the more offshore water mass is reflected by variations in productivity, species composition and nutrient uptake among the different water types (Foster *et al.*, 1982a, b; 1983; 1984; Voltolina *et al.*, 1986) with the magnitude of the phytoplankton standing stocks depending on advective dispersal processes and respective intensity (Spencer, 1985).

The high phytoplankton standing stock period starts at the beginning of the spring with high densities of diatoms in water surface, originating from standing crops which survived the winter in plume areas. This diatom bloom is dominated by different species in distinct areas within the Bay, with *Navicula pelagica* being predominant along the North Wales coast. The bloom is sustained by the high nutrient concentrations, in particular silicate and nitrate, allowed to accumulate in the coastal waters by the front mentioned above, which acts as a barrier limiting exchanges between inshore and offshore waters (Foster *et al.*, 1978a; 1982a). However, the initial phase of the development of the vernal blooming of phytoplankton is much more influenced by the presence of the different water types in the bay than by the density discontinuity, the effect of which is only pronounced towards the later stages, by limiting diatom growth in the inshore waters when silicate depletion occurs. Consequently, whilst during the start of the bloom no high concentrations of chlorophyll or phytoplankton biomass were found associated with the front, towards the declining phase the opposite had occurred as a result of the contrasting nutrients composition of the two juxtaposing water masses (Foster *et al.*, 1982b).

Concurrent with the decline of the diatoms bloom, a quasi-monospecific population of *Phaeocystis* (*P. pouchetti*) develops, constituting more than 80% of the total phytoplankton, further eroding the nutrients pool in Liverpool Bay waters (Jones & Haq, 1963; Lennox, 1979; Foster *et al.*, 1982b, 1983, 1985). High numbers of the diatom *Rhizosolenia deliculata* can occasionally be present, contemporary with the *Phaeocystis* peak (Spencer, 1987). This *Phaeocystis* bloom can take place between April and July, most

commonly around the end of May, beginning of June, and it appears to be anticipated by a short period of favourable meterological conditions featuring a rise in water temperature, long periods of sunshine and predominantly easterly winds (C.P. Spencer, pers. commun.).

Similarly, at the offshore discontinuity, the earlier diatomaceous prominence is progressively replaced by high densities of *Phaeocystis* dominating more than 90% the total phytoplankton biomass. A similar situation is found in the waters along the North Wales coast, with total phytoplankton cell numbers larger than  $5 \times 10^6$  dm<sup>-3</sup>, whilst in more central areas of the bay the numbers decline as a result of nutrient exhaustion (Foster *et al.*, 1983).

With the gradual warming of both coastal and offshore waters during summer, the magnitude of the difference of density between these two water masses decreases to a point in early autumn where no thermal or saline induced stratification occurs (Foster *et al.*, 1984). This allows western Irish Sea water to ingress inshore replenishing to some extent the nutrient pool of eastern waters, which can give rise to autumnal blooms of opportunistic species whose development is, nonetheless, quickly compromised by the low nutrient availability and decreasing light intensity (Foster *et al.*, 1984; Voltolina *et al.*, 1986). At this time of the year, phytoplankton numbers are highest along the southern and eastern coasts of the Irish Sea with diatoms particularly well represented in the waters of the North Wales coast. Since silicate regeneration occurs earlier than that of nitrate, the growth of a diatom population in autumn is more likely to be nitrogen than silicon limited, a situation inverse to that occurring in spring (Voltolina *et al.*, 1986).

Towards the winter, the increased river discharges in Liverpool Bay, with fresh water rich in nutrients, induces an increase in both vertical and horizontal stability, thus providing the conditions for the re-establishment of the front, allowing the nutrient reservoirs to be regenerated in the coast.

"What we have to learn to do, we learn by doing." (Aristotle)

# CHAPTER III General Methodology

This chapter deals with the general methods and techniques employed for the determination of physical, chemical and biological parameters used in this study. Whilst a number of the methods are extensively used, with minimum variation, others are in a state of evolution, a variance exists and their interpretation may not be simple or widely agreed. Thus, in the present section some methods will be reviewed in more detail, including essential background, reasons beyond the adoption of a particular procedure and discussion of the limitation to interpretation.

# **3.1. SAMPLING**

Seawater was collected from the Menai Strait having been sampled from St. Georges Pier, Menai Bridge (Figure 2.1), within two hours of high tide. During the 1995 seasonal study, sampling was undertaken fortnightly between February and August and monthly from September until November, between 7 and 10 am; in 1994, it took place before dawn and less regularly than in 1995. A hand bilge pump (5 dm<sup>3</sup> min<sup>-1</sup>) was used to collect the water from approximately 1m depth into three  $25 dm^3$  opalescent polypropylene aspirators which had been washed with a solution of hydrochloric acid (10% v/v) and thoroughly rinsed with distilled water, and sample-like seawater at the moment of sampling. During 1994, the sea water was drawn through a 200 µm mesh attached to the pump. In the following year this practice was discontinued as it incurred in a significant loss of part of the phytoplankton community during the *Phaeocystis* bloom (S. Blight, pers. commun.). The water temperature was measured at each sampling occasion by means of a digital thermometer (Digitron T208). Following collection, samples were protected from the sunlight by covering the aspirators with thick black plastic sheets.

# **3.2. SAMPLE SIZE FRACTIONATION**

### 3.2.1. Background

Given the difficulty in separating completely the bacterial and the phytoplanktonic components of natural assemblages due to the overlap in classes of particle size, studies comparing nitrogen utilisation by both types of organisms have not been often attempted. Wheeler & Kirchman (1986) have used inhibitors of protein synthesis to separate prokaryotic from eukaryotic nitrogen uptake but despite their apparent success, this approach has not been adopted and fractionation continues to be the most widely used method for this purpose, regardless its limitations.

For the seasonal study carried out in the Menai Strait (CHAPTER IV) and for the 1995 laboratory experiments (CHAPTER V) the community size compartment containing organisms smaller that  $2\mu m$  was chosen to represent the bacterioplankton. In 1996 laboratory experiments (CHAPTER V), the 0.8  $\mu m$  instead of the <2 $\mu m$  fraction was studied in order to attempt a clearer distinction between eukaryotic and prokaryotic processes.

The initial choice to study the picoplanktonic fraction was based on the structure of the pelagic ecosystem suggested by Sieburth et al., (1978). According to these authors, the

picoplankton consists of organisms with diameter between 0.2 and  $2\mu m$ , comprising mainly unattached unicellular bacteria. The problem of appending the description "bacterial" to such a fraction is that small autotrophs may be able to pass through the  $2\mu m$  membrane pores during fractionation, precluding the purely heterotrophic character of this fraction to be realised. Cautiously, the  $<2\mu m$  fraction is at times referred to as "bacterial" with acknowledgement of these uncertainties.

### 3.2.2. Limitations of the Size Fractionation Procedure

One of the main drawbacks encountered when applying this technique is the contamination of the smaller fractions with larger organisms which may be able to pass through the membrane pores, resulting in an uneffective separation of the different size components of the plankton and consequently of their metabolic processes.

On the opposite hand, filter clogging may cause the loss of the organisms of interest, specially bacteria, possibly causing biological processes to be underestimated in the fractionated samples due to increased retention of material by the membrane. This is particularly the case when studying small size compartments of the planktonic community such as the  $<0.8\mu m$  or the  $<2.0\mu m$  fractions, as a significant proportion of bacteria live attached to detritus, having the highest chances of being retained by the filter.

In conditions of high suspended matter, such small size fractionations may be troublesome or even impossible to carry out as filters burst frequently. To prevent this, membranes have to be replaced very often, seriously increasing the cost of this procedure, in terms of money and time. If a study demands large volumes of fractionated sample, these can be so slow to obtain under these conditions that the design of the experiment is compromised and/or the accuracy of the estimates lessened as a result of the prolonged stress to which the organisms are subjected and of their loss due to membrane clogging.

### 3.2.3. Experimental Procedure

Sample size fractionations were performed by means of a custom made, gravity driven, reverse flow filtration system using large diameter (142 mm), polycarbonate membrane filters (Poretics Inc., Livermore, CA, USA) of the desired porosity (2 or  $0.8 \mu$ m). When

outdoor fractionations were required, they were performed shielded from direct sunlight and completed within two hours of sampling. During intense bloom of phytoplankton (particularly *Phaeocystis*) or increased suspended load arising from severe wind conditions in the Menai Strait, membranes were replaced frequently to prevent them from bursting and to minimise the aforementioned potential errors associated with their clogging.

As this work demanded large volumes of fractionated sample, the decision to study the  $<2\mu$ m fraction was considered to provide the best compromise between plankton size separation and efficiency and cost of the procedure, in the light of the limitations of the technique described above. The chlorophyll content of the fractionated samples was estimated so that metabolic processes could be corrected for the presence of autotrophs and the heterotrophic contribution realised.

# **3.3. CHEMICAL DETERMINATIONS: INORGANIC NITROGENOUS NUTRIENTS**

The concentration of dissolved inorganic nitrogen in the form of ammonia ( $NH_3 + NH_4^+$ ), nitrate and nitrite was determined colorimetrically according to the techniques described by Parsons *et al.*, (1984). All chemicals used were purchased from BDH and were of Analar® grade, except where otherwise specified. During the seasonal work of 1994 and 1995, seawater was taken from the Menai Strait approximately one and a half hours before the sampling routine for the seasonal study took place. The determination in advance of the ambient ammonia and nitrate concentrations was necessary in order to establish the concentration of their <sup>15</sup>N-labelled forms to be used in the <sup>15</sup>N tracer technique.

**Nitrite:** In the present work the classical diazo reaction of Bendschneider & Robinson (1952), as described by Parsons *et al.*, (1984), was adopted for nitrite determinations with the only exception that a calibration curve of optical density (Y) versus concentration (X) ( $\mu$ mol N dm<sup>-3</sup>) was drawn (Y = 0.0007 + 0.052 X; R = 0.9998; n=6; p<0.001). Since there is no interference by salinity in the determination of nitrite at concentrations lower than 10  $\mu$ mol dm<sup>-3</sup> in seawater (Grasshoff, 1983a), the calibration was performed with deionised

water. The lower limit of detection ["the analyte concentration giving a signal equal to the blank signal plus two standard deviations of the blank", (Miller & Miller, 1984)] of this technique was  $0.01\mu$ mol dm<sup>-3</sup>.

**Nitrate:** The cadmium reduction procedure of Morris & Riley (1963), Grasshoff (1964) and Wood *et al.*, (1967), as described in Parsons *et al.*, (1984), was followed without modification. As for the nitrite method, a calibration curve of optical density (Y) versus nitrate concentration (X) was obtained (Y = 0.013 + 0.045 X; R = 0.9998; n=6; p < 0.01). Because of a small salt effect, standard solutions of potassium nitrate were prepared with synthetic seawater of a composition given in Parsons *et al.*, (1984).

The preparation and reactivation of the reducing column were carried out according to the recommendations of Grasshoff (1983b). The efficiency of the column was checked periodically and as soon as it fell below 95% it was reactivated. Under normal working conditions the column had an efficiency between 95 and 98%. It was found that the use of a buffered ammonium chloride solution, which is absent from the Parsons' *et al.*, (1984) protocol, improved the performance of the reductor, increasing its efficiency and the reproducibility of the results. The time during which the sample was in contact with the metal was found to be critical for the precision of the technique, hence, a flow rate was set such that  $50 \text{ cm}^3$  of sample were collected in 4 to 6 minutes.

The lower limit of detection of this technique, calculated as described above was  $0.025\mu$ mol dm<sup>-3</sup>. For the best possible degree of accuracy, nitrate analysis should be carried out immediately after sampling. This condition was observed during the course of the work under consideration.

Ammonia  $(NH_4^+ + NH_3)$ : The classical methodology to determine ammonia in seawater is based on Solorzano's (1969). Although this method offers the advantage of being specific for ammonia, it involves the use of a particularly toxic phenol reagent whose handling requires a fume cupboard, precluding its convenient use in field work. Consequently, in this study, an alternative technique by Matsunaga & Nishimura, (1974) was adopted, in which ammonia is reduced to nitrite with hypochlorite in alkali, using a large excess of potassium bromide as a catalyst. The nitrite formed is then determined as described above. The original nitrite concentration must be subtracted after dividing it by
bloom of diatoms may be due to the *Phaeocystis* requiring the water to be pre-conditioned by the former organisms.

The magnitude of the *Phaeocystis* population is variable, with the highest number of cells per litre between  $4.6 \times 10^5$  and  $3.4 \times 10^7$  (Jones & Spencer, 1970; Lennox, 1979; Newton, 1986; Blight *et al.*, 1995) normally recorded towards the middle stages of the bloom (Lennox, 1979), which can last for 50 or more days (Lennox, 1979; Spencer, 1987). The maximum number of bladders could be up to  $6 \times 10^3$  per litre, decreasing sharply towards the end of the bloom when the colonies disintegrate (Lennox, 1979; Blight *et al.*, 1995).

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Although the seasonal sequence of phytoplankton growth in the Menai Strait, described above, follows a regular inter-annual pattern, some exceptions to it have been recorded (Spencer, 1987), with diatoms dominating throughout the productive season and *Phaeocystis* exhibiting a less marked and short-lived presence. Among other factors, wind direction and intensity play an important role in determining the magnitude of the

acetone (90% v/v dilution) for a period longer than or equal to 18 hours, but not exceeding 24 hours, during which samples were kept in the dark and refrigerated (4°C).

The chlorophyll concentrations (C) were calculated according to the following expression:

$$C (\mu g dm^{-3}) = \frac{K_{f} (fo-fa) \times E}{V}$$
(3.1)

where **fo** is the fluorescence of the sample at 670 nm and **fa** is the fluorescence of the sample at 670 nm, both in instrument units, after the addition of 2N HCl; **E** is the extract volume (8 cm<sup>3</sup>); **V** is the volume of sample filtered (dm<sup>3</sup>); **K**<sub>f</sub> is a constant resulting from instrument calibration and depending on its sensitivity, determined according to the instructions given by Tett (1987) and equal to 0.67 in ( $\mu$ g chl *a* equivalent) (fluorometer unit)<sup>-1</sup> (cm<sup>3</sup> extract)<sup>-1</sup>.

This method is considered to be quite effective, extracting at least 90% of the pigments from most phytoplankton samples. However, it does not measure exclusively chlorophyll a but also its derivatives such as chlorophyllide a and some of the phaeopigments, specially pheophytin a and pheophorbide a (Tett, 1987). For this reason, the property measured is called "chlorophyll" and not chlorophyll a.

### 3.4.2. Total Bacteria Numbers

#### 3.4.2.1. Background

Despite the methodological variations that may result in different estimations of bacteria abundance, the current thought is that the use of the widely spread fluorochrome staining techniques have revolutionised the field of aquatic microbial ecology by providing more accurate estimations of bacterial abundance and standing crops than previous methods, such as the spread plating or most probable numbers (MPN) techniques.

The two most frequently used staining techniques for the enumeration of total bacteria employ the fluorochromes DAPI (4'6 - diamino - 2 phenylindole) and acridine orange according to Porter & Feig's (1980) and Hobbie *et al*'s., (1977) methods, respectively.

Compared with that using acridine orange, the DAPI technique offers the advantage of extended times between preparation of the microscope slides and counts, since the fluorescence of the cells does not fade as quickly. Porter & Feig (1980) report no significant decrease in the number of cell counts over a period of 24 weeks if slides are kept refrigerated (4°C), whilst the acridine orange technique requires slides to be prepared and counted within 2 weeks of sample collection. Moreover, DAPI is highly bacteria specific producing very little non-specific staining of detrital matter (Sieracki *et al.*, 1985). This is in contrast with acridine orange which can bind to small detritus and colloids, appearing as bacteria cells and resulting in overestimated counts. This situation is particularly problematic in waters carrying a heavy load of suspended matter, such as the Menai Strait.

Despite these arguments some authors still consider acridine orange as the stain of choice. Suzuki *et al.*, (1993) report underestimations of bacterial abundance and average cell size by the DAPI method, with bacteria numbers 57 to 82% of those determined with the acridine orange technique, and cell volumes on average 59% of those determined from measurements of acridine orange-stained organisms. These authors consider that DAPI, by only staining nucleic acid, in contrast to acridine orange which also binds to other structures as the cell wall, would make bacteria appear smaller in size. Also, if a cell did not have sufficient DNA (for instances, it had been subjected to a period of starvation), it would not be detectable by the use of DAPI but still be visible with acridine orange staining. It could be case that Suzuki *et al.*, (1993) over-estimated the numbers of acridine orange-stained cells by counting non-bacterial particles due to the aforementioned stain non-specificity, and this could be the reason why some of the samples which were double-stained with both fluorochromes showed no DAPI fluorescence when excited with UV light. However, this was not discussed by the authors.

Recent evidence, however, suggest that only 2 to 32% of the particles that are enumerated as bacteria by traditional epifluorescence techniques are truly nucleoid-containing cells (NUCC). According to Zweifel & Hagstrom (1995) up to 98% of the total bacteria counts are cell residues of virus-lysed bacteria "(ghost)" or remains of protozoan grazing. The above authors offer a procedure based on fixing the samples with sodium azide diluted with 0.2µm-pore-size filtered "Milli-Q water" prior to staining with DAPI, which allows the bacterial nucleoid to be distinguished, thus providing the bases for differentiation between active cells (NUCC) and "ghosts", giving a more accurate scenario of bacteria abundance and activity in aquatic environments. If the evidence presented by Zweifel & Hagstrom (1995) is proven right, current ideas on the role of bacteria in aquatic microbial ecology may need readjusting in a near future, with the productivity being currently measured in the sea having to be attributed to a much lower number of cells growing at much higher rates than previously estimated.

#### 3.4.2.2. Sources of Error in Bacteria Counts

The direct count method for estimating bacteria numbers carries a significant degree of uncertainty which arises from errors introduced at different stages of the technique. The way to minimise this variance would be through replication. However, since there are several levels at which it can be performed, rules for replication were vague, if not confusing, before Kirchman et al.,'s (1982) thorough statistical analysis of the sources of variation in this technique. These authors have studied the statistical significance of replication at different steps of the method namely at the subsample, filters and microscopic fields levels, defined as follows: "subsample" (<20 to 100 cm<sup>3</sup>): normally taken from a large sample of the water body in study and preserved with an aldehyde until staining; "filter": include an even smaller subsample (<2 to 50 cm<sup>3</sup>) from preserved water filtered onto it and stained with a fluorochrome; "microscopic fields": examined to count number of cells; it can be the whole field of view of the microscope or a section of it, normally a grid with a known area. Kirchman et al., (1982) found that the highest source of variation was at the "microscopic field" level with a contribution of 62 to 80% of the total variance. The "filters" contributed with 16 to 27% of the total, whereas the preserved "subsamples" introduced a variance of 0 to 10%.

Since the highest variation arises at the counting step, to minimise it most investigators perform their counts on several microscopic fields, usually until a given number of cells, typically 200 to 400, have been counted. However, normally only one filter from a single subsample is examined which is not sufficient to determine the total uncertainty associated with a bacteria number estimate.

Sokal & Rohlf (1969) suggest that the ideal procedure offering less variance is to replicate at the highest level with only one replicate at all lower levels, *i.e.*, counting bacteria in 10 subsamples with one filter per subsample and one field per filter. This would produce a smaller variance than counting ten fields on one filter per subsample. However, this procedure is costly both in time and money. Therefore, based on measured variance components, Kirchman *et al.*, (1982) calculated an optimal scheme which enters into consideration with time and financial cost, guaranteeing the lowest error for a specific cost. A sampling strategy is suggested where two subsamples are preserved, one filter per subsample should result in approximately 30 cells per field in order to minimise the coefficient of variation. Kirchman (1993) further considers that the largest source of errors in the direct count method is between - operator variation and unless care is taken to "standardise" the recognition of cells, differences in counts among operators can be quite significant.

### 3.4.2.3. Experimental Procedure

The total number of bacteria in fractionated and non-fractionated samples was determined using the DNA specific stain DAPI and epifluorescence UV microscopy (Leitz Ortoplan Microscope; x1250 magnification). The choice to use this fluorochrom is based on the arguments associated with bacteria specificity and longer preservation times discussed above.

Subsamples of 20 cm<sup>3</sup> were preserved with 50% solution glutaraldehyde (0.5% final concentration) and stored at 4°C for a maximum period of 1 month, prior to the preparation of the slides. Aliquots of 0.5 to 2.5 cm<sup>3</sup> were then filtered under gentle vacuum ( $\leq$  100 mm Hg) onto black 25mm polycarbonate filters (Poretics Inc.) and stained with DAPI (Sigma, USA, final concentration of 1.25 µg cm<sup>-3</sup>) for 10 minutes. Contrary to Porter & Feig's (1980) indications, it was found that an extended staining time of 10 minutes, instead of the recommended 5, improved counting ease due to a more effective colouring of the cells.

The stained preparations were mounted onto microscope slides using immersion oil (PAN SCAN) and coverslips and subsequently stored at -20°C until counts were carried out,

which was achieved within the following three months. This is well within the period of 24 weeks indicated by Porter & Feig (1980) during which counts remained consistent in slides stored 4°C. At -20°C a longer storage time is to be expected although this was not tested in this study.

Despite the above recommendations for minimising errors when estimating bacteria abundance, given by Kirchman *et al.*, (1982) and Sokal & Rohlf (1969), due to logistic limitations in the present study only one filter from a single 20 cm<sup>3</sup> subsample was examined. A total number of bacteria equal or exceeding 300 was counted per filter and a minimum of 30 fields (1009.4  $\mu$ m<sup>2</sup> Grid. Patterson and Circle NGI) were examined. The volume of sample stained was such to provide a maximum number of cells per field not exceeding 30 since this would probably incur in errors as a result of operator fatigue (Kirchman *et al.*, 1982). To avoid between-operator variation, all counts were performed by the author.

The analysis of a single subsample is considered to be acceptable (Kirchman, 1993) in the case where the study of the temporal fluctuations in bacteria abundance is concerned which was the case in part of the work under consideration, since absolute values are of less importance than those variations. However, this procedure precluded the use of formal statistical analysis to assess the total uncertainty of the bacteria number estimates.

## **3.4.3.** Oxygen Flux Measurements: Respiration, Gross and Net Community Production

### 3.4.3.1. Background

During 1994 and 1995 seasonal studies in the Menai Strait, rates of primary production were estimated using the oxygen Winkler technique. This classical methodology has been widely used and discussed in the literature and its theory can be found elsewhere.

In these seasonal studies, *in vitro* measurements of changes in the oxygen concentration of whole and fractionated samples, were performed based on the procedure originally introduced by Gaarder & Ran (1927). These changes in the oxygen concentration of the

samples, due to its utilisation and production are expressed in terms of primary production  $(\mu mol dm^{-3} d^{-1})$  according to the following formulae:

**Gross community production** = oxygen concentration in the samples incubated in the light + oxygen concentration in the samples incubated in the dark.

Net community production = oxygen concentration in the samples incubated in the light - oxygen concentration in the samples at time zero (fixed samples at the start of the incubations).

**Respiration** = oxygen concentration in the samples at time zero - oxygen concentration in the samples incubated in the dark.

## 3.4.3.2. Sources of Error in the Oxygen Technique

According to Carrit & Carpenter (1966) and Williams & Jenkinson (1982), in order to attain maximum performance from this method, particular attention needs to be paid at critical steps of the technique, otherwise errors introduced at these stages will significantly compromise the overall accuracy and precision of the oxygen determinations. These include:

a) Volume of sample titrated - needs to be known with an accuracy higher than 0.03%, which means that, since the whole bottle contents is being titrated, its volume needs to be determined to the  $0.001 \text{ cm}^3$  level. Preferably the calibration of the bottles should be carried out in a constant temperature room and in the case of high precision estimations volumes should be corrected to a standard temperature (usually 20°C).

b) Fixing of the samples - the samples should be fixed within 1°C of one another or a correction should be applied for temperature differences. Normally this does not pose a problem as the bottles are incubated under the same conditions and temperature differences are less than 1°C.

c) Standardisation of thiosulphate - since thiosulphate is not a primary analytical standard it needs to be standardised with a solution of known molarity, conventionally potassium iodate. Under acid conditions, iodate reacts with iodine ions to liberate iodide which is then titrated with the thiosulphate. The accurate molarity of the thiosulphate can then be calculated with the following expression:

$$M_{\text{Thio}} = \frac{6 \times V_{\text{I}} \times M_{\text{I}}}{V_{\text{Thio}}}$$
(3.2)

where  $M_{Thio}$  is the molarity of the thiosulphate;  $V_I$  is the volume of iodate added (cm<sup>3</sup>);  $V_{Thio}$  is the volume of thiosulphate used in the titration (cm<sup>3</sup>) and  $M_I$  is the molarity of the iodate standard.

d) Reagent addition - all chemicals involved in this technique (with the exception of the sulphuric acid) need to be added with a precision higher than 0.03 cm<sup>3</sup>, since pipetting errors in excess of 3% introduce a significant error in the overall estimation of oxygen concentration.

Finally the use of borosilicate rather than soda glass bottles offer better optical consistency which is needed to ensure that the automatic titration does not either overshoot or fail to reach the end point. On the other hand, borosilicate bottles are more robust than soda glass ones lessening the risk of breakages, which are always faced with a great sense of loss as the careful preparation and calibration of each bottle attribute them an intrinsic value well above their actual cost.

#### 3.4.3.3. Experimental Procedure

Glass bottles  $(50 \text{ cm}^3)$  were used to carry out the 24 hour incubations, with a replication of 3 to 5 bottles per determination. The incubations took place outdoors in water cooled incubators with one incubator kept in the dark for the whole 24 hour period whilst the other was subject to the diel cycle over the same period of time. The incubation temperature was kept as close as possible to that of *in situ* and it did not vary more than 3°C above it. The irradiance was attenuated to approximately 60% of the total incident light by a neutral density mesh, so that it was close to the *in situ*. The oxygen concentration of fixed samples at the beginning of the incubation period (time zero) was also determined.

To comply with the above requirements for high precision and accuracy, borosilicate bottles were used, having been pre-calibrated to a precision level of 0.001 cm<sup>3</sup>. At fixation, the temperature of the samples did not vary by more than 0.2°C. The molarity of the thiosulphate reagent was standardised using a 0.09-0.12M potassium iodate solution. All

reagents were added with a BCL 8000 repetitive pipette with an accuracy of  $\pm 0.6\%$  and precision between  $\pm 0.6\%$  for volumes between 0.5 and 1.0 cm<sup>3</sup>. The titrant was dispensed with an automatic burette (Metrohm 665 Dosimat) with a nominal precision of at least 0.3% for volumes as small as 0.2 µl

Titrations were performed by means of an automated, PC-based system with a photometric endpoint detector based on the design described by Williams & Jenkinson (1982). The overall precision of this system is reported to be between 0.03 and 0.1% (coefficient of variation). Such high precision is of paramount importance when estimates of oxygen fluxes are to be undertaken in waters where only small changes in oxygen concentrations occur, such as in oligotrophic environments. Although accuracy is a lesser requirement in those studies, the above authors believe it to lie between 99.5 and 99.8%. Despite every effort having been made to minimise experimental errors, in this study such high precision was not attained, with the majority of the determinations having been made within a relative standard error of approximately  $\pm 10\%$ . The lowest relative standard errors of 1-2% were obtained during periods of high production; outside the most productive months of the seasonal cycle, when oxygen changes were small, relative standard errors were very high (50-90%) in a few occasions, thus these measurements should be regarded with due caution.

## 3.4.4. Inorganic Nitrogen Uptake: The <sup>15</sup>N Technique

### 3.4.4.1. Background

Although the importance of inorganic nitrogen in the biological production cycle in the marine environment has been the subject of many years of research, the most valuable information that has contributed to the present knowledge about the utilisation and recycling of nitrogenous nutrients is based on the use of the stable isotope <sup>15</sup>N, introduced by Dugdale & Goering (1967). During the late 1960s and 1970s, the emphasis of <sup>15</sup>N tracer work was on the determination of nitrogen uptake by phytoplankton in the water column (Dugdale & Goering, 1967; Goering *et al.*, 1970; Eppley *et al.*, 1973) whereas in the next decades the focus has been on the nitrogen characteristics of nutrient starved phytoplankton (Glibert & Goldman, 1981; Goldman *et al.*, 1981) and on the coupling between nitrogen

uptake and regeneration over short periods of time (Goldman & Glibert, 1982; Wheeler *et al.*, 1982; Glibert & Garside, 1989; Glibert *et al.*, 1991) and between nitrogen and carbon uptake (Slawyk *et al.*, 1977; Collos & Slawyk, 1979; Slawyk & Collos, 1982).

In general, a nitrogen tracer experiment involves the addition of a known quantity of a  $^{15}$ N isotopically labelled compound (substrate) and the subsequent monitoring of this tracer into different particulate or dissolved pools by following changes in the isotopic enrichment (ratio of  $^{15}$ N/ $^{14}$ N) in the substrate and particulate fractions. In  $^{15}$ N uptake studies, the progressive increase in isotopic enrichment of the particulate fraction as a function of time is measured, most commonly by mass spectrometric analysis.

Despite the limitations of the <sup>15</sup>N tracer technique (which will be dealt with in the next section) there is not at present an alternative technique capable of providing the type of information regarding nitrogen fluxes that is possible with the <sup>15</sup>N tracer technique. One possibility may be the use of the nitrogen radioisotope <sup>13</sup>N. However, its half-life is of approximately 10 minutes only, restricting its application, particularly in field work. Radioactive nitrogen has been applied in studies of denitrification (Gersberg *et al.*, 1976) and nitrate uptake by natural phytoplankton populations (Axler & Goldman, 1981; Suttle *et al.*, 1990). The advantage of using this radioisotope is the possibility of making true tracer additions since the radionuclide can be produced in very high specific activities, and of reducing the incubation times during experiments. However, this advantages are overtaken by the impracticability of having to produce the radionuclide whenever is necessary and to use it within a short period of time.

## 3.4.4.2. Assumptions Made in <sup>15</sup>N Experiments

The practical application of the <sup>15</sup>N tracer technique is based on a number of basic assumptions about the behaviour of the tracer relative to that of the biological system under study, which Harrison (1983a) has summarised as follows:

- (1) The tracer undergoes the same metabolic changes as the unlabelled substance, *i.e.* isotopic discrimination is negligible.
- (2) The addition of a tracer will not disturb the system, either to compartments of it or their transformations.

- (3) The tracer is initially not in equilibrium with the system and its changes over time are quantifiable and reflect transformations of the labelled compound.
- (4) There is no exchange of the isotope between the tracer and other compounds in the system.

For isotopes used in nitrogen studies, assumptions (1) and (4) are not seriously violated. Isotopic discrimination is usually not considered in <sup>15</sup>N tracer work and unlike in the <sup>14</sup>C technique, a correction factor is not applied. This does not mean that there are no "isotope effects" - the case is that the mass spectrometric techniques used in tracer work are not sufficiently sensitive to detect very small variations in the <sup>15</sup>N/<sup>14</sup>N ratios. There are experimental conditions under which discrimination may lead to measurable changes in the atom% of the dissolved nitrogen pool. For example, during uptake, the dissolved nitrogen remaining will become progressively enriched if little or no simultaneous isotope dilution takes place. However, in natural samples, regeneration is likely to occur in most of the cases and the effect of isotope discrimination may be ignored (Glibert & Capone, 1993). For assumption (2), true tracer additions are not always possible to perform and the systems under study can often be disturbed to a lesser or greater extent, as will be discussed later. The validity of assumption (3) may also be compromised in many tracer studies. As it will be seen, this may be a potential problem in long term incubations due to substrate recycling, inducing substantial changes in the relative and absolute concentration of the tracer.

## **3.4.4.3.** Limitations of the <sup>15</sup>N Tracer Technique and Practical Considerations on its Application.

The main limitations of the <sup>15</sup>N technique are associated with the necessity to confine samples in a bottle for a more or less extended period of time (during which substrate depletion, recycling and isotopic equilibrium are likely to occur), its lower sensitivity in relation to the radioisotopic techniques and potential errors due to the impossibility of, in many cases, performing true tracer additions. Due to these, in the application of the <sup>15</sup>N tracer technique two crucial questions have to be addressed with care:

i) determination of the tracer additions;

ii) duration of the incubation;

Whichever practical procedure is adopted regarding either of these, it will influence the N flux estimates and their interpretation.

#### i) Determination of tracer additions

When initiating a tracer experiment one of the most important preliminary steps is the evaluation of the amount of tracer to be used, as the addition will have to be sufficiently large so as to provide a detectable signal but at the same time small enough not to disturb the system. According to Dugdale & Goering's (1967) recommendation, an isotopic addition which enriched the ambient by approximately 10% would not greatly perturb the system and this has become common practice in the application of the <sup>15</sup>N-tracer technique. The specific uptake rate (V) of nitrogen by marine phytoplankton can be related to the substrate concentration by a rectangular hyperbolic function analogous to Michaelis-Menten kinetics (Dugdale, 1967; Eppley *et al.*, 1969) therefore the higher the concentration of tracer added, the higher the increase in the uptake rates measured (until  $V_{max}$  is attained) and so the greater the disturbance in the system. Hence, even a 10% increase in the ambient pool could stimulate the rate of uptake by up to 10%.

A real problematic situation arises when the nutrient ambient concentrations fall below the detection limits making it impossible to perform true tracer additions. In these circumstances, investigators make additions that range from 0.03 to 0.1 $\mu$ mol dm<sup>-3</sup> which results in an incalculable enrichment lying between 50 and >100% (McCarthy, 1980), with the consequent disturbance of the *in situ* conditions. At the moment it appears that under "below detection limit" nutrient concentrations, there is no completely satisfactory way to determine "real" uptake rates. To overcome the errors associated with the classical procedure used by the scientific community, it would be necessary to introduce corrections based on the uptake kinetics information but this requires knowledge of the concentration dependent uptake for a given community which may vary with time (Harrison *et al.*, 1996). Time course, uptake kinetics and isotopic dilution experiments would have to be carried out simultaneously in all determinations, which is clearly unrealistic.

The <sup>15</sup>N additions performed in this work, according to the ambient concentration of ammonium or nitrate, are specified in the following section. When the ambient concentration of these nutrients fell below detection limits, an addition of labelled material of 0.01  $\mu$ mol dm<sup>-3</sup> was made. For the calculation of the uptake rates, the lower limit of

detection of these nutrients (0.030  $\mu$ M for ammonium; 0.025  $\mu$ M for nitrate) was used in the equation which requires a value for the ambient concentration of the nutrient in question (equation 3.5; section 3.4.4.5.). This can result in serious overestimations of the uptake rates principally when the real ambient concentration of nutrients are very low (in the order of a few nanomoles). Therefore, the uptake estimates obtained under these circumstances can only be considered, at best, as approximations and too much emphasis should not be put on these data.

#### ii) Duration of the incubations

Depending on how long for samples are incubated, different cellular metabolic processes may be measured (Glibert & Goldman, 1981; Wheeler *et al.*, 1982): for example, while transport of the materials across the cell membrane can occur in minutes, with time, these will be incorporated into the organisms compounds and, in the case of nitrogen, into amino acids and proteins.

Short incubation periods (2 to 6 hours) will generally give an estimate of the potential uptake of a nutrient by a community and are normally used in coastal areas where substrate turnover rates are high. It has been found that phytoplankton can assimilate ammonium from the environment, in a matter of minutes (McCarthy, 1980; Glibert & Goldman, 1981; Goldman *et al.*, 1981; Goldman & Glibert, 1982), showing the inadequacy of long term incubations. However, extrapolations from these measurements to a daily uptake may not necessarily compare well with flux estimates from longer incubations and rates could be overestimated.

Long term incubations provide estimates of the net uptake of a nutrient and they have been favoured by some investigators (Dugdale & Goering, 1967; McCarthy, 1972 a,b; Eppley *et al.*, 1979a,b; MacIsaac *et al.*, 1979; Olson, 1980; Harrison *et al.*, 1982) because they avoid the uncertainties involved in extrapolating from results of a shorter period to daily rates. However, the drawbacks of long incubations are plenty and mostly related to a) "bottle effects" and b) non-linearity of nitrogen uptake during the incubation period due to substrate depletion and recycling.

a) "Bottle effects": the effects of confining a sample to a bottle for a limited period of time are well known to scientists trying to investigate, in particular, microscale processes in the marine environment. They vary from alterations in the relative species composition of phytoplankton in the samples, to contamination from the incubation containers, and they have been extensively dealt with in the literature (Venrick *et al.*, 1977; Gieskes *et al.*, 1979; Fitzwater *et al.*, 1982; Goldman & Dennet, 1984, among others). Certain measures can be adopted to minimise these effects, such as the use of short incubations; polycarbonate containers instead of glass which may leach toxic compounds; and large volumes of sample which reduce the chances of contact between the organisms and the wall surface of the bottle. As far as it was possible, these measures were followed in this work.

b) Non-linearity of nitrogen uptake rates: one of the principles behind tracer work is that the rate at which the label is incorporated into the particulate matter must be constant for the duration of the experiment. In fact, and unless the incubation period is kept very short (in the order of minutes to a few hours), this may not be so. Significant decreases in the maximum specific uptake rates of ammonium over short periods of time have been reported both in laboratory cultures (Conway & Harrison, 1977; Goldman & Glibert, 1982) and for natural phytoplankton populations (Glibert & Goldman, 1981; Wheeler *et al.*, 1982; Harrison, 1983b). The two main reasons for this change in nitrogen uptake rates during the course of the incubation period are related to the depletion of the nitrogen substrate and progression towards isotopic equilibrium (MacIsaac & Dugdale, 1969; Fisher *et al.*, 1981; Goldman *et al.*, 1981).

Substrate depletion has been considered a potential problem for <sup>15</sup>N tracer incubations in low-nutrient waters particularly during long incubations, contributing to underestimations of nitrogen turnover by an order of magnitude or more when compared with estimates from short incubations (2 hours or less), during which uptake is linear (Goldman *et al.*, 1981). Nitrogen depletion in incubation bottles may also contribute to the frequently observed high C:N assimilation ratios of surface phytoplankton (Harrison, 1983a).

Although one of the assumptions of isotope techniques states that the tracer is not in equilibrium with the system, a progression towards an equilibrium between the tracer in the dissolved and in the particulate phases, will take place, possibly within a 24 hours period incubation (Harrison, 1983a). This is the result of the uptake of labelled substrate by the particulate matter and of isotopic dilution of the liquid phase by the production of non-labelled nitrogen by microzooplankton (in the case of  $NH_4^+$ ) and/or bacterial

remineralisation. The decrease of <sup>15</sup>N in solution due to remineralisation processes is exponential with time (Caperon *et al.*, 1979; Blackburn, 1979) and this remineralisation of the substrate constitutes another violation of the assumptions of tracer work. If happening, it will lead to underestimations of the nitrogen uptake rates. With the increase of labelled material in the particulate matter, the chances of remineralisation of the <sup>15</sup>N also increase, again contributing to lower estimations of N fluxes. The effects of this recycling errors are particularly important for rapidly recycling substrates such as NH<sub>4</sub><sup>+</sup>. For slow recycling forms of nitrogen such as NO<sub>3</sub><sup>-</sup>, errors in uptake measurements from isotopic dilution or equilibrium are, normally, minimal (Harrison, 1983a).

Based on the above arguments, it was decided that short (6 hours) and long (24 hours) term incubations were to be performed during the seasonal studies of phytoplanktonic and "bacterial" inorganic nitrogen uptake in Menai Strait waters. Short incubations are particularly suitable in the case of ammonium, given its rapid recycling, specially under bloom conditions, minimising the aforementioned errors associated with substrate recycling and depletion and with isotopic equilibrium. Long term incubations were necessary in order that samples dually labelled with <sup>13</sup>C in addition to <sup>15</sup>N, were submitted to identical experimental conditions as those employed for the measurement of oxygen fluxes. Furthermore, outside the productive season (November to March), uptake and substrate recycling rates are not as fast, and longer incubation periods may be required to allow sufficient accumulation of label in the particulate matter to be measured. The estimations of nitrogen uptake in both sets of incubations will be presented and discussed in CHAPTER IV.

At the beginning of the seasonal studies (February), an experiment was carried out to test the linearity with time of  ${}^{15}N$  [( ${}^{15}NH_4$ )<sub>2</sub>SO<sub>4</sub> and Na ${}^{15}NO_3$ ] accumulation in the particulate matter of the whole community and organisms contained in the 2µm size fraction. Incubations were carried out for 24 hours over the diel cycle in outdoor incubators.

The results have shown (Figure 3.1) that although there was a progressive increase in the concentration of labelled material in the particulate matter, this increase was not always linear with time (except, perhaps, for the uptake of ammonium by the  $<2\mu$ m fraction), *i.e.*, the uptake rates of ammonium and nitrate were not constant over time.



- dark period of the incubation

Figure 3.1. Uptake of <sup>15</sup>N-ammonium and nitrate by the whole community (WC) and "bacterioplankton" ( $<2\mu$ m size fraction), over a diel cycle.

Shifts in the uptake rates did not appear associated with the dark period of the incubation (between the 8<sup>th</sup> and the 20<sup>th</sup> hour), during which the uptake of inorganic nutrients continued. These results show the inadequacy of using short incubations for period the estimation of nitrate uptake by the whole community and "bacteria", outside the productive season. It appears that no substrate depletion or dilution took place during this experiment perhaps as a result of the slow uptake and recycling rates expected at this time of the year.

## 3.4.4.3. Experimental Procedure in the Field

During part of the 1994 and in the 1995 seasonal studies in the Menai Strait, the uptake of <sup>15</sup>N-labelled ammonium [( $^{15}NH_4$ )<sub>2</sub>SO<sub>4</sub>; 99.3 atom%; Europa Scientific, Ltd] and nitrate (Na<sup>15</sup>NO<sub>3</sub>; 99.4 atom%; Europa Scientific, Ltd) by the whole community and organisms smaller than 2µm ("bacteria") was estimated.

Seawater was collected from the Menai Strait following the procedure already described (section 3.1) and two sub-samples of 10 dm<sup>3</sup> were taken. One was inoculated with  $Na^{15}NO_3$  and the other with  $({}^{15}NH_4)_2SO_4$ . The isotopic additions were made according to the ambient concentration of nitrate and ammonia, following the scheme presented in Table 3.1. Half of each of the 10 dm<sup>3</sup> subsamples were incubated in the dark and the other half subjected to the natural diel cycle. Samples were incubated in polycarbonate bottles of 1dm<sup>3</sup> capacity. Although the utilisation of large containers is advisable in order to minimise the so called "bottle effect", in the present study their use was precluded by the restrictive dimensions of the incubators.



**Figure 3.2.** Schematic representation of the experimental procedure applied for the measurement of <sup>15</sup>N uptake by the whole community (WC) and  $<2\mu$ m fraction.

Ambient Concentration of nitrate or ammonia (µM)	Added concentration of <sup>15</sup> NH <sub>4</sub> <sup>+</sup> or <sup>15</sup> NO <sub>3</sub> (µM)				
[below detection limit to 0.1]	0.01				
[0.1 - 0.3[	0.01				
[0.3 - 1.0]	0.03				
[1.0 - 3.0[	0.1				
[3.0 - 10.0[	0.3				
≥ 10	1.0				

**Table 3.1.** Addition of <sup>15</sup>N-ammonium and -nitrate according to the ambient concentration of these nutrients in the seawater.

Incubations were carried out for six and twenty four hours under identical conditions to those described for the oxygen technique (section 3.4.3.). The bottles subjected to the twenty four hour incubation period were also inoculated with NaH<sup>13</sup>CO<sub>3</sub> (section 3.4.6.). Following the six and twenty four hours incubations, identical samples were pooled so that any differences amongst bottles arising during those periods were eliminated.

A picoplankton fraction ( $<2\mu$ m) was then isolated from the whole community by passive reverse flow fractionation (section 3.2.) and the particulate matter in these two fractions collected onto glass fibre filters for further elemental and isotopic analysis (section 3.4.5.). The decision to perform the fractionation post incubations instead of prior to them was based on two main reasons: i) to avoid submitting the organisms to the fractionation stress, which could affect their physiology and consequently their subsequent uptake of nutrients; ii) as the comparison of nitrogen uptake by phytoplankton and bacteria was of interest in this research, by allowing these two communities to co-exist during the incubation, a more realistic scenario of their interactions *in situ* was expected. However, this procedure has its disadvantages, as pointed out by Wheeler & Kirchman (1986), such as the presence in the fractionated samples of labelled fragments from larger cells, which may result in overestimations of the uptake by the picoplankton.

The flow diagram depicted in Figure 3.2. summarises the practical procedure described in this section.

## 3.4.4.5. Calculation of the Uptake Rates

Nitrate and ammonium uptake rates by the whole community and by the organisms present in the fractionated samples were calculated following equations based on those introduced by Dugdale & Goering (1967) and later given by Grunseich *et al.*, (1980). Based on these original equations, several approaches have been considered, as thoroughly discussed by Collos (1987) and Dugdale & Wilkerson (1986), but the two more commonly used expressions to calculate nitrogen **absolute uptake rates** ( $\rho_{(0)}$  and  $\rho_{(t)}$ ) are

$$\rho_{(0)} = \frac{PON_0 \ (C_p - C_0)}{(C_d - C_n) \ \Delta T} \quad \text{and} \quad (3.3)$$

$$\rho_{(t)} = \frac{PON_t \left(C_p - C_0\right)}{\left(C_d - C_n\right) \Delta T}$$
(3.4)

where PON<sub>0</sub> and PON<sub>t</sub> are respectively the particulate organic nitrogen at the start and at the end of the incubation time ( $\mu$ mol dm<sup>-3</sup>); C<sub>p</sub> is the concentration of the label in the particulate phase after the incubation (<sup>15</sup>N-atm%); C<sub>0</sub> is the concentration of <sup>15</sup>N in the particulate phase prior to the addition of labelled material (<sup>15</sup>N-atm%); C<sub>n</sub> is the <sup>15</sup>N natural abundance (0.3663 atm%);  $\Delta$ T is the duration of the incubation; C<sub>d</sub> is the concentration of the label in the dissolved phase (<sup>15</sup>N-atm%) at the beginning of the incubation and estimated according to the following equation:

$$Cd = ([^{14}N] \times 0.3663) + ([^{15}N] \times A)$$
(3.5)  
$$[^{14}N] + [^{15}N]$$

Where  $[^{14}N]$  is the ambient concentration of the nitrogen form under study (ammonium or nitrate) in µmol dm<sup>-3</sup>;  $[^{15}N]$  is the added concentration of labelled nitrogen ( $^{15}N$ -ammonium or  $^{15}N$ -nitrate) in µmol dm<sup>-3</sup>; A is the  $^{15}N$ -atm% of the labelled nitrogen (normally in excess of 99 atom%).

The term absolute uptake rate ( $\rho$ ), given in units of mass volume<sup>-1</sup> time<sup>-1</sup> was introduced by Dugdale (1977) to differentiate it from the **specific uptake rate** (V) given in (units of time)<sup>-1</sup>, where

$$\rho_{(t)} = V_t x PON_t \tag{3.6}$$

$$\rho_{(0)} = V_0 \times PON_0 \tag{3.7}$$

$$V_{(t)} = (C_p - C_0)$$

$$(C_d - C_n) \Delta T$$
(3.8)

and

$$V_{(0)} = (C_{p} - C_{0})$$
(3.9)  
(C\_{d} - C\_{p})  $\Delta T$ 

with PON<sub>t</sub>, PON<sub>0</sub>, C<sub>p</sub>, C<sub>0</sub>, C<sub>d</sub>, C<sub>n</sub> and  $\Delta T$  as before. In the present study, absolute uptake rates were calculated, using expression (3.6) following Collos' (1987) recommendations.

The use of expressions (3.6) and (3.7) is based on the following assumptions:

- i) there is only a single nitrogen source used by the organisms;
- ii) there is no excretion of nitrogen by the organisms;
- iii) Cd remains constant over the incubation time;
- iv) the particulate organic carbon does not vary through the incubation time;
- v) isotope discrimination is negligible.

The problem associated with the uptake of other, non labelled, sources of nitrogen is that the isotopic composition of the particulate matter will be diluted and, consequently, the specific uptake rates underestimated. This could be the reason why some authors report an inhibition of labelled nitrate uptake by the presence of unlabelled ammonium: the generally reported decrease in  $V_{NO3}$  as the ammonium concentration is increased, may simply reflect the increasing ammonium uptake relative to that of nitrate and the resulting dilution of <sup>15</sup>Natoms in the final particulate nitrogen by <sup>14</sup>N-atoms from the unlabelled ammonium. In experiments where more than one source of nitrogen is available, the estimation of absolute uptake rates given by equation (3.6), will yield the most accurate results, as this expression is insensitive to unlabelled nitrogen uptake. This is because the first bias due to the contribution of the unlabelled compound is exactly compensated by a second bias introduced by the final particulate nitrogen. If equation (3.7) is used to calculate absolute uptake rates, this cancellation does not occur since the initial particulate nitrogen is used. This same argument applies to the second assumption: the production of nitrogen would dilute the <sup>15</sup>N - atom% of the substrate; consequently, assumption iii) would not be verified over the incubation time, and uptake rates would be underestimated (Collos, 1987).

The fact that particulate organic nitrogen may vary during the incubation time is taken into consideration by the use of the final concentration of particulate nitrogen in the sample, so that assumption (iv) is corrected for if not held. Another advantage of using the final concentration of particulate nitrogen, instead of the initial, is that it can be determined from

the same sample as the isotopic composition ( $^{15}N$  - atom%), therefore providing the most accurate estimate of the amount of  $^{15}N$  incorporated in the particulate fraction during the experiment.

## **3.4.5.** Nitrogen (and Carbon) Content and Isotopic Composition of the Particulate Organic Matter.

#### 3.4.5.1. Background

The nitrogen and carbon content and isotopic composition (<sup>15</sup>N and <sup>13</sup>C) of the particulate matter were determined by means of an elemental analyser ("Roboprep"; Europa Scientific, Ltd, Cheshire, UK) interfaced with a mass spectrometer ("TracerMass"; Europa Scientific, Ltd, Cheshire, UK) (Figure 3.3.).



**Figure 3.3.** Schematic diagram illustrating <sup>15</sup>N and <sup>13</sup>C analysis by an ANCA-MS (Europa Scientific Ltd, Cheschire, UK). GC stands for gas chromatography; CV for control valve and MF for magnetic field. Adapted from instrument's operating manual.

This technique is based on the principle of the design and use of the system described by Preston & Owens (1983), offering the advantages of allowing not only the determination of

nitrogen and carbon in the same sample but also the isotopic composition of both elements, contributing to a higher level of accuracy of the measurements.

The elemental analyser is based on the Dumas combustion technique where a sample is introduced automatically into a combustion furnace (1000°C) in an atmosphere of oxygen, burning exothermically. The complete oxidation of the sample to  $N_2O$ ,  $H_2O$  and  $CO_2$  is achieved by passing the products of the combustion through chromium trioxide, using He as carrier gas. These products are carried into a reduction furnace (600°C) containing elemental copper, where the nitrogen oxides are converted to  $N_2$  and excess oxygen is absorbed.

The mixture of gases then passes through a trap of magnesium perchlorate to remove water and of "Carbosorb" to remove  $CO_2$  if this element is not to be analysed; otherwise the  $CO_2$ trap can be bypassed. A gas chromatographic column separates  $N_2$  from  $CO_2$  and low molecular weight hydrocarbons which may be formed during poor combustion and could interfere with the subsequent mass spectrometric analysis. Finally the purified gases are carried into the mass spectrometer through a continuous stream of helium where they are ionised by an electron beam source which bombards the gas molecules. The positively charged ions are accelerated and collimated into a fine beam which enters a magnetic field deflecting the ions into circular paths whose radii are proportional to the masses of the isotopes, according to the fundamental equation of magnetic deflection mass spectrometry (Larsson & Odham, 1984):

$$m/z = (H^2 \times r^2)/2V$$
 (3.10)

Where m/z is the mass-to-charge ratio of the particle; H is the strength of the magnetic field; r is the curvature of the pathway (instrumentally dictated) and V is the voltage used to accelerate the ions.

The beam is divided into its component masses and these charged particles travel on to strike a series of 3 collectors. This triple-collector mass spectrometer was tuned to give outputs of the ion intensities of m/z 28, 29, 30 for nitrogen and 44, 45 and 46 for carbon. Amplifiers attached to the collectors transform the ionic impacts into voltage which is then converted into a frequency. From the ratio of the isotopic species the levels of <sup>15</sup>N and <sup>13</sup>C are calculated.

## 3.4.5.2. Sample Preparation

For the determination of particulate organic nitrogen (and carbon) and their <sup>15</sup>N (and <sup>13</sup>C) isotopic composition, a known volume of sample (0.05 to 0.15 dm<sup>3</sup> of unfractionated and 0.3 to 0.5 dm<sup>3</sup> of fractionated) was filtered onto precombusted (500°C for 4 hours), 25mm diameter glass fibre filters (Whatman, GF/F; see appendix) under gentle vacuum ( $\leq$  100 mm Hg). The filters were frozen (-15°C) until their preparation for mass spectrometric analysis could be carried out, which normally took place within the following two or three weeks. The filters were then dried at 40°C overnight, the used surface of the filters removed with a 20mm diameter metallic punch and the non-used rim discarded. The cut filters were wrapped in precombusted (500°C, 4 hours), 30mm diameter aluminium discs (Elemental Microanalysis, Ltd) to form small spheres, after which they were stored in a desiccator until analysed.

Care was taken to avoid contamination of the samples with organic matter, so gloves were used at all times during their handling and all material in contact with the filters, such as forceps and punch, were wiped clean with acetone. To account for any contamination that could have remained after the filters were pre-combusted or that was introduced at the sample preparation level, at least 5 "blanks" consisting of filters handled similarly to the samples, were prepared and analysed and their mean values for nitrogen and carbon contents subtracted from the samples.

There is a choice between using aluminium or tin discs to enclose the filters. Tin combusts more completely producing less ash and so it is preferred by some investigators. However, it cannot be pre-combusted, as it melts, and it was found that the commercially available tin discs carried unacceptable and variable levels of carbon and nitrogen, producing high "blanks". Although the combustion of aluminium leaves a higher residue than that of tin, the discs can be safely pre-combusted, thus minimising contamination, hence their preferred use in this work.

## 3.4.5.3. Instrument Performance and Calibration

The internal precision of the instrument originally described by Preston & Owens (1983) was  $\pm 0.00026$  atom % for <sup>15</sup>N. This level of precision has been maintained ( $\pm 0.00037$ 

atom %) in the Europa Scientific elemental analyser "Roboprep" coupled with the mass spectrometer "Tracermass", at the level of 100  $\mu$ g of nitrogen (7.14  $\mu$ mol). For carbon, at the 100  $\mu$ g level (8.3  $\mu$ mol), the precision quoted by the manufacturers is  $\pm$  0.00038 atom % (John Davies, Pers. Commun.; Europa Scientific, Ltd).

To ascertain the precision of the instrument in use during this study, a test was performed where the reproducibility of <sup>15</sup>N-atom % at natural abundance levels (0.3663 atom%), using urea as reference material in concentrations ranging from 0.5 to 7.5  $\mu$ mol of nitrogen (7 to 105  $\mu$ g N), was determined for 12 replicates of each standard. The results are presented in Table 3.2.

Table 3.2 Reproducibility of  $^{15}$ N-atom % (0.3663) in reference material of varying nitrogen content.

Nitrogen Content (µmol)	0.5	1.0	2.5	5.0	7.5
Mean ( <sup>15</sup> N-atom %)	0.36690	0.36683	0.36688	0.3660	0.3664
SD ( <sup>15</sup> N-atom %)	0.00052	0.00041	0.00030	0.00031	0.00016
Relative error (%)	0.142	0.112	0.082	0.085	0.044

For these concentrations, the precision was of the same order of magnitude as that quoted by the manufactures and it appeared to improve with increasing the size of the sample. However, the application of an analysis of variance (ANOVA; 95% significance level) showed that the precision of <sup>15</sup>N measurements was not significantly different for nitrogen concentration between 0.5 and 5  $\mu$ mol (F=1.47; p=0.235; the residuals were normally distributed and there was no significant heterogeneity of variance), but it increased significantly for larger concentrations (7.5 µmol N) (F=3.66; p=0.010; the residuals were normally distributed and there was no significant heterogeneity of variance). It is expected that this precision will decrease again for very high concentrations of nitrogen. This was not investigated in this study as excessively high nitrogen concentrations were not expected in the natural samples. For an identical instrument, Owens (1988) has found poorer precision at similar nitrogen levels: he determined the reproducibility of <sup>15</sup>N-atom % at natural abundance levels using (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in concentrations ranging between 0.5 and 2.0  $\mu$ mol of nitrogen and reports a precision of 0.0038 atom % (standard deviation, n=12). Later, Owens & Rees (1989) tested the same reproducibility in much lower nitrogen concentrations (10-100 nmol) and found similar results, with the standard deviation

showing a tendency to decrease with increasing the size of the reference (standard deviation of 0.0036 atom % for the lowest concentrations and of 0.0016 atom % for the highest).

For carbon, a similar test was performed and the results are shown in Table 3.3.

Table 3.3. Reproducibility of  ${}^{13}$ C-atom % (1.108) in reference material of different carbon content.

Carbon Content (µmol)	1	2.5	5	10	15	20
Mean ( <sup>13</sup> C-atom %)	1.107	1.107	1.108	1.106	1.107	1.108
SD ( <sup>13</sup> C-atom %)	0.0004	0.0005	0.0005	0.0006	0.0003	0.0001
Relative error (%)	0.036	0.045	0.045	0.054	0.027	0.009

Identical tests regarding the precision of <sup>13</sup>C-atom % measurements have not been reported in the literature, therefore performance comparisons between instruments cannot be drawn. In relation to the manufacturers specifications, who quote a precision of 0.0038 atom % at a level of 100  $\mu$ g (8.3  $\mu$ mol), the precision of the instrument used in this study was an order of magnitude higher, showing a tendency to increase for levels of carbon between 15.0 and 20  $\mu$ mol C. An analysis of variance (ANOVA; 95% significance level) revealed that the precision of <sup>13</sup>C-atom% for concentrations between 1 and 5  $\mu$ mol was not significantly different (f=0.54; p=0.591; the residuals were normally distributed and there was no significant heterogeneity of variance) and that the increase in precision for concentrations higher than 10  $\mu$ mol was, indeed, real (f=18.17 to 31.06; p<0.001; the residuals were normally distributed and there was no significant heterogeneity of variance).

These data refer only to the performance of the mass spectrometer since the reference material was inorganic and did not provide a realistic test for the combustion step. The efficiency of the combustion was checked by examining the mass spectra of the samples. By-products of poor combustion are resolved chromatographically after the sample nitrogen and carbon peaks, and are detectable if present. An inefficient combustion will lead to the underestimation of the nitrogen and carbon content and overestimation of the <sup>15</sup>N-, <sup>13</sup>C- atom %. The latter, results from the generation of CO and organic molecules of relatively low mass, which are detectable, as they interfere with the masses of interest (for instances, CO has a m/z of 28 or 29 which could be resolved with the nitrogen peak). Also,

the oxidation capacity of the catalysts decreases during the analysis of a large number of samples; so they were replaced as soon as a decline in their oxidising efficiency was detected.

As far as the accuracy of atom % measurements is concerned, this is currently impossible to determine, since there is no internationally certified standards enriched with the heavy isotopes of interest (<sup>15</sup>N and <sup>13</sup>C).

In this technique the measurement of total nitrogen was made by calibrating the integrated area of the ion beams (m/z 28 + 29 + 30 for nitrogen; m/z 44 + 45 + 46 for carbon) against standards of known concentration by means of a calibration curve of nitrogen or carbon concentration (X) versus ion beam intensity (Y). For concentrations of nitrogen between 0.5 and 7.5  $\mu$ mol, the absolute response of the mass spectrometer in use was linear (Y = - $4.73 \times 10^{-10} + 2.36 \times 10^{-8}$  X; R = 0.9997; P<0.001) with a precision [coefficient of variation] (%), n=10] varying between 1.4 and 5%. This is in accordance with Owens & Rees (1989) who have also found that this level of precision is maintained at concentrations as low as 100 nmol of nitrogen with the coefficient of variation increasing to 12% at the 10 nmol level. The calibration of the instrument for nitrogen analysis revealed the existence of a background signal shown by a Y axis intercept. This is due to the presence of nitrogen in the carrier gas provenient from the oxygen pulse which is added at the combustion stage to ensure complete oxidation of the sample. The amount of nitrogen introduced this way is constant. Its contribution can be assessed from "blank" analysis and automatically subtracted from the samples. This contamination can preclude the use of this technique for the analysis of samples of very low nitrogen content (in the order of nanomoles). However, Owens & Rees (1989) report on the possibility of avoiding the contribution of such contamination by timing differently the admittance of the sample and the oxygen pulse into the combustion furnace. By manipulating the normal working conditions of the instrument, the authors were able to perform analysis of organic reference material with a total nitrogen content as low as 1 nmol and in natural samples of phytoplankton with a nitrogen content between 45 and 73 nmol, without loss of sensitivity. However, during the present study their suggested manipulation was not followed since nitrogen contamination from the oxygen pulse was found to be negligible (normally less or equal to 5% of the total nitrogen in the samples) and easily corrected for.

The calibration of the mass spectrometer for the determination of carbon showed a linear relationship (Y=  $-1.278 \times 10^{-8} + 4.6139 \times 10^{-8}$  x; R=0.99925; p<0.001) between concentration of carbon and absolute response of the instrument for a range between 1 and 20 µmol of carbon, with a precision [coefficient of variation (%); n=10) varying between 2 and 6%. The presence of carbon in the carrier gas did not pose a problem to the analysis of the samples in question since the carbon background levels were normally less than 0.5% of their measured carbon content.

## 3.4.6. The <sup>13</sup>C-Technique

## 3.4.6.1. Background

This technique was first applied to the determination of photosynthetic production rates by micro-organisms by Slawyk et al., (1977, 1979). Contrary to the other carbon isotope technique (<sup>14</sup>C), the stable <sup>13</sup>C has had more restricted use. Reasons for this could be its lower sensitivity, requiring the use of larger volumes of sample and longer incubation periods; the complexity of maintenance and operation of the required analytical instrumentation; and cost of heavy isotopes. However, since some countries (such as Japan) are introducing tighter environmental regulations against the use of radioisotopes, alternative procedures had to be considered. For the study of microbial carbon dynamics in the aquatic environment, the use of <sup>13</sup>C instead of <sup>14</sup>C appears to be the appropriate choice as it involves no hazardous handling. Furthermore, the <sup>13</sup>C-technique has been shown to be highly compatible with the <sup>14</sup>C, yielding very proximate estimates of production both in cultured phytoplankton (Slawyk et al., 1979) and in natural phytoplankton populations (Hama et al., 1983; Sakamoto et al., 1984; Slawyk et al., 1984). Combined with the <sup>15</sup>N technique, it provides an outstanding tool in the study of interactions between population and uptake of inorganic nutrients (Slawyk et al., 1977; Collos & Slawyk, 1979; Slawyk & Collos, 1982).

## 3.4.6.2. Interpretation of the <sup>13</sup>C-Technique

The <sup>13</sup>C-technique shares with that of <sup>14</sup>C the doubt of whether it measures gross or net community production and although comments below refer to the <sup>14</sup>C, with no doubt they will apply to the <sup>13</sup>C-technique as well. This "whether ... or" view may be over simplistic

as discussed by Williams (1993), who states that if we wish to interpret the <sup>14</sup>C technique measurements in terms of which type of production it determines, than it would be necessary to know how to correct these estimates for organisms of different and varying physiological states. Moreover, it appears that prevailing physiological conditions, in particular the net growth rate, may be determined by the depth of the water column which is in accordance with Ryther's (1954) suggestion that the <sup>14</sup>C-technique could be measuring different processes under different environmental conditions. Other factors which may influence the position of <sup>14</sup>C uptake rates in relation to gross or net carbon assimilation, are the floristic composition of the phytoplankton community and experimental design aspects such as the duration of the incubation (Williams, 1993).

The interpretation of the <sup>14</sup>C-technique measurements has been debated since the introduction of the method itself by Steemann Nielsen in 1952 and although it has been more than 45 years since, a clear answer has not yet been formulated. The reason for this appears to be the incomplete understanding by the researchers of the intracellular fluxes of carbon. The main areas of uncertainty are the extent of (1) respiration of the phytosynthetically fixed <sup>14</sup>CO<sub>2</sub> and (2) recycling of respired CO<sub>2</sub> and its preferential use over external CO<sub>2</sub>. If there is little subsequent metabolism of the photosynthetically fixed <sup>14</sup>C and minimal recycling of respiratory CO<sub>2</sub>, then the <sup>14</sup>C-technique measures gross production, but it is doubtful that these conditions are ever met. On the other hand, if there is respiration but no recycling of CO<sub>2</sub>, initially a rate close to that of gross production will be measured, eventually approaching net production rates. If, on the contrary, there is extensive recycling of respiratory CO<sub>2</sub>, this technique will initially measure net production but as time progresses, gross production will be approximated (Williams & Lefèvre, 1996; Williams *et al.*, 1996).

The intracellular carbon fluxes are not easily measured; consequently, a series of models appear in the literature, attempting to provide a better understanding of these fluxes and interpretation of <sup>14</sup>C measurements (Dring & Jewson, 1982; Smith *et al.*, 1984; Smith & Platt, 1984; Williams 1993). However, none of these has succeeded in providing a definitive conclusion, for a number of different reasons. More recently, Williams & Lefèvre (1996) have proposed a model to define an experimental strategy to establish the separate effects of respiration and recycling on the time course of net <sup>14</sup>C uptake. These

authors have produced a series of equations to describe the time course of <sup>14</sup>C assimilation if respiration of photosynthetically fixed <sup>14</sup>C was significant or if recycling of respiratory  $CO_2$  was extensive. To parameterise their model, experiments were carried out where the net and gross inorganic carbon assimilation and <sup>14</sup>C uptake were compared and rate constants for photosynthesis and respiration derived (Williams *et al.*, 1996). Combined with the use of high precision techniques for the measurement of TCO<sub>2</sub> (Robinson & Williams, 1991) and the <sup>14</sup>C-technique, this model appears to provide the basis for rigorous analysis of net, gross CO<sub>2</sub> fixation and net <sup>14</sup>C uptake and could finally lead to the development of algorithms for the full interpretation of the <sup>14</sup>C uptake in the field (Williams *et al.*, 1996).

#### 3.4.6.3. Errors Inherent to the Technique

The <sup>13</sup>C-technique can present some problems inherent to any tracer methodology (for instances, substrate enrichment; discrimination against labelled substances by the organisms) as in the case of <sup>15</sup>N-technique, and others specific to the metabolism of carbon, such as dark carbon fixation. These have been extensively dealt with in the literature and a very brief account of only the most commonly referred to potential sources of error is given here.

#### **Isotope Discrimination**

It is well known that phytoplankton prefer <sup>12</sup>C to <sup>13</sup>C or <sup>14</sup>C (Falkowski, 1991). For this reason, a correction factor of 1.05 has been applied for isotope discrimination in the <sup>14</sup>C-technique (Slawyk *et al.*, 1977; Sondergaard, 1988). Similarly, Hama *et al.*, (1983) among others have suggested the introduction of a factor of 1.025 to be applied. In practice, however, this correction has not been used since its effect is small on the estimated primary production rates when compared to that of the <sup>14</sup>C-technique (Hama & Handa, 1992a, b). Furthermore, the application of a factor to a phytoplanktonic community can incur in inaccuracies as the discrimination of carbon by these organisms depend on the specific metabolism of different species (Falkowski, 1991).

#### Substrate Enrichment Effect

As discussed for the <sup>15</sup>N-technique, one of the major drawbacks of the heavy isotope techniques is that, because of their lesser sensitivity in relation to those which use

radioisotopes (e.g. the  $^{14}$ C), higher concentrations of the labelled material have to be added. This may result in a significant enrichment of the substrate what can disturb the metabolism of the substance in question particularly in environments where it is limiting.

In the case of the <sup>13</sup>C-technique, there is little cause for concern despite the fact that a higher enrichment of the substrate (5-15%) than in the <sup>14</sup>C-technique is applied. Hama *et al.*, (1983) found no significant effect of additions of 4.4 to 17.7% of the existing inorganic carbon on the photosynthetic rate. This is because dissolved inorganic carbon is not photosynthesis limiting in the great majority of aquatic environments. Exception to this may be found in hypertrophic lakes in Summer, where the supply of dissolved inorganic carbon may, under these circumstances, influence production rates (Miyazaki *et al.*, 1985a).

#### **Correction for Dark Uptake**

The manner in which researchers have used dark fixation values in the <sup>14</sup>C-technique has not always been consistent. In most cases the rate of dark fixation is subtracted as prescribed by the method; in other cases it is simply ignored or assumed to be a small percentage of the carbon fixation in the light. Morris *et al.*, (1971), for instances, recommended that until more is known about the mechanisms of dark carbon fixation this process should be ignored.

For the <sup>13</sup>C-technique, where normally a "dark" incubation is carried out, it is not expected that this inconsistent attitude towards the use of carbon dark fixation estimates will be different. One reason for this inconsistency could be that whilst some authors find no or insignificant dark carbon assimilation (Sakamoto *et al.*, 1984), others (Prakash *et al.*, 1991; Tuttle & Jannasch, 1977) have shown that this process should not be ignored, as it can represent 50% or more of the carbon assimilation in the light. This variable uptake of <sup>14</sup>C or <sup>13</sup>C in the dark has been attributed in large part to bacterial fixation whose higher uptake rates in the dark than in the light could be caused by photoinhibition of these organisms (Tuttle & Jannash, 1977). Therefore, the subtraction of the dark carbon fixation to that in the light may result in serious underestimations of primary production as this process can have a heterotrophic origin. This has been also confirmed by Li & Dickie (1991) who observed net increase in bacterial abundance of a magnitude appropriate to account for the measured dark <sup>14</sup>C fixation suggesting that incubations should be kept for a minimum

period of time so as to minimise the complications of declining chlorophyll *a* and increasing bacterial abundance, which make interpretation of the measurements difficult. In addition to bacterial effects, some of the dark uptake could also result from the continuation of phytoplankton light-initiated reactions, in the dark (Taguchi & Platt, 1977).

#### 3.4.6.4. Experimental Procedure

The experimental procedure for the determination of <sup>13</sup>C-uptake rates by the whole community and organisms  $<2\mu$ m was in all identical to that applied to the <sup>15</sup>N-technique, with seawater samples simultaneously labelled with both isotopes. A 5% addition of labelled material was used, *i.e.* 100 µmol of <sup>13</sup>C per litre of sample (as NaH<sup>13</sup>CO<sub>3</sub>; 99.0 atom %; Europa Scientic, Ltd), assuming an ambient TCO<sub>2</sub> concentration of 2000 µmol dm<sup>-3</sup>. This dual labelling is particularly advantageous as it allows the microbial carbon and nitrogen dynamics to be followed in the same sample, contributing to a higher accuracy of the estimates. Moreover, it saves duplicating incubations, filtrations and analysis of two different samples, which is not only costly and time consuming but also introduces a larger error due to inter-sample variability.

The <sup>13</sup>C isotopic composition of the samples and their particulate organic carbon content were analysed by means of the elemental analyser ("Roboprep"; Europa Scientific, Ltd, Cheshire, UK) interfaced with the mass spectrometer ("TracerMass"; Europa Scientific, Ltd, Cheshire, UK), as already described.

#### **3.4.6.5.** Calculation of Primary Production rates

Primary production rates based on the uptake of  ${}^{13}C$  were calculated according to the formulae suggested by Hama *et al.*, (1993):

$$\rho_{c}(t) = \underline{\Delta \text{ POC } (t)}_{t} = \underline{(a_{is} - a_{ns})}_{t} \times \underline{\text{POC } (t)}_{t}$$
(3.11)  
t (a\_{ic} - a\_{na}) t

Where,  $\rho_c$  (t) is the production rate (µmol C dm<sup>-3</sup> hr<sup>-1</sup>);  $\Delta$  POC (t) is the increase in POC concentration during the incubation time (µmol C dm<sup>-3</sup>); t is the incubation time (hours);  $a_{is}$  is the <sup>13</sup>C-atm% of particulate matter at the end of the incubation (atom %);  $a_{ns}$  is the <sup>13</sup>C-atm% of particulate matter in the non- incubated samples (atom %);  $a_{ic}$  is the <sup>13</sup>C-atm% in

the dissolved phase at the beginning of the incubation (atom %) (equation 3.12);  $a_{na}$  is the <sup>13</sup>C-atm% of naturally occurring dissolved inorganic carbon (1.108 atom %); and POC(t) is the particulate organic carbon in the sample at the end of the incubation time (µmol dm<sup>-3</sup>).

$$a_{ic} = \underbrace{([^{12}C] \times 1.108) + ([^{13}C] \times B)}_{[^{12}C] + [^{13}C]}$$
(3.12)

Where,  $[^{12}C]$  is the concentration of dissolved inorganic carbon in the sample; a value of 2000 µmol dm<sup>-3</sup> was used in the calculations; 1.108 atom% is the natural abundance of  $^{13}C$ ;  $[^{13}C]$  is the concentration of added isotope (100 µmol dm<sup>-3</sup>); B is the  $^{13}C$ -atom% of the added heavy isotope (99.0 atom%).

In this study, a 24 hour incubation period was performed and rates per day were calculated  $(\mu mol C dm^{-3} d^{-1})$  with dark C fixation not substracted to that over the diel cycle.

Equation (3.11) is a modification of that originally suggested by Slawyk *et al.*, (1977, 1979) which used the concentration of particulate organic matter at the beginning of the incubation, [POC (0)] (equation 3.13).

$$\rho_{c}(0) = \underline{\Delta \text{ POC } (0)} = \underline{(a_{is} - a_{ns})}_{t} \times \underline{\text{POC } (0)}_{t}$$
(3.13)

This meant that the particulate organic matter had to be measured from another filtered sample obtained at the start of the incubation. This can contribute to more serious experimental errors than when equation (3.11) is used, since in that case the <sup>13</sup>C-atom% and the concentration of POC (t) are measured from the same sample, accounting to changes in the particulate matter during the incubation and resulting in more accurate estimates. Another advantage of using equation (3.11) instead of (3.13) is that the dilution of the isotopic ratio of the incubated sample due to the uptake of unlabelled compounds can be cancelled out by the increase in the particulate organic carbon (Hama *et al.*, 1983). The same applies in the case of overestimations of particulate organic carbon due to the retention of non-phytoplanktonic organic carbon by the filters used to collect the particulate matter. Hama *et al.*, (1983) have shown that particulate organic carbon overestimations due to the absorption of dissolve organic carbon to the filter was overcome by the corresponding dilution effect on a<sub>is</sub>. In equation (3.13) the use of POC (0) and decreased a<sub>is</sub> due to isotopic dilution will result in underestimation of the production rate.

"The sea is calm to-night, the tide is full, the moon lies fair upon the straits"

(Mattew Arnold)

## CHAPTER IV Menai Strait Seasonal Study

In this chapter, results of the seasonal work undertaken in the coastal waters of the Menai Strait during 1994 and 1995 will be presented and discussed.

The objectives of this field study were,

- 1- to describe the seasonality of the inorganic nitrogen fluxes in the Menai Strait with special reference to its relation to the primary production cycle;
- 2 to assess the contribution of a bacteria dominated size fraction (<2μm) to the total (unfractionated) inorganic nitrogen uptake;</li>
- 3- to investigate the relationship between carbon and nitrogen uptake by the bacterial and phytoplanktonic populations;

Although part of a team study within the research programmes M.E.I.C.E. (Microbiological Elemental Cycling in Coastal Environments) in 1993 and in 1994, and P.R.I.M.E. (Plankton Reactivity in the Marine Environment) in 1995, virtually all determinations concerned with the whole community and the  $<2\mu$ m size fraction were carried out by the author. Exception to this were the oxygen flux measurements and

chlorophyll determinations during the spring and summer of 1994. When used, data obtained by other workers will be appropriately acknowledged. The contribution of the author was, therefore, as follows:

- assisted with sampling (from May 1993 onwards);
- undertook 'in situ' temperature measurements (1995);
- performed determinations of inorganic nitrogenous nutrients (from May 1993 onwards);
- determined oxygen fluxes in the whole community and  $<2 \mu m$  size fraction (in 1994 between September and November and in 1995);
- estimated bacterial abundance and chlorophyll contents in both size fractions (autumn 1994 and in 1995);
- designed and carried out all <sup>15</sup>N and <sup>13</sup>C uptake experiments (1994 and 1995);
- filtered, prepared and analysed all samples for isotopic and particulate organic carbon and nitrogen determinations (1994 and 1995) using the Europa Scientific Carbon and Nitrogen analyser "Roboprep" coupled with the Mass Spectrometer "Tracermass".

The study of the seasonal variation of microbial processes in the Menai Strait as part of the M.E.I.C.E. Programme started in 1993. Results of part of this investigation, which continued in 1994 and to which the author contributed particularly with nutrients data, are reported in the paper of Blight *et al.*, (1995) (appended to this thesis).

To accomplish the proposed objectives, the following parameters were determined, according to the procedures described in CHAPTER III: temperature; ambient concentration of ammonium, nitrite and nitrate; oxygen production and consumption by the whole community and  $<2\mu$ m fraction; chlorophyll, particulate organic carbon and nitrogen contents of both size fractions; bacteria abundance; <sup>15</sup>N-labelled ammonium and nitrate and <sup>13</sup>C (as Na<sup>13</sup>HCO<sub>3</sub>) uptake by the whole community and  $<2\mu$ m fraction over the diel cycle and in dark only conditions. The set of data relative to the uptake of <sup>15</sup>N nitrogenous nutrients in 1994 is incomplete due to the participation of the author in mesocosms experiments which took place in Denmark during June and July as part of the aforementioned M.E.I.C.E. Programme. As soon as it was possible the work in the Menai Strait was resumed. Consequently, the herein described seasonal dynamics of the various

parameters measured in the Menai Strait is based on the more complete set of data obtained during 1995.

# 4.1. MEASURED PHYSICAL AND CHEMICAL FEATURES OF THE STUDIED ENVIRONMENT

## 4.1.1. Temperature

The Menai Strait water temperature measured between February and November 1994 and 1995, followed a seasonal trend which is in good agreement with historical records taken since 1955 (CHAPTER II) (Figure 4.1). The year of 1994 featured a particularly low value at the end of February (3.4°C), with the highest (available) measurement in June (14.7°C). The more complete data set from 1995 showed highest temperatures in August (20.2°C) and minimum in March (7.0°C) as recorded in previous years.



Figure 4.1. Menai Strait surface water temperature during 1994 and 1995. Comparison with historical data from 'The Menai Database'.

## 4.1.2. Dissolved Inorganic Nitrogen

**Nitrate** - The seasonal cycle of nitrate concentration in the waters of the Menai Strait followed much the same trend in the three year period during which measurements were undertaken (Figure 4.2a). As found by others (Ewins, 1964; Ewins & Spencer, 1967; Lennox, 1979), this pattern was characterised by a build up of nitrate from October onwards, to reach a maximum in March, followed by a sharp decrease leading to below detection levels in May. During the summer months, nitrate was depleted from these waters, with concentrations rarely rising above detection limits (0.025  $\mu$ M).

During the three year period the highest determination  $(13.58 \pm 0.047 \,\mu\text{M})$  was recorded in 1994, on day 82, roughly a month later than in 1995 (8.79 ± 0.00  $\mu$ M; day 58). The 1994 spring decline was phased into two periods and was more abrupt than that in the following year.

The regeneration of nitrate started in October in all three years but at a higher rate in 1993 than in 1994 or particularly in 1995, having had already reached relatively high concentrations by day 343 (10.87  $\pm$  0.094  $\mu$ M).

**Nitrite** - Nitrite concentrations were generally low ( $\leq 0.010$  to  $0.78 \pm 0.007\mu$ M) and showed an irregular seasonal pattern of variation which was not analogous for the three sampled years (Figure 4.2b). Despite this fact, a general trend characterised by maximum levels in the autumn could be identified. The lowest records were observed during summer, where levels declined to below detection limits (0.010  $\mu$ M), particularly in 1993.

**Ammonia** - Data from 1994 and from the more complete set of measurements carried out during 1995 put in evidence a pattern of the seasonal variation of ammonium concentration that is variable from year to year, featuring short intervals of ammonium accumulation in the water, followed immediately by periods of near complete exhaustion, mirroring the fast turnover rate of this compound (Figure 4.2c). Concentrations remained below 1  $\mu$ M for most of the season, falling to below detection limits (0.030  $\mu$ M) frequently. A general trend of increased concentrations towards the autumn could be perceived during 1995 but not in 1994. In both years but particularly in 1994, a peak in the ammonium concentration was


Figure 4.2. Seasonal variation of dissolved inorganic nitrogen in Menai Strait waters between 1993 and 1995. Bars represent standard errors.

observed in May (4.70  $\pm$  0.171  $\mu$ M; day 23 in 1994; 0.70  $\pm$  0.015  $\mu$ M; day 130 in 1995), although it is possible that the unusually high value of 1994 may have resulted from contamination during the experimental procedure.

# 4.2. WHOLE COMMUNITY RELATED CHARACTERISTICS AND METABOLIC PROCESSES

# 4.2.1. Chlorophyll

The seasonal fluctuations of the chlorophyll concentration in the years of 1994 and 1995 are depicted in Figure 4.3. In 1994, the  $<200\mu$ m size fraction was studied whereas in the following year the whole community was considered.





The year of 1994 characterised was by three main chlorophyll peaks between April and June, corresponding vernal to the blooming of phytoplankton, and later by a one, occurring at the end of October (day 295), of a much smaller magnitude earlier than the

peaks (2.68  $\mu$ g dm<sup>-3</sup>). The first peak (day 107; 3.58  $\mu$ g dm<sup>-3</sup>) was composed by a mixed diatom population; the second (day 131; 8.45  $\mu$ g dm<sup>-3</sup>) comprised primarily diatoms but a

few *Phaeocystis* bladders could already be detected; the third and highest chlorophyll peak, at the end of May (day 145; 15.36  $\mu$ g dm<sup>-3</sup>), consisted mainly of *Phaeocystis* although diatoms, particularly *Rhizosolenia deliculata* were still abundant (Blight *et al.*, 1995). No community characterisation of the autumn peak was carried out but it is likely that it consisted mainly of diatoms, particularly of *Thalassiosira spp* (Al-Hasan *et al.*, 1975).

The seasonal fluctuations of the chlorophyll concentration in 1995 followed a similar pattern to that recorded in the previous year, with the main peak in spring and a secondary one towards autumn. The magnitude of the spring peak was considerably lower than that measured in 1994 despite the fact that a larger size fraction was analysed. The seasonal highest concentration (8.34  $\mu$ g dm<sup>-3</sup>) was reached at very much the same time (day 143) as in 1994, again coinciding with the *Phaeocystis* bloom. Diatoms dominated the early stages of this single spring peak and were present, though less markedly, throughout its duration.

In both years, the summer months (July until September) featured low standing crops of phytoplankton. The timing of the autumn peaks and their magnitude were proximal (day 295 in 1994, 2.68  $\mu$ g dm<sup>-3</sup>; day 298 in 1995, 1.52  $\mu$ g dm<sup>-3</sup>).

# 4.2.2. Particulate Organic Matter

## 4.2.2.1. Measurements in 1995

#### **Particulate Organic Carbon**

During 1995 the concentration of POC varied between  $17.6 \pm 1.30$  and  $62.7 \pm 11.31\mu$ M, an 8 fold increase which took place between March (day 73) and June (day 158). Three, not particularly distinct peaks (May, June and October) characterised the pattern of seasonal variation of POC in these waters and they appeared to be associated with the spring and autumn chlorophyll blooms (Figure 4.4a).

The concentrations observed in 1995 were substantially lower than those from 1993 determined by Blight *et al.*, (1995), despite the facts that in 1993 a smaller size fraction ( $<200\mu$ m) was considered, and that the chlorophyll concentrations were similar in both years. Furthermore, the year of 1993 featured a well marked spring peak, in contrast to the



Figure 4.4. Seasonal variation of the a) particulate organic carbon; b) particulate organic nitrogen and c) C:N ratio of the particulate matter of Menai Strait waters, during 1995. Comparison with 1993 data from Blight *et al.*, (1995). Bars represent standard errors.

year of 1995 situation. In both years an increase in POC concentrations towards the autumn could be perceived, with peaks recorded at the same time (day 298, 36.9  $\mu$ M in 1993 and 55.4  $\pm$  1.85  $\mu$ M in 1995). During 1995, POC concentrations were relatively low during summer with values comparing well to those from 1993. After the autumnal peak, concentrations declined.

#### **Particulate Organic Nitrogen**

In 1995, the seasonal pattern of the variation of PON was analogous to that of POC, with the exception that the autumnal peak was absent. Concentrations oscillated between a minimum of  $2.4 \pm 0.05 \,\mu\text{M}$  in March (day 73) and a maximum of  $8.3 \pm 0.89 \,\mu\text{M}$  in June (day 158) (Figure 4.4.b). After this spring peak they declined remaining fairly constant throughout summer and autumn when a subsequent decrease lead to the lowest levels in winter. This seasonal pattern was distinct from that observed by Blight *et al.*, (1995) in 1993, with concentrations consistently lower throughout the season and absence of an autumn peak, well marked in 1993. However, maximum concentrations were detected between the end of April and the end of May in both, years, as well as a definite decrease during June and July.

#### **C:N Ratio of the Particulate Organic Matter**

In 1995 the C:N ratio of the particulate matter varied between  $5.2 \pm 0.69$  (day 233) and  $15.8 \pm 2.67$  (day 88), following a seasonal pattern characterised by two distinct peaks: one of  $15.8 \pm 2.67$  in March and the second of  $12.1 \pm 1.24$  in October (day 298), the latter coinciding with the autumn POC and chlorophyll maxima (Figure 4.4c).

The high C:N ratios appear to result from a non-proportional increase in the POC relative to PON: comparing Figures 4.4a and 4.4b, it can be seen that the raised POC concentrations on day 233 and 298 did not correspond to a build up of PON. This could be due to the presence of aged detritus, rich in C but depleted in N.

The seasonal mean C:N ratio of 7.9 is close to Redfield's suggesting that despite the depleted nutrient conditions for part of the season, the particulate organic material (believed to be mostly of phytoplanktonic origin) did not appear to be generally, severely nitrogen limited. The 1995 C:N ratios were consistently higher than those estimated from Blight's *et al.*, (1995) data from 1993 (seasonal mean of 5.67). Their highest ratios

(between 7.01 and 8.43) were concomitant with the spring chlorophyll peak, a situation not encountered in 1995.

## 4.2.2.2. Measurements in 1994

Discontinuous measurements of POC and PON taken during 1994 (Table 4.1) are in good agreement with those described for both 1993 and 1995.

Day of the year	PON (μM) mean ± se	POC (μM) mean ± se	C:N ratio mean ± se
108	$7.9 \pm 0.42$	40.3 ± 1.64	$5.1 \pm 0.34$
123	$3.7 \pm 0.13$	34.2 ± 1.38	9.3 ± 0.50
137	$7.3 \pm 0.28$	$126.2 \pm 4.95$	$17.2 \pm 0.94$
152	$5.5 \pm 0.81$	$120.2 \pm 20.22$	$21.7 \pm 4.7$
257	$3.6 \pm 0.20$	$23.1 \pm 1.98$	$6.5 \pm 0.66$
295	3.4 ± 0.63	34.4 ± 4.44	$10.1 \pm 2.26$
318	$7.0 \pm 0.54$	$27.1 \pm 6.16$	$3.9 \pm 3.86$

**Table 4.1.** Particulate organic carbon and nitrogen measurements undertaken during1994 and C:N ratio of the particulate matter.

Particularly high C:N ratios during the productive season, well in excess of Redfield's, suggest that the phytoplankton was nitrogen limited. Despite some availability of nitrate in the waters at that time of the year (May), the concentrations were probably not high enough to balance the C:N uptake ratios by the algae. On the other hand, these high C:N ratios during May, coincident with the bloom of the flagellate *Phaeocystis*, could result from the presence of mucopolysaccharides excreted by *Phaeocystis* which could have been retained in the filters, and subsequently analysed as particulate material.

# 4.2.3. Oxygen Flux Measurements: Gross and Net Community Production and Dark Respiration

The annual productive season in the Menai Strait waters has been briefly characterised in CHAPTER II and extensively dealt with by Blight *et al.*, (1995).



Figure 4.5. Seasonal variation of a) gross and b) net community oxygen production and c) dark community oxygen respiration, during 1995. Comparison with 1994 data from Blight *et al.*, (1995) and author's own determinations. Bars represent standard errors.

The 1995 gross and net production cycles followed patterns comparable to those observed in previous years featuring well demarked peaks during spring and summer, evolving to smoother curves towards the autumn (Figure 4.5a and b).

Gross and net production rates started to increase from the end of March to reach maxima of  $47.6 \pm 0.66 \ \mu\text{mol} \ O_2 \ dm^{-3} \ d^{-1}$  and  $37.6 \pm 0.76 \ \mu\text{mol} \ O_2 \ dm^{-3} \ d^{-1}$  on day 143, coinciding with the season's highest concentration of chlorophyll (Figure 4.3). It followed a period characterised by a sharp decline leading to rates of gross community production as low as  $3.8 \pm 1.03 \ \mu\text{mol} \ O_2 \ dm^{-3} \ d^{-1}$  (day 173) and to negative rates of net community production (-9.4 ± 1.13 \ \mumol \ O\_2 \ dm^{-3} \ d^{-1}, also on day 173). After approximately a month, community production rised again exhibiting two distinct peaks on days 188 and 219. At the end of September and October, increases in both gross and net community production rates were also detected but of a much lower significance than the spring and summer peaks.

Contrasting with the profiles of 1993 and 1994 given by Blight *et al.*, (1995), the main 1995 spring productive period did not appear to be constituted by two phases. This could have been due to the less frequent sampling programme carried out during this year, resulting in a rougher definition of the seasonal pattern. The maximum registered rates were very close, both in magnitude and time, to those measured in 1993 (34.6µmol  $O_2 \text{ dm}^{-3} \text{ d}^{-1}$ ; day 146) and 1994 (30.1 µmol  $O_2 \text{ dm}^{-3} \text{ d}^{-1}$ ; day 153) by the above authors.

The occurrence of the summer net production peaks of 1995 was somewhat anticipated in relation to that of 1993, and the measured rates (up to  $21.0 \pm 1.23 \mu mol O_2 dm^{-3} d^{-1}$ , day 219) were considerably higher than those reported by Blight *et al.*, (1995) (11.2  $\mu mol O_2 dm^{-3} d^{-1}$ ; day 237). Despite low, net production rates in the autumn of 1995 (6.2  $\pm$  0.34 and 2.7 $\pm$  0.39  $\mu mol O_2 dm^{-3} d^{-1}$  on day 265 and 298 respectively) were higher that those determined in 1994 (maximum of 1.08 on day 263).

The seasonal variation of oxygen respiration during 1995 followed a pattern characterised by two discrete peaks in May (day 143;  $10.0 \pm 0.65 \mu mol O_2 dm^{-3} d^{-1}$ ) and June (day 173;  $13.1 \pm 1.19 \mu mol O_2 dm^{-3} d^{-1}$ ) and a smoother increase in August ( $6.2 \pm 0.53 \mu mol O_2 dm^{-3} d^{-1}$ ; day 219), followed by fairly constant rates until the end of October, declining thereafter (Figure 4.5c). The May respiration peak coincided with the seasonal maximum net production whereas the June rise was concurrent with negative net production rates. Other occasions when oxygen respiration exceeded net oxygen production were on days 250, 279 and 320. During summer, in July and August, the respiration profile did not follow closely that of the net production but high rates of respiration were coincident with the peaks of net community production.

The 1995 seasonal profile of respiration was quite dissimilar to that reported by Blight *et al.*, (1995) for the years of 1993 and 1994, with maximum rates well below those measured (particularly) in 1993. During 1993, a single, well marked peak in the beginning of June characterised the seasonal pattern; this peak reached a value of 39.4  $\mu$ mol O<sub>2</sub> dm<sup>-3</sup> d<sup>-1</sup> and was observed with a lag period of two weeks in relation to that of net community production. Respiration rates then declined sharply, remaining below 5  $\mu$ mol O<sub>2</sub> dm<sup>-3</sup> d<sup>-1</sup> from the end of July. A lag period of about three weeks between the early net community growth maximum (24.7  $\mu$ mol O<sub>2</sub> dm<sup>-3</sup> d<sup>-1</sup>; day 107) and that of respiration (20.8  $\mu$ mol O<sub>2</sub> dm<sup>-3</sup> d<sup>-1</sup>; day 131) was also detected in 1994 (Blight *et al.*, 1995). However, at a later stage, such phasing was absent with the second respiration peak coinciding with that of net community production (day 153). Such an interval between net community production and respiration peaks was not so evident in the seasonal pattern of the oxygen flux measurements undertaken in 1995. In 1995, as in the two previous years, there was no respiratory response to the increased rates of net growth in late summer/autumn.

Based on the 1995 results, the seasonal cycle of production in these waters can be divided into four different phases:

- I. Primary net autotrophic phase (April and May) PNAPh
- II. Net heterotrophic phase (June) NHPh
- III. Secondary net autotrophic phase (July and August) SNAPh
- IV. Regenerative phase (from September onwards) RPh

# 4.2.4. Uptake of <sup>15</sup>N- Nitrate and <sup>15</sup>N- ammonium

# 4.2.4.1. Determinations in 1995

## Uptake of Nitrate Over the Diel Cycle

The uptake of nitrate by the whole community was low throughout the 1995 seasonal study, with rates generally below 0.05  $\mu$ M d<sup>-1</sup>. Exception to this was the rapid increase in May, leading to maximum uptake rates between 0.4856 ± 0.02326 and 0.7400 ± 0.01643  $\mu$ M d<sup>-1</sup> (as given by the 6 and 24 hours incubation experiments respectively) (Figure 4.6.a). This nitrate uptake peak was associated with the primary net autotrophic phase defined above, coinciding with the seasonal highest net production rates (Figure 4.5.b) and chlorophyll concentrations (Figure 4.3), and resulting in the rapid decline of ambient nitrate (Figure 4.2. a).

The lowest uptake was recorded at the beginning of both July (day 188; between 0 and  $0.0073 \pm 0.00042 \ \mu M \ d^{-1}$ ) and August (day 219; between  $0.0025 \pm 0.00003$  and  $0.0037 \pm 0.000021 \ \mu M \ d^{-1}$ ) mirroring the low availability of ambient nitrate in these waters (below detection levels to  $0.2 \ \mu M$ ) at this time of the year.

To the autumn chlorophyll peak (day 298) a slight increase in nitrate uptake was detected in the 6 hours incubation experiment (from  $0.0099 \pm 0.00030 \,\mu\text{M} \,d^{-1}$  on day 265 to  $0.0400 \pm 0.0000 \,\mu\text{M} \,d^{-1}$  on day 298). For the 24 hour experiment this was not evident, possibly as the result of substrate recycling which could have resulted in the dilution of the particulate matter <sup>15</sup>N-atom %. Unfortunately, this cannot be proven since regeneration experiments were not carried out concomitantly.

The results also show that for the majority of the cases there were no marked discrepancies between daily rates estimated from the 6 and 24 hour incubation experiments. This suggests that in either case uptake was linear with time and/or that errors resulting from the 6 hours extrapolations cancelled out those which may have occurred in the longer incubations.

On five occasions (days 101, 143, 233, 298 and 320) daily uptake rates obtained from the 6 hour incubation extrapolations exceeded those resulting from the 24 hour experiments by

up to 7 times. It is possible that under conditions of substrate depletion, the addition of even a small concentration of (labelled) nutrient may induce surge uptake which, in the present circumstances, would have been perceived mostly in the samples subjected to short incubations, resulting in the overestimation of the extrapolated daily rates.



**Figure 4.6.** Seasonal variation of <sup>15</sup>N-nitrate uptake by the whole community **a**) over the diel cycle and **b**) in dark only conditions. Results from the 6 and 24 hour incubation experiments. Bars represent standard errors.

During longer incubations, the <sup>15</sup>N-atom% in the particulate matter taken up during the surge uptake could decrease as a result of the dilution of the <sup>15</sup>N-atom% of the substrate due to its regeneration, leading to lower estimates of the uptake rates.

Dilution of the <sup>15</sup>N atomic percentage of the particulate matter could also have happened as a result of substrate exhaustion, on day 143, when uptake rates based on the 6 hour incubation experiment exceeded those based on the 24 hour ones, by a 2 fold factor. According to the uptake rates measured, the low ambient nitrate (0.6  $\mu$ M) could have been consumed over the 24 hour incubation period, substrate exhaustion could have taken place, leading to low <sup>15</sup>N-atom% in the particulate matter at the end of the incubation period resulting, therefore, in underestimated rates.

When the contrary happens, *i.e.*, when the 24 hour incubation based uptake rates largely exceed those based on shorter incubations (as it was the case of day 208:  $0.0659 \pm 0.00306$   $\mu$ M d<sup>-1</sup> against  $0.312 \pm 0.00173 \mu$ M d<sup>-1</sup> respectively) it is possible that the organisms exhibited a lag period when the uptake was particularly slow, so that adaptive changes in their metabolism could take place to allow the uptake of the nutrient in question. This is particularly the case when other preferential forms of nitrogen are present, such as ammonium, which phytoplankton will use until it is exhausted, before switching to the uptake of other less energetically efficient forms of nitrogen (such as nitrate).

It is important at this point to recall that on five occasions (days 158, 173, 188, 219 and 233), uptake rates were estimated assuming the ambient nitrate concentration ( $0.025\mu$ M - the lower limit of detection of the method used to determine ambient NO<sub>3</sub><sup>-</sup>) since the actual levels were below detection limits. Therefore the calculated rates constitute overestimations of the true values, and the results obtained for these dates should be regarded with due uncertainty.

#### Nitrate Uptake in Dark Only Conditions

The seasonal profile of dark nitrate uptake by the whole community was similar to that in the light with peaks in May ( $0.0504 \pm 0.00115$  to  $0.1088 \pm 0.00611 \mu M d^{-1}$ ; day 143), July ( $0.0244 \pm 0.00257$  to  $0.0277 \pm 0.00167 \mu M d^{-1}$ ; day 208) and end of October ( $0.0282 \pm 0.00379 \mu M d^{-1}$ ; day 298). However, the highest rates were observed early in the year ( $0.0585 \pm 0.00711$  to  $0.1168 \pm 0.00637 \mu M d^{-1}$ ; day 59), unlike the uptake over the diel cycle (Figure 4.6.b).

The proportion of dark uptake in relation to that in the light varied widely between values as low as 2% (e.g., on day 131; 24 hour incubation experiments) and those higher than

100% (e.g., on day 173; 24 hour incubation experiments) with the majority laying below 40%. The lowest percentages were observed at times of high net production and increased levels of chlorophyll (e.g., on days 131, 143, 188, 219, 233, 265 - for results obtained with the 24 hours incubations experiments). On the contrary, dark uptake assumed higher importance at less productive stages of the seasonal cycle, with rates as high as or higher than those measured in the light (e.g., from day 59 to 115 and on days 158 and 173). These results suggest that lower percentages of nitrate uptake in the dark in relation to those in the light are associated with a dominance of autotrophic processes in these waters (primary and secondary net autotrophic phases) whereas the contrary seem to be concurrent with periods when the heterotrophic processes become more important (net heterotrophic phase and regenerative phase).

This analysis of the results regarding the dark nitrate uptake by the whole community was based mostly on the 24 hour incubation experiments. In some cases (*e.g.*, on days 173, 158, 143, 88) the discrepancy between these and estimates given by the 6 hour incubation experiments was not marked but in others the two procedures lead to diverging results (*e.g.*, days 219, 265 and 298). Extrapolated daily rates obtained with the 6 hour incubation experiments were generally higher (up to 25 times; day 298) than those from the 24 hour incubation experiments, with the exception of the period between the beginning of June and end of August when they were in close agreement. Once more, reasons for underestimations resulting from the 24 hour incubation experiments are based on the possibility of substrate depletion (particularly on those occasions when the nitrate ambient concentration was below  $0.025\mu$ M) and recycling. Extrapolations from the 6 hour incubation experiments assume that the uptake would have been linear with time which is often not the case, resulting in overestimated uptake rates. Surge uptake caused by <sup>15</sup>N-labelled substrate addition could also have taken place leading to inflated, extrapolated daily rates, as explained before.

#### Uptake of Ammonium Over the Diel Cycle

The uptake of ammonium by the whole community (Figure 4.7a) followed a seasonal pattern which exhibited three distinct peaks: the first in the beginning of May (day 131;  $0.4226 \pm 0.00761$  to  $0.6884 \pm 0.00040 \,\mu\text{M} \,\text{d}^{-1}$ ); the second, of similar magnitude, at the end of July ( $0.6167 \pm 0.00924$  to  $0.6564 \pm 0.01256 \,\mu\text{M} \,\text{d}^{-1}$ ; day 208), and finally the third and

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slightly lower peak, at the end of August, (day 233), with a maximum of 0.4800  $\pm$  0.00822  $\mu M~d^{-1}$  .



Figure 4.7. Seasonal variation of  $^{15}$ N-ammonium uptake by the whole community a) over the diel cycle and b) in dark only conditions. Results from the 6 and 24 hour incubation experiments. Bars represent standard errors.

Whilst the duration of the first and second peaks was short, with rates rising and declining sharply over a period of one month, for the last one, to the steep increase in the uptake, followed a long period (from 2 to 3 months) when the rates fell slowly, never reaching very low values.

The first, second and part of the third ammonium uptake maxima were associated with the primary and secondary net autotrophic phases and coincided with periods of increased concentration of ambient ammonium (Figure 4.2.c). From August, the prolonged availability of this nutrient appeared to be sufficient to sustain the last and protracted uptake period, well into the regeneration phase. The May peak was registered a fortnight earlier than the net community oxygen production and chlorophyll maxima as it had happened with the highest nitrate uptake rate. The second and part of the third ammonium uptake peaks occurred at a time of low phytoplankton biomass (July and August) but the later stages of the third peak appear to have sustained the minor autumn chlorophyll bloom (day 298).

The spring and summer ammonium uptake peaks were separated by a period of very low uptake rates (0.0155  $\pm$  0.00112 to 0.1052  $\pm$  0.00063  $\mu$ M d<sup>-1</sup>) between the end of May (day 143) and beginning of July (day 188). This period was characterised by decreased chlorophyll concentrations (Figure 4.3) and levels of ambient ammonium between below detections limits (0.030  $\mu$ M) and 0.1 $\mu$ M. In all but one (day 188) of the four uptake measurements undertaken during this period, the ambient ammonium concentration had to be assumed to be at the lower detection limit level (0.030 $\mu$ M) and consequently the uptake rates calculated must be considered as overestimated.

The results have also shown that the uptake rates calculated from the 6 and 24 hour incubation experiments were close, with the former generally exceeding the later by no more than 2.6 times (day 173). This difference was obtain at a time when the ambient concentration of ammonium was below detection limits, therefore discrepancies were expected for the reasons already presented. During the spring peak (day 131), rates were 1.6 times overestimated by extrapolating from the 6 hour incubation experiments.

#### Uptake of Ammonium in Dark Only Conditions

The dark ammonium uptake by the whole community followed a seasonal pattern very close to that over the diel cycle, with peaks on days 131 (0.2489  $\pm$  0.01394  $\mu$ M d<sup>-1</sup>), 208 (0.1960  $\pm$  0.00181  $\mu$ M d<sup>-1</sup>) and 233 (0.2406  $\pm$  0.00425  $\mu$ M d<sup>-1</sup>) (Figure 4.7.b). This seasonal profile was consistent for rates estimated from both the 6 and 24 hour incubation experiments, with the former exceeding the latter generally by two fold.

The percentage of dark uptake in relation to that in the light varied between values as low as 1% (day 188; 24 hour incubation experiments) and more than 100% (day 88; 6 and 24 hour incubation experiments; day 173, 6 hour incubation experiment). However, the majority of the percentages fell between 40 and 90%, with the lowest values found during the primary and secondary net production phases and the highest early in the year (day 88) and during the net heterotrophic phase (day 173). The regenerative phase of this seasonal cycle exhibited dark uptake rates which were approximately 50% of those measured in the light.

# 4.2.4.2. Determinations in 1994

## **Uptake of Nitrate**

The nitrate uptake rates by the whole community during 1994 were of the same order of magnitude as those determined in 1995, with a maximum of  $0.4590 \pm 0.03585 \ \mu M \ d^{-1}$  on day 108 (Table 4.2).

	DIEL CYCLE		DARK	
	Ammonium	Nitrate	Ammonium	Nitrate
Day of the Year	mean ± se	mean ± se	mean ± se	mean ± se
108	-	$0.4590 \pm 0.03585$	-	$0.0000 \pm 0.00000$
123	-	$0.1079 \pm 0.00723$	-	$0.0039 \pm 0.00049$
137	$0.0209 \pm 0.00000$	$0.0220 \pm 0.00049$	$0.0295 \pm 0.00016$	$0.0000 \pm 0.00000$
152	$0.0260 \pm 0.00065$	$0.0207 \pm 0.00065$	$0.0498 \pm 0.00225$	$0.0227 \pm 0.00000$
257	$0.0872 \pm 0.00086$	-	$0.0209 \pm 0.00122$	-
295	$0.0427 \pm 0.00111$	-	$0.0246 \pm 0.00257$	-
318	$0.0261 \pm 0.00388$	$0.0388 \pm 0.00000$	$0.0200 \pm 0.00037$	$0.0439 \pm 0.00151$

**Table 4.2.** <sup>15</sup>N uptake rates ( $\mu$ M d<sup>-1</sup>) by the whole community, over the diel cycle and in dark only conditions in 1994.

The spring peak was recorded approximately one month in advance to that in the following year. It coincided with the first peak of chlorophyll and of net community oxygen production in these waters. Over the next month and a half, nitrate uptake decreased to rates of  $0.0207 \pm 0.00065 \,\mu\text{M} \,\text{d}^{-1}$ , despite the heavy blooming of phytoplankton, illustrated by the high chlorophyll concentrations (Figure 4.3) and net community production (Figure

4.5b). Over this period of time the ambient nitrate levels declined sharply (in agreement with the measured uptake rates) reaching concentrations below detection from mid May (Figure 4.2.a) An estimate of nitrate uptake of  $0.0388 \pm 0.0000 \ \mu M \ d^{-1}$ , by the whole community, in the autumn (day 318) is in close agreement with measurements undertaken for the same period of time in the following year.

Dark nitrate uptake during the spring of 1994 was only detectable on three occasions: day 123,  $(0.0039 \pm 0.00049 \,\mu\text{M d}^{-1})$ , day 152,  $(0.0227 \pm 0.00000 \,\mu\text{M d}^{-1})$  and day 318  $(0.0439 \pm 0.00151 \,\mu\text{M d}^{-1})$ , constituting 3.6% of that over the diel cycle on the first occasion and more than 100% in the others.

#### **Uptake of Ammonium**

The ammonium uptake by whole community started to be determined by the middle of May, therefore, only two measurements exist for the spring of 1994 (Table 4.2). The measured rates were identical to those of nitrate uptake, and were observed at a time of high ambient ammonium availability (4.7  $\mu$ M). The uptake of this nutrient was also measured between September (day 257) and November (day 318), roughly at monthly intervals. Over this period of time, rates decreased from 0.0872 ± 0.00086  $\mu$ M d<sup>-1</sup> (day 257) to 0.0261 ± 0.00388  $\mu$ M d<sup>-1</sup> (day 318). During spring, dark ammonium uptake exceeded that in the light and in the autumn it made up to 77% (day 318) of that (28% on day 257) and 53% on day 295). The autumn peak of ammonium uptake (day 257) coincided with the ambient ammonium maximum for this time of the year (0.54 $\mu$ M; Figure 4.2.c) and it preceded the autumn chlorophyll peak (day 295; Figure 4.3).

# 4.2.5. "f-Ratio"4.1.

One way of comparing the relative contribution of each form of inorganic nitrogen to the primary production measured in a body of water, is to calculate the f-ratio, an index defined by Eppley & Peterson (1979) as the quotient of new to total nitrogen uptake, as follows:

$$f = \rho_{\text{NO3}} / (\rho_{\text{NO3}} + \rho_{\text{NH4}} + \rho_{\text{urea}})$$
 (4.1)

<sup>&</sup>lt;sup>4.1.</sup> The reason we have chosen to place the *f*-ratio between "" is described in section 4.5.1.1.

where  $\rho_{NO3}$ ,  $\rho_{NH4}$  and  $\rho_{urea}$  are the rates of uptake of nitrate, ammonium and urea, respectively. Thus f is the proportion of the "new nitrogen" that is assimilated by phytoplankton and 1-f is the proportion of regenerated nitrogen equally assimilated. In Figure 4.8a) the seasonal variation of the "f ratio" in the Menai Strait waters for the year of 1995 is depicted.



Figure 4.8. a) Seasonal variation of the "f ratio" during 1995. Estimations derived from the 6 and 24 hour incubation experiments. b) Analogy between the phases defined for the "f ratio" and those for the net community oxygen production with PNAPh, NHPH, SNAPh and RPh as defined before.

Uptake rates estimated from both the 6 and 24 hour incubation experiments were used. Since the uptake of urea was not measured in this study, that of nitrate and ammonium was considered to be the total nitrogen uptake for the calculation of the "*f*-ratio".

There was a progression with the time from the predominant use of nitrate ("f-ratio" > 0.5) to that of ammonium ("f-ratio" <0.5), which is in agreement with the seasonal availability of these nutrients in this environment. Accordingly, the seasonal cycle can be divided into three distinct phases as far as the temporal distribution of the "f-ratio" is concerned:

**Phase I,** until the end of May, characterised by "*f*-ratios" greater than 0.5 (and up to 0.9), indicating a predominant use of new nitrogen; **Phase II**, during June, featuring decreased ratios, between 0.2 and 0.45. This indicates that although nitrate was still being used, the uptake of ammonium was becoming more important than previously. Thus, this phase can be seen as a transitional period; **Phase III**, from July, distinguished by its low "*f*-ratios", in the majority of cases below 0.25, implying a preponderant uptake of regenerated forms of nitrogen.

A parallel can be drawn between **phase I** and the primary net autotrophic phase defined before (Figure 4.8.b). During this periods, 40 to 95% of the net growth was supported by the uptake of nitrate constituting, according to Dugdale & Goering (1967), "new production". To the maximum of net production on day 143 corresponded an uptake of new nitrogen of 87% in relation to the total nitrogen uptake. Likewise, **phase II**, matches with the net heterotrophic period, characterised by negative net oxygen production and high oxygen respiration, suggesting that during June, nitrogen uptake could have been heterotrophic. **Phase III**, can be divided into two periods, the first comprehending the months of July and August, and the second from September onwards. The months of July and August correspond to the secondary net autotrophic phase and according to the "fratios" determined, more than 80% of the net production was supported by ammonium thus, "regenerated production" in the sense of Dugdale & Goering (1967). For the period after September and equivalent to the regenerative phase, the low net production registered was also predominantly (more than 75%) supported by the uptake of ammonium ("regenerated production").

# 4.2.6. <sup>13</sup>C uptake

# 4.2.6.1. Determinations in 1995

# Uptake Over the Diel Cycle

The uptake of <sup>13</sup>C by the whole community followed a seasonal pattern characterised by three peaks which occurred in May (maximum of  $10.31 \pm 0.616 \mu M d^{-1}$  on day 131); beginning of July (maximum of  $6.55 \pm 0.482 \mu M d^{-1}$  on day 188); and end of August (maximum of  $5.77 \pm 0.844 \mu M d^{-1}$  on day 233) (Figure 4.9.a). Being the seasonal highest, the May maximum rate was registered a fortnight earlier than the peaks of chlorophyll (Figure 4.3) and net community oxygen production (Figure 4.5.b)

The timing of the summer uptake maxima agreed well with that of the phytoplankton net growth, and fluctuations in the <sup>13</sup>C uptake exhibited a profile identical to that of ammonium uptake (Figure 4.7.a), indicative of the close relationship between the uptake of this form of regenerated nitrogen and production at this time of the year. Similarly, the May <sup>13</sup>C uptake peak coincided with that of nitrate uptake (Figure 4.6.a) confirming the importance of this nutrient to the early stages of primary production in these waters.

Based on these production measurements and on the "*f*-ratios" estimated previously, a seasonal distribution of the "new" and "regenerated production" in terms of carbon was calculated and is presented also in Figure 4.9.b. The three phases defined in the previous section can be clearly distinguished with "new production" dominating phase I (up to 7.80  $\mu$ M d<sup>-1</sup> on day 143) and "regenerated production", phase III (up to 6.13  $\mu$ M d<sup>-1</sup> on day 188). During the transitional phase (phase II), the co-occurrence of "new" and "regenerated" production is also evident, although production was very low (approximately 0.5  $\mu$ M d<sup>-1</sup>).

# **Uptake in Dark Only Conditions**

Uptake of <sup>13</sup>C by the whole community in dark only conditions was detected (Figure 4.9.c) and although it constituted less than 13% (seasonal mean) of that over the diel cycle for most of the year, during April (day 115) and June ( days 158 and 173) this percentage increased to nearly 50% (Figure 4.9.c). The high dark uptake of <sup>13</sup>C in June agrees well with the net heterotrophic character of this period, as indicated before, and it is possible



Figure 4.9. a) Seasonal variation of  ${}^{13}$ C uptake by the whole community over the diel cycle. b) Total, "new" and "regenerated" C production, with the "new" and "regenerated" C production calculated with basis on estimated "*f*-ratios". c) Seasonal variation of  ${}^{13}$ C uptake by the whole community in dark only conditions: absolute and relative (as percentage of C uptake over the diel cycle) estimates. Bars represent standard errors.

that it was carried out by heterotrophic bacteria (as will be discussed further on). A similar explanation can be attributed to the relatively high uptake at the end of April since the primary net autotrophic phase had not yet fully developed. It is interesting to note that during the primary and secondary net autotrophic periods, the percentage of dark uptake in relation to that over the diel cycle was at its lowest,  $\leq 2.5\%$ , whereas it increased during the regenerative phase and was particularly high at the net heterotrophic phase, as indicated above.

Figure 4.9.c) shows that the seasonal profile of dark uptake does not entirely agree with that of the uptake over the diel cycle. Although it features two main periods of relatively high uptake, these peaks are not always concurrent with those observed over the diel cycle. For instances, whereas there is a well defined maximum of light <sup>13</sup>C uptake in May, followed by a sharp decrease and low rates in June, to the May increase in <sup>13</sup>C dark uptake follows an extended period of high rates which only start to decline by July. Contrary to the light uptake profile, that of dark uptake does not exhibit well defined peaks in July and August. Furthermore, to the linear decrease in the diel uptake rates observed from the end of August does not correspond such a rapid fall in dark uptake rates which only becomes evident from October.

# 4.2.6.2. Determinations in 1994

The scarce measurements of <sup>13</sup>C uptake undertaken during the spring of 1994 revealed a peak between the end of May (day 137; 20.61  $\pm$  1.566  $\mu$ M d<sup>-1</sup>) and the beginning of June (day 152; 23.92  $\pm$  1.277  $\mu$ M d<sup>-1</sup>) (Table 4.3). This maximum coincided with that of net community oxygen production for this season (27.86 to 30.12  $\mu$ M d<sup>-1</sup>) (Figure 4.5.b) but not with the chlorophyll peak which was registered on day 145 (15.36  $\mu$ g dm<sup>-3</sup>) (Figure 4.3). The value of 23.92  $\mu$ M d<sup>-1</sup> is more than the double of that measured in the following year, despite the fact that in 1995 net community production exceeded that in 1994 (37.62  $\mu$ M d<sup>-1</sup> in 1995 against 30.12  $\mu$ M d<sup>-1</sup> in 1994). However, chlorophyll maximum concentration reached only 8.34  $\mu$ g dm<sup>-3</sup> in 1995, approximately half than in 1994, which could be the cause for the difference in C primary production between these 2 years. The uptake rates observed in the following year for the same stage of the seasonal cycle.

	DIEL CYCLE	DARK	
Day of the Year	mean ± se	mean ± se	
108	•	-	
123	$2.47 \pm 0.340$	$0.1236 \pm 0.000$	
137	$20.61 \pm 1.566$	$0.3881 \pm 0.042$	
152	$23.92 \pm 1.277$	1.0867 ± 0.017	
257	$0.85 \pm 0.105$	$0.0440 \pm 0.013$	
295	$0.19 \pm 0.032$	$0.0243 \pm 0.008$	
318	$0.56 \pm 0.064$	$0.0368 \pm 0.004$	

**Table 4.3.** <sup>13</sup>C uptake rates ( $\mu$ M d<sup>-1</sup>) by the whole community, over the diel cycle and in dark only conditions, in 1994.

Uptake of C in the dark was also detectable and it constituted between 2 and 13% of that over the diel cycle, with the lowest percentages observed during the highest productive periods and the highest during autumn.

# 4.2.7. C:N Uptake Ratio

The seasonal profile of the variation of the C:N uptake ratio by the whole community is shown in Figure 4.10. Total N uptake (ammonium and nitrate) estimates based on the 6 and 24 hour incubation experiments were used to calculate the ratios. The uptake ratios calculated from <sup>15</sup>N uptake estimates based on the 6 and 24 hour incubation experiments showed no major discrepancies. On day 143, however, the C:N uptake ratio based on the 6 hour incubation experiment was approximately half of that for the 24 hour incubation experiment mirroring the difference between the <sup>15</sup>N uptake rates obtained with each experimental procedure. Throughout the season their patterns followed the same trend, with virtually the same ratios at certain parts of the year (*p.e.*, March, August and November).

Figure 4.10 shows that throughout the year the C:N uptake ratio oscillated more or less broadly around the Redfield ratio of 6.6, except in the following occasions when it increased to anomalously high values: on days 143, 188 and 219, ratios of 32.5 (24 hour experiments), 88.6 (6 hour experiments) and 182.2 (6 hour experiments) were,

respectively, obtained. These peaks coincided with the primary and secondary net autotrophic phases suggesting that at these stages primary production was nitrogen limited and more so in the summer than in spring.



Figure 4.10. a) Seasonal variation of the whole community uptake and compositional C:N ratio; b) detail of the lower part of a).

During July and August, nitrate levels were between below detection and 0.2  $\mu$ M and although some ammonium was present (0.18 to 0.89  $\mu$ M), concentrations did not appear to be sufficiently high to result in a "balanced" uptake ratio. At this time of the year, C:N

uptake ratios could, in reality, be higher because the  ${}^{15}NO_3$  uptake rates were possibly overestimated since the ambient nitrate concentration had to be assumed for the purpose of rate calculation. At the end of May (day 143) nitrogen depletion was as severe as in July and August, with nitrate concentrations down to 0.6  $\mu$ M and ammonium below detection levels. However, these conditions were preceded by a long period of nutrient abundance, resulting in lower C:N uptake ratio than those observed in the summer. Excluding the three highest estimates, the seasonal mean of the C:N uptake ratio was around 8.5, very close to the Redfield ratio and according to Goldman *et al.*, (1979) indicative that the phytoplankton population was not nutrient limited and was being able to grow at maximum rates.

Compared to the compositional (Figure 4.10), the uptake C:N ratio was more variable throughout the year and its seasonal pattern did not follow that of the former. Even during the secondary net autotrophic phase, when the uptake C:N ratios where particularly high, the measured compositional C:N ratio of the particulate matter oscillated only between 5.2 and 8. This discrepancy between compositional and uptake ratios is indicative of a loose coupling between photosynthesis and inorganic nitrogen uptake (MacIsaac & Dugale, 1972).

# 4.3. PICOPLANKTONIC (<2µm) CHARACTERISTICS AND METABOLIC PROCESSES

# 4.3.1. Bacteria Abundance

The seasonal variation of bacteria abundance (Figure 4.11.) in the waters of the Menai Strait during 1995 followed a profile featuring a main peak in June (day 173;  $7.3 \times 10^6$  cells cm<sup>-3</sup>) followed by a lesser one a month later (day 207;  $2.1 \times 10^6$  cells cm<sup>-3</sup>). The number of bacteria then slowly declined towards the autumn reaching minimum values in winter (day 59;  $0.42 \times 10^6$  cells cm<sup>-3</sup>).



Figure 4.11. Seasonal variation of the bacterial abundance in the Menai Strait during 1995. Comparison with 1994 data from Blight *et al.*, (1995) - as circles and author's own determinations - as triangles.

The occurrence of a bacteria maximum during June is in good agreement with the predominant

heterotrophic character this of month, coinciding with the net heterotrophic phase defined before. It followed the spring of primary peak production with a lag period of one month. The following bacteria

abundance peak, in July (day 207), was associated with the initial part of the secondary net autotrophic phase, with maximum cell numbers occurring a fortnight later than the first peak of net community production at this stage of the seasonal cycle (day 188). On the contrary, bacteria did not appear to respond to the second net community production peak on day 219, with cell numbers starting to decline after day 207.

Data from 1994 (Figure 4.11) compared well with those from 1995 for the early part of the year, with the highest counts observed in May. However the magnitude of the peak of bacteria abundance in 1994 was approximately half of that in 1995. Furthermore, in 1994 an autumn peak was evident, occurring on day 273 ( $3.09 \times 10^6$ ), which was not seen during the 1995 seasonal cycle. A reason for the 1994 spring peak to be lower than that of 1995 could be that counts were performed in the <200µm size fraction of the community whereas in 1995 the whole community was considered. Sample pre-screening could have resulted in the exclusion of a considerable number of cells attached to large particles or even of free living cells, which could have been retained by the clogged mesh.

Size fractionation of the samples also lead to further loss of bacteria cells. Numbers in the  $<2\mu m$  size fraction amounted to 64 to 100% of those in the whole community, with

differences between 10 to 50% more frequently found. Due to the large errors associated with this technique (already discussed in CHAPTER III) some of the smaller differences may not be statistically significant. However, this was not possible to ascertain since replicate counts were not possible to carried out.

# 4.3.2. Chlorophyll Content of the Picoplanktonic Fraction

Ideally the  $<2\mu$ m fraction would not contain autotrophic organisms, hence no chlorophyll, so the character of this size fraction could be regarded exclusively as heterotrophic.



The of presence small phytoplankters (or photoautotrophic bacteria such as cyanobacteria) in this fraction is, however, unavoidable when the described method of sample size fractionation is employed, and it needs to he quantified. As it can be seen from Figure 4.12, the presence of

Figure 4.12. Seasonal variation of the chlorophyll absolute and relative contents (as a percentage of that of the whole community) in the  $<2\mu$ m size fraction.

chlorophyll in the picoplanktonic fraction was always detectable throughout the 1995 season, in the waters of the Menai Strait, with the highest concentration registered in May  $(0.43\mu g \text{ dm}^{-3}; \text{ day } 143)$  and lowest at the beginning of the year  $(0.05\mu g \text{ dm}^{-3}; \text{ day } 73)$ .

The spring chlorophyll peak in the  $<2\mu$ m fraction coincided with that in the whole community (Figure 4.3) but the seasonal patterns of variation of these two size fractions were dissimilar, with the picoplanktonic exhibiting two peaks, in the beginning of July (day

188; 0.29  $\mu$ g dm<sup>-3</sup>) and end of August (day 233; 0.39  $\mu$ g dm<sup>-3</sup>), which were absent from the whole community profile. The summer peaks of chlorophyll in the <2 $\mu$ m fraction were concomitant with the two community production maxima (Figure 4.5a and b) suggesting that part of the primary production at this time of the year could be attributed to the autotrophic picoplankton. For this same period of time, the percentage of chlorophyll in the <2 $\mu$ m fraction in relation to the unfractionated varied between 25% (day 207) and 67% (day 233) (Figure 4.12). Interestingly, on day 233, gross oxygen production by this fraction was only 1.8% of that by the whole community.

Throughout the season, the lowest percentages of chlorophyll in the picoplanktonic fraction in relation to that in the whole community were registered between April and June, varying between 1% (day 158) and 13% (day 101). Clogging of the fractionation membranes by the high concentration of phytoplankton in the water at this time of the year, in particular by *Phaeocystis*, could have prevented higher contamination of the  $<2\mu$ m fraction with phytoplankton cells. The absence of such severe conditions in the autumn allowed more chlorophyll containing organisms to pass the membrane.

# 4.3.3. Oxygen Gross and Net Production and Dark Respiration

Oxygen gross production by the  $<2\mu$ m fraction was only significant during July and August having reached a maximum of 2.81 ± 0.84 µmol O<sub>2</sub> dm<sup>-3</sup> d<sup>-1</sup> on day 208 (Figure 4.13). It constituted up to 16% of the unfractionated gross production and coincided with the secondary net autotrophic phase of the seasonal cycle. Earlier in the year (day 73) a small gross production peak was also detected. It only amounted to 1.05 ± 0.95 µmol O<sub>2</sub> dm<sup>-3</sup> d<sup>-1</sup>, but represented 64% of the unfractionated gross production. During the most productive period of the seasonal cycle (May), gross oxygen production by the  $<2\mu$ m fraction remained very low, practically inexistent, with rates only reaching 0.34 ± 0.73 µmol O<sub>2</sub> dm<sup>-3</sup> d<sup>-1</sup>. This is in accordance with the low levels of chlorophyll found in this size fraction during this month (Figure 4.12).



Figure 4.13. Seasonal variation of the oxygen fluxes by the  $<2\mu$ m size fraction. Net and gross production should be read, in the graph, as rates of oxygen production. In this case, net production was always negative indicating that, in fact, no oxygen was produced during the seasonal cycle; gross production of oxygen ocurred mostly during July and August and early in the year (March). Dark respiration, should be read, in the graph, as rates of oxygen consumption.

For the whole of the season, net oxygen production remained negative suggesting a predominantly heterotrophic character of this fraction. Further confirmation of this arises from the dark oxygen respiration rates, which remained high between May and end of October when the season's highest rate was registered (day 298;  $8.76 \pm 2.470 \mu mol O_2 dm^{-3} d^{-1}$ ). Throughout the year, several peaks of respiration were detected, namely on days 143 (7.97  $\pm$  0.73  $\mu mol O_2 dm^{-3} d^{-1}$ ) and 219 (7.60  $\pm$  0.49  $\mu mol O_2 dm^{-3} d^{-1}$ ). During June, coinciding with the net heterotrophic phase, high respiration rates persisted, (up to 7.37  $\pm$  0.42  $\mu mol O_2 dm^{-3} d^{-1}$ ).

The seasonal profile of picoplanktonic respiration was distinct from that of the whole community (Figure 4.5c). Although a peak in May was characteristic of both profiles, respiration by the  $<2\mu$ m fraction extended to the regenerative phase of the seasonal cycle whilst that by the whole community started to decrease from the beginning of August (Figure 4.5.c). Respiration by the  $<2\mu$ m fraction generally exceeded that by the whole community, by up to 3 times (day 298). The highest contribution of the  $<2\mu$ m respiration to

the unfractionated was registered in the summer and autumn, whereas earlier in the year the unfractionated respiration appeared to be associated with organisms larger than  $2\mu m$ .

Periods of high picoplanktonic respiration followed or coincided with those of high primary production. For instances, the May production peak was accompanied by high respiration rates by the  $<2\mu$ m fraction which were sustained until the end of June, well after the decline of the phytoplankton bloom. In August, the secondary net production phase was accompanied by a peak of picoplanktonic respiration, as was a small, late peak of primary production at the end of September, but in the latter case with a lag period of approximately one month. This phasing of autotrophic and heterotrophic plankton metabolism has been extensively dealt with in the paper of Blight *et al.*, (1995) and it will not be further addressed here.

# 4.3.4. Particulate Organic Carbon and Nitrogen Contents of the <2μm Fraction.

#### 4.3.4.1. Measurements in 1995

#### Particulate Organic Carbon (POC)

The picoplankton particulate organic carbon seasonal variation (Figure 4.14a) followed a pattern close to that of the whole community (Figure 4.4a), similarly featuring the season's maximum in June (day 158;  $30.1 \pm 5.80 \mu$ M) and representing a contribution to the unfractionated particulate organic carbon of 48% (Figure 4.14b). Other peaks of picoplanktonic POC were observed on days 101 (18.0 ± 1.80  $\mu$ M), 219 (13.1 ± 1.08  $\mu$ M) and 298 (15.5 ± 0.22  $\mu$ M) comprising 53, 41 and 28% of the unfractionated POC, respectively (Figure 4.14b). The lowest contribution of the fractionated POC to the whole community's was recorded earlier in the year (day 88): 17%. However, contributions lower than 30% were not frequent, with the great majority of the estimates lying between 30 and a maximum of 52%.

The  $<2\mu$ m POC maximum in June appeared to be mainly associated with bacteria (rather than with phtyoplankton), as it coincided with the season's peak of bacteria abundance



Figure 4.14. Seasonal variation of the  $<2\mu m a$ ) absolute, b) relative (percent of the unfractionated particulate matter) particulate organic carbon and nitrogen and c) of the corresponding C:N ratio. Comparison with 1994 data. Bars represent standard errors.

(Figure 4.11). On the other hand, at this time of the year, chlorophyll concentrations were low, with levels in the  $<2\mu$ m fraction amounting to 0.03-0.24 µg dm<sup>-3</sup>. Therefore, the contribution of phytoplankters or photoautotrophic bacteria to the  $<2\mu$ m POC peak at this time of the year is believed to be low. Since the contribution of the  $<2\mu$ m POC to the unfractionated was at most 52% at the POC season's peak, and since the chlorophyll concentration amounted merely to 1.95 µg dm<sup>-3</sup> on this occasion, it is possible that the unfractionated POC had a significant component other than phytoplankters or bacteria. In fact, Blight *et al.*, (1995) have reported that at this time of the year the seasonal highest abundance of ciliates and dinoflagelates could be found.

The early August  $<2\mu$ m POC peak (day 219; 13.1 ± 1.08  $\mu$ M) was recorded 12 days after the summer bacteria abundance peak (day 207). Phytoplankters could have contributed to this peak, as the chlorophyll levels in the  $<2\mu$ m fraction showed an increase at this time of the year (Figure 4.12). On the contrary, the autumn POC peak (day 298) did not appear to have developed as a response to an increase in bacteria abundance or chlorophyll concentration in this fraction (Figures 4.11 and 4.12).

## Particulate Organic Nitrogen (PON)

The  $<2\mu m$  PON (Figure 4.14a) did not vary widely over the studied seasonal cycle, with the exception of the month of June when an increase of 7.3 times in relation to the lowest recorded concentration (0.6 ± 0.08  $\mu$ M; day 88) was observed. This maximum concentration reached 4.2 ± 0.65  $\mu$ M on day 158 and was concurrent with the seasonal peak of bacteria abundance. Apart from this situation, picoplanktonic PON fluctuated between the aforementioned minimum and 2.4 ± 0.17  $\mu$ M, with values between 1 and 2  $\mu$ M more commonly found.

The seasonal variation of picoplanktonic PON exhibited a profile very close to that of the whole community (Figure 4.4b) suggesting that organisms in the  $<2\mu$ m fraction constituted a significant proportion of the unfractionated PON. In fact, the seasonal mean contribution of the picoplanktonic PON to that of the whole community amounted to 36%, with approximately two thirds of the contributions above 35% and attaining a maximum of approximately 50% in June (Figure 4.14b).

#### **Picoplankton Compositional C:N Ratio**

The C:N ratio of the  $<2\mu$ m particulate matter varied between a minimum of 4.9 (day 233) and a maximum of 13.2 (day 143), with a seasonal mean of 7.9, a value equal to that of the unfractionated particulate matter (Figure 4.14c). Given that this size fraction is assumed to be predominantly constituted by bacteria, which have a high proportion of N in relation to C in their composition, lower compositional ratios would have been expected, according to other investigators' observations (Nagata, 1986). Three main departures from the mean were observed throughout the seasonal cycle, on days 73 (11.4), 143 (13.2) and 298 (9.8), with the early spring and autumn peaks coinciding with C:N ratio maxima of the whole community. It also suggests a predominantly non-bacterial composition of the  $<2\mu$ m fraction on those particular occasions. Given the concentration of chlorophyll present in this fraction and their percentage in relation to whole community levels, (day 73: 0.05 µg dm<sup>-3</sup>; 13%, Day 143: 0.46 µg dm<sup>-3</sup>; 6%, day 298: 0.09µg dm<sup>-3</sup>; 6%), it appears that a marked phytoplanktonic composition was unlikely. Such high C:N ratios could also be indicative that bacteria were growing in nitrogen depleted conditions.

#### 4.3.4.2. Measurements in 1994

Data from 1994 were generally in good agreement with that collected in 1995 (also Figures 4.14a, b and c) following very approximate seasonal trends. Particulate organic carbon concentrations were, however, higher than those recorded in the following year, with the spring maximum in May (day 137;  $33.0 \pm 8.22 \mu$ M) instead of June (Figure 4.14a). This peak was concurrent with that of the whole community (Table 4.1) constituting up to 26% of it (Figure 4.14b). The lowest concentration was registered in the autumn (day 318), as in 1995 (day 320), with very approximate estimates in both years 7.0 ± 4.18  $\mu$ M and 8.4 ± 2.304  $\mu$ M respectively.

The concentrations of picoplanktonic PON showed less fluctuation than the POC, varying between  $1.3 \pm 0.03 \mu M$  (day 295) and  $2.6 \pm 0.22 \mu M$  (day 137) and were very close to the 1995 estimates (Figure 4.14a). This resulted in generally slightly higher compositional C:N ratios in 1994 than in 1995 (Figure 4.14c), since as seen above, POC estimates of 1994 for the most part exceeded those of 1995.

The contribution of the picoplanktonic PON to the unfractionated varied seasonally between 26% (day 318) and 57% (day 123) whereas the picoplanktonic POC amounted to a maximum of 66% of the whole community's (day 257), with a minimum contribution of 23% on day 152. These percentages of both picoplanktonic PON and POC relate very closely to those found in 1995.

# 4.3.5. Uptake of <sup>15</sup>N-Nitrate and <sup>15</sup>N-Ammonium by the Picoplankton

In this section, results regarding the uptake of nitrate and ammonium by the  $<2\mu$ m fraction obtained in 1994 and 1995 will be presented. An attempt will be made to separate the uptake by the heterotrophic bacteria present in this size fraction from that by autotrophic micro-organisms, (either bacteria or small algae), with basis on the chlorophyll content of the  $<2\mu$ m fraction, as will be described later. This treatment will be applied to the 1995 results only, since this is the more complete set of data available. Results from the intermittent measurements taken in 1994 are presented together with those from 1995 but will receive much less detailed consideration than the latter.

## 4.3.5.1. Uptake Over the Diel Cycle

#### Nitrate uptake

Nitrate uptake by the picoplankton over the natural diel cycle in 1995 was very low throughout the season with rates not exceeding 0.1  $\mu$ M dm<sup>-1</sup> (Figure 4.15a). Estimates from the previous year were, in most cases, lower than those from 1995, exhibiting a similar trend of highest rates during the months of May and June.

The 1995 seasonal variation of nitrate uptake by the  $<2\mu$ m size fraction followed a pattern approximate to that of the unfractionated uptake (Figure 4.6a) exhibiting a main peak in May. However, whereas the whole community maximum uptake was recorded on day 131 that of the  $<2\mu$ m fraction occurred 12 days later (day 143).





Similarly to the unfractionated uptake, other peaks of picoplanktonic nitrate uptake were observed on days 101, 208 and 233, having been generally more perceptible from the estimates derived from the 6 hour incubation experiments than from the 24 hour ones. These peaks were, however. of much lesser importance than that in May. This similarity of seasonal trends between the picoplanktonic nitrate uptake and the unfractionated

suggests a weighty contribution from the <2μm organisms to the total uptake. Figure

4.15b) shows that this contribution was, at times, as high as 84% (day 73, 24 hours incubation experiments). However, for 22 out of 31 determinations the percentage contribution of picoplanktonic uptake to the unfractionated was below 30%, with minimum relative values during May (4.3 to 4.7%), registered on the date of highest nitrate uptake by the whole community (day 131). The peak of nitrate uptake by the <2 $\mu$ m fraction on day 143, corresponded to 16-19% of the unfractionated uptake. Estimates from the previous year (Figure 4.15b) were in agreement with those just described.

Due to the presence of autotrophs in the  $<2\mu$ m fraction indicated by detectable levels of chlorophyll (Figure 4.12), the uptake of the inorganic nitrogen by the organisms in this fraction cannot be guaranteed to be purely heterotrophic. But an absence of a relationship between the chlorophyll contents of the picoplanktonic fraction and the magnitude of the nitrate uptake rates (Figure 4.15c) is indicative that the uptake of nitrate by these organisms must also have a heterotrophic component.



Figure 4.15. c) Relationship between the chlorophyll content and the uptake of nitrate by the  $<2\mu$ m size fraction. Results from the 6 and the 24 hour incubation experiments were averaged.

Assuming that the nitrate uptake measured the unfractionated in sample can be attributed mostly to the phytoplankters, chlorophyll specific rates were calculated. Based on these estimates it was possible to estimate the contribution of chlorophyll associated nitrate uptake (autotrophic uptake) to the total measured by the <2µm fraction, and this was done so for the 6 and 24 hour incubation experiments sets of data, according to the following expressions:

- i) Total  $<2\mu$ m uptake =  $<2\mu$ m autotrophic uptake +  $<2\mu$ m heterotrophic uptake (bacterial).
- ii)  $<2\mu$ m heterotrophic uptake = total  $<2\mu$ m uptake  $<2\mu$ m autotrophic uptake.
- iii)  $<2\mu$ m autotrophic uptake = whole community's chlorophyll specific uptake x  $<2\mu$ m chlorophyll content.

From Figures 4.15d) to g) it can be seen that the May peak of picoplanktonic nitrate uptake started by being due to autotrophic micro-organisms (day 131), despite the low concentrations of chlorophyll in this fraction (0.25  $\mu$ g dm<sup>-3</sup>; 9% of that of the unfractionated), but this was followed by predominant heterotrophic uptake (up to 71% on day 143, the occasion of picoplanktonic nitrate uptake maximum), which was sustained throughout June, when virtually all uptake (98%) could be attributed to heterotrophic micro-organisms.


Figure 4.15. Seasonal variation of the picoplanktonic autotrophic and heterotrophic nitrate uptake over the diel cycle, in 1995. Results from the d) 6 and the e) 24 hour incubation experiment.

This high contribution of bacterial uptake to the picoplanktonic total is in accordance with the net heterotrophic character attributed earlier to this month, coinciding in time with the bacteria abundance peak in these waters.

During July and August the two picoplanktonic nitrate uptake peaks were chlorophyll mostly associated. with only 21.8% having the possibility of being bacterial. Chlorophyll concentrations in this fraction were among the seasonal highest (0.39 µg dm<sup>-3</sup>: day 233) constituting up to 67% of that in the unfractionated samples (Figure 4.12), and some gross

production was detectable (Figure 4.13). This confirms a high contribution by the autotrophic component of the picoplankton at this time of the year, despite a peak in bacteria abundance at the end of July (Figure 4.11). In the autumn (September to November) a strong contribution by the autotrophs to the total picoplanktonic nitrate uptake was found. Despite low ( $\leq 0.18 \ \mu g \ dm^{-3}$ ), picoplanktonic chlorophyll constituted up to 45% of that of the whole community (day 320). However, inconsistent results from the 6



Figure 4.15. Proportion of autotrophic and heterotrophic picoplanktonic nitrate uptake over the diel cycle during 1995. Results from the f) 6 and the g) 24 hour incubation experiments. N.U. - no measurable uptake.

and 24 hour incubation experiments regarding October estimates suggest that during this month up to 80% of the uptake could have been heterotrophic. Chlorophyll concentrations of 0.09  $\mu$ g dm<sup>-3</sup> (6% of those in the unfractionated sample) (Figure 4.12), negative net production rates and the season's maximum of <2 $\mu$ m dark oxygen respiration (Figure 4.13) appear to support the heterotrophic character of October nitrate uptake.

Comparing the purely heterotrophic nitrate uptake with that by the whole community (Figure 4.15h), it can be seen that for the great majority of determinations, the former constituted less than 20% of the latter.



Figure 4.15. h) Seasonal variation of the contribution of the heterotrophic picoplanktonic (bacterial) nitrate uptake over the diel cycle to the unfractionated uptake, in 1995. N.D.- no data available. Results from the 6 and the 24 hour incubation experiments.

In particular, no heterotrophic uptake was associated with the peak of unfractionated nitrate uptake on day 131 (Figure 4.15a); on day 143 the contribution of bacteria to the high uptake rates observed amounted only to a maximum of 13.6%. Deviations from this were found early in the year (day 73) when up to 70% of the unfractionated nitrate uptake was heterotrophic and especially during June, by definition the net heterotrophic stage of the seasonal cycle, when up to 68% of the nitrate uptake was considered to be bacteria associated. Differences in the profiles of Figures 4.15b and 4.15h illustrate the

predominantly low contribution of bacteria to both the picoplanktonic and unfractionated nitrate uptake over the diel cycle.

# Uptake of ammonium

Ammonium uptake by the picoplankton in 1995 varied between  $0.0009 \pm 0.00019 \mu mol dm^{-3} d^{-1}$  (day 88; 6 hour incubation experiments) and  $0.4471 \pm 0.00983 \mu mol dm^{-3} d^{-1}$  (day 208; 24 hour incubation experiments) as shown on Figure 4.16a), being generally higher than those measured in the previous year. In 1995, the uptake followed a seasonal pattern similar to that exhibited by the unfractionated ammonium uptake (Figure 4.7a), characterised by periods of high uptake in early May, July and between the end of August and October. The seasonal profiles followed by the two sets of data regarding the 6 and 24 hour incubation experiments agreed well but on a few occasions rates differed by up to 2.5 times (for instance, on day 158).

The contribution of picoplanktonic uptake to the unfractionated was generally high (Figure 4.16b), hence the similarity of the profiles exhibited in Figures 4.7a and 4.16a. It varied between a minimum of 22% (day 131; 6 hour incubation experiments) and a maximum of 76.8% (day 173; 24 hour incubation experiments) with the majority of determinations above 40%. To the three peaks of unfractionated ammonium uptake registered on days 131, 208 and 233 the <2 $\mu$ m organisms contributed with a maximum of 35.5%, 72.5% and 45.4% respectively. Estimates from 1994 were generally in good agreement with those from 1995.

Similarly to the  $<2\mu$ m uptake of nitrate, chlorophyll specific uptake rates were calculated so that it was possible to separate the autotrophic from the heterotrophic picoplanktonic ammonium uptake. From Figures 4.16c) to 4.16f), a general predominance of picoplanktonic heterotrophic uptake over the autotrophic can be perceived, despite some divergence between the 6 and the 24 hour incubation experiments sets of data. Exceptions to this markedly heterotrophic character of the picoplanktonic ammonium uptake were particularly evident on day 88, when 50 to 100% of the uptake was autotrophic, and on days 233 and 320 when virtually all uptake was chlorophyll associated.



Figure 4.16. a) Seasonal variation of the <sup>15</sup>N-ammonium uptake by the picoplankton over the diel cycle, in 1994 (24 hour incubations) and 1995 (6 and 24 hour incubation experiments); b) picoplanktonic uptake as a percentage of that by the whole community. Bars represent standard errors.

During the summer July months of and ammonium August, uptake was also predominantly autotrophic but to the peak of uptake by the <2µm organisms at end of July, corresponded 65.6% of heterotrophic uptake (day 208; Figure 4.16c to 4.16f). The of prevalence autotrophic uptake during this period had also been observed regarding the uptake of nitrate (Figures 4.15d; e) coincided and with increased chlorophyll concentrations in the <2µm size fraction. which day on 233 constituted up to 67% of the unfractionated (Figure 4.12).

Other maxima of picoplanktonic ammonium uptake, registered in May, June and September can be attributed mostly to the heterotrophic micro-organisms with maximum contributions of 74.7%, 98.2% and 61.6% on days 131, 158 and 265, respectively. The predominant heterotrophic character of the autumn peak was still evident in October with the purely bacterial uptake constituting up to 87% of the total picoplanktonic. This is a

similar situation to that regarding the uptake of nitrate where up to 80% of the picoplanktonic uptake on day 298 could be attributed to bacteria (Figure 4.15f). Likewise, June was the month when virtually all picoplanktonic ammonium uptake was associated with the heterotrophic component.



Figure 4.16. Seasonal variation of the picoplanktonic autotrophic and hetrotrophic ammonium uptake over the diel cycle, during 1995. Results from the c) 6 and d) 24 hour incubation experiments.

The fraction of the ammonium uptake by the whole community that could be attributed to the heterotrophic picoplankton (bacteria) varied seasonally, as shown in Figure 4.16g), having reached a maximum (68.7%) during June, concomitant with the season's bacteria abundance peak and low phytoplanktonic biomass in these waters. The lowest of contributions the picoplanktonic heterotrophic ammonium uptake to the total were registered on days 88 (0.42%) and 233 (0%). However, the large majority of the contributions were found to be above 30%.

To the three distinct unfractionated ammonium uptake peaks (Figure 4.7a) on days 131, 208 and 233,

corresponded a maximum bacterial uptake of 26.5%, 47.6% and 0%, respectively. This



Figure 4.16. Proportion of autotrophic and heterotrophic picoplanktonic ammonium uptake over the diel cycle during 1995. Results from the e) 6 and the f) 24 hour incubation experiments. N.D. - no data available.

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relatively low contribution of the heterotrophic picoplankton to the total uptake during the spring peak had already been found in the case of uptake of nitrate, suggesting that in the early stages of the productive season (primary net autotrophic phase) autotrophs are the main consumers of inorganic nitrogen. However, this situation changes quickly with strictly bacteria uptake dominating the next stage of the cycle (net heterotrophic phase), when phytoplankton biomass declines and bacteria bloom.



Figure 4.16. g) Seasonal variation of the contribution of the heterotrophic picoplanktonic (bacterial) ammonium uptake to the unfractionated uptake over the diel cycle during 1995. N.D. - no data available. Results from the 6 and 24 hour incubation experiments.

Both the secondary net autotrophic phase and the regenerative phase were dominated by autotrophic uptake with the heterotrophic uptake never contributing with more than 47.6% to the total ammonium uptake.

#### Relative Uptake of Ammonium and Nitrate by the Heterotrophic Picoplankton

In Figure 4.17 the seasonal variation of the relative uptake of nitrate in relation to the total inorganic N uptake (nitrate + ammonium) by the heterotrophic bacteria is depicted. Nitrate uptake dominated the total heterotrophic inorganic nitrogen uptake early in the year, with



**Figure 4.17.** Seasonal variation of the relative heterotrophic uptake of nitrate in relation to the total heterotrophic inorganic nitrogen uptake (nitrate + ammonium) over the diel cycle, in 1995. Results from the 6 and the 24 hour incubation experiments.

uptake rates of nitrate exceeding those of ammonium by up to a factor of eight (day 73; 24 hour incubation experiments), until early April (day 101). Between days 101 and 158 the uptake of either form was not prevalent and this could be considered as a transitional phase to the next period, comprehended between June and November, when the uptake of ammonium became progressively more predominant. This pattern is in accordance with the profiles of abundance of nitrate and ammonium in these waters, with nitrate uptake predominating during the period of high ambient nitrate concentration (Figure 4.2a) and ammonium uptake becoming more important with the increased availability of this nutrient in these waters (Figure 4.2c), towards the summer and autumn.

The ambivalent situation observed between day 101 and 158 could be due to the simultaneous availability of both nutrients. Of particular interest is day 131 when a peak of ambient ammonium was registered; despite still available, nitrate did not appear to have been utilised by bacteria (Figure 4.17), indicating clearly the preference of these organisms for the more reduced forms of inorganic nitrogen. With the exhaustion of this short lived ammonium pool, nitrate uptake by the heterotrophic picoplankton was resumed (day 143).

# Contribution of the Picoplanktonic Heterotrophic Uptake of Inorganic Nitrogen to the Unfractionated Uptake

In Figure 4.18 the seasonal variation of the contribution of the heterotrophic bacteria to the total unfractionated uptake of inorganic nitrogen (ammonium and nitrate) is shown.

This contribution was quite significant throughout the year having reached a maximum (68.6%) during June, the net autotrophic phase of the seasonal cycle. During the regenerative phase, particularly in September, October and March, the heterotrophic uptake also assumed a preponderant part in the total inorganic nitrogen uptake. The contribution of the bacterial uptake during the primary and secondary net autotrophic phases was low and the nitrogen taken up at these times of the year, even that by the picoplankton, was mostly chlorophyll associated and had therefore an autotrophic character. For partitioned contributions of nitrate and ammonium heterotrophic uptake to the unfractionated total see Figures 4.15h) and 4.16g).



Figure 4.18. Seasonal variation of the contribution of the heterotrophic uptake (bacterial) to the total unfractionated nitrogen uptake over the diel cycle in 1995. Results from the 6 and the 24 hour incubation experiments. PNAPh stands for primary net autotrophic phase; NHPh for net heterotrophic phase; SNAPh for secondary net autotrophic phase and RPh for regenerative phase of the seasonal cycle.

#### 4.3.5.2. Uptake in Dark Only Conditions

#### Nitrate Uptake

The uptake of nitrate in the dark by the picoplankton (Figure 4.19a) varied seasonally with a pattern approximate to that exhibited for the uptake over the diel cycle (Figure 4.15a). Maximum rates were observed on days 59 (0.0306  $\pm$  0.00149  $\mu$ M d<sup>-1</sup>), 143 (0.0271  $\pm$ 0.00297  $\mu$ M d<sup>-1</sup>) and 265 (0.0255  $\pm$  0.00000  $\mu$ M d<sup>-1</sup>) and, in general, estimates derived from the 6 hour incubation experiments were higher than those from the 24 hour ones. At certain sampling days (59, 101, 115, 208, 219, 265 and 298) the uptake in dark only conditions exceeded that measured over the natural diel cycle whilst in other cases it constituted 10% or less of it (for instance, day 143 and 188; 24 hour incubation experiments). Measurements from the previous year agreed well with those obtained in 1995 but were lower until the end of May.



Figure 4.19. a) Seasonal variation of the <sup>15</sup>N-nitrate uptake by the picoplankton in dark only conditions, in 1994 (24 hour incubations) and 1995 (6 and 24 hour incubations); b) picoplanktonic uptake as a percentage of that by the whole community. Bars represent standard errors.

The contribution of the picoplanktonic to the unfractionated dark nitrate uptake is shown in Figure 4.19b) and as it can be seen, on day 265 all of the uptake measured in the whole community could be accounted for by that in the  $<2\mu m$  fraction. As shown in Figure 4.7b) the whole community dark nitrate uptake seasonal pattern exhibited peaks on days 59, 143, 208 and 298, being the earliest the highest, similarly to the picoplankton uptake situation. On these occasions, the contribution of the picoplankton to the unfractionated was 26.2-38.5% on day 59; 7.9-

25% on day 143; 29.5-51.6% on day 208 and 0-37.6% on day 298. The percent contribution estimates from 1994 were within the range obtained in 1995.

As before, chlorophyll specific uptake rates were calculated and the picoplanktonic uptake of nitrate in the dark divided into an autotrophic and a heterotrophic component.



Figure 4.19. Seasonal variation of the picoplanktonic autotrophic and heterotrophic nitrate uptake in the dark, during 1995. Results from the c) 6 and the d) 24 hour incubation experiments.

in the former date and between 30.% (24 hour experiments) and 77.9% (6 hour incubation experiments) in the latter. To the peaks of dark nitrate uptake by  $<2\mu$ m fraction, the heterotrophic component contributed with 71-80% on day 59; 30.5-77.9% on day 143 and with 77.3-87.8 on day 265.

Figures 4.19c) to f), show that the picoplanktonic uptake of nitrate was predominantly

heterotrophic, particularly during June and October, contributing to the total picoplanktonic uptake with a maximum of 98% (day 158). The lowest contributions were registered on days 88 and when 320, no heterotrophic uptake was detected in the 6 hour experiments set of data, and on day 233, when it constituted 0-10% of the picoplanktonic total. On days 208 and 143 the two sets of data differed quite markedly with these percentages varying between 15.5 (6 hour incubation experiments) (24)and 51.7% hour incubation experiments)



Figure 4.19. Proportion of autotrophic and heterotrophic picoplanktonic nitrate uptake in dark only conditions during 1995. Results from the e) 6 and the f) 24 hour incubation experiments. N.D. - no data available. N.U. - no measurable uptake.

Finally, picoplanktonic heterotrophic nitrate uptake in the dark as a percentage of the unfractionated in the same conditions, is shown in Figure 4.19g).



**Figure 4.19 g)** Seasonal variation of the contribution of the heterotrophic picoplanktonic (bacterial) nitrate uptake to the unfractionated uptake during 1995 in dark only conditions. N.D. - no data available. Results from the 6 and the 24 hour incubation experiments.

The profiles presented agree well with those exhibited in Figure 4.19b) since, as seen above, the picoplanktonic dark nitrate uptake was mostly associated with the heterotrophic component. The contribution of the heterotrophic picoplanktonic to the unfractionated dark nitrate uptake was at times very low or even undetectable (for instances on days 88, 143, 188, 218 and specially 233), but in other occasions accounted for virtually all of the uptake measured in the larger size fraction (day 265). To the peaks of dark nitrate uptake by the whole community (Figure 4.6b), the heterotrophic uptake constituted 18.6-30.9% on day 59; 2.4-19.4% on day 143; 4.6-26.7% on day 208 and finally 0-31.7% on day 298 (Figure 4.19g).

#### **Ammonium Uptake**

The picoplanktonic uptake of ammonium in the dark (Figure 4.20a) varied seasonally. It exhibited peaks on days 131 (0.1010  $\pm$  0.00333  $\mu$ M d<sup>-1</sup>), 208 (0.0944  $\pm$  0.00028  $\mu$ M d<sup>-1</sup>)



and 233 (0.1530  $\pm$  0.00134  $\mu$ M d<sup>-1</sup>), following a profile close to that of the uptake over the diel cycle (Figure 4.16a).

Figure 4.20. a) Seasonal variation of the <sup>15</sup>N-ammonium uptake by the picoplankton in the dark, during 1994 (24 hour incubations) and 1995 (6 and 24 hour incubations); b) picoplanktonic uptake as a percentage of that by the whole community. Bars represent standard errors.

The dark uptake generally constituted more than 50% of that measured over the diel cycle (for instances, on days 88, 115, 208, 320 for both sets of data) exceeding it on two occasions.

The seasonal pattern of variation of picoplanktonic uptake of ammonium in the dark was also identical to that followed by the whole community uptake in identical conditions (Figure 4.7b) with peaks observed on the same occasions. The contribution of the picoplanktonic to the total unfractionated uptake in these cases was 40.6-54.7% on day

131; 48.2-54.3 on day 208 and 62.1-63.6% on day 233 (Figure 4.20b). Throughout the season this contribution was in most cases above 50%, indicative of the importance of the organisms in this small fraction to the overall dark ammonium uptake.



Measurements from 1994 (Figure 4.20a and b) agreed well with those taken in the following year.

Figure 4.20. Seasonal variation of the picoplanktonic autotrophic and heterotrophic ammonium uptake in the dark, during 1995. Results from the c) 6 and the d) 24 hour incubation experiments.

In Figures 4.20c) to f). the dark uptake of ammonium by the  $<2\mu m$ fraction has been divided into its heterotrophic and autotrophic components, based on chlorophyll specific estimates of the uptake rates, as The described before. uptake of ammonium was markedly associated with the heterotrophic picoplankton (as it had happened with the dark nitrate uptake by the <2µm fraction). This was particularly evident on days 59, 73 and 158 when more than 90% of the total picoplanktonic uptake could be attributed to bacteria. Exceptions this to predominant

heterotrophic character of the dark ammonium uptake were observed on days 88 (when up to 76% of the uptake was autotrophic), 208 (when both types of uptake co-occurred), 233 (when all measured uptake was chlorophyll associated) and 320 (when up to 68% was autotrophic).



Figure 4.20. Proportion of autotrophic and heterotrophic picoplanktonic ammonium uptake in dark only conditions during 1995. Results from the e) 6 and the f) 24 hour incubation experiments. N.D. - no data available.

To the peaks of total dark ammonium uptake by the  $<2\mu$ m size fraction (Figure 4.20a) the heterotrophic component contributed with 77.9-83.6% on day 131; 48.2-54.2% on day 208 and made no contribution to the day 233 peak since, as mentioned above, on this occasion all uptake was chlorophyll associated (Figure 4.20e and f). Given this inferred high affinity of the heterotrophic component of the picoplankton for ammonium, it is not surprising that a high proportion of the dark ammonium uptake measured in the unfractionated samples could be attributed to these organisms, as can be seen in Figure 4.20g.



Figure 4.20. g) Seasonal variation of the contribution of the heterotrophic picoplanktonic (bacterial) ammonium uptake in dark only conditions to the unfractionated uptake during 1995. N.D. - no data available.

On day 73, all unfractionated dark uptake of ammonium could be accounted for by the picoplanktonic heterotrophs according to the results of the 6 hour experiments. However, on this occasion the two studied sets of data differed markedly, as it also occurred on days 101 and 188.

To the peaks of unfractionated uptake, the picoplanktonic heterotrophic uptake contributed with 31.6%-45.7% on day 131; 23.2%-29.5% on day 208, and that registered on day 233

was purely related to autotrophic organisms. These relatively low contributions are not surprising since these uptake maxima were registered at times of predominant autotrophic activity (primary and secondary net autotrophic phases of the seasonal cycle). For the rest of the year the heterotrophic uptake constituted mostly between 30 and 75% of the unfractionated.

# 4.3.6. <sup>13</sup>C Uptake by the Picoplankton

### 4.3.6.1. Uptake Over the Diel Cycle

Rates of <sup>13</sup>C uptake by the  $<2\mu$ m size fraction during the 1995 seasonal cycle were between 0.0380 ± 0.00291 µM d<sup>-1</sup> and 2.4514 ± 0.12805 µM d<sup>-1</sup> (Figure 4.21a, total picoplankton uptake curve).





The seasonal fluctuations of the total picoplankton uptake rates followed a pattern identical to that of the unfractionated <sup>13</sup>C uptake, featuring peaks on day 131 (0.8438 ± 0.01228  $\mu$ M d<sup>-1</sup>), 188 (2.4514 ± 0.09055  $\mu$ M d<sup>-1</sup>) and 233 (1.7540 ± 0.03130  $\mu$ M d<sup>-1</sup>). This profile was also close to that of the seasonal variation of the chlorophyll contents in the <2 $\mu$ m fraction (Figure 4.12) with the peaks coinciding with the net autotrophic phases of the seasonal cycle.

Based on chlorophyll specific rates, it was possible to separate the <sup>13</sup>C uptake associated with the autotrophs present in the  $<2\mu m$  size fraction from that by the heterotrophic microorganisms (bacteria) (Figure 4.21b).



Figure 4.21. b) Contribution of the heterotrophic (bacterial) and autotrophic picoplanktonic inorganic carbon uptake to the total uptake by the  $<2\mu$ m size fraction, over the diel cycle, in 1995.

At times the purely heterotrophic <sup>13</sup>C uptake amounted to up to 97% of that by the picoplankton (day 158), contrasting with other times when the picoplanktonic uptake appeared to be virtually all autotrophic (days 88, 131, 219, 233 and 320). In fact, two of the observed picoplanktonic <sup>13</sup>C peaks (day 131 and 233) were not at all related to the heterotrophic component and only 29% of the season's maximum (day 188) was associated

with bacteria. On these three occasions (days 131, 233 and 188), increased chlorophyll concentrations in the  $<2\mu m$  fraction were observed (Figure 4.12).

The contribution of the strictly heterotrophic  $^{13}$ C uptake to the total unfractionated was estimated and is shown also in Figure 4.21a) (percentage of unfractionated uptake curve). This contribution was highest during June, in accordance with the net heterotrophic character of this month, amounting to up to 43.8% on day 158. The remaining contributions were of 18% or less (with the large majority below 10%) except on day 115 when it constituted 30% of the whole community uptake.

#### 4.3.6.2. Uptake in Dark Only Conditions

The dark uptake of <sup>13</sup>C by the total picoplankton varied seasonally between 0.0022  $\pm$  0.00044  $\mu$ M d<sup>-1</sup> and 0.1321 $\pm$  0.00784  $\mu$ M d<sup>-1</sup> (Figure 4.22a).





The highest rates were observed during June (day 158) and beginning of July but throughout the rest of the summer and in the autumn, smaller peaks were also detected

(day 219 and 265). However, whilst in June the dark uptake of <sup>13</sup>C constituted between 55 and 62% of that registered over the diel cycle, the later peaks of uptake amounted only to a maximum of 8 % of the uptake in those conditions. This suggests a predominant heterotrophic character of the earlier uptake. In fact, when correcting these uptake rates to the amount of chlorophyll detected in the  $<2\mu$ m fraction, it can be seen (Figure 4.22 b) that during June (as in the preceding months) most of the picoplanktonic uptake of <sup>13</sup>C in the dark could be attributed to heterotrophic organisms, constituting 98% of the total uptake by this size fraction on day 158.



Figure 4.22. b) Contribution of the heterotrophic (bacterial) and autotrophic picoplanktonic uptake to the total uptake by the  $<2\mu$ m size fraction, in the dark, during 1995.

Exceptions to this preponderant heterotrophic uptake of <sup>13</sup>C in the dark were observed on days 88, 188, 208, 233 and 320 when it amounted to a maximum of 46% of the total picoplanktonic uptake, with the rest associated with the presence of autotrophs in this fraction. The possibility of dark carbon uptake by the primary producers has also been

reported by other investigators (Carpenter & Lively, 1980 and references therein) and does not appear to be unusual.

The highest contribution of the heterotrophic <sup>13</sup>C dark uptake to the unfractionated was in June reaching a value of 60% on day 158. However, the great majority of the values were below 30% (Figure 4.22a).

# 4.3.7. C:N Uptake Ratio

The seasonal variation of the C:N uptake ratio by the  $<2\mu$ m fraction is depicted in Figure 4.23a). It follows a pattern very close to that by the unfractionated phytoplankton (Figure 4.10). This similarity of profiles suggests a heavy contribution of the smaller organisms to the overall C:N uptake ratio.



Figure 4.23. a) Seasonal variation of the  $<2\mu$ m fraction C:N uptake ratio over the diel cycle, during 1995. Comparison with the  $<2\mu$ m POM compositional C:N ratio. For the calculation of the C:N uptake ratio, the results from the 6 and 24 hour incubation experiments were averaged.

Identically to the unfractionated seasonal pattern, that of the picoplankton exhibited minor peaks in the early spring (day 101; C:N uptake ratio between 7.6 and 8.7; and day 143, C:N uptake ratio between 6.1 and 13.9). Likewise, the main feature of this profile was the two extremely high values registered during summer, namely on days 188 (ratios between 66.4 and 90.5) and 219 (ratios between 86.0 and 108.0), coincident with the secondary net autotrophic phases. This suggests that the autotrophic picoplankton was severely nitrogen limited at this stage of the seasonal cycle, or that the N uptake rates were highly underestimated.

The lowest estimates were obtained outside the productive season, in particular on days 320 (between 2.2 and 3.3) and 115 (between 1.6 and 2.4).

Discarding the two anomalously high ratios of days 188 and 219, the seasonal mean of the picoplankton C:N uptake ratio was approximately 5.5, considering both the 6 and 24 hour incubation experiments sets of data. This value is lower than that of the picoplankton compositional C:N ratio (7.9) and the temporal profiles of both these parameters differed, (Figure 4.23a). The particularly high C:N uptake ratios observed in summer were not reflected on the compositional ratio indicating a very high turnover rate of carbon and/or a loose coupling between the uptake of nitrogen and photosynthesis.

Based on the rates of <sup>13</sup>C and total <sup>15</sup>N uptake by the heterotrophic component of the picoplankton, C:N uptake ratios for this fraction were estimated and their seasonal variation is presented in Figure 4.23b). The heterotrophic C:N uptake ratios were, in general, lower than those for the total picoplankton, with a mean of approximately 4 (when heterotrophic <sup>13</sup>C uptake could not be detected, the C:N uptake ratios were plotted as zero in Figure 4.23b but these values were not considered in the estimation of the seasonal mean. The anormalously high ratios verified on day 208 were also discarded from this calculation).

Similarly to the  $<2\mu$ m and whole community C:N uptake ratio seasonal patterns, that of the heterotrophic picoplankton exhibited a particularly high value on day 208, suggestive of a weighty contribution of bacteria to the overall carbon and nitrogen uptake; however the main interest in this seasonal profile resides in the absence of a peak on day 219, as observed in the other two. This is due to the fact that on this occasion all <sup>13</sup>C uptake by the

 $<2\mu$ m fraction was chlorophyll associated constituting, therefore, autotrophic uptake. On day 233, no heterotrophic uptake of either carbon or nitrogen could be detected and on day 320, the null C:N ratio is due to the absence of <sup>13</sup>C uptake by the heterotrophic picoplankton.



Figure 4.23. b) Seasonal variation of the bacterial C:N uptake ratio during 1995.

On a few occasions, the C:N uptake ratios of the total and heterotrophic picoplankton were identical (days 59, 73, 115, 158, 173, 188, 208 and 298), suggesting that on these the picoplankton had days, mainly heterotrophic a composition. In these cases picoplanktonic chlorophyll constituted between 1 (day 158) and 25% (day 208) of the unfractionated.

# 4.4. CHARACTERISATION OF THE FOUR PHASES OF THE MENAI STRAIT SEASONAL CYCLE: A SUMMARY

The detailed presentation of the results regarding the seasonal study of the uptake of inorganic nitrogen by the whole phytoplanktonic community and by the picoplankton (namely by its heterotrophic component) and its relation with other ecological factors, in particular with the primary production, lead to the division of the Menai Strait seasonal cycle into four different phases which will be characterised below:

# I. Primary Net Autotrophic Phase (PNAPh: April-May)

This phase was characterised by the season's main phytoplankton growth which normally started in April, reaching maximum development towards the end of May. This vernal bloom was initially diatomaceous, evolving to a predominance of *Phaeocystis* as it matured.

Chlorophyll concentrations rose accordingly, attaining the season's maximum at the end of May, concurrent with the bloom of the aforementioned flagellate. The contribution of the picoplanktonic chlorophyll to the unfractionated varied between 2.0 % and 12.6 %, being lowest at the time of the whole community's peak.

Gross and net community oxygen production increased markedly during this period with the season's maxima coinciding with the chlorophyll peak. Virtually no picoplanktonic production was detected at this time of the year. Dark community oxygen respiration peaked on the same occasion, with picoplanktonic respiration constituting 80 - 100% of the whole community's. An increase in bacteria numbers in April but specially during May was observed.

During the PNAPh, particulate organic matter built up with the possibility of the season's maxima being registered during May. The picoplankton contributed with 25.6-52.6% and 26.6-57.4% to the unfractionated POC and PON, respectively. The whole community C:N ratio fluctuated closely to Redfield's whilst that of the picoplankton showed wider variation.

The predominant form of inorganic nitrogen taken up by both the whole community and (less markedly) the  $<2\mu$ m fraction was nitrate, with up to 87% of the production being nitrate based. Consequently, ambient nitrate declined abruptly during this phase becoming exhausted at the end of May. Both the whole community and the  $<2\mu$ m fraction exhibited uptake rates of ammonium and nitrate which were among the season's highest, with a peak of ammonium uptake coinciding with a short lived accumulation of this nutrient in the water.

Both nitrate and ammonium were taken up in dark only conditions and the uptake by either size fraction peaked during May. Rates of dark nitrate uptake by the whole community varied between 2.2 and 73% of those measured over the diel cycle, and by the  $<2\mu$ m fraction between 50 and 92%. The contributions of the  $<2\mu$ m fraction uptake to that by the whole community and of the heterotrophic picoplankton (bacteria) to both the total

picoplanktonic and unfractionated uptake over the diel cycle and in dark only conditions are summarised in Tables 4.4 and 4.5.

**Table 4.4.** Contributions of the  $<2\mu$ m fraction inorganic nitrogen uptake to that by the whole community and of the bacterial to the total picoplanktonic and unfractionated uptake over the diel cycle, during the PNAPh.

Nutrient taken up	Contribution of the	Contribution of the	Contribution of the
	picoplanktonic	bacterial	bacterial
	uptake to the	uptake to the	uptake to the
	unfractionated (%)	picoplanktonic (%)	unfractionated (%)
Nitrate	4.7 - 25.4	0 - 71.2	0 - 17.7
Ammonium	21.9 - 56.5	58.8 - 86.4	12.9 - 43.8
Nitrate + Ammonium	14.7 - 34.7	51.0 - 75.7	7.5 - 24.2

Table 4.5. Contributions of the  $<2\mu$ m fraction inorganic nitrogen uptake to that by the whole community and of the bacterial to the picoplanktonic and unfractionated uptake in dark only conditions, during the PNAPh.

Nutrient taken up	Contribution of the	Contribution of the	Contribution of the
	picoplanktonic	bacterial	bacterial
	uptake to the	uptake to the	uptake to the
	unfractionated (%)	picoplanktonic (%)	unfractionated (%)
Nitrate	7.9 -59.1	30.5 - 78.4	2.4 - 46.3
Ammonium	30.1 -83.0	58.0 - 88.3	17.6 - 70.3
Nitrate + Ammonium	18.2 - 66.6	58.2 - 84.3	12.6 - 53.8

This phase featured high <sup>13</sup>C uptake by the whole community, with a peak (season's highest) in May which did not coincide with peaks of net or gross community oxygen production or chlorophyll but which was concurrent with maximum inorganic nitrogen uptake. The uptake of <sup>13</sup>C by the <2 $\mu$ m fraction increased on this occasion constituting between 8.2 and 38% of the unfractionated. The heterotrophic component of the picoplankton did not appear to contribute to the peak of <sup>13</sup>C uptake by the picoplankton and by the whole community but, on other occasions it accounted for as much as 79.6% of the uptake by the former and 30.1% of the latter.

The unfractionated <sup>13</sup>C uptake in the dark also peaked in May whereas that by the  $<2\mu$ m organisms remained relatively low throughout this phase, increasing slowly towards the

end of that month. At the end of April, rates of dark uptake by both fractions were approximately 50% of those measured over the diel cycle. For the rest of this phase, dark uptake was between 0.8 and 19% of that in the above conditions. The picoplankton uptake was responsible for up to 42% of that by the whole community and 69.5 % - 79.1 % could be attributed to its heterotrophic component, the contribution of which to the unfractionated amounted to a maximum of 29.4%.

The C:N uptake ratios by the whole community, total picoplankton and bacteria during this phase are summarised in Table 4.6. A decrease of C:N uptake ratio with the size fractions could be detected in accordance with a richer relative composition of the smaller organisms in nitrogen than in carbon (Nagata, 1986). Higher uptake ratios were generally found towards the end of May suggesting conditions of N insufficiency, which agrees with the depleted levels of ambient nitrate and ammonium at this time of the year.

**Table 4.6.** C:N uptake ratios by the whole community, total picoplankton and heterotrophic component of the  $<2\mu$ m size fraction during the PNAPh.

Whole	community	Total pic	oplankton	Heterotrophi	c picoplankton
range	$mean (n=8 \pm SE)$	range	$mean (n = 8 \pm SE)$	range	$mean (n = 6 \pm SE)$
1.3 - 32.5	12.1 ± 7.079	1.6 - 13.9	$6.2 \pm 2.77$	1.6 - 6.32	3.8 ± 1.34

# II. Net Heterotrophic Phase (NHPh: June)

During this phase, heterotrophic metabolism became predominant, coinciding with the seasons' highest peak of bacteria abundance.

Ambient concentrations of inorganic nitrogen were low with nitrate remaining below detection levels and ammonium just rising above them towards the end of the month ( $\leq 0.2 \mu$ M). Nitrite was present throughout this phase though not markedly ( $\leq 0.1 \mu$ M).

Chlorophyll concentrations decreased sharply accompanying the decline of the spring bloom, with picoplankton accounting for 1.2 to 12.3% of the unfractionated phytoplankton. Accordingly, gross and net community oxygen production fell rapidly with the latter

attaining negative rates, concomitant with the season's maximum of respiration. For the first part of the month picoplankton accounted for all of the unfractionated respiration with its contribution declining to 54.6% on the second half. No picoplanktonic oxygen production was detected.

Both unfractionated and  $<2\mu$ m particulate organic matter peaked during this phase with the picoplanktonic constituting approximately 50% of the whole community's POC and PON.

The uptake of the inorganic nitrogen by the two size fractions was much lower than that registered in the previous phase, in agreement with the observed depletion of ambient ammonium and nitrate. Ammonium was the predominant form of nitrogen taken up by both the whole community and the  $<2\mu$ m fraction, with 55.6 to 80% of the production being ammonium based. Ammonium also dominated the total inorganic nitrogen uptake by the heterotrophic picoplankton although some bacterial uptake of nitrate could still be detected (12.6 to 45% of the total).

Dark uptake of ammonium and nitrate by both size fractions decreased during this phase, with rates by the whole community between 60 - >100% of those measured over the diel cycle and those by the  $<2\mu$ m between 47.4 - >100%. The distribution of the uptake of ammonium and nitrate between the whole community, total picoplankton and heterotrophic picoplankton over the diel cycle is shown in Table 4.7 and in dark only conditions in Table 4.8.

Nutrient taken up	Contribution of the	Contribution of the	Contribution of the
	picoplanktonic	bacterial	bacterial
	uptake to the	uptake to the	uptake to the
	unfractionated (%)	picoplanktonic (%)	unfractionated (%)
Nitrate	41.0 - 76.4	70.0 - 98.2	28.6 - 68.3
Ammonium	63.3 - 70.0	80.6 - 98.2	51.0 - 68.7
Nitrate + Ammonium	58.8 - 76.7	79.1 - 98.3	46.5 - 68.6

**Table 4.7.** Distribution of the uptake of ammonium and nitrate between the whole community, total picoplankton and bacteria over the diel cycle, during the NHPh.

In relation to the previous phase, the contribution of both the total picoplanktonic and bacterial uptake to the unfractionated both in dark conditions and over the diel cycle, increased markedly. Therefore, whilst during the primary net autotrophic phase the autotrophic uptake of inorganic nitrogen was predominant, in this phase, uptake by the  $<2\mu$ m heterotrophs assumed higher importance.

**Table 4.8.** Distribution of the uptake of ammonium and nitrate between the whole community, total picoplankton and heterotrophic picoplankton in dark only conditions, during the NHPh.

Nutrient taken up	Contribution of the	Contribution of the	Contribution of the
	picoplanktonic	bacterial	bacterial
	uptake to the	uptake to the	uptake to the
	unfractionated (%)	picoplanktonic (%)	unfractionated (%)
Nitrate	40.5 - 68.0	71.1 - 98.2	30.3 - 66.8
Ammonium	53.3 - 80.3	76.9 - 98.4	40.9 - 74.4
Nitrate + Ammonium	48.9 - 72.0	76.0 - 98.2	39.3 - 70.8

The uptake of <sup>13</sup>C by the whole community and the  $<2\mu$ m fraction decreased sharply in relation to that observed in the PNAPh, in agreement with the low phytoplankton biomass and net and gross oxygen production. On the contrary, but in accordance with the predominantly heterotrophic character of this period, unfractionated and picoplanktonic uptake in dark only conditions reached seasonal maxima rates, constituting up to 57% of the uptake measured over the diel cycle, and coinciding with the peak of bacteria abundance. The contributions of the  $<2\mu$ m fraction uptake to that by the whole community and of the bacterial to the picoplanktonic and unfractionated over the diel cycle and in dark only conditions are shown in Table 4.9.

Table 4.9. Contributions of the  $<2\mu$ m fraction <sup>13</sup>C uptake to that by the whole community and of the heterotrophic to the picoplanktonic and unfractionated uptake over the diel cycle and in the dark only conditons, during the NHPh.

Incubation conditions	Contribution of the	Contribution of the	Contribution of the
	picoplanktonic	bacterial	bacterial
	uptake to the	uptake to the	uptake to the
	unfractionated (%)	picoplanktonic (%)	unfractionated (%)
Diel Cycle	45.0	72.0 - 97.3	31.6 - 43.8
Dark Only	48.0 - 61.4	74.4 - 98.0	35.7 - 60.2

The contribution of smaller fractions to the unfractionated <sup>13</sup>C uptake both during the diel cycle and in dark only conditions increased in relation to the PNAPh, with the heterotrophic uptake accounting, at times, for virtually all picoplanktonic uptake in either situation. The uptake in the former conditions remained predominantly autotrophic as in the previous phase but the uptake in the latter conditions was up to 60% related to the heterotrophic component of the <2 $\mu$ m fraction, being approximately the double of that observed in the PNAPh.

The C:N uptake ratios by the whole community, total picoplankton and bacteria during this phase are summarised in Table 4.10.

Table 4.10. C:N uptake ratios by the whole community, total picoplankton and heterotrophic component of the  $<2\mu$ m size fraction (bacteria) during the NHPh.

Whole community		Picoplankton		Heterotrophi	c picoplankton
range	mean (n =4)	range	mean (n =4)	range	mean (n =4)
4.7 - 14.9	9.3 ± 4.21	3.4 - 8.5	5.9 ± 2.08	3.3 - 7.3	5.5 ± 1.63

As in the previous phase, there was a decrease of the C:N uptake ratios with the size fractions, although not so markedly, with the ranges of variation narrower in the present situation. The picoplankton C:N uptake ratios were similar to those of its heterotrophic component, suggesting a weighty contribution of the uptake by the latter to the uptake by the former. As seen before, this was the case during this net heterotrophic phase, for both the uptake of carbon and nitrogen.

# **III.** Secondary Net Autotrophic Phase (SNAPh: July and August)

Throughout this phase, autotrophic metabolism prevailed, for the second time during the Menai Strait seasonal cycle. However, whilst the PNAPh was characterised by the metabolism of meso- and microplankton, during this phase nano and picoplankton became more predominant, with picoplanktonic chlorophyll constituting between 21.6 and 67.2% of the unfractionated. This was accompanied by a rapid decrease in the bacteria abundance. A small peak of bacteria was detected at the end of July but for the remaining of this phase cell numbers declined.

Ambient nitrogen remained depleted with nitrate concentrations  $\leq 0.7 \mu M$ , most often below detection limits. Ammonium levels increased substantially in relation to the previous phase but were still  $\leq 1\mu M$ .

With the continued decrease in phytoplanktonic and bacterioplanktonic biomass, unfractionated and  $<2\mu$ m particulate organic matter concentrations were much lower than those in the preceding phase, remaining fairly stable throughout this period after the initial fall. Picoplanktonic particulate matter made up 27.4 - 40.7% and 33.0 - 45.7% of the unfractionated POC and PON, respectively.

The SNAPh featured two peaks of community oxygen production and it was the only time of the year when gross oxygen production by the picoplankton could be detected (1.8 to 16.1% of the unfractionated), despite low chlorophyll concentrations and inorganic nitrogen depletion. After initially decreasing in July, dark community and picoplanktonic oxygen respiration peaked at the beginning of August, concurrent with a small increase in the bacteria abundance. On this occasion, picoplanktonic respiration accounted for all the unfractionated, and throughout the rest of this phase it constituted 54.2 to 250.7% of that by the whole community.

This period was characterised by a markedly predominance of ammonium uptake in relation to that of nitrate with 83-100% of the production constituting ammonium based production. Peaks of ammonium uptake by either the whole community or the  $<2\mu$ m fraction were associated with increased ambient availability of this nutrient but did not coincide with net oxygen production or respiration maxima by those two communities.

Of the total nitrogen uptake by the bacteria, virtually all was of ammonium. However, the contribution of the bacterial uptake to the unfractionated and total picoplanktonic decreased substantially in relation to the previous phase with values closer to those observed in the PNAPh. The maximum contributions were simultaneous with the July peak of bacteria.

Dark uptake of nitrate, but mostly that of ammonium, peaked during this phase with the relative unfractionated and picoplanktonic uptake varying widely in relation to that over the diel cycle. The distribution of ammonium and nitrate uptake between the whole

community,  $<2\mu$ m fraction and heterotrophic picoplankton over the diel cycle and in dark only conditions is shown in Tables 4.11 and 4.12.

Table 4.11. Contributions of the  $<2\mu$ m fraction uptake to that by the whole community and of the bacterial to the total picoplanktonic and unfractionated uptake over the diel cycle, during the SNAPh.

Nutrient taken up	Contribution of	Contribution of	Contribution of
	picoplanktonic	bacterial	bacterial
	uptake to the	uptake to the	uptake to the
	unfractionated (%)	picoplanktonic (%)	unfractionated (%)
Nitrate	13.5 - 46.3	0 - 21.8	0 - 6.9
Ammonium	33.4 - 72.5	0 - 65.6	0 - 47.6
Nitrate + Ammonium	32.8 - 68.6	0 - 63.7	0 - 43.7

The SNAPh was characterised by a decrease in the contribution of both the picoplanktonic and bacterial uptake to the unfractionated, in relation to the antecedent phase. This decrease was more marked over the diel cycle than in the dark and it was particularly striking regarding the uptake of nitrate.

Table 4.12. Contributions of the  $<2\mu$ m fraction uptake to that by the whole community and of the bacterial to the picoplanktonic and unfractionated in dark only conditions, during the SNAPh.

Nutrient taken up	Contribution of the	Contribution of the	Contribution of the
	picoplanktonic	bacterial	bacterial
	uptake to the	uptake to the	uptake to the
	unfractionated (%)	picoplanktonic (%)	unfractionated (%)
Nitrate	29.5 - 78.6	0 - 72.6	0 - 57.3
Ammonium	38.5 - 98.5	0 - 74.9	0 - 71.7
Nitrate +Ammonium	37.5 - 98.3	0 - 74.4	0 - 72.0

Also, whilst in the two previous phases the  $<2\mu$ m uptake was in most occasions markedly heterotrophic in character (specially in the NHPh), during this phase the situation was less clear-cut with the contribution of the bacterial uptake to the picoplanktonic varying widely.

 $^{13}$ C uptake by the whole community and <2 $\mu$ m fraction increased considerably in relation to the previous phase, exhibiting two peaks: one at the beginning of July, coinciding with maxima of net and gross community oxygen production, anteceding an ammonium uptake

peak, and corresponding to the picoplanktonic seasonal highest; and the other at the end of August, registered on the same occasion as the second ammonium uptake peak. Dark <sup>13</sup>C uptake by both size fractions fell during July to increase again during the following month. It constituted between 1.1 and 7.7% of that measured over the diel cycle. Such low relative values compare well with those found during May (PNAPh), being much lower than those in the NHPh. The contributions of the <2 $\mu$ m fraction uptake to that by the whole community and of the bacterial to the picoplanktonic and unfractionated over the diel cycle and in dark only conditions are summarised in Table 4.13.

**Table 4.13.** Contributions of the  $<2\mu$ m fraction <sup>13</sup>C uptake to that by the whole community and of the heterotrophic uptake to the picoplanktonic and unfractionated uptake over the diel cycle and in the dark only conditions, during the SNAPh.

Incubation conditions	Contribution of picoplanktonic uptake to the unfractionated (%)	Contribution of heterotrophic uptake to the picoplanktonic (%)	Contribution of heterotrophic uptake to the unfractionated (%)
Diel Cycle	21.0 - 37.4	0 - 28.9	0 - 10.8
Dark Only	15.9 - 62.9	0 - 65.7	0 - 41.4

During this phase, the contribution of the  $<2\mu$ m fraction and of the bacterial to the unfractionated uptake decreased substantially in relation to that in the NHPh. <sup>13</sup>C uptake over the diel cycle and in dark only conditions remained predominantly chlorophyll associated, particularly in the former case, a situation similar to that encountered in the PNAPh. Moreover, whilst in the previous phase most of the  $<2\mu$ m uptake in either incubation condition could be accounted for by the bacteria, the circumstances in the SNAPh were more ambiguous with picoplanktonic autotrophic uptake prevailing over the heterotrophic over the diel cycle and assuming a higher importance than in any of the two preceding phases, in the dark.

Regarding the C:N uptake ratios by the whole community,  $<2\mu$ m fraction and heterotrophic picoplankton, this phase was mainly characterised by abnormally high values registered on days 188 and 219, coinciding with the peaks of primary production. These inflated C:N uptake ratios could be perceived in all three communities, except for the bacterial on day 219 since all the <sup>13</sup>C uptake registered on that occasion was chlorophyll associated. Such high C:N uptake ratios suggest severe nitrogen depletion or underestimation of N uptake rates due to methodological interferences as will be discussed later. Composition C:N ratios of both the whole community and the  $<2\mu$ m fraction did not appear to be affected by this "unbalanced" uptake, with the ratios remaining as in the previous phase.

## IV. Regeneration Phase (RPh: September-November; February-March)

This phase can be divided into two periods, one comprising the months of September to November - autumn; and the other, the months of February and March - winter. Although these two periods are similar in many aspects, they also present differing features as it will be noted below.

In accordance to its name, one of the main features of this phase was the progressive regeneration of ambient inorganic nitrogen, with the season's highest concentrations of nitrite and ammonium registered in the autumn and those of nitrate in winter. Despite this, low phytoplanktonic biomass featured in this phase, with the lowest concentrations registered during the winter months, possibly as a result of the growth limiting low temperatures of the water. However, a small peak of chlorophyll in the autumn (normally at the end of October) was normally encountered. The contribution of the picoplanktonic chlorophyll to the unfractionated varied between 6% and 45% constituting between 6% (1995) and 8% (1994) of the aforementioned unfractionated chlorophyll peak.

Bacteria abundance may (1994) or may not (1995) respond to this late burst of phytoplankton growth, with cell numbers decreasing thereafter to reach the season's lowest values during the early months of the year.

The season's lowest concentrations of particulate organic matter were observed over the winter months, whereas the autumn featured rised unfractionated and fractionated POC, concomitant with the chlorophyll peak at the end of October. This was not accompanied by an increase in the PON, resulting in high compositional C:N ratios of both the whole community and  $<2\mu$ m fraction. Picoplanktonic particulate matter accounted for 17 to 40.6% and 23.5 to 37.3% of the unfractionated POC and PON, respectively.

Whilst practically no primary production was registered during the winter months, two minor peaks of net and gross community production characterised the autumn period, associated with rised chlorophyll concentrations, with the unfractionated oxygen
respiration decreasing throughout this period. Virtually no picoplanktonic oxygen production could be detected during this phase but picoplanktonic respiration was at its season's highest, exceeding, by up to 3 times, that of the whole community. The highest rates were registered in the autumn, coinciding with the peak of unfractionated primary production.

The division of this phase into an autumn and a winter period is well justified as far as the uptake of inorganic nitrogen is concerned: during autumn, the estimated "f-ratios" indicate a predominant use of ammonium over nitrate with up to 97% of the production being ammonium based whilst in winter, any production measured was predominantly based on the uptake of nitrate (77 to 94.2%). The predominant uptake of ammonium during autumn occurred despite the equal ambient availability of this nutrient and that of nitrate. This trend was also observed for the bacterial uptake of inorganic nitrogen, with the heterotrophic picoplankton taking up only ammonium between September and November and predominantly nitrate during winter (67 to 89% of its total nitrogen uptake).

Another important characteristic of this phase was that the uptake of nitrate in dark only conditions reached its season's peak during winter, with rates as high as or even higher than those measured over the diel cycle. This trend was featured by both the whole community and the picoplankton. An identical situation was not seen regarding the uptake of ammonium by either size fraction which is in agreement with the much higher availability of nitrate than ammonium at this time of the year. The distribution of the ammonium and nitrate uptake among the whole community,  $<2\mu$ m fraction and heterotrophic picoplankton over the diel cycle and in dark only conditions is shown in Tables 4.14 and 4.15.

Maxima contributions of the  $<2\mu$ m fraction and of bacteria to the unfractionated over the diel cycle, were higher in winter than in the autumn. In dark only conditions this difference was not as evident. As in the previous phase, the contribution of the bacterial uptake to the picoplanktonic and unfractionated varied widely between none at all to high relative values. However, maxima contributions were higher than those observed in the SNAPh with the highest estimates, particularly during the winter period, comparing better to those encountered in the NHPh.

**Table 4.14.** Contributions of the  $<2\mu$ m fraction uptake to that by the whole community and of the bacterial uptake to the picoplanktonic and unfractionated uptake over the diel cycle, during the RPh.

Nutrient taken up	Contribut	ion of the	on of the Contribution on		Contribution of the	
	picopla	nktonic	hktonic bacterial		bacterial	
	uptake	to the	to the uptake to the		uptake to the	
	unfraction	nated (%)	nated (%) picoplanktonic		unfractionated (%)	
	autumn	winter	autumn	winter	autumn	winter
Nitrate	7.5-9.1	19.8-83.7	0-79.7	0-84.0	0-23.2	0-70.2
Ammonium	38.0-59.2	34.6-73.6	11.0-86.9	0-85.9	5.5-39.2	0.59.9
Nitrate + Ammonium	30.2-51.4	21.3-74.4	10.6-86.7	0-82.0	5.2-38.7	0.61.0

**Table 4.15.** Contributions of the  $<2\mu$ m fraction uptake to that by the whole community and of the bacterial to the picoplanktonic and unfractionated in dark only conditions, during the RPh.

Nutrient taken up	Contribution of the		Contribution of the		Contribution of the	
	picoplanktonic		bacterial		bacterial	
	uptake to the		uptake to the		uptake to the	
	unfractionated (%)		picoplanktonic (%)		unfractionated (%)	
	autumn	winter	autumn	winter	autumn	winter
Nitrate	0-100	26.2-66.4	0-87.8	0-80.3	0-100	0-53.4
Ammonium	46.8-77.9	30.8-100	31.9-87.3	24.0-92.4	21.0-45.8	10.4-100
Nitrate + Ammonium	46.1-100	27.6-63.0	19.1-85.5	8.6-81.2	21.0-47.0	2.6-49.6

Therefore, it appears that although an exclusively heterotrophic character cannot be associated with this regenerative phase (as, at times, no heterotrophic nitrogen assimilation appeared to have taken place), bacteria can account greatly to the total picoplanktonic and unfractionated nitrogen uptake, with maxima contributions among the season's highest. Of particular interest is the uptake of nitrate, normally associated with the autotrophs (see PNAPh and SNAPh), which was, at times, related mostly to the bacteria, both over the diel cycle and in dark only incubations.

The uptake of <sup>13</sup>C over the diel cycle by the whole community and  $<2\mu$ m fraction decreased linearly during the autumn, to reach the season's lowest values during winter. Dark uptake by both size fractions also declined, though not so rapidly, constituting 3.6-13.6% of that measured in the former conditions. The distribution of the <sup>13</sup>C uptake by the whole community,  $<2\mu$ m fraction, and the heterotrophic picoplankton over the diel cycle and in dark only conditions are summarised in Table 4.16.

Incubation conditions	Contribution of	Contribution of	Contribution of
	picoplanktonic	bacterial	bacterial
	uptake to the	uptake to the	uptake to the
	unfractionated (%)	picoplanktonic (%)	unfractionated (%)
Diel Cycle	14.2 - 35.4	0 - 75.2	0 - 18.0
Dark Only	10.7 - 65.5	0 - 84.1	0 - 31.6

**Table 4.16.** Contributions of the  $<2\mu$ m fraction <sup>13</sup>C uptake to that by the whole community and of the heterotrophic uptake to the picoplanktonic and unfractionated uptake over the diel cycle and in the dark only conditions during the RPh.

The contribution of the picoplankton to the unfractionated was similar to that observed in the previous phase with the lowest relative values characteristic of the winter period, for both incubation conditions. The uptake of carbon by the  $<2\mu$ m fraction was, at times, markedly heterotrophic. Despite this fact, total <sup>13</sup>C uptake remained a predominantly autotrophic process with the heterotrophic contribution to the unfractionated uptake among the season's lowest.

The C:N uptake ratios by the whole community, picoplankton and bacteria during this phase can be found in Table 4.17.

Table 4.17. C:N uptake ratios by the whole community, total picoplankton and heterotrophic component of the  $<2\mu m$  size fraction during the RPh.

	Whole c	ommunity	To Picopl	otal lankton	Hetero picopla	trophic ankton
	range	mean	range	mean	range	mean
Autumn	2.8 - 13.8	$9.2 \pm 5.08$ (n = 5)	2.2 - 10.0	$5.6 \pm 2.82$ (n = 6)	0.7 - 9.4*	$5.5 \pm 4.42$ (n = 3)
Winter	5.2 - 10.3	$6.7 \pm 2.61$ (n = 6)	1.5 - 5.5	$3.5 \pm 1.34$ (n = 6)	1.4 - 5.6*	$3.0 \pm 1.81$ (n =4)

\*excluding the cases when no <sup>13</sup>C heterotrophic uptake was detected.

The C:N uptake ratios were much lower than those estimated in the previous phase. The mean values obtained for the autumn period compared particularly well with those from the NHPh for all three size fractions, whilst those obtained in winter were among the season's lowest. This decline between the autumn and winter periods of this phase agree with higher nitrogen availability in the latter than in the former and with a weighty contribution of the heterotrophic community to the nitrogen metabolism, at this time of the year.

### 4.5. DISCUSSION

The study in coastal waters of the seasonal N dynamics of phytoplankton is of interest for various reasons; from a physiological aspect it may increase the understanding of the nutritional strategy of algae and of the influence of a range of local environment factors on the utilisation of the nutrients as well as of the interactions with other microbial organisms of the same ecosystem. The higher biomass levels in coastal waters allow experimental studies that may be precluded in the open ocean where biomass estimates are low. The coastal waters of the Menai Strait were chosen as the study site not only because convenient but most importantly because this body of water has been extensively studied over the past 45 years, and a vast data base is available covering a wide range of environmental parameters, such salinity, as temperature, nutrient availability. phytoplankton biomass seasonal variations, and more recently autotrophic and heterotrophic production of the microbial community as discussed and referenced in CHAPTER II. However, the understanding of the seasonal dynamics of the microbial community in this environment would be incomplete without observations regarding the nitrogenous nutrition of phytoplankton and bacteria, therefore the work reported in this chapter attempted to fill the existing gap concerning data on this important aspect of the microbial ecology of these waters.

The primary production cycle in the Menai Strait, its timing, the environmental controls and its relation to the micro-heterotrophic processes has been the subject of recent, extensive investigations by S.P. Blight [see Blight *et al.*, (1995) and Blight (1996)] as summarised in CHAPTER II. Consequently, these aspects of the Menai Strait's phytoplanktonic and bacterial seasonal cycles will not be addressed in depth in this part. This discussion will rather attempt to interpret the data obtained on the nitrogen dynamics of the whole community and the "bacterial" size fraction, according to the objectives proposed in the introduction of this chapter. All considerations will be made with regard to the 1995 data set; this constitutes the more complete observations but comments apply to both 1994 and 1995 seasonal studies since results were consistent in both years.

Long term work normally aims at describing and understanding the sequence of ecological events in a given area (e.g. Paasche & Kristiansen, 1982; Glibert et al., 1982b; Carpenter &

Dunham, 1985; Kristiansen *et al.*, 1994; Maguer *et al.*, 1996), such as the work under consideration. However, despite the relevance of this type of investigations, the number of reports in the literature is low, which is understandable given the considerable cost and effort incurred. Most of the work published concentrate on short term measurements taken over intervals of a few days/weeks, during the periods of the season of most relevance for the study being undertaken, normally involving the spring/summer and/or the autumn phytoplankton blooms. These short term investigations frequently aim at answering very specific questions (*e.g.* Horrigan *et al.*, 1990; Smith, 1993; Wheeler, 1993; Selmer *et al.*, 1996; Slawyk *et al.*, 1997, among many others), therefore leaving the sequential events that take place over the whole seasonal cycle and that characterise the microbial ecology of a given marine system, poorly documented.

## **4.5.1.** Seasonal Variation of the Nitrogenous Nutrition of Menai Strait's Phytoplanktonic Community.

The results presented before showed that the seasonal cycle of primary production in the Menai Strait is multi-phasic with a vernal bloom of phytoplankton occurring between early April and the end of May; a summer bloom taking place in July and/or August; and finally an autumn bloom between the end of September and October. The magnitude of these maxima of primary production decreases through the summer and this seasonal trend has been found to be characteristic of temperate coastal waters (Glibert *et al.*, 1982b; Carpenter & Dunham, 1985; Dauchez *et al.*, 1991; Petterson, 1991; Maguer *et al.*, 1996).

This seasonal cycle of primary production is accompanied by variations in the rates of utilisation of inorganic nitrogen, where the bulk of nitrate and ammonium uptake takes place concurrently with the spring bloom, with the summer/autumn months characterised by low nitrate uptake whilst ammonium continues to be utilised at relatively high rates sustaining the primary producers at this time of low inorganic N availability.

#### 4.5.1.1. Relative Uptake of Nitrate and Ammonium: the Calculation of "f-Ratios"

In section 4.2.5., "*f*-ratios" were calculated in order to show the relative contribution of each of these two forms of inorganic nitrogen to the nutrition of the primary producers (Figure 4.8) in the environment under study.

When considering the nitrogenous nutrition of phytoplankton it is convenient to use the quotient known as f-ratio. This quotient has its origins in the concepts of "new" and "regenerated" production derived from deep ocean work (Dugdale & Goering, 1967; Eppley & Peterson, 1979): "new" production is sustained by allochthonous forms of N such a nitrate whilst "regenerated" production is based on locally regenerated nitrogen (e.g. ammonium and urea). In the open ocean such concepts are clear and the distinction between them fairly straightforward since N utilisation and presumed regeneration are separate in time and space. In coastal waters, however, where there is a likelihood of nitrification occurring in the sediments, the discrimination between the two types of production based on the assimilation of nitrate and ammonium becomes less secure, for nitrate can be autochthonous in origin and ammonium can have an allochthonous provenience such as sewage discharges and land run-off. This is the case in the Menai Strait where due to the shallow water column, the production zone in not only in close contact with the sediments where nitrate regeneration may occur throughout the year, but it also receives ammonium inputs from local sewage discharges. Therefore, though useful in the present case as a means for discussion, the calculated "f-ratios" do not bear the legitimate meaning associated with the terms "new" and "regenerated" production and they should be regarded in the strict sense of facilitating the distinction between nitrate and ammonium supported production.

Furthermore, in the present study of the seasonal variation of the N nutrition of the Menai Strait phytoplankton assemblages, the uptake and availability of urea was not determined and therefore not included in the "*f*-ratio" estimations. As evidence suggest (McCarthy *et al.*, 1977; 1982; Kaufman *et al.*, 1983; Furnas 1983; Harrison *et al.*, 1985; Probyn & Painting, 1985; Horrigan *et al.*, 1990; Metzler *et al.*, 1997), the importance of this form of N can be quite considerable for the nutrition of planktonic algae, particularly after the nitrate based spring bloom, *i.e.* in summer and autumn, when, as with ammonium, urea is

recycled rapidly enough to maintain high phytoplankton growth rates (Kristiansen *et al.*, 1994). The exclusion of urea from the calculation of *f*-ratios obviously leads to the overestimation of "new production" and to the underestimation of "regenerated production" in any given area where its uptake may be significant (Harrison *et al.*, 1987; Probyn, 1988; Wafar *et al.*, 1995). Based on several sets of data, Wafar *et al.*, (1995) concluded that *f*-ratios that did not account for the uptake of urea were overestimated in the range of 6% in upwelling waters, through 17, 24 and 42% in coastal, polar and oceanic waters to a maximum of 55 % in estuaries. Actual field measurements suggest that urea increase "regenerated production" by about 50% in coastal waters (McCarthy, 1972b; Furnas, 1983; Kaufman *et al.*, 1983) although the contribution may be even higher where urea concentrations are high (Harrison *et al.*, 1985).

The "f-ratio" estimates in the study under discussion fell within a wide range (0.02-0.9) because measurements were taken over the whole of the production season in the Menai Strait, with the two forms of nitrogen studied assuming varying importance with time. For each of the three phases defined in Figure 4.8a, the values obtained agree well with those from published studies in comparable circumstances (Olson, 1980; Garside, 1981; Paasche & Kristiansen, 1982; Wheeler & Kokkinakis, 1990; Dauchez *et al.*, 1991; Kristiansen *et al.*, 1994; Tamminen, 1995). In particular, the high estimates of nitrate based production during the spring bloom are in accordance with Platt & Harrison's (1985) suggested maximal value of 80% in coastal areas. However, during the period between the end of winter-beginning of spring, the "f-ratio" estimates for the Menai Strait were rather high (approaching unity), well in excess of those discussed, for instance, by Eppley & Peterson (1979), Glibert *et al.*, (1982a), Cochlan (1986), Eppley (1989) and especially by Maguer *et al.*, (1996), who included urea and nitrite uptake in their seasonal study.

As described above, the exclusion of urea uptake from the calculations of the "f-ratio" may have contributed to these inflated figures of nitrate based production in the Menai Strait but other errors inherent to the conventional <sup>15</sup>N technique may have also affected the "f-ratio" estimates (Harrison *et al.*, 1987; Harrison, 1990). Major among these are thought to be the effects of isotope dilution on ammonium uptake experiments due to the recycling of ammonium during the course of the incubation, and the "pulsing effect" of the tracer addition (*i.e.*, short lived enhancement of uptake) on both nitrate and ammonium uptake.

Significant isotope dilution will result in an underestimate of the ammonium uptake rates and consequently on overestimated "f-ratios". Glibert et al., (1982c) suggested that in cases where the isotopic dilution is not accounted for, rates of ammonium uptake can be underestimated by a factor of 2 to 3. "Pulsing", on the other hand, will result in an overestimate in nitrate and ammonium uptake and consequently the effects on the "f-ratio" are indeterminate. In this study, ammonium isotope dilution was not estimated, although it is likely to have occurred at times in the longer incubations carried out (24 hours) in contrast with the shorter ones, where isotope dilution was less probable. As far as "pulsing" is concerned, the trace additions performed in this seasonal work involved, in most occasions, very low concentrations of labelled ammonium and nitrate (below 10% of ambient concentrations) except when the ambient nutrient concentrations were below detection limits in which cases <sup>15</sup>N additions may have been in excess of 100% of the ambient concentrations. In these circumstances enhancement of uptake is highly likely to have occurred. The effects of these additions were possibly diluted out throughout the 24 hour incubation period, but they would have been more perceptible in the shorter incubations. For evident reasons, isotope dilution and "pulse effects" were the main causes of the discrepancy observed at times, between the 6 and the 24 hour incubation sets of data, with the former influencing more the 24 hour estimates and the latter those based on the 6 hour incubation measurements.

Finally, our confidence in using the "f-ratio" to estimate nitrate based production depends on the validity of the assumption that the uptake of nitrate and reduced N forms is mediated by autotrophs only, mainly phytoplankton. However, as it will be discussed in the next section, there is evidence that the uptake of inorganic N by bacteria can be significant, in particular that of ammonium, representing a large proportion of that measured in unfractionated samples (Wheeler & Kirchman, 1986; Kirchman *et al.*, 1989; 1991; 1994; Kirchman, 1994). A reduction in the autotrophic contribution to the ammonium based production would result in an elevation of the "f-ratio", potentially of the same order of magnitude as the reduction envisaged for the effects of urea utilisation and isotope dilution (Harrison *et al.*, 1987) whereas the exclusion of heterotrophic uptake from "f-ratio"

#### Seasonal variation of the "f-ratio"

It is now apparent that independently of the geographical situation of the marine environment in consideration, "new production" will be characteristic of the seasonal periods comprised between winter and the end of spring whilst "regenerated production" is a feature of the summer and autumn months. This characteristic seasonal variation of the *f*ratio within a region has been described by several authors (*e.g.* Garside, 1981; Glibert *et al.*, 1982a,b; Paasche & Kristiansen, 1982; Furnas, 1983; Cochlan, 1986; Wheeler & Kokkinakis, 1990; Dauchez *et al.*, 1991; Smith, 1993; Kristiansen *et al.*, 1994; Tamminen, 1995). Small scale temporal variations can also be expected, with marked decreases occurring over short periods of time ( $\leq 1$  month), in particular during phytoplankton blooms, as reported by Smith (1993), Sambroto *et al.*, (1993) and Tamminen (1995).

The measurements taken in the Menai Strait showed that the trend followed by the "fratios" temporal variations agreed with that expected from the above cited reports, with the highest values in winter and early spring, followed by a decrease towards summer and autumn. This pattern was very similar to that described by Kristiansen et al., (1994) for the Barents Sea, despite the differing environmental conditions. The time of the year covered by their measurements was from April to September with the seasonal cycle characterised by a phytoplankton bloom in April and May, a transition stage in June and a post-bloom phase thereafter, as observed in the Menai Strait. Kristiansen and collaborators reported percent "new production" during the spring pre-bloom and bloom periods of 94% which decreased to 53% during the transition period and continued to fall during the post-bloom time to reach values of 17%. The relative nitrate based production estimates from the Menai Strait at corresponding stages of the seasonal cycle are also in close agreement with those reported by Kristiansen et al., (1994). Small scale temporal variations were not as evident in the Menai Strait with decreases in the "f-ratios" more progressive thus less marked than those described by Smith (1993), who report a decline in that quotient from 0.8 to <0.20 during a spring bloom of Phaeocystis in the Greenland Sea, over a period of one month (April-May). Over a similar time scale, Tamminen (1995) was able to identify three stages in the bloom period between the end of April and the middle of June, in the Baltic, which resembled the seasonal pattern of relative N uptake by the phytoplankton in the Menai Strait, at that stage of the seasonal cycle. As in the present work, the bloom reported by Tamminen (1995) started during a period of high nitrate concentration which was used at higher rates than ammonium-bloom initiation phase; during the following phase- bloom peak- although the community was adapted to nitrate assimilation, the increased availability of the energetically more favourable ammonium caused a reorganisation of prevailing N assimilation patterns and resulted in decreased *f*-ratios, with this decline being more accentuated in the Baltic than in the Menai Strait. The Baltic bloom peak showed equal uptake of the two nitrogen sources, a situation which in this work only occurred in the post-bloom period (phase II in Figure 4.8). However, in both cases, the post bloom stages were characterised by increasing ammonium uptake and consequently rapidly decreasing "*f*-ratios".

The timing of the transition from nitrate dominated production to that supported by the uptake of ammonium could be dependent upon a) the relative abundance of, and interactions between the various N forms available; b) the size of the cells of the predominant phytoplankton assemblage in the area under consideration and their preference for a given form of inorganic N, and finally c) other processes leading to the recycling of inorganic N occurring concomitantly with the uptake, as will be discussed below.

a) Relative Abundance of, and Interactions Between the Various Nitrogenous Forms Available - The seasonal transition from nitrate based to ammonium based production can be, in part, associated with varying ambient nutrient conditions: as seen above, the high abundance of nitrate during the early months of the year coincided with highest "f-ratios" whereas "f" values decreased with decreasing nitrate concentration and increased ambient ammonium. In fact, the recognition of a strong relationship between "new production" and ambient nitrate has led Platt & Harrison (1985) to propose a model to predict the former with basis on the knowledge of the latter. These authors have reported a general pattern featuring an asymptotic increase in the f-ratio with increase in ambient nitrate concentration which was best described by the equation

$$f = f_{\text{max}} \left[ 1 - e^{-(m [\text{NO3}] / f \text{max})} \right]$$
 4.2

where  $f_{\text{max}}$  is the maximum attained *f*-ratio (*f*) and *m* is the slope of the curve that describes the rate at which the *f*-ratio increased at low nitrate concentration ([NO<sub>3</sub><sup>-</sup>]).

In the present work, the equation of Platt & Harrison (1985) was applied to both the 6 and the 24 hour incubation sets of data (Figure 4.24) and an optimisation routine ("Solver" in the programme EXCEL version 5.0 for MICROSOFT OFFICE) was used to determine the best fitted line, *i.e*, the line that minimised the difference between the sum of squares between the observed and the calculated data points.



Figure 4.24. Relationship between percent "f-ratio" and ambient nitrate concentration in the Menai Strait. calc.-6hr and calc.-24hr refer to the calculated hyperbolic curves with basis on the Platt & Harrison model for the 6 and 24 hour sets of data, respectively.

The obtained values of  $f_{\text{max}}$ and m approached closely those reported by Harrison et and the  $f_{\rm max}$ al., (1987) reported by Dugdale et al., (1992) although their m value was much lower than that for the Menai Strait (Table 4.18). Figure 4.24, shows that the curves obtained for both sets of data coincide, not reaching, however, the asymptote stage for the concentrations of ambient nitrate detected obviously, further

determinations are needed to document better this relationship at the higher concentrations.

**Table 4.18.** Comparison of the values of  $f_{\text{max}}$  and *m* for the asymptotic curves relating "new production" and ambient concentrations of nitrate, obtained in the present study and those reported in the literature.

f <sub>max</sub>	m	Reference
90.6 <sup>b)</sup> -91.4 <sup>a)</sup>	24.6 <sup>a)</sup> -25.3 <sup>b)</sup>	present study
59-86	0.65-30.02	Harrison et al., (1987)
83	5.48	Dugdale et al., (1992)

a) and b)- results from the 6 and 24 hour incubation experiments, respectively.

Vezina (1994) have also found that at the 50% light level, the values of f were non-linearly related to ambient nitrate, with a relationship between the two approaching that described

by Platt & Harrison (1985); Kristiansen *et al.*, (1994) report a hyperbolic relationship between percent "new production" and ambient nitrate, as well.

It is now apparent from the wide range of values of  $f_{\text{max}}$  and *m* obtained by the various authors mentioned above, that a set of parameters will only be applicable to a limited area. Despite consistency in general features among data sets, an expression with a single, universally applicable set of parameters to describe the relationship between *f* and nitrate concentration is unlikely to materialise. This applies mainly to regions characterised by low concentrations of this nutrient, since, as obtained by Harrison *et al.*, (1987), *m* varied by more than one order of magnitude in such circumstances. These variations are not unexpected since various factors can influence *f*-ratio estimates, such as light, ambient concentration of ammonium, heterotrophic uptake of inorganic N and errors inherent to the <sup>15</sup>N technique, as discussed by Harrison *et al.*, (1987).

Besides resulting from low availability of nitrate, the low assimilation of this nutrient observed in the Menai Strait during the summer and autumn (resulting in the low "f-ratios" observed during this period of the seasonal cycle) could have also been caused by ammonium inhibition of nitrate uptake. The inhibitory effect of ammonium on nitrate uptake by phytoplankton has been well documented in laboratory experiments (Eppley et al., 1969; Conway, 1977; Syrett, 1981) and many field studies, have also shown that increases in ammonium uptake are associated with shifts in nutrient preference towards ammonium (MacIsaac & Dugdale, 1972; McCarthy al., 1977; Eppley et al., 1979a,b; Olson, 1980; Garside, 1981; McCarthy, 1981; Glibert et al., 1982a; Harrison et al., 1987; Wheeler & Kokkinakis, 1990, among many others). The multiple effects of ammonium on nitrate uptake include inhibition of ammonium transport, inhibition of nitrate reductase and repression of the synthesis of nitrate reductase in phytoplankton (Syrett, 1981) but the concentration at which this effect occurs has not been ascertained since it appears to vary widely. For instances, McCarthy (1981) suggests nearly complete inhibition at ammonium concentrations of 1  $\mu$ M, in accordance with previous studies by Conway (1977), but also notes that it often occurs at lower concentrations. Wheeler & Kokkinakis (1990), observed complete inhibition at ammonium concentrations of 0.1-0.3 µM in the Subarctic Pacific whereas Paasche & Kristiansen (1982) report strong inhibition by ammonium concentrations greater than 1-2  $\mu$ M.

Figure 4.25 shows a negative relationship between the phytoplankton nitrate uptake and the ambient ammonium concentrations found for Menai Strait's waters.



ammonium concentration (µM)

Figure 4.25. Relationship between nitrate uptake by the whole community and the ambient concentration of ammonium. Results from the 6 and the 24 hour incubation experiments (as circles and triangles, respectively).

Little nitrate was taken up by the phytoplankton for ambient ammonium concentrations near unity  $(\mu M)$  which is in agreement McCarthy's with (1981) and Conway's (1977) suggestion and also with the observations of Harrison et al. (1987), although some of the observations plotted in Figure 4.25 are from times when ambient nitrate was below detection limits (June and August), hence a certain degree of inaccuracy may be attached to the estimated rates at those times of

the seasonal cycle, as discussed in CHAPTER III.

Additionally, the highest nitrate uptake occurred at the lowest ambient ammonium. An identical trend has been observed by Garside (1981), when the uptake of nitrate as a percentage of total N uptake was plotted against ambient ammonium. Olson (1980), Glibert *et al.*, (1982a) and Wheeler & Kokkinakis (1990), among others, also describe such negative association between these two parameters but the relationship they report was a linear one, unlike that shown in Figure 4.25. Glibert *et al.*, (1982a) have shown than when NH<sub>4</sub><sup>+</sup> concentrations were >1 $\mu$ M, nitrate accounted for <40% of the total N uptake in the Scotia Sea, thus even when nitrate was present in quantities that approached the upper limit for near-surface open-ocean waters, the processes that recycled ammonium locally made a major contribution to the nutrition of the phytoplankton during the austral summer.

Wheeler & Kokkinakis (1990) have also obtained a strong negative correlation between percent inhibition and Chlorophyll a concentration suggesting that when autotrophic

biomass is relatively high, ammonium concentrations and inhibition of nitrate uptake are low; conversely when autotrophic biomass is relatively low, ammonium concentrations are higher and cause significant inhibition of nitrate assimilation. This describes well the situation in the Menai Strait in the spring when nitrate uptake and autotrophic biomass were high but ambient ammonium low, whereas at the end of summer beginning of autumn, the increased ammonium concentrations could have inhibited the uptake of the increasingly available nitrate by the low phytoplankton biomass.

**b)** Phytoplanktonic Preference for a Given Form of Inorganic Nitrogen - The shift from a predominance of "new production" early in the year to that of "regenerated production" can also be influenced by the varying preferences of the dominant phytoplanktonic assemblage for a particular form of inorganic N.

In order to ascertain the order of preference by the Menai Strait phytoplankton populations of the inorganic nitrogen forms studied (nitrate and ammonium) in relation to their availability, Relative Preference Indexes (RPI) were estimated according to McCarthy *et al.*, (1977) following the expression given in CHAPTER I (section 1.2.3.).

Figure 4.26.a) shows the seasonal variation of ammonium and nitrate RPIs calculated for the Menai Strait phytoplankton population. As found by others, ammonium was preferred over nitrate throughout the seasonal cycle but the magnitude of both RPIs varied. In spring (February-May), RPI<sub>NH4</sub> were well in excess of unity, clearly showing the preference of the phytoplankton for this form of inorganic N, despite the fact that at this time of the year ambient nitrate concentrations were at their highest, well above those of ammonium. For the same period of time, nitrate was shown to be either discriminated against (end of Aprilbeginning of May) or, more often, to have been used equitably with its availability. It is interesting at this point to note that although both the ambient concentrations and uptake rates of nitrate were higher than those of ammonium, the latter was nonetheless the preferred form of inorganic N by the *Phaeocystis* bloom that occurred at this time of the year.



Figure 4.26. a) Seasonal variation of ammonium and nitrate RPI of Menai Strait phytoplankton. b) Relationship between nitrate RPI and ambient ammonium concentration.

During June and August both RPIs (RPI<sub>NH4</sub> and RPI<sub>NO3</sub>) were close to unity<sup>4.2.</sup> whereas for the remainder of the year, nitrate was clearly rejected as a nutrient, in particular during the early part of the summer phytoplankton bloom (July) and during the smaller autumn bloom, despite increasing concentrations of this nutrient at the later part of the seasonal cycle. An inverse relationship between nitrate RPI values and ammonium concentrations has been reported in the literature, including the sharp curtail of nitrate uptake for ambient concentrations  $>1\mu M$ , which reflects the above discussed ammonium inhibition of nitrate assimilation.(McCarthy et al.. 1977; Eppley et al., 1979b; Glibert al., 1982a,b; Paasche et & Kristiansen, 1982, Furnas, 1983). In the present case, that inverse

relationship was not particularly evident (Figure 4.26b) but there seemed to be a certain inhibition of nitrate uptake for concentrations higher than 1 $\mu$ M. In short, nitrate appears to be utilised equitably with its availability, when ambient ammonium concentrations are low but it is rejected as a N source when ambient ammonium are relatively high (normally >1 $\mu$ M).

<sup>&</sup>lt;sup>4.2.</sup> Some discrepancies between the 6hr and the 24hr sets of data can be detected and reasons for this have already been discussed.

A similar consistent, higher affinity for ammonium than for nitrate has been observed for natural phytoplankton from Oslofjord, Norway (Paasche & Kristiansen, 1982). These authors have observed that nitrate was never preferred over ammonium and at low concentrations was only used in proportion to its share in the total N pool. Glibert et al., (1982b) also report a general ammonium preference in relation to nitrate by natural populations from the Vineyard Sound, USA and the values of RPI<sub>NH4</sub> and RPI<sub>NO3</sub> obtained in the present work compare well with those obtained by these authors, with RPI<sub>NO3</sub> consistently lower than unity and RPI<sub>NH4</sub> reaching values as high as 6.75, in the spring. In the Scotia Sea, despite the domination of the nitrogenous pool by nitrate, ammonium was still the preferred N form (Glibert et al., 1982a), a situation similar to that observed in the Menai Strait in early spring, and to that described by Cochlan (1986) on the Scotian shelf. where even the samples from the nitrate rich waters of March had values of RPI<sub>NO3</sub> lower than unity, with the phytoplankton showing only a slight preference for ammonium. The RPI<sub>NH4</sub> values reported by Glibert et al., (1982a) reached up to nearly 100, showing the non-proportioned assimilation of this nutrient in relation to its availability. Values as high as those obtained in the work under discussion (up to 22) were reported by Probyn & Painting (1985), for a <200 µm size fraction in Antarctic coastal, surface waters.

As shown above, preferential use of the more reduced forms of inorganic N has been extensively referred to in studies of marine phytoplankton (Dugdale & MacIsaac, 1971; McCarthy, 1981; Syrett, 1981; Glibert *et al.*, 1982b; Paasche & Kristiansen 1982; Harrison *et al.*, 1982; Furnas, 1983; Cochlan, 1986; Metzler *et al.*, 1997 among others) even when nitrate is present in excess (MacIsaac & Dugdale, 1969, 1972; Conway, 1977; McCarthy *et al.*, 1977; Glibert *et al.*, 1982a) and it is expected on energetic grounds, although the physiological and biochemical processes regulating it are still not well understood. Furthermore, other studies have shown simultaneous and comparable uptake of both compounds (Eppley & Renger, 1974; Eppley *et al.*, 1969; Conway, 1977; McCarthy *et al.*, 1977) but this will tend to happen only when insufficient ammonium is available to meet the entire nitrogen requirements of the phytoplankton. Additionally, other forms of inorganic N can be preferred: for instances, Horrigan *et al.*, (1990) described how nitrite was usually preferentially utilised over ammonium and nitrate, making a greater contribution to the total N uptake than to the total pool of inorganic N of Cheasapeak Bay (USA) in late spring. Boyer *et al.*, (1994) have also reported many instances when nitrate

was the preferred nitrogen source in the Neuse river estuary (USA). Therefore, it appears that the near universal preference of phytoplankton for ammonium in relation to the more oxidised forms of inorganic N is not always observed, with the possibility of many factors interacting to give rise to the modifications of the above generalisation, such as concentration of the different forms of inorganic N in the water, size class composition of the phytoplanktonic assemblage, light and temperature (McCarthy *et al.*, 1977; Malone, 1977; Glibert *et al.*, 1982b; Maestrini *et al.*, 1982; Probyn & Painting, 1985; Koike *et al.*, 1986; Glibert *et al.*, 1995).

The overall discrimination against nitrate, generally observed in the waters of the Menai Strait does not imply that on an absolute scale nitrate was unimportant for the nutrition of the phytoplankton of this environment. As seen from the estimated "f-ratios", a very large proportion (up to 87%) of the primary production registered early in the year, including the vernal bloom of phytoplankton, was nitrate based. This proportionately greater utilisation of nitrate early in the year in comparison to that in summer and autumn could be related to the predominant composition of the phytoplankton assemblage at those times. As reported earlier and also as described by Blight et al., (1995), the composition of the early blooms of phytoplankton in the Menai Strait was dominated by meso- and microplankton (particularly by diatoms) whilst that in summer comprised mainly nanoplankton. It has been hypothesised for a variety of systems that large cells such as diatoms tend to bloom when environmental conditions such as upwelling, mixing or runoff increase the flux of nitrate. Although there is no evidence to support or refute a physiological basis for this, there are many examples in the literature that demonstrate that diatom growth and nitrate uptake are favoured when nitrate concentrations are high. For instance, Glibert et al., (1982b) reported that during a large winter bloom of the diatom Rhizosolenia deliculata (also a predominant species in the Menai Strait during spring), nitrate appeared to be used preferentially and Maestrini et al., (1982) found that some natural assemblages of phytoplankton constituted mostly by diatoms could utilise nitrate even when ammonium was present at concentrations over an order of magnitude higher than values reported to suppress nitrate reductase activity and nitrate uptake. Furthermore, Koike et al., (1986) as well as Probyn & Painting (1985) have also observed that the larger sized phytoplankton took up proportionately more nitrate that did the picoplankton and the nanoplankton fractions. This was confirmed by Bouteiller (1986) who describe how phytoplankton of the  $<3\mu m$  fractions presented a lower affinity for nitrate than larger cells contained in the  $<35\mu$ m and  $<200\mu$ m fractions, with organisms  $<3\mu$ m showing a clear preference for ammonium that for nitrate. Additionally, Sambroto *et al.*, (1993) refer to "new production" in spring and summer being predominantly associated with particles  $>5\mu$ m in the surface waters of the Iceland Basin and Owens *et al.*, (1991) report that small phytoplankton ( $<20\mu$ m) exhibited a statistically significant greater preference for ammonium than larger cells.

In conclusion, it appears that in addition to the previously given reasons for the seasonal shift from high to low "*f*-ratios" (relative abundance of, and interactions between the various nitrogenous nutrients available and phytoplanktonic preference for a given N form), this phenomenon could also be associated with the different composition of the phytoplankton assemblage in terms of the size of the organisms and their distinct utilisation of the various inorganic N nutrients.

Environmental factors such as temperature appear to influence too, the utilisation of inorganic N by an algal assemblage. For instance, Glibert *et al.*, (1995) hypothesised that diatoms may be better able to exploit the high concentrations of nitrate available early in the year than smaller sized phytoplankton due to their capacity to function at the low temperatures characteristic of early spring (*ca.*  $5^{\circ}$ C). However, it seemed that when temperature increased above 18 °C (normally at the end of May), the diatom population declined quickly to give place to other types of algae such as cyanobacteria, chrysophytes, chlorophytes, etc. Data from the Menai Strait seasonal study support the observations of Glibert *et al.* (1995), with the diatom dominated spring bloom occurring at times when temperatures did not exceed 18 °C, in early spring and autumn but especially in March and April when temperatures ranged between 6 and 10 °C.

c) Processes Leading to the Recycling of Inorganic Nitrogen - The seasonal switch from nitrate to ammonium based production observed in the Menai Strait after the spring bloom could also be a consequence of increased respiration and production of ammonium by copepods and microzooplankton (Kristiansen *et al.*, 1994). Grazing activity, specially high during this period, combined with degradation of organic material by bacteria could be the contributing processes to the *in situ* regeneration of inorganic N (attested by the increasing ammonium concentrations from July onwards- Figure 4.2-, and high rates of respiration by

the  $<2\mu$ m fraction -Figure 4.14). As discussed before, regenerated forms of N are favoured by the phytoplankton, hence the switch from high to low "*f*-ratios" detected at this time of the year.

The increase in ambient regenerated nitrogen observed during the summer and autumn as a result of the aforementioned bacterial and zooplanktonic respiration appear to have been sufficient to sustain the gross community  $O_2$  production by the low standing stocks of phytoplankton observed at this times of the seasonal cycle, despite ambient ammonium concentrations lower than 1.5  $\mu$ M. This in agreement with the observations of McCarthy (1977) who report that because of the rapid remineralisation of ammonium, only a low ambient concentration is required to meet the needs of a productive phytoplankton assemblage. The high chlorophyll-specific uptake rates of ammonium from July onwards (Figure 4.27) argue against a seriously deprived phytoplanktonic population with high ratios obtained in the spring, summer and autumn; rather, such rates reflect that despite the low phytoplanktonic biomass (Figure 4.3), ammonium was assimilated at high rates. Similarly to Slawyk's *et al.* (1997) observations, where the highest chlorophyll - specific ammonium uptake was always concurrent with impoverished ambient nitrate conditions, in the Menai Strait the highest chlorophyll - specific ammonium uptake rates were observed in summer and autumn a time of depleted ambient nitrate.

A similar situation has been found by Kristiansen *et al.*, (1994) in the Barents Sea MIZ between the spring and the autumn, where, despite a decrease with time in the phytoplanktonic standing stocks, accompanied by a reduction in total N uptake and f-ratios, chlorophyll-specific rates remained high and nearly constant, arguing against any severe N-limitation of the phytoplankton. The chlorophyll specific N uptake rates obtained in the present study compare well with those obtained by Kristiansen *et al.*, (1994) for the bloom and transition periods, but were much higher (approximately 2-5 times) during the pre- and post-bloom phases than the reported ones.

In the Menai Strait, the only period characterised by low nitrate and ammonium chlorophyll-specific uptake rates corresponded to the month of June, previously characterised as the predominantly heterotrophic phase of the Menai Strait seasonal cycle.



**Figure 4.27.** Seasonal variation of the chlorophyllspecific nitrogen uptake rates. Results from the 6 and 24 hour incubation experiments.

During this month. both ambient nitrate and ammonium concentrations were below or near detection limits (Figure 4.2) and the decreasing phytoplankton population was obviously N depleted. Competition with bacteria for the scarcely available inorganic N could have aggravated the situation (as will be further discussed in the following section). This one month lag period between the exhaustion of ambient nitrate and the satrt of the accumulation of ammonium in the seawater, which was more evident from July onwards, may have been the principal factor causing the decline of the main phytoplankton spring bloom,

coupled with increased grazing pressure. As soon as ammonium levels were raised, primary production increased again, as seen by the two peaks in gross and net  $O_2$  community production, which took place in July and August 1995 (Figure 4.5). Later peaks of chlorophyll of comparable magnitudes of those observed in spring and summer were possibly prevented in the autumn and winter by the declining temperature and light.

## 4.5.1.2. Relationship Between Regenerated Production and Bacterial Respiration.

Since it is now apparent that the phytoplankton sampled in the Menai Strait have a clear preference for ammonium than for nitrate and that "regenerated production" predominates in this environment for a great part of the seasonal cycle, it seems pertinent to explore whether or not the "regenerated production" in these waters is driven by the bacterial respiration of organic matter, of which ammonium is a subproduct.

"Regenerated production" as a function of bacterial  $O_2$  respiration ( $<2\mu$ m  $O_2$  respiration corrected for the presence of chlorophyll in a similar fashion to that employed for the calculation of  $<2\mu$ m heterotrophic N uptake) is represented in Figure 4.28 a, b). Two parts of this plot are evident, one showing a relatively linear relationship between the two parameters, the other, enclosed in the ellipse, escaping that correlation. The linear relationship put in evidence in figure b), has shown that only a maximum of 67% of the regenerated production is actually driven by bacterial respiration. Other processes may therefore be important as sources of this regenerated nutrient, such as sloppy feeding by grazers, reduction of nitrate and nitrite to ammonium in the sediments (Val Klump & Martens, 1983), antropogenic inputs *via* sewage discharges and land runoff (well present in the studied area), zooplankton excretion (Bidigare, 1983), etc. Whilst reduction reactions and antropogenic inputs may constitute a continuous source of ammonium into these waters, the importance of grazing and zooplankton excretion may be more seasonal, with the highest contribution in late spring and summer, when these organisms are most abundant in the Menai Strait (Blight *et al.*, 1995).

The data outside the linear relationship established in Figure 4.28b) and encircled by the ellipse in figure a), was obtained on days 143, 158, 173, 219 and 298. The lack of correlation between the observed regenerated production and bacterial respiration was due to the particularly low uptake rates of ammonium suggesting that in these occasions ammonium uptake was severely underestimated. Indeed, this could have happened due to the experimental errors previously discussed, in particular to the non-correction of the estimated uptake rates for isotope dilution caused by *'in vitro'* ammonium regeneration during the incubation period. Another indication that the uptake of ammonium was severely underestimated on the above occasions (and also on day 73) is that at those times the estimated O/N ratio was extremely high (>100) whereas it averaged 11.7 for the points included in the straight line; this average O:N estimate is relatively close to the Redfield value of 8.6 (Anderson, 1995) indicating in this circumstance coupling between these two parameters which would be expected according to the Redfield's model.



underestimation This of the ammonium uptake rates could have been particularly serious in August and October (days 219 and 298) when, according to the increase in ambient ammonium and measured high bacterial respiration, the processes responsible for ammonium regeneration were operative at this time of the year. On days 143 and 219, which corresponded to peaks of primary production, it could have also been the case that due to the high inorganic N demand by the phytoplankters,  $^{15}NH_{4}^{+}$ exhaustion occurred, resulting in the underestimation of the uptake rates. The lack of correspondence between these two parameters on day 143 can also be associated with the fact that on this occasion, regenerated production represented less than 15% of the total N production

which was essentially nitrate based ("*f*- ratio" of 87%). In June, (days 158 and 173), more than 50% (maximum of ~70%) of the total ammonium uptake could be accounted for by bacterial uptake, hence the observed discrepancy between "regenerated" production and bacterial respiration.

In order to ascertain with more confidence the processes driving regenerated production particularly during summer and autumn in the Menai Strait, more studies need to be undertaken, involving more accurate ammonium uptake measurements by the phytoplankton. However, these preliminary observations indicate that bacterial respiration appear to have a preponderant role in supporting "regenerated production" in these waters and the data have a general stoichiometry consistence with the Redfield model.

#### 4.5.2. Dark Uptake of Nitrate and Ammonium

Results from the 6 and 24 hour incubations in the dark showed that there was uptake of nitrate and ammonium by the whole community and the  $<2\mu$ m fraction in these conditions. Contrary to what may have been expected, dark uptake of inorganic N was not essentially associated with the bacterial metabolism, suggesting that the uptake of nitrogenous nutrients by the algae is not necessarily associated with the photosynthetic process. Figure 4.29 a and b) illustrates the distribution of ammonium and nitrate dark uptake between the algae and the bacteria. Bacterial dark nitrate uptake constituted a seasonal mean of 30% ( $\pm$  25.0%) of that by the whole community, with these organisms contributing with 41% ( $\pm$  17.8%) to the total dark ammonium uptake. For clarity, the two sets of data (6 and 24 hour incubations) were averaged.

Until the end of May-mid June, nitrate uptake by both the algae and the bacteria predominated over that of ammonium, which became a more important source of N for both these populations for the remaining of the seasonal cycle (Figure 4.30). This is in accordance with the ambient availability of these nutrients in the Menai Strait and with the pattern observed in the diel incubations.

For identical periods of incubation, nitrate and ammonium dark uptake rates varied between 0 and >>100% of those measured over the diel cycle, with the majority of estimates of percent dark uptake in relation to that over the diel cycle being >50%; whilst for the algae the median percent values were 46 and 36.7%, for nitrate and ammonium, respectively, for the bacteria the corresponding medians were much higher: 117 and 70.6%.

These results are also suggestive of a higher sensitivity of ammonium uptake to the light than that of nitrate which is in contradiction with reports from the literature defending a higher dependence of nitrate uptake on irradiance than that of ammonium. MacIsaac & Dugdale (1972) were the first to report a strong dependence of algal nitrate uptake on light, with rates of nitrate in darkness conditions being nearly negligible;





ammonium uptake while found to be also dependent irradiance on was significant the in dark (40% of the uptake in the light). More recently, dark of inorganic uptake nutrients have been studied particularly in Polar areas, for instances by Olson (1980), Glibert al.. et (1982a), Smith & Harrison (1991), Smith (1993), Kristiansen et al., (1994) more temperate and in (Conway & waters Whitledge, 1979).

In all these published significant works, algal uptake of ammonium, and to a lesser extent, nitrate, could be detected in dark only conditions, constituting in some cases a substantial fraction of the uptake in the light: for instance, Smith & Harrison

(1991) report that the ratio of uptake in darkness to the maximum rate of nutrient uptake in the light varied from 0-0.39 for nitrate and 0.23-0.77 for ammonium. Kristiansen *et al.*,

(1994) report lower percentages with dark ammonium uptake representing  $28 \pm 4\%$  and dark nitrate uptake  $4 \pm 1\%$  of light uptake of the respective nutrients.





Reasons for this discrepancy between the Menai Strait results and those from the literature mentioned above, could be the underestimation of the ammonium uptake rates which, for reasons already discussed could have occurred at times. contributing to the lower percent dark uptake of ammonium than that of nitrate. Furthermore. the results obtained in the environment under discussion are not entirely comparable with those from the literature, as in this the "light" case measurements were taken over the natural diel cycle which include dark a period of seasonally varying duration, possibly resulting in lower uptake rates. Thus, the percent

dark estimates in relation to those in the "light" may constitute overestimates of the true values and hence the much higher results than those of, for instance, Kristiansen *et al.*, (1994).

A consequence of high dark inorganic N uptake would be that of reduced C:N uptake ratios (Harrison *et al.*, 1982, 1985; Collos & Slawyk, 1986) by the algae and bacteria, since the assimilation of C is highly reduced in darkness. However, this must be a non-steady state situation, because if a steady-state was being approached, the C:N elemental ratios would also decrease accordingly, which is against the constant C:N composition of Redfield's. Although an imbalance between uptake and elemental ratios of the phytoplankton may occur over short time-scales, it is unlikely to be maintained on a scale of weeks to months (Smith & Harrison, 1991). The expectation would be that over a natural diel cycle the cells would achieve their C:N quota. A decrease in the particulate matter C:N composition in darkness could be due to the synthesis of protein which, according to Li & Harrison (1982) occur in the dark whereas that of lipids and polysaccharides appears to virtually cease.

## 4.5.3. Seasonal Variation of the Picoplankton Nitrogenous Nutrition

#### 4.5.3.1. Characterisation of the <2µm Fraction

At the start of the seasonal study under discussion, the  $<2\mu$ m size fraction was chosen to represent the bacterial component of the plankton, according to the structure of the pelagic ecosystems described by Sieburth *et al.*, (1978). The choice of a  $<2\mu$ m porosity instead of a lower one to perform the size fractionations, such as  $1\mu$ m or even  $0.8\mu$ m used in other studies of bacterial ecology studies (for instance by Harrison & Wood, 1988; Kirchman *et al.*, 1994), was also made having in consideration that, at certain times of the seasonal cycle in the Menai Strait, very fine size fractionations of the planktonic community would be unfeasible not only due to the great increase in the phytoplanktonic biomass but to the nature of the blooming species, as well: during the well documented *Phaeocystis* bloom in the spring, large quantities of mucillage are produced by this phytoplankter, rapidly clogging the membranes when fractionation is attempted. Consequently, it was thought that in order to make measurements possible at this time of the year, fractionation to the lowest size would have to be sacrificed. The immediate consequence of this was that the size fraction originally intended to be representative of only the bacterial population, was "contaminated" with phototrophs to an extent that was variable during the studied period of

time (Figure 4.12). Therefore, the interpretation of the results regarding the  $<2\mu$ m fraction was not as straightforward as expected since the processes could not be considered to be carried out exclusively by bacteria.

An attempt was made, however, to quantify the proportion of the <sup>15</sup>N and <sup>13</sup>C<sup>\*</sup> uptake associated with the autotrophic and the heterotrophic (bacterial) component of the  $<2\mu$ m fraction: as explained in section 4.3.5.1., the picoplanktonic autotrophic uptake rates were obtained by multiplying the concentration of chlorophyll in the  $<2\mu$ m fraction by the whole community's chlorophyll specific uptake rates; the difference between the total uptake by the  $<2\mu$ m fraction and the autotrophic uptake was considered to be due to bacteria and thus denominated heterotrophic uptake.

This procedure has also been adopted by Kirchman *et al.*, (1994) to correct for the presence of chlorophyll containing organisms in a <0.8  $\mu$ m fraction which chlorophyll content varied between 5 and 16% of the unfractionated samples, during a spring bloom in the North Atlantic ocean. These percentages are very similar to those obtained in the Menai Strait study at the time of the *Phaeocystis* dominated spring bloom in 1995.

The estimates obtained by means of this simplistic approach can only be considered as approximations because the calculations described above make two basic assumptions:

1) The relative content of the chlorophyll containing organisms in the  $<2\mu$ m is the same as that in larger phytoplankters. In reality, this assumption may not be held as it appears that smaller algae contain more chlorophyll per unit of biomass than the larger cells (Tett, P.; pers. comm.). Therefore, the picoplanktonic autotrophic rates obtained were possibly overestimated and consequently the uptake rates by the heterotrophic component, underestimated.

2) Uptake rates of  $<2\mu$ m phototrophs are identical to those of larger phytoplankters. In fact, it is generally regarded that the rates of metabolic processes of living organisms are inversely related to size and that size dependence of the growth rate of microorganisms can be described by an allometric relation of the form:

$$rate = aW^b$$
 4.3

where rate is the growth rate or other metabolic process; a (time<sup>-1</sup>), is a proportionality coefficient; and b (dimentionless), a constant which value has been the subject of some variability in the literature, with Fenchel (1974) reporting a figure of -0.28 for heterotrophic microbes, Banse (1982), values of -0.13 and -0.15 for diatoms and dinoflagellates, respectively, and Joint (1991), an estimated -0.15 for picophytoplankton productivity. In the present work however, allometric estimates of N uptake rates were not performed so, the uptake rates by the autotrophic component of the  $<2\mu$ m fraction probably constituted underestimates of the real value, and accordingly, the heterotrophic uptake was overestimated.

Moreover, with the chlorophyll-based correction, it is also assumed that the  $<2\mu$ m phytoplankton use ammonium and nitrate equally well as larger phytoplankters. This may lead to overestimating nitrate uptake by small phytoplankton and thus underestimating nitrate uptake by the heterotrophic bacteria because, as discussed before, it is currently believed that nitrate uptake is mostly associated with large phytoplankton species like diatoms.

The use of a smaller pore sized membrane in the present work may have not resulted in a better separation of the heterotrophic bacteria from phototrophic organisms than that obtained, since there is always some overlap in classes of particle size. For instances, the presence of cyanobacteria is common in marine environments (although not markedly during most of the seasonal cycle of the Menai Strait) and are functionally part of the phytoplankton component despite closer structural affinity with heterotrophic bacteria.

For a more effective separation between heterotrophic and autotrophic picoplankton, Wheeler & Kirchman (1986) have used a combination of size fractionation ( $<1\mu$ m) with inhibitors of prokaryotic and eukaryotic protein synthesis. Although the authors report this as a successful strategy, it has not become popular to the extent that it has not been adopted in more recent ecological studies. Size fractionation continues to be the method of choice, despite the fact that even the smallest size fractions are often well "contaminated" with phototrophs, as indicated by the relatively high concentrations of chlorophyll that may be detected (*e.g.* Harrison & Wood, 1988).

# 4.5.3.2. Seasonal Variation of the Contribution of the Heterotrophic Component of the <2 $\mu$ m Fraction (bacteria) to the Total Uptake of Inorganic Nitrogen

If long term studies of the phytoplankton nitrogenous nutrition in a given marine environment are not very abundant, by comparison those regarding the seasonal variation of the uptake of inorganic N by the bacteria- or by a picoplanktonic size fraction - are scarce in the extreme, with the work by Hoch & Kirchman (1995) being one such example. This is understandable considering that until relatively recently, bacteria were considered to be unimportant consumers of ammonium and nitrate. The acknowledgement that bacterial uptake of inorganic N is relevant, has led scientists to study this facet of the heterotrophic metabolism, its environmental regulations and ecological repercussions, but the most of the published work has been of short duration. For example, Horrigan *et al.*, (1988) and Kirchman *et al.*, (1991,1992) have shown that bacteria are indeed capable of taking up nitrate in laboratory conditions and also *in situ* (Kirchman *et al.*, 1994), despite earlier reports that such uptake was negligible (Wheeler & Kirchman, 1986); conversely, the heterotrophic uptake of ammonium was found to be ubiquitous, thus less controverse (Wheeler & Kirchman, 1986; Harrison & Wood, 1988; Kirchman *et al.*, 1990; Hoch *et al.*, 1992; Selmer *et al.*, 1993; Hoch & Kirchman, 1995, among many others).

Such studies have demonstrated that the bacterial uptake of inorganic N can be significant and that the percent of total ammonium uptake by the picoplankton in relation to that by the whole community can be large, with the uptake of nitrate less so. However, there is considerable variability between environments (Probyn & Painting, 1985; Probyn, 1985; Glibert, 1982; Suttle *et al.*, 1990) and the investigation of the contribution of bacteria to the total unfractionated inorganic N uptake in the waters of the Menai Strait has shown that, even in the same environment, such variability can be extensive over the seasonal cycle.

As it may be seen from Figure 4.31, the relative bacterial uptake of total N (ammonium and nitrate) was highest during the net heterotrophic phase (NHPh-June: up to ~70% of that by the whole community) and the regeneration phase (RPh-September to April; maximum of 60% of that by the whole community obtained in March).



#### month of the year

Figure 4.31. Seasonal variation of the contribution of the bacterial uptake to the total unfractionated inorganic N utilised. S., O., N., stand for September, October and November, respectively. Results from the 6 and the 24 hour incubation were averaged whereas values in the text refer to both data sets individually. The lowest contributions of the bacterial fraction to the total inorganic Ν uptake were registered during periods of high primary production (maximum of 24%) in the PNAPh and 43.6% in the SNAPh), with the peak of bacterial relative uptake observed in June being concurrent with the bacterial abundance seasonal maximum (Figure 4.11), peaks of  $<2\mu m$  $O_2$  respiration (Figure 4.13) and <2µm POC and PON (Figure

#### 4.14) and with decreased concentrations of chlorophyll.

Until the end of May, the uptake of nitrate by bacteria exceeded that of ammonium, constituting between ~ 40-90% of the total heterotrophic uptake. From June onwards there was a sharp decrease in the uptake of this nutrient and for the remainder of the year, ammonium was virtually the only inorganic N form utilised by bacteria (Figure 4.17 a, b). The peak of bacterial nitrate uptake was at the end of May, a week later than that of the whole community and constituting only ~12% of the nitrate unfractionated uptake on that occasion. The maximum ammonium uptake by the bacteria was observed on days 208 and 265 constituting on those dates between 36.5 and 47.5% of the uptake by the whole community. This is in agreement with the seasonal availability of nitrate and ammonium in the waters of the Menai Strait, when ambient nitrate was high in early spring and ammonium increased in summer and autumn.

These percentages of bacterial uptake in relation to that by the whole community are well within the range reported in the literature in identical circumstances, in particular to those estimated by Kirchman *et al.*, (1994) in the North Atlantic during the spring bloom. These authors reported that after correction for phytoplankton activity (in an identical fashion to

that used in the present work), heterotrophic bacteria (<0.8µm fraction) accounted for 22-39% and 4-14% of the total ammonium and nitrate, respectively, which are very similar estimates to those obtained during the Phaeocystis bloom in the Menai Strait, as indicated above. In the NW Atlantic, Harrison & Wood (1988) observed that between July and August the <1µm size fraction accounted for 0.6-93% and 7-66% of the total nitrate and ammonium uptake. This unusually high percent nitrate uptake could have been due to the fact that ~40% of the total chlorophyll could be found in the picoplanktonic fraction, therefore a large proportion of the  $NO_3^-$  uptake (as well as that of  $NH_4^+$ ) measured in this fraction is highly likely to have represented autotrophic uptake. In the more oligotrophic waters of the NW Mediterranean, Selmer et al., (1993) found that at the time of the seasonal chlorophyll maxima, 40-50% of the total ammonium uptake could be accounted for by the  $<1\mu$ m size fraction; however, the authors did not correct for the presence of chlorophyll which amounted to ca. 20% of that in the unfractionated samples, consequently their estimates of "bacterial" uptake may have also been overestimated. In Antarctic coastal waters, similar percentages to those found by Selmer et al., (1993) were reported by Probyn & Painting (1985), with the  $<1\mu$ m fraction responsible for  $\sim30-50\%$  and 0-55% of the total ammonium and nitrate uptake, respectively, in March and April.

The seasonal measurements of Hoch & Kirchman (1995) in the Delaware estuary (USA) are also of relevance, as these authors found that bacterial uptake of ammonium varied not only spatially but temporally, with NH<sub>4</sub><sup>+</sup> accounting for a large percentage of bacterial N requirements in spring and fall (30-50%) but mostly in summer (50-90%); in winter, ammonium was a minor source of N for the bacteria. These results agree with the trend observed in the Menai Strait, with comparable relative values. Furthermore, in summer, bacteria accounted for only 10-20% of the total uptake at the more coastal areas of the Delaware estuary, in agreement with the earlier measurements of Glibert (1982) who reported a 30% contribution of bacterial uptake to the total. These relatively low percentages of bacterial ammonium uptake at this time of the year compare well with some of those obtained in the Menai Strait at the time of the summer primary production peaks, during which bacterial uptake of inorganic N was low, as discussed before. Conversely, Fuhrman *et al.*, (1988) and Suttle *et al.*, (1990) report an increase in the contribution of the bacteria to the total ammonium uptake from 10% in winter to 40% in summer, in the Long Island Sound (USA) which contrasts from the trend observed in the Menai Strait, where the

fractional uptake of ammonium by bacteria fluctuated considerably throughout the year (Figure 4.17b).

## 4.5.3.3. Inorganic Nitrogen Uptake by Bacteria in Relation to that by the Phytoplankton

The obtained results suggest a general trend characterised by a certain mutual exclusivity of the algal and the bacterial inorganic N uptake, *i.e.*, at times of high uptake of ammonium and nitrate by the phytoplankton, that by bacteria was either low or below detection limits (Figure 4.32). Exceptions to this generalisation were the data from days 131 and 208 when peaks of bacterial and algal ammonium uptake coincided, with the bacterial uptake constituting respectively, 22 and 40% of the algal uptake on those two occasions.



Figure 4.32. Seasonal variation of the algal and bacterial (bact.) ammonium (ammon.) and nitrate (nitra.) uptake (uptk.) in the Menai Strait, during 1995.

This apparent mutually exclusive behaviour of the phytoplankton and bacteria regarding the uptake of inorganic N, may have various reasons. For instance, it can be the case that in the Menai Strait, during the exponential phase of phytoplankton growth when the lowest

relative bacterial uptake was found, bacteria metabolism was inhibited by the algae. In fact, Sieburth (1960) reports that during rapid growth, *Phaeocystis* releases acrylic acid as a result of dimethyl sulphide metabolism, which has antimicrobial proprieties. This has been confirmed by Guillard & Hellebust (1971) who also found relatively few bacteria in exponentially growing Phaeocystis pouchettii cultures but dense bacterial growth in and around the gel material of old cultures. Eberlein et al., (1985) suggested that the accumulation of dissolved organic substances during a *Phaeocystis* bloom in the North Sea may have been due to low decomposition by bacteria whose activity could have been reduced by acrylic acid. The presence of this substance during the Menai Strait spring Phaeocystis bloom could have suppressed to a certain extent the bacterial N uptake (in particular that of nitrate), which was resumed once the phytoplankton bloom entered its senescence phase (end of May, beginning of June). It is possible that *Phaeocystis* may have developed this strategy in order to minimise competition with the bacteria for inorganic nutrients, at times when they become limiting. However, other mechanisms would have to have been in operation in summer, at the time of the second phytoplankton bloom, since relative bacterial ammonium uptake during that phytoplankton maxima at this time of the seasonal cycle was below detection, and this bloom was not predominantly constituted by that flagellate.

It could also be possible that the bacterial inorganic N demand was easily met by the available ambient ammonium, nitrate or other sources of N at the earlier stages of the phytoplankton blooms, hence the relatively low bacterial uptake compared to that by the phytoplankton; Billen & Fontigny (1987), suggested that during phytoplankton blooms, or at least during their early phases, bacterial N needs can be mostly satisfied by the uptake of dissolved free amino acids which appear to be abundantly produced by *Phaeocystis* (Eberlein *et al.*, 1985), and this could have been the case in the Menai Strait at the time of the spring bloom.

The main reason for the increased N demand by the bacteria following the algae blooms may be the inflated availability of DOM resulting from both phytoplankton exudation and cell lysis (self or viral) and breakage (for instance induced by sloppy feeding by grazers) (Ducklow *et al.*, 1993; Kirchman *et al.*, 1994; Blight *et al.*, 1995). The first process (exudation) introduces mainly low molecular weight (LMW) materials, such as dissolved

free amino acids (DFAA), and constitute ~65% of the phytoplankton produced DOC (Amon & Benner, 1996; Biddanda & Benner, 1997); the latter (cell lysis and breakage) is a source of detritus and high molecular weight (HMW) compounds. These two pools of materials have distinct biochemical compositions: LMW substances are normally highly bioreactive (but see Amon & Benner, 1996), N-rich hence their C:N ratio is relatively low (3-11) and are turned over in a matter of hours to <1day. Conversely, HMW matter are constituted predominantly of polymeric carbohydrates, therefore C-rich compounds of high C:N ratios (19-25) which can take days to weeks to be turned over (Lancelot, 1984; Lancelot & Billen, 1985; Suttle *et al.*, 1990; Keil & Kichman, 1991; Benner *et al.*, 1992; Amon & Benner, 1996; Biddanda & Benner, 1997). Whereas LMW materials support high bacterial growth efficiencies, HMW compounds support high rates of bacterial respiration (Amon & Benner, 1996; Gardner *et al.*, 1996), and appear to be utilised to a greater extent than LMW products (Amon & Benner, 1996).

Exudation of photoassimilated C tends to increase with time during a phytoplankton bloom; Guillard & Hellebust (1971) found that the percentage of photoassimilated C that was released as exudates by *Phaeocystis pouchettii* increased from 3% at the early singlecell stage to 15-64% during the later colony phase, with the progression being from LMW to HMW. Therefore, in order to be able to utilise these compounds, bacteria may need to assimilate increasingly additional N from the environment, particularly if the high C:N ratio compounds are to be metabolised and the bacterial C:N quota kept constant<sup>4.3.</sup> . In fact, Kirchman *et al.*, (1990) observed that the addition of glucose stimulated NH<sub>4</sub><sup>+</sup> depletion in unfractionated water and in the <0.8µm size fraction, indicating that the metabolism of C-rich compounds may need to be accompanied by concomitant N uptake. Contrastingly, the LMW materials produced at the earlier stages of phytoplankton growth may not require extensive utilisation of ambient N by the bacteria since these compounds are already N-rich and of C:N ratio close to that of these organisms. In these circumstances, production of ammonium may occur (see CHAPTER V).

In this lag period of the availability of these two pools of DOM of differing bioreactivities, may, therefore, reside the explanation for the phasing of phytoplanktonic  $NO_3^-$  and  $NH_4^+$ 

<sup>&</sup>lt;sup>4.3.</sup> The regulation of inorganic N uptake by bacteria by the C:N ratio of the available organic substrate will be further explored and discussed in CHAPTER V and has been studied by Goldman *et al.*, (1987) and Goldman & Dennett, (1991).

uptake (associated with photosynthesis) and that by bacteria (coupled with DOM metabolism), and indeed for the phasing of autotrophic and heterotrophic plankton O<sub>2</sub> metabolism observed in the Menai Strait, as suggested by Blight et al., (1995). According to these authors, during the early spring phytoplankton bloom (diatom dominated) and possibly the early stages of the *Phaeocystis* bloom, the flux of material to the bacteria appeared to be by a LMW pool which is in accordance with the low bacterial N demand found in the present study at those times of the year and with the sharp increase in bacteria cell numbers observed between the beginning of May and end of June. During the senescence stages of the Phaeocystis bloom and subsequent weeks, HMW seemed to prevail. This and other temporary storage of non-living organic material (Williams, 1995) are likely to have supported bacterial metabolism after the decline of photosynthesis, resulting in the protracted inorganic N heterotrophic uptake throughout June. During the summer phytoplankton blooms (July and August), the transfer of organic matter to the bacterioplankton appear to have been mediated predominantly through a HMW pool, since the heterotrophic uptake of ammonium was even higher than at the time of and after the Phaeocystis bloom. Once again, the peaks of bacterial N uptake did not coincide with those of gross community O<sub>2</sub> production at this time of the year. The ammonium uptake increased on day 208 and then after day 233, when the peak of autotrophs had already declined, and remained relatively high until the end of October, declining slowly afterwards. LMW materials may have also supported bacterial metabolism at the late stage of the seasonal cycle (end of September and October), with origin on the small bursts of photosynthetic production which were detected on days 265 and 298 (Figure 4.5a). In agreement with the observations of Gardner et al., (1996), high bacterial respiration rates were detected during the post bloom periods, both in spring and from summer to autumn (Figure 4.13).

Kirchman *et al.*, (1994) had also attributed the uncoupling between the phytoplankton and bacterioplankton production and biomass maxima during the spring bloom in the North Atlantic ocean (a situation identical to that observed in the Menai Strait) to the supply of DOM used by bacteria: in one hand bacterial production could be supported by the DOM released during grazing, which would explain the lack of coupling of bacterial production to primary production on a day time-scale; the week-scale lag between bacteria and phytoplankton biomass maxima could be caused by the lag between phytoplankton and

herbivore activity. On the other hand (and as previously discussed by Ducklow *et al.*, 1993), high bacterial production at the end of the bloom may have been supported by DOM or detritus that had accumulated during the early stages of the phytoplankton bloom.

In N-limited systems such as the Menai Strait after May, the increased bacterial demand for inorganic N induced by the enrichment in high C:N organic could have contributed to the acceleration of the decline of the primary producers or even for the near inhibition of their metabolism. This happened more obviously during June in the Menai Strait when, in the space of a fortnight, gross community oxygen production decreased 10 fold and the concentration of chlorophyll 4 fold. Given their higher capacity for exploiting the low ambient nutrient concentrations than phytoplankton (Suttle *et al.*, 1990), bacteria may have outcompeted phytoplankton at this stage of the seasonal cycle. In accordance to this suggestion, Parsons *et al.*, (1981) found that very high glucose additions inhibited primary production by two to three fold and caused a three fold decrease in chlorophyll.

The relatively high bacterial uptake of inorganic N, mostly in the form of nitrate, at the beginning of March, which constituted 40-60% of the total N uptake on this occasion, may have taken place to fulfil bacterial N demands in order to utilise C-rich compounds with sources other than phytoplanktonic exudates and cell lysis/breakage, such as land runoff and sewage discharges. At this time of the year, autotrophic N uptake was low, possibly limited by low light and temperature which precluded extensive algae growth; of the total nitrate uptake at this time of the year, 50- 84% was accounted for by the  $<2\mu$ m fraction of which only a maximum of 27% was autotrophic uptake.

#### 4.5.3.4. Bacterial Inorganic Nitrogen Preference

The preponderant uptake of nitrate by the bacteria early in the year does not imply that bacteria preferred this form of inorganic N in relation to ammonium. In fact, when RPIs for ammonium and nitrate were calculated, nitrate was always rejected by the bacteria except in February and March when RPI values of 0.8 and 0.9 revealed utilisation of nitrate nearly equitable with its ambient availability (Figure 4.33a). On day 233 only, was the value of RPI slightly above unity (1.5) for the 6 hour incubation set of data and this could have been due to an overestimation of the nitrate uptake rate, since the corresponding rate for the 24 hour incubations rendered a much lower RPI.


Figure 4.33. a) Seasonal variation of ammonium and nitrate RPI for Menai Strait bacteria. b) Relationship between bacterial nitrate RPI and ambient ammonium.

It is also apparent form the relationship between nitrate RPIs and ambient ammonium that concentrations above 1µm inhibit nitrate uptake by the bacteria (Figure 4.33b) discussed as for the phytoplankton uptake. Results obtained by Kirchman et al., (1989, 1992, 1994) have also shown that nitrate was the least preferred N source in relation to both amino acids (which were preferred over ammonium) and ammonium. Again, this order of preference can be explained by considering the energetics of using each N source for macromolecular synthesis, as discussed by Kirchman et al., (1989) for dissolved free amino acids vs ammonium use. Based on this argument, nitrate would be expected

to be the least preferred N source since it must be reduced to the oxidation state of  $R-NH_2$ , a reaction that is energetically expensive.

The RPI<sub>NH4</sub>, NO3 values obtained for the Menai Strait bacterial population (Figure 4.33a) are similar to those obtained by Kirchman *et al.*, (1994) during the spring bloom (RPI<sub>NH4</sub>: 2.3-24.0; RPI<sub>NO3</sub> : 0.1-0.3). The higher uptake of nitrate than ammonium until the end of May (Figure 4.17a and b) could be due to the fact that at this time of the year ambient ammonium concentrations were not sufficiently high to fulfil bacteria needs in inorganic N with these organisms having to resort to taking up a less preferred source of N but which was amply available.

# **4.5.4.** Relationship Between Carbon and Nitrogen Uptake by the Phytoplankton and Bacteria

### 4.5.4.1. Algal Uptake

The development of the <sup>15</sup>N, <sup>14</sup>C and more recently the <sup>13</sup>C techniques has made it possible to determine the uptake rates of both N and C by the phytoplankton in plankton ecology studies. Of particular importance has been the possibility of measuring <sup>15</sup>N and <sup>13</sup>C uptake in the same sample, which has brought many advantages, as discussed in CHAPTER III.

### **Uptake Ratios**

In section 4.2.7. the seasonal variation of the C:N uptake ratio by the whole community was presented (Figure 4.10). The general seasonal pattern presented is coarsely in agreement with that described by Carpenter & Dunham (1985), with ratios below the theoretical 6.6 during early spring and late fall, but above (in some cases well above!) that value in late spring and summer. The C:N uptake ratios obtained are within the range reported originally by MacIsaac & Dugdale (1972) who found values between 6.4 and 178 in eutrophic waters. With the exception of the abnormally high ratios obtained in summer, the remaining values measured throughout the Menai Strait seasonal study, closer to Redfield's theoretical value of 6.6, were in the same range as others reported more recently in the literature, in eutrophic freshwaters (Miyazaki et al., 1985a, b; Miyazaki & Ichimura, 1988; Sakamoto, 1989), in different marine systems (Eppley et al., 1977; Carpenter & Dunham, 1985; Collos & Slawyk, 1986; Harrison, 1986; Horrigan et al., 1990; Dauchez et al., 1991; Hama et al., 1993; Bury et al., 1995) and in cultures (Slawyk et al., 1977; Sakshaug et al., 1983). Only a few of these measurements, however, involved the use of simultaneous incubations with <sup>13</sup>C and <sup>15</sup>N (e.g. Slawyk et al., 1977; Miyazaki et al., 1985a, b; Collos & Slawyk, 1986; Hama et al., 1993; Bury et al., 1995) with the majority having employed the <sup>15</sup>N and <sup>14</sup>C techniques in parallel.

Apart from MacIsaac & Dugdale's (1972) reports of extremely high C:N uptake ratios, it appears that the abnormally high values obtained in the present study on days 143 (16.1-32.5), 188 (58.3-88.6) and 219 (175.5-182.2) have also been found by others. For instance, Metzler *et al.*, (1997) report C:N uptake ratios of 150.05 in an inshore station in the South

Atlantic off Brazil, whereas Petterson (1991) obtained estimates up to 57 in the coastal areas of Skagerrak, Sweden.

Those particularly high estimates from the Menai Strait were registered at the time of the Phaeocystis dominated spring bloom (day 143) and of the summer primary production maxima (days 188 and 219) (Figure 4.10) and are suggestive of seriously N depleted phytoplankton, particularly in the latter case when primary production was supported by ammonium, available then, at low concentrations ( $<1\mu$ M). In environments where N limits production, a high ratio would be expected (MacIsaac & Dugdale, 1972; Carpenter & Dunham, 1985). For example, Eppley et al., (1977) found an average C:N uptake ratio of 22.2 in the North Pacific Gyre and McCarthy (1972) report a ratio of 12.4 for southern California coastal waters in which N was limiting. High C:N uptake ratios were also obtained by Dauchez et al., (1991) at the end of the spring when N limitation began, in a coastal eutrophic ecosystem (Bay of Brest, France). However, high C:N uptake ratios may not always be indicative of a N depleted phytoplankton population; if the algae used other sources of N (such as urea, which as discussed before can be an important source of N for these organisms) but not the studied ones (in this case nitrate and ammonium), then elevated ratios may be obtained despite the nutrient repletion state of the population. The Menai Strait results are also in agreement with Williams (1995) who considers that C:N uptake rates will be high during mid- to- late summer due to the accumulation of C-rich DOM resulting from N depletion at that time of the year; low availability of ambient N would contribute to C-rich phytoplanktonic exudates and preclude bacterial decomposition of the accumulated organic matter.

An additional and likely explanation for the observed high C:N uptake ratios could be that, during summer, N uptake rates were underestimated; this is particularly true for the measured ammonium uptake since isotope dilution corrections were not made to account for *in vitro* ammonium recycling, which is likely to have occurred. Furthermore, due to the high N demand at that time, inferred from the high ammonium rates of uptake obtained and from what would be expected under a phytoplankton bloom situation, <sup>15</sup>N-ammonium substrate exhaustion may have occurred particularly in the longer incubations, resulting in rate underestimation. However, results from the 6 and 24 hour incubations were virtually identical therefore this last explanation may not apply.

"Excess" photosynthesis and/or high rate of C turnover could have also resulted in the observed inflated C:N uptake ratios: on days 188 and 219 the phytoplanktonic population exhibited peaks of gross  $O_2$  community production but the ambient ammonium was possibly not high enough to sustain  $V_{max}$  and this uncoupling between photosynthesis and N uptake may have resulted in "unbalanced" C:N uptake ratios. Furthermore, it appears that high C:N ratios are related to high photosynthetically available radiation (PAR) to which C uptake is directly related and N uptake inversely related (Collos & Slawyk, 1986). Consequently, in light saturating conditions as those likely to be found in summer, photosynthesis could have been maximum (and hence <sup>13</sup>C uptake) whilst N uptake could have been repressed to some extent. Accordingly, Slawyk *et al.*, (1978) have found that algae submitted to increasing light intensities showed increased C:N uptake ratios when growing on a single source of N. Similar patterns have also been shown in the field by Harrison *et al.*, (1982).

Conversely, low C:N ratios (well below Redfield's 6.6) such as those found on days 115 (1.3-1.5) and 320 (2.8-4.6) could have been caused by low C uptake as a result of light limitation. Values as low as 1.8 have been reported by Harrison (1986) in the eastern Canadian Arctic and ratios of 0.1 were estimated for the Antarctic by Slawyk (1979). These values were taken at 25-50% and 1-4% light depths, however, which was not the case in the Menai Strait study where samples came from surface waters and were incubated at surface-like light intensity. Consequently, factors other than light may have operated in those occasions to cause such low C:N uptake ratios. For instance, a predominance of heterotrophic N uptake could be suggested (Metzler et al., 1997; Bury et al., 1995) but, as revealed in Figure 4.18, bacterial N uptake constituted a maximum of 24% of the total N uptake on day 115 and 5.5% on day 320, only. An alternative explanation could be that although C uptake is highly reduced in the dark (Hama et al., 1993) that of nitrogen may continue. If dark N uptake was extensive on the occasions when the lower C:N uptake ratios were observed (not only on days 115 and 320 but also 59, 88 and 208 when ratios of 4.3-5.2, 4.7-5.9, and 5 were, respectively, obtained), this could have given rise to lowered C:N uptake ratios. This is further aggravated if it is considered that during the dark periods, C is also respired by the autotrophs, although on the particular occasions indicated above, dark community respiration was not high. From Figures 4.6b) and 4.7b) it could be seen that dark N uptake occurred and in some cases contributed quite extensively to the total N

uptake measured over the 24 hour incubation period, therefore, this explanation appears probable. Accordingly, Glibert *et al.*, (1995) reported for the Cheasapeak Bay, that C:N uptake ratios determined for short-term incubations from mid-morning approached Redfield values but were considerably lower when integrated over the full day period. However, short-term uptake experiments can lead to an over-estimation of C uptake; incubations for 24 hours carried out for C and N are more appropriated for comparisons because nocturnal respiration and C excretion and N uptake are included (Slawyk *et al.*, 1978).

#### **Relationship Between C:N Uptake Ratios and C:N Cell Quota**

In Figure 4.10, the phytoplanktonic C:N uptake ratios were compared with the C:N biomass composition. For convenience, this relation is displayed again in Figure 4.34 and there appears to be no relationship between the two parameters except in the fall, when the C:N uptake and composition ratios covaried. Even on the occasions when C:N uptake ratios largely exceeded that of Redfield's on days 143 (mean ratio of 24.2), 188 (mean ratio of 73.5) and 219 (mean ratio of 179), the C:N composition of the phytoplankton biomass remained low (6.8, 6.1 and 8, respectively).



**Figure 4.34.** Comparison between C:N composition and uptake ratio for the Menai Strait phytoplankton in 1995. Nitrogen uptake rates from the 6 and the 24 hour incubation experiments were averaged.

Some discrepancy these between two parameters is, however, expected since uptake ratios are only indicative of the cellular metabolism of the biomass present in the the time water at of sampling -thus. an instantaneous propertywhereas in the best case POC/PON composition time-averaged are the of this resultant

metabolism (Dauchez *et al.*, 1991) - thus, an integrated property. Only under steady state the ratio of C:N uptake by phytoplankton is expected to be equal to their internal C:N content (Eppley, 1981) a situation seldom believed to be obtained in nature, since there are many factors affecting the uptake processes, possibly leading to the uncoupling between the uptake of C and N (Flynn, 1991).

During October, such balanced growth appears to have taken place in the Menai Strait with the two parameters showing identical numerical values and temporal fluctuations.

Uncoupling between C:N compositional and uptake ratios has been observed by others, following the general pattern of the former being normally lower than the latter (e.g. Slawyk et al., 1978; Miyazaki et al., 1985a, b; Collos & Slawyk, 1986; Miyazaki & Ichimura, 1988; Dauchez et al., 1991; Petterson, 1991; Metzler et al., 1997). A reason for this could be that C is turned over in the energy processes in the cell and is not just used for growth, therefore C uptake may need to be higher than expected from elemental composition. Moreover, Collos (1986) presented a theory with two different strategies for algae concerning the relationship between nutrient uptake and growth: the first deals with the species which are not able to store nutrients intracellularly and hence have a close coupling between uptake and growth; in this circumstances, C:N uptake and composition ratios will be approximate. The other strategy is to store nutrients intracellularly, thus having a time lag between uptake and growth. While the species having the latter strategy can grow if they get one single addition of nutrients, the former need nutrients more or less continuously. From this a greater degree of uncoupling between not only C:N composition and uptake ratios but also between C and N uptake may result, in periods when phytoplankton species with high storing capacity are dominating.

Another explanation for the uncoupling between the C:N content and uptake ratios of the Menai Strait phytoplankton could be the presence of detritus. Figures 4.35 show the relationship between chlorophyll and POC (a) as well as PON (b). As illustrated, a large proportion of the POC (~65%) and PON (~76%) seasonal variation could be accounted for by fluctuations in the phytoplankton biomass. However, if one considers the intercept of the given straight lines as corresponding to the detrital component, then there is a marked presence of particulate matter that is not part of the phytoplankton biomass.



Figure 4.35. Relationship between a) POC and b) PON and chlorophyll concentrations in the Menai Strait during the 1995 seasonal cycle. Statistic analyses revealed that in both cases, the intercept was significantly different from zero (p<0.001).

estimated C:N The composition of this detrital matter (given by the ratio of the y intercept of the POC and PON straight lines, respectively) of 8.55 is higher than the calculated C:N ratio of the chlorophyll related material, given by the ratio of the slopes of those (6.3), straight lines indicating that indeed, the C:N ratio of the particulate matter could, at times, be inflated due to the presence of detritus. The above relationships were obtained, however, by excluding the data point for day 143, at the of the chlorophyll time seasonal maximum, associated with the The **Phaeocystis** bloom. POC and PON estimates obtained, 34.5 and 5.04 µM

respectively, are considerably lower than could be anticipated in such circumstances and an experimental error is possibly to blame: at this time of the seasonal cycle, the collection of particulate matter onto the glass fibre filters (GF/F) was problematic and almost impossible due to the smothering of the filters' surface with *Phaeocystis* produced mucilage; consequently, only a small volume of sample was able to pass through the filters despite the higher than normally used negative pressure applied, which could have resulted in the loss of particulate material ultimately inducing the observed underestimations of POC and PON.

Similar proportions of non-phytoplanktonic matter in the PON to those found in the Menai Strait were reported by Furnas (1983) who obtained a value of 26% in unfractionated samples from Narragansett Bay (USA) and Dickson & Wheeler (1995) with a 20% contribution of non phytoplanktonic N to the total PON. The obtained PON/chlorophyll ratios for the Menai Strait particulate matter are however, considerably higher (2.9-11.6) that those described by Horrigan *et al.*, (1990) in the spring (0.55-2.38) and in the autumn (0.67-1.85) reflecting a higher contribution of detrital N to the particulate matter in the Menai Strait than in the Cheasapeake Bay (USA). As discussed in CHAPTER II, Menai Strait waters carry a heavy load of inorganic and organic suspended matter, tending to increase during the autumn-winter months, which may have resulted in the inflated C:N composition ratios observed on days 298 (12.1) and 88 (15.8) and in the seasonal mean of 7.9, above the theoretical Redfield's ratio of 6.6.

### 4.5.4.2. Bacterial Uptake

Analysis of the <sup>13</sup>C uptake observations have revealed that a variable proportion of the <sup>13</sup>C uptake by the whole community could be, in fact, attributed to the  $<2\mu$ m fraction (8.2-45%); not all of this picoplanktonic uptake was necessarily photosynthesis associated since a large percentage of it (up to 97%) could be considered to be bacterial, after correcting the uptake rates for the presence of chlorophyll in that size fraction. Over the diel cycle, the contribution of heterotrophic uptake to the total unfractionated amounted to a maximum of 44% (day 158; Figure 4.21 a). In dark only conditions, this percentage increased to a value of 60%, measured on the same occasion (Figure 4.22a).

### 4.5.4.2. a) Association Between Carbon and Nitrogen Bacterial Uptake

Although inorganic carbon uptake in natural waters has been considered to be due to algal photosynthetic activity (Steeman Nielsen, 1952), it has also been shown that bacterial C fixation may occur (Romanenko, 1963, 1964). However, the presence of tracer from added  $CO_2$  in the bacterial fraction is conventionally regarded to be due to transfer *via* release of algal photosynthetic products (Williams & Yentsch, 1976), with the heterotrophic fixation ignored in the vast majority of studies (*e.g.* Derenbach & Williams, 1974, based on Kusnetsov & Romanenko's, 1966, observations that bacterial dark fixation appeared to be

low compared with the total bacterial metabolism). In the present section of this discussion, the presence of  $^{13}$ C in the bacterial fraction is discussed assuming that heterotrophic C fixation is negligible whereas, over the next section (4.5.4.2.b), the data obtained will be analysed having in considerations the observations of Romanenko (1963, 1964).

In Figures 4.36, the seasonal variation of the algal and heterotrophic <sup>13</sup>C uptake over the diel cycle (a) and in the dark (b) is presented, as well as the percent C incorporation into the bacterial biomass in relation to that into the phytoplankton (c), in both incubation conditions. This percent bacterial uptake may be interpreted as the proportion of the C fixed through photosynthesis that has been made available to, and utilised by the bacteria in the form of low molecular weight compounds of low C:N ratio (3-11) such as amino acids, and high molecular weight substances of high C:N ratio (15-22) such as polymeric carbohydrates (Benner *et al.*, 1992; Lancelot, 1984; Lancelot & Billen, 1985). The assumed C transfer estimates from the autotrophs to the microheterotrophs fall within the range reported by others, namely Derenbach & Williams (1974) (1-30%), Ducklow *et al.*, (1993) (15-80%), Cole *et al.*, (1988) (15-50%) regarding data gathered from the literature collected in different ecosystems, and Kirchman *et al.*, (1994), who observed that <sup>14</sup>C in the 0.8  $\mu$ m fraction was between 12-25% of that in the whole community.

From Figure 4.36 a) and b), it can be observed that in both incubation conditions, peaks of  $^{13}$ C by the algae generally coincided with those by the bacteria, suggesting rapid and extensive transference of materials from the former to the latter. However, this C transference appeared not only to decrease with time after the spring phytoplankton bloom particularly according to the diel incubations (Figure 4.36c), but, with the progression of the seasonal cycle, a longer lag interval between maxima phytoplankton and bacterial C uptake is apparent (Figure 4.35a,b). For instances, whilst early in the year, prior to the *Phaeocystis* dominated spring bloom the lag time was not particularly evident, in May, beginning of June, it had increased to 15 days and at the end of the summer, to a maximum of 75 days (between days 233 and 298, diel incubations). The duration of these lag intervals is obviously influenced by the frequency of sampling, higher in the spring and summer (up to day 233) than in the autumn; a more frequent sampling programme would have been desirable in order that a finer definition of the relationships between autotrophic and



Figure 4.36. Seasonal variation of the algal and bacterial a) diel and b) dark uptake of inorganic C and, c) of the inferred percent "transference" of algal assimilated C to the bacteria.

heterotrophic C and N metabolism could have been attained but such was not a practical proposition.

Both these features of the bacterial C uptake, (decreased bacterial C utilisation and increase in the duration of the period between maxima phytoplanktonic and bacterial uptake, with the progression of the seasonal cycle), may be explained by taking into consideration the seasonal variation of the inorganic N availability in this environment, the assumed chemical composition of the available DOM of phytoplanktonic origin, and the basic assumption, that in order to keep their C:N quota constant, the bacterial C and N uptake metabolism would have to be intrinsically linked (Lancelot & Billen, 1985; Anderson, 1992). An increase with time in the duration of the interval between maxima phytoplanktonic and bacterial C uptake, is consistent with increased production and accumulation of HMW compounds at the later stages and decline of the phytoplankton blooms, particularly during summer (Williams, 1995), which requires the simultaneous uptake of inorganic N, contrary to the LMW, which utilisation may result in N regeneration as observed by Gardner et al., (1996). Therefore, the high availability of inorganic N in the Menai Strait until the end of May may have contributed not only for the shorter lag times between the above mentioned events, but it could also have made possible the bacterial utilisation of high C:N ratio compounds (Kirchman et al., 1990; 1991; Gardner et al., 1996). Relative bacterial C utilisation was, consequently, higher in the spring than at the later stages of the seasonal cycle, when the assimilation of DOC may have been N limited.

The high utilisation of DOM by the bacteria immediately after the decline of the *Phaeocystis* dominated bloom, inferred from the relatively high  $^{13}$ C uptake in June (Figures 4.21.a) and b), resulted in the observed sharp increase in bacteria numbers (Figure 4.11). It is possible that the heterotrophs utilised mostly HMW rather than LMW compounds in the Menai Strait during this month, in accordance with Gardner's *et al.*, (1996) observations that bacterial abundance and growth rates increased more in the presence of HMW than in the presence of LMW DOM. Similarly, in July an increase in the bacteria cell numbers was detected, possibly as a response to the photosynthesis peak (Day 188; Figure 4.5a), with a lag period of 15 days. In contrast, despite subsequent primary production maxima, no bacterial response in terms of increased biomass or C uptake over the diel cycle was detected. This suggests that either the utilised C was from LMW compounds or if from

HMW substances, these were not being utilised to the same extent as they were in the spring, resulting in lower bacterial growth. Ambient accumulation of ammonium throughout summer and early autumn is consistent with utilisation of LMW compounds which could have been produced by the observed bursts of phytoplankton growth in August (day 219), September (day 265) and end of October (day 298).

### **Bacterial C:N Uptake Ratios**

Despite the assumption that bacteria would require a closely coupled C and N metabolism in order to maintain their C:N quota constant, Figure 4.37 shows that the patterns of temporal variation of bacterial C and N uptake did not always coincide, for incubations both over the diel cycle (a) and in darkness (b). For instance, in the former case the N and C uptake closely accompanied each other early in the year, when inorganic N was amply available and prior to the spring phytoplankton growth (March and April). It did not do so at the time of the main bloom (May), when nutrients started to become depleted and competition with the phytoplankton for N is thought to have been increased.

In July, coinciding with the first maxima of the summer phytoplankton growth (day 188; Figure 4.5a), a peak of bacterial C uptake could be detected, which preceded that of bacterial N uptake by a fortnight (days 188 and 208). No C or N bacterial uptake maxima could be seen at the time of the second summer phytoplankton peak (day 213), but in the autumn, a peak of bacterial N uptake (day 265) preceded that of C by a month. In the dark, there seemed to be a better co-ordination between C and N bacterial uptake throughout the seasonal cycle, mainly in March, April, June and September, perhaps due to a decreased competition with the phytoplankton for inorganic N and to the prevention of phytoplanktonic DOM production in these incubations by the absence of light.

Such unparalleled C and N uptake resulted, at times, in apparent C:N uptake ratios as high as 93.7, observed at the beginning of July in the incubations carried out over the diel cycle (Figure 4.37a) or as low as  $\leq 1$ , observed in several occasions, in either incubation condition. Both situations are severe deviations from the Redfield ratio, despite the fact that bacterial uptake ratios lower than that predicted by Redfield (6.6) would be expected given their low C:N composition ratios of 4-5 (Nagata, 1986).



Figure 4.37. a) Seasonal variation of the total N and C uptake by the bacteria and resulting C:N uptake ratios, over the diel cycle and b) in the dark.

In fact, apart from those exceptions, the obtained bacterial C:N uptake ratios (2.7-7.7) were between expected values.

The extremely high ratio obtained on day 188 could artefact have been an caused by the means by which these ratios were obtained and that had two major shortcomings. The first is associated with the determination of nitrogen uptake which could have been severely underestimated at this time of the year, leading to inflated estimates of the C:N uptake ratios. The second is associated with <sup>13</sup>C uptake for it the ignores firstly the source of

DOC other than recently exudated photosynthetic products and secondly the possibility of  $CO_2$  fixation by the microheterotrophs (discussed below). Furthermore, contamination of the bacterial fraction with autotrophs which presence was not adequately corrected for by the mathematical procedure given before, could have also resulted in overestimations of the C uptake attributed to bacteria.

Contrary to what was observed for the whole community, the C:N bacterial uptake ratios were, in general much lower than the bacterial C:N composition ratio (data not plotted) which varied between 2.1 (day 208) and 15 (day 143), with variations in the former

appearing not to have much bearing on those in the latter. The majority of the compositional C:N estimates were somewhat, but not severely, higher than 6.6, which may be considered to be high for bacterial biomass (Nagata, 1986) and they may have resulted from the presence of detritus, contributing to a higher discrepancy between uptake and compositional C:N ratios.

### 4.5.4.2. b) Heterotrophic Fixation of Inorganic Carbon

The considerations in the previous part of this section regarding the C bacterial metabolism were made assuming that heterotrophic fixation of C was negligible or very low compared with the total bacterial metabolism, in accordance with Kusnetsov & Romanenko, (1966). However, Romanenko's (1963, 1964) work with heterotrophic bacteria (both with individual species and mixed assemblages of natural microflora from water reservoirs) has shown that dark heterotrophic fixation of carbon dioxide occurred simultaneously with oxygen consumption, with an average estimate of 0.0077  $\mu$ mol of C fixed per  $\mu$ mol of O<sub>2</sub> consumed. A higher ratio of CO<sub>2</sub> to O<sub>2</sub> uptake of 0.015 has been found by Bryan (1979) for mixed assemblages of marine bacteria.

In Table 4.19, Romanenko's (1964) and Bryan's (1979) ratios were applied to the Menai Strait seasonal study data, where, with basis on the dark bacterial oxygen respiration, rates of bacterial  $CO_2$  fixation were predicted. Figure 4.38 shows the relationship between the actual C assimilation by the bacteria and that predicted by Romanenko's and Bryan's ratios.

The plotted data show that the estimates of bacterial C uptake obtained in this field study were generally lower that those predicted by the two authors. Thus, it can be concluded that the C found in the bacterial biomass, in the great majority of the cases, could be accounted for by the direct C fixation instead of indirect assimilation *via* utilisation of photosynthetic products. Hence, in these circumstances, the presumed C transference from the phytoplankton to the bacteria discussed in part a) of this section, is likely to constitute an overestimation.

Day of the year	measured bacterial dark respiration	predicted C fixation according to Romanenko (1964)	predicted C fixation according to Bryan (1979)	measured C uptake in the dark	"excess" C uptk. in relation to predicted by Romanenko (1964)	"excess" C uptk. in relation to predicted by Brycen (1979)
		· · · · ·			(1704)	Digan (1779)
73	1.52	0.0116	0.0228	0.0049	0	0
101	0.37	0.0028	0.0055	0.0265	0.0237	0.0210
115	0.45	0.0034	0.0068	0.0176	0.0142	0.0108
131	5.3	0.0403	0.0795	0.0202	0	0
143	4.42	0.0564	0.0663	0.0305	0	0
158	4.29	0.0555	0.0645	0.1294	0.0739	0.0649
173	5.55	0.0422	0.0832	0.0847	0.0425	0.0015
188	1.80	0.0136	0.027	0.0371	0.0235	0.0101
208	6.24	0.0474	0.0936	0.0069	0	0
219	6.26	0.0476	0.0939	0.0422	0	0
234	1.16	0.0088	0.0174	0.0000	0	0
265	5.20	0.0395	0.0780	0.0434	0.0038	0
298	8.58	0.0652	0.1287	0.0338	0	0
320	0.52	0.004	0.0078	0.0068	0.0028	0

**Table 4.19.** Calculation of the predicted heterotrophic C fixation in the dark, from bacterial  $O_2$  consumption, with basis on Romanenko's (1964) and Bryan's (1979) ratios.

Bacterial dark respiration:  $\mu$ mol O<sub>2</sub> dm<sup>-3</sup> d<sup>-1</sup>; Predicted and observed C uptake:  $\mu$ mol C dm<sup>-3</sup> d<sup>-1</sup>; "excess" C uptake: C taken up in excess of that predicted to be fixed by the bacteria; uptk. stands for uptake.



Figure 4.38. Relationship between the observed C assimilation by Menai Strait bacteria and that predicted by Romanenko's (1963) and Bryan's (1979) ratios of  $CO_2:O_2$  uptake.

The times of the year when the observed dark C uptake exceeded expected according that to (1964)Romanenko's and Bryan's (1979) ratio, were during April (days 101 and 115), Juneearly July (days 158, 173 and 188), September (day 265) and November (day 320). It can be considered that during these periods only, did the presumed bacterial utilisation of photosynthetic materials occur. The observed high C uptake in April is difficult to explain as no photosynthetic activity (as given by the gross  $O_2$  community production) was observed during this month, hence the expected negligible exudation of <sup>13</sup>C-labelled photosynthetic products. Nevertheless, a small peak of <sup>13</sup>C by the algae was detected on day 101 and it is possible that part of this uptake was transferred to the bacteria through exudation/cell breakage or lysis even though this did not appear to have been accompanied by corresponding bacterial  $O_2$  consumption.

The bacterial <sup>13</sup>C uptake in excess of that expected between days 158 and 188 could reflect utilisation of <sup>13</sup>C-labelled algal products with origin in the main spring phytoplankton bloom. On the other hand, the absence of "excess" C uptake during most of summer and early autumn, suggesting non utilisation of such products at this time of the year despite the primary production peaks in July and August, is consistent with the depleted levels of inorganic N in the environment which, as discussed before, would preclude the utilisation of the presumed available organic matter. Accordingly, the increase in C uptake in November in "excess" of that predicted by Romanenko's (1964) and Bryan's (1979) ratios could have been made possible by the increased ambient inorganic N at that time of the year (similarly to that in September) was low compared to that earlier in the year (spring, early summer).

It is possible, however, that the C/O ratio reported by Romanenko (1964) and Bryan (1979) may overestimate heterotrophic C fixation since it is highly unlikely that according to their predicted C assimilation estimates, from mid July until November, Menai Strait bacteria virtually did not utilise algal products in the dark, given the high primary production at this time of the seasonal cycle. Although ambient inorganic N was scarce at this time of the seasonal cycle, phytoplankton did not appear to be particularly N-starved, as discussed before; consequently, with their greater capacity to exploit nutrients at lower concentrations than the autotrophs, bacteria would presumably be able too, to make use of the available inorganic N which, in turn, would make possible the utilisation of C-rich organic matter of phytoplankton origin. Furthermore, bacterial respiration was high at times during this period (days 208, 219, 265 and 298), which is in accordance with extensive organic matter utilisation.

On the other hand, if bacterial C fixation had been recognised as significant by marine scientists it would have received more attention. Obviously, this issue needs further investigation and it is surprising that it has not received recent attention, given the present acknowledgement of the importance of bacterial processes in the C and N dynamics in the marine environment. Fractionation techniques available at the present would reduce some of the uncertainties inherent to the earlier studies of Romanenko (1963, 1964) making it possible to assess more accurately this process.

## 4.6. CONCLUSIONS

In respect to the three objectives given at the beginning of this chapter, the conclusions reached from the seasonal field study in the Menai Strait were as follows:

- 1) Primary production patterns in the Menai Strait followed a seasonal trend characterised by nitrate dominated production in the spring, comprising the diatom and *Phaeocystis* dominated spring bloom characteristic of these waters (PNAPh), evolving to ammonium based production in the summer and autumn (SNAPh and part of the RPh), after a transitional phase (NHPh) during which both forms of production co-occurred. This trend, generally characteristic of temperate waters, followed the seasonal variation pattern of the predominant forms of inorganic N available in these waters, with nitrate marked presence in these waters, early in the year being replaced by that of ammonium from the end of June onwards.
- 2) Ammonium was consistently the inorganic N source preferred by both the algae and bacteria. Nitrate was used equitably with its abundance in the spring but was normally rejected for the remainder of the seasonal cycle and never favoured over the more reduced form of inorganic N.
- 3) A maximum of 67% of the ammonium based production was estimated to be driven by bacterial respiration, with other processes such as zooplankton excretion, land runoff and

antropogenic input *via* sewage discharges, by default important sources of ammonium, fuelling the remainder of this type of production in this environment.

- 4) Bacteria were responsible for up to 70% of the total inorganic N uptake (ammonium and nitrate) throughout the Menai Strait seasonal cycle, with the heterotrophic uptake generally exceeding the algal during the NHPh (June). The lowest contributions of the bacterial to the total inorganic N uptake were registered at times of high primary production (up to 24% in the PNAPh and up to 43.6% in the SNAPh).
- 5) It has been hypothesised that bacteria took up inorganic N in order to be able to utilise C-rich, deficient in N compounds of high molecular weight, whilst endeavouring to maintain a constant C:N cell quota. Based on this assumption, the increased bacterial N uptake in the weeks that followed a phytoplankton peak were attributed to the inflated availability of HMW compounds with origin in the breakage/lysis of the algae cells. In these circumstances, bacteria form a biological sink for inorganic N. Conversely, relatively low bacterial inorganic N uptake during the phytoplankton blooms was speculated as being due to the presence of phytoplankton exuded low molecular weight substances such as amino acids which do not require further assimilation of N by the bacteria in order to be utilised.
- 6) Throughout the seasonal cycle, the phytoplankton did not appear to be severely N starved with the exception of the month of June when competition with bacteria for nitrate and ammonium may have led to the observed rapid decline of the autotrophs spring bloom. This was the only period when there is reason to suspect the existence of competition for inorganic N between these two populations: nitrate had recently become exhausted and ammonium regeneration had yet not been sufficient to lead to its ambient accumulation, with ambient levels of both these nutrients below detection limits.
- 7) The ambient availability of inorganic N seemed to influence the proportion of C fixed by the autotrophs that was transferred to the bacteria. The assumed C transference from the autotrophs to the heterotrophs was highest in the spring, following a period of ample availability of inorganic N, and lowest during summer (despite peaks of primary production), associated with depleted ambient N conditions. The utilisation of low molecular weight compounds, less dependent on inorganic N availability, was thought to

have taken place instead, with origin in the summer primary production peaks, leading to the observed high  $O_2$  respiration rates, accumulation of ammonium in the environment, high C:N uptake ratios and higher than expected C:N composition ratios (although the presence of detritus may have also contributed to the latter).

Finally, the interpretation of the calculations of the N and C dynamics in this work, as well as the phasing of autotrophic and heterotrophic metabolic processes of the phytoplankton in the Menai Strait by Blight *et al.*, (1995) was based on observations reported in the literature regarding the nature of the DOM accessible to bacterial use at different stages of the primary production seasonal cycle. These reports refer to the predominance of LMW compounds, present in the exudates during the early stages of phytoplankton growth, and that of HMW substances concurrent with the declining phases of the blooms, with origin in the breakage/lysis of the algae cells. Consequently, it would be desirable that future studies in this and other marine environments endeavoured not only to quantify but also to characterise chemically the composition of the available DOM, as well as its sources and relative use by the heterotrophs, since certain compounds of HMW, previously regarded as unavailable to bacterial degradation, are actually important sources of C for the growth of these organisms, as long as inorganic N exists to be taken up concomitantly, to "balance" the cell C:N quota.

From this and other seasonal studies, it has become apparent that in investigating the microbial ecology of a given marine environment it is futile to consider the autotrophic and the heterotrophic compartments separately as it is now clear that their dynamics are intrinsically interwoven. As far as it was possible, the study of the C and N relationships between these two populations (phytoplanktonic and bacterial) in the Menai Strait has confirmed and deepened the present understanding of those processes and their regulations.

"The study of microbial ecology has until recently been persued either by visionaries or fools"

(Azam et al., 1994)

# CHAPTER V

# LABORATORY EXPERIMENTS

## **5.1. INTRODUCTION**

This chapter deals with laboratory experiments carried out during the Menai Strait's productive seasons of 1995 and 1996. These were aimed at investigating the influence of bacterial organic substrates of varying C:N ratios on the uptake of ammonium and nitrate by the whole phytoplanktonic community (1995 and 1996 experiments), the picoplankton ( $<2\mu$ m size fraction; in 1995) and the organisms contained in the 0.8µm size fraction (in 1996).

These experiments were based on the assumption that, when mineralising organic matter, heterotrophic bacteria endeavour to maintain a constant C:N quota. When the C:N ratio of the available organic substrate is low, the heterotrophs are presumed to excrete the excess nitrogen as ammonium. When this quotient is above that necessary for balanced growth (C:N>10; Lancelot & Billen, 1985), in principle two alternative strategies exist:

- i) to remedy the imbalance by taking up inorganic nitrogen; thus, the C growth yield should be maintained (Hypothesis I).
- ii) to remedy the imbalance by respiring the excess C, thus the C growth yield would fall in high C:N ratio situations, and although no assimilation of inorganic N will occur, N retention efficiency will increase (Hypothesis II).

There would be competition between algae and bacteria in the former case but not in the latter.

The conceptual models of Lancelot & Billen (1985) and Anderson (1992) assume Hypothesis I and this strategy is presented in Figure 5.1a. The alternative situation, not explicitly stated but supported by the observations of Andrews & Williams (1971) is illustrated in Figure 5.1b.



Figure 5.1. a) *Hypothesis I.* Control of the bacterial inorganic nitrogen uptake by the C:N ratio of the DOM and cell C:N quota. Model of conservation of a constant proportion of carbon and competition for nitrogen.

The predicted metabolic and/or ecological consequences associated with both proposed hypotheses are summarised in Table 5.1.



Figure 5.1.b) *Hypothesis II*. Bacteria inorganic nitrogen metabolism is not controlled by the C:N ratio of the DOM substrate and cell C:N quota. Model of disposal of excess carbon determining bacteria growth yield.

Table	5.1.	Summary	of	the	predicted	metabolic	and/or	ecological	consequences	of	the	two
propos	ed hy	potheses.										

		Physiological Responses									
C:N rations substra	o of ite	Increased organic Carbon mineralisation	Carbon yield	Use of inorganic Nitrogen	Organic Nitrogen mineralisation	Nitrogen yield	Competition for inorganic Nitrogen				
Hypothesis I	Low	No	Fixed	No	Yes	Reduced	No				
ngpoulosis i	High	No	Fixed	Yes	No	Maxim.	Yes				
Hypothesis II	Ĺow	No	Fixed	No	Yes	Reduced	Na				
	High	Yes	Reduc.	No	No	Maxim.	No				
Value in te alternative hy	sting pothesis	Useful but not testable in the experiments	Useful and testable	Useful and testable	not useful	not useful	Useful and indirectly testable				

High and Low refers to the C:N ratio of the substrates available to the bacteria; Maxim. is maximum and Reduc. is reduced.

In order to test these hypotheses, experiments were set up with the objective of measuring the uptake of <sup>15</sup>N- ammonium and <sup>15</sup>N- nitrate by the whole and  $<2\mu m$  (1995) or  $<0.8\mu m$  (1996) communities in the presence of bacterial organic substrates of high (20), low (2)

(1995 &1996) and "balanced" (1995) C:N ratios. A "balanced" bacteria substrate is considered to have a C:N ratio of 10, assuming a bacteria C:N ratio of 4 (Nagata, 1986) and a growth yield of 40% (Lancelot & Billen, 1985; Collos, 1987).

The practical strategies followed in the 1995 and 1996 experiments differed and they will be detailed in the following sections.

### **5.2. EXPERIMENTAL STRATEGY**

### 5.2.1. Experiments in 1995

To investigate the effects of the C:N ratio of a bacterial organic substrate on the uptake of inorganic N by the "bacterial" ( $<2\mu$ m fraction) and the phytoplanktonic communities in conditions of nutrients depletion, four different factorial experiments were set up:

- **EXPERIMENT** I The uptake of <sup>15</sup>N- ammonium by the above communities was determined in the presence of organic substrates (mixtures of glycine and glucose) of high (20) and low (2) C:N ratios;
- **EXPERIMENT II** The uptake of <sup>15</sup>N- nitrate was determined in the same conditions as above;
- **EXPERIMENT III** The uptake of <sup>15</sup>N- ammonium and nitrate was determined in the presence of an organic substrate of "balanced" C:N ratio (10);
- **EXPERIMENT IV** The uptake of these <sup>15</sup>N labelled nutrients was determined in samples with no organic additions ("control" of the set of experiments).

### **Inorganic and Organic Additions**

The inorganic N additions were 0.020  $\mu$ M and 0.010  $\mu$ M of <sup>15</sup>N- ammonium and <sup>15</sup>Nnitrate, respectively. The seasonal period during which these assays were undertaken was characterised by ambient nitrate concentrations below detection limits (0.025  $\mu$ M). Ammonia was also undetectable for most of the time (<0.030  $\mu$ M) but concentrations increased to 0.094  $\mu$ M on the occasion of experiment IV. The added organic substrate was composed by a mixture of glycine ( $^{13}$ C- gly and  $^{12}$ C- gly) and glucose ( $^{12}$ C- glu) as sources of organic carbon and nitrogen, except in the case of the low C:N ratio substrate, constituted only by the former. The final concentration of C added was of 15  $\mu$ M; this addition was made considering that bacteria consume approximately 50% of the photosynthetically fixed C (Andrews & Williams 1971; Larsson & Hagström, 1982; Lancelot & Billen, 1984) and that a typical photosynthetic rate at this time of the year was 30  $\mu$ M d<sup>-1</sup>. A single pulse addition of 15  $\mu$ M was made at the beginning of the experiments.

The quantities of glycine and glucose added in order to obtain a substrate mixture of the required C:N ratio and C content were calculated using the following equations:

$$\frac{2 \operatorname{Gly} + 6 \operatorname{Glu}}{1 \operatorname{Gly}} = \frac{C}{N}$$
5.1

$$2 \operatorname{Gly} + 6 \operatorname{Glu} = \operatorname{C_T}$$
 5.2

where, (2Gly + 6Glu) corresponds to the number of moles of carbon from glycine and glucose, respectively;  $C_T$  is the total carbon amount added: 15µM; and (1Gly) is the numbers of N moles provided by 1 mole of glycine.

In all experiments the glycine added was uniformly <sup>13</sup>C-labelled except in the low C:N substrates where 50% of the glycine was unlabelled. A summary of the chemical conditions at the start of each Experiment is presented in Table 5.2.

#### **Incubation Conditions**

These experiments were undertaken using Menai Strait sea water, collected at high tide according to the procedure described in CHAPTER III.

Samples of 25 dm<sup>3</sup> were incubated in transparent polycarbonate containers, at a constant temperature of 15°C, under moderate aeration which ensured thorough mixing of the cultures. The air was filtered prior to admission to the incubating containers by means of a glass fibre (GF/F) filter and a 0.8µm polycarbonate membrane located on line. A photoperiod of 14 hours light and 10 hours darkness was provided, simulating early summer diel conditions.

Laboratory Experiments

Table 5.2. Chemical experimental conditions at the start of the 1995 Experiments.

		Organic Ac	Iditions				Inorganic N (uN	Additions		
Date	Experiment	Glycine	Glucose	<sup>13</sup> С added (µМ)	% of <sup>13</sup> C	Substrate C:N ratio	<sup>15</sup> NH4	<sup>15</sup> NO <sub>3</sub>	Minimum <sup>15</sup> NH4 ( <sup>15</sup> N- atom%)	Minimum <sup>15</sup> NO <sub>3</sub> ( <sup>15</sup> N- atom%)
27/05/95	Ι	0.75	2.25	1.5	10	20	0.02	ı	40	I
02/06/95	П	0.75	2.25	1.5	10	20		0.01	ı	28.7
17/06/95	Ш	1.50	2.0	3.0	20	10	0.02	0.01	40	28.7
17/06/95	Ш	1.50	2.0	3.0	20	10	0.02	0.01	40	28.7
27/05/95	I	7.50	·	7.5	50	7	0.02	ı	40	ı
02/06/95	П	7.50	,	7.5	50	7	·	0.01	ı	28.7
21/06/95	IV	I	•	ı	۱		0.02	0.01	17.7	28.7
27/06/95	Ŋ	١	t	•	•	•	0.02	0.01	17.7	28.7

Chapter V

211

Harvesting of 2.5 dm<sup>3</sup> subsamples from these containers took place immediately after the organic and inorganic additions had been made, after 3.5, 7, 14 hours and then at the change of the photoperiod phase, until 72 hours.

#### **Parameters Measured**

At each harvesting occasion, the concentrations of bacteria, chlorophyll, particulate organic carbon and nitrogen and <sup>15</sup>N and <sup>13</sup>C isotopic composition of the whole phytoplanktonic and fractionated (<2 and 0.8  $\mu$ m) communities were determined. Whilst bacteria counts and chlorophyll quantifications were performed on a single sample, the remaining determinations were made either in triplicates or, more often, in duplicates. This limitation of replication was due to the small volume of sample possible to harvest at each time, as only 25dm<sup>3</sup> incubations were feasible according to the available experimental conditions. The sample size fractionation and experimental protocols have been described in CHAPTER III.

### 5.2.2. Experiments in 1996

The laboratory experimental work carried out in 1996 took place in May and June as in the previous year, coinciding with the most productive period of the seasonal cycle in the Menai Strait. Ambient concentrations of ammonium and nitrate were below detection limits (0.030 and 0.025  $\mu$ M, respectively).

Since the main aim of this study was to extend the observations made in 1995, the general experimental procedure was maintained. Some modifications were introduced, principally the substitution of a  $<0.8\mu$ m fraction for the  $<2\mu$ m used in the earlier experiments, and the introduction of a "control" culture run in parallel with the other samples. Harvesting was undertaken less frequently than before, having taken place in this study after 6, 12, 24, 48 and 72 hours of incubation.

In the 1996, the study of the influence of an organic substrate of "balanced" C:N ratio (10) was not performed due to limiting resources. Therefore, priority was given to the

investigation of the effect of an organic substrate of high and low C:N ratio on the uptake of inorganic nitrogen by the above mentioned communities.

In addition to the parameters investigated in 1995, changes in the concentration of nitrite, nitrate and ammonium were determined throughout the course of the incubations. Three experiments were therefore set up as follows:

- **EXPERIMENT I** The uptake of <sup>15</sup>N- ammonium by the whole community and <0.8μm size fraction was measured in the presence of organic substrates (glycine ± glucose) of high (20) and low (2) C:N ratios and in a "control" situation (no organic additions).
- **EXPERIMENT II** -The uptake of <sup>15</sup>N- nitrate was determined in the same conditions as above;
- **EXPERIMENT III** The uptake of <sup>15</sup>N- ammonium was determined as in Experiment I but in samples supplemented with 2.5 μM of NH<sub>4</sub><sup>+</sup>. The more specific objective of this assay was to compare the <sup>15</sup>N -ammonium uptake in those substrates in conditions of **no inorganic nitrogen depletion**, with that observed in Experiment I (**nitrogen depletion**). Unfortunately, unforeseen difficulties in the utilisation of the Mass Spectrometer, which could have not been solved within the time allowed for this project, precluded the analysis of the samples for POC, PON, <sup>13</sup>C and <sup>15</sup>N atomic composition. Results regarding all other measured parameters are reported.

The initial organic additions were identical to those performed in 1995. The inorganic nitrogen added was modified to  $0.025\mu$ M of <sup>15</sup>N-nitrate and <sup>15</sup>N- ammonium, except in Experiment III where  $0.25\mu$ M of <sup>15</sup>N- ammonium were used. The experimental conditions at the start of the incubations are summarised in Table 5.3.

Laboratory Experiments

Chapter V

Table 5.3. Chemical experimental conditions at the start of the 1996 Experiments.

	Minimum <sup>15</sup> NH4 ( <sup>15</sup> N- atom%)	45	ı	6	45	ı	6	45	ı	6
	Minimum <sup>15</sup> NO <sub>3</sub> ( <sup>15</sup> N- atom%)	ı	49.5	ı	ĭ	49.5	ı	ł	49.5	•
litions	14NH4	ı	ı	2.5	•	,	2.5	•	ı	2.5
nic N Add (µM)	<sup>15</sup> NH4	0.025	•	0.25	0.025	•	0.25	0.025	·	0.25
Inorga	<sup>15</sup> NO <sub>3</sub>	ı	0.025		,	0.025	·		0.025	
	Substrate C:N ratio	20	20	20	7	7	7	ı	ı	ı
	% of <sup>13</sup> C	10	10	10	50	50	50	·	ı	ı
lditions )	1 <sup>3</sup> C added (µM)	1.5	1.5	1.5	7.5	7.5	7.5	ı	ı	ı
	Glucose	2.25	2.25	2.25	•	•	•	•	۰	ı
Organic A (µN)	Glycine	0.75	0.75	0.75	7.5	7.5	7.5	•	•	•
	Experiment	I	П	Ш	Ι	п	Ш	ບັ	Сп	CB
	Date	17/05/96	22/05/96	29/05/96	17/05/96	22/05/96	29/05/96	17/05/96	22/05/96	29/05/96

 $C_{\rm b}$   $C_{\rm II}$  and  $C_{\rm III}$  are the "control" incubations for Experiments I, II and III, respectively.

214

### **5.3. RESULTS AND DISCUSSION**

Due to the repetitive and factorial character of the Experiments carried out, and of the data obtained, the strategy adopted to report the results was to refer mostly to those observations which have shown to be relevant to the discussion of the proposed Hypotheses and to examine them in the light of the available literature. Although at times not all results are considered, anomalies are noted and commented. This strategy is prefered to the systematic and exhaustive description of every observation regarding the temporal variation of the various variables investigated, which would result in a protracted text and would not provide any better grounds for the discussion of the theoretical models under consideration. The discussion of the results is done in parallel with their presentation, relating to the headings of Table 5.1, where the metabolic and/or ecological consequences of the Hypotheses are described. Using this approach, a more focused discussion of the data was achieved.

### 5.3.1. Mineralisation of Organic Carbon

### **Expected Results According to the Two Alternative Hypotheses**

According to Hypothesis I no change in the extent of mineralization of organic carbon is expected in response to varying C:N ratios (high, low and "balanced"). By contrast, Hypothesis II predicts an increase in C mineralisation in high C:N ratio substrates. Carbon mineralization could not be directly tested in the experiments, as no  $CO_2$  measurements were made, but an attempt can be made to infer it from "C conservation yields" as a basis to test the two hypotheses.

The C conservation yields (%) were calculated according to the following expression:

$$Y = \frac{{}^{13}C- \text{ glycine assimilated } (\mu M) \times 100}{\text{Total} {}^{13}C- \text{glycine added } (\mu M)}$$
5.3

where

<sup>13</sup>C- glycine assimilated (
$$\mu$$
M) = [13C (atom%)<sub>tx</sub> - 13C (atom%)<sub>t0</sub>] POC<sub>tx</sub>, 5.4

 $^{13}$ C (atom%)<sub>tx</sub> and  $^{13}$ C (atom%)<sub>t0</sub> are the  $^{13}$ C atomic composition of the POC at a certain time (tx) of the incubations and at the start of the experiments (t0) prior to the addition of the labelled material and POC<sub>tx</sub> is the concentration of particulate organic matter ( $\mu$ M) at a certain time (tx) of the incubation.

#### **Observations**

Figures 5.2 a) to e) illustrate the temporal variation of the percentage of <sup>13</sup>C-glycine assimilated into the "bacterial" biomass in relation to that added, in substrates of high, low and "balanced" C:N ratio.

The plotted curves show a greater glycine accumulation in the fractionated particulate matter at high C:N ratios than at low, with the lag period before maximum carbon accumulation in the particulate material (24-48 hr) generally shorter in the former case than that in the latter ( $\geq$  48 hours). The temporal profile observed in the "balanced" C:N ratio substrate was closer to that at high C:N ratio (1995), with a lag interval between 24 and 48 hours and with similar "C conservation yields" attained; this suggests that the C:N organic additions made in this experiment may not have been as "balanced" as intended and that the initial assumptions on the bacterial C:N ratio and growth yield, to determine the C:N ratio of a "balanced" substrate may not be appropriate to natural bacterial populations.

This decrease in the lag period in substrates containing a C rich compound ("balanced" and high C:N ratio cases) in addition to just amino acids (low C:N ratio) was also observed by Hollibaugh (1978) who report a significant decrease in the period taken from the addition of the substrate until half the N regenerated is released, when compounds such as D-glucose, citrate and succinate were added to L- arginine incubations. The lag period decreased from approximatly 40 hours (L- arginine only) to c. 20 hours in amino acid substrates amended with glucose. These time scales are in agreement with those obtained in the 1995 and 1996 Experiments (Figures 5.2), although in the present case the lag period





incubation time (hrs)

Figure 5.2. Variation with the incubation time of the net uptake of  ${}^{13}$ C by the "bacterial" fraction in the 1995 (a to c) and 1996 (d & e) laboratory experiments. "H", "L" and "balanced C:N" stand for substrates of high, low and "balanced" C:N ratios, respectively. Bars represent standard errors.



Figure 5.2. Continuation

was defined to be the time interval before maximum amino acid-C was detected in the particulate matter.

According to Hollibaugh (1978), the lag period before the rapid increase in assimilation of organic substrate is not due to

"increases in cell numbers as a result of the growth of either the whole population or a subset of the population but rather to enzyme induction in pre-existing cells or activation from a resting state".

Results from the present work support this view since no consistent increase in the bacterial abundance could be detected during the time course<sup>5.1</sup> (Figures 5.3). An initial multiplication could be observed in all the incubations, but, except in the case of 1996-Experiment III where there was an increase in the cell numbers with time, particularly at low C:N ratios, in all other cases bacterial growth was not sustained. The bacterial abundance either fluctuated throughout the incubation period (*e.g.* 1995-Experiment I; 1996-Experiment I) or decreased (1996-Experiment II) after the initial growth. Due to the large uncertainty associated with the bacteria counts, some of these temporal fluctuations are likely to be due to experimental errors and not too much emphasis should be placed on them. These considerations apply to Experiments I ( $^{15}$ N-ammonium added) and II ( $^{15}$ N-nitrate added) in both years, showing identical uptake and metabolism of C independently from the available forms of inorganic N. It is also apparent from the Figures 5.2. that the heterotrophic C uptake was not influenced by the photoperiod as no specific pattern associated with the light/dark cycle could be distinguished.

Two possible explanations for the observations of an apparent higher net uptake of glycine at high C:N ratios than at low are that: i) less glycine was taken in the absence of glucose; ii) a comparable amount was taken up at the two C:N ratios but there was less mineralization of C in the presence of glucose. Lacking data on the quantity of glycine remaining and that of  ${}^{13}CO_2$  produced, does not allow the unequivocal determination of which of the two alternatives is correct. The time courses which characteristically reached either a plateau or a maximum do not favour the former explanation, the second appearing to be more probable. However, this interpretation is at first sight not consistent with either of the two proposed strategies and is diametrically opposed to the predictions of

<sup>&</sup>lt;sup>5.1</sup> - No bacteria abundance estimated are available for the 1995-Experiment II.



Figure 5.3 a) to d) Variation of the bacterial abundance in high and low C:N ratio substrates during a 72 hour incubation.

Hypothesis II. But before rejecting the two Hypotheses, it needs to be taken into consideration that nothing was known of the fate of glucose since, for reasons of imposed economy, <sup>13</sup>C-labelled glucose was not used. When an organic substrate is taken up, it can be used for biomass synthesis and/or as a source of energy; amino acids can serve as multiple C, N and energy sources (Brown *et al.*, 1972). Therefore, glycine could be used directly in bacterial protein synthesis (Kirchman & Hodson, 1984) whereas glucose would be preferentially utilised for energy purposes. It is, therefore, possible to envisage that at high C:N ratios, created in the experiment by the addition of glucose, the bacteria used this compound preferentially as a source of energy, saving glycine for biosynthesis, thus a high glycine yield would be observed in this case. In the low C:N situation, where there is an absence of added C-rich substrates at comparable concentrations, glycine was used for both energetic requirements and biosynthesis, hence the encountered reduced yields. The use of amino acids as a source of energy for maintenance has been observed by Goldman & Dennet (1991).

The above explanation is also supported by the particulate organic carbon data (some examples illustrated in Figures 5.4 a to c), where a general tendency for higher concentrations at low C:N ratio than at high could be verified rather consistently in the two sets of Experiments (1995 and 1996)<sup>5.2</sup>. This is in accordance with a more extensive C respiration in the latter case than in the former. Thus, it appears possible to reconcile the present observations with Hypothesis II.

The work of Goldman *et al.*, (1987) support the above interpretation and conclusions by showing higher C growth efficiencies when a mixture of glucose and amino acids (A) was provided rather than when individual amino acids (B) were used as substrates of varying C:N ratio (1.5-10). For example, for the same C:N ratio substrate of 3, in case (A) the growth efficiency fell between 80 to 90% whereas in (B) the growth efficiency was between 50 and 60%. This appears to be in agreement with the explanation given above for the 1995 and 1996 experiments, that when a combination of amino acids and glucose (as in A) is given to the bacteria, they will use the amino acids directly for biomass synthesis leaving glucose to serve energetic purposes, whereas when only amino acids are available (as in B) they have to fulfil biosynthetic and energetic purposes; hence the higher C yields

<sup>&</sup>lt;sup>5.2</sup> In 1995-Experiment I (data not plotted) this pattern was not followed with the POC concentrations throughout the incubation being identical in both substrates.



Figure 5.4. a) to c) Temporal variation of the fractionated and unfractionated particulate organic carbon in 1995 and 1996 Experiments. High and Low refer to the C:N ratio of the added organic substrates. WC- Whole Community. Dark periods of the incubation. "Bacterial" refer to the  $<2\mu$ m size fraction in the 1995 experiments and to the  $<0.8\mu$ m size fraction in the 1996 experiments. Bars represent standard errors.
in the former than in the latter case, observed by Goldman et al., (1987). Moreover, in Goldman's et al., experiments, the percentage of total ammonium regenerated was lower in the combined (61%) than in the single substrate (81%), suggesting a more extensive catabolism of the amino acids in the latter case, which seems to support our interpretation of the results. With increasing the substrate C:N ratio to 10, those authors have observed that the carbon growth efficiency gap between the two treatments decreased, with C yields around 40%, but still slightly higher in the case of substrate (A) than in (B). The total  $NH_4^+$ regenerated in these circumstances was also identical in both cases: approximately 31%. Goldman et al., (1987) results show, therefore, that C conservation increases in proportion to the degree of C limitation and that a stoichiometric quantity of N is utilised under such circumstances. These results were later confirmed by Goldman & Dennet (1991) and Caron et al., (1988) who found high C assimilation efficiencies (>50%) and low N assimilation efficiencies in cultures amended with glycine, with low C assimilation efficiencies (<30%) and high N assimilation efficiencies (100%) in those cultures to which glucose had been added. These results also agree with the predictions of Hopkinson's et al., (1989) model and experimental observations of increased C mineralisation with increasing the C:N ratio of the added bacterial substrate (accompanied by a decrease in the percentage of N mineralised). These reports are in close agreement with the proposed Hypothesis II as far as the C metabolism is concerned. The N metabolism will be addressed in the following section.

The growth efficiencies at the lowest C:N ratios reported by Goldman *et al.*, (1987) are similar to those found by others such as Williams (1970), Williams & Yentsch (1976) and Carlucci *et al.*, (1984) but they are much higher than those reported by Hollibaugh (1978) and Bjornsen & Kuparinen (1991) (with most estimates between 30 and 40%) and than those found in the present work (maximum of 5-10%; Figures 5.2). The results from Hollibaugh's experiments (1978) also contrast with those from Goldman's *et al.*, (1987) by showing lowest C conservation percentages associated with the uptake of arginine (C:N=1.5) in relation to other substrates of higher C:N ratio such as glutamamte and lysine, which is also opposed the findings of the present work.

High C growth yields (>40%) such as those obtained by Williams (1970), Williams & Yentsch (1976), Carlucci et al., (1984) and Goldman et al., (1987) among others, are

however, thought to constitute overestimates since those authors have used radiolabelled substrates such as amino acids and monosaccharides which are taken up with low energetic cost and can directly enter anabolic pathways. Lower values of growth yields (for instance between 21 and 45% as reported by Middelboe *et al.*, 1992; between 11 and 27% obtained by Bjornsen, 1986 and a mean of 25% found by Bell & Kuparinen, 1984), have been obtained from batch experiments on natural DOC substrates usually involving longer incubation times compared to the radiotracer technique, as shown by Bjornsen (1986) with a compilation of data from published literature. So, it appears that the results obtained with the radiotracer techniques may be measuring something more like *uptake efficiency* than true growth yields, more realistically obtained with longer incubation times (days) using natural substrates (Bjornsen, 1986). As it can be seen from the above, the reports on bacterioplankton growth yield is characterised by controversy mainly because of the use of different substrates, methods to estimate bacterial growth, experimental design and incubation times, resulting in the estimation of different processes.

The C conservation yields calculated for the 1995 & 1996 experiments cannot be considered true C growth yields as nothing is known of the amount of C respired; they are rather an expression of the net utilisation of the added substrate (Glycine). However, the C conservation values obtained at low C:N compare well with the very low growth yields reported by Kirchman *et al.*, (1991) (<10%). Possible explanations for these low values could be that the microbial assemblage sampled contained very low numbers of individual bacteria species that possessed the enzyme systems necessary for transport or metabolism of glycine. Goldman *et al.*, (1987) observed that the affinity of bacteria for this amino acid was lower than to other amino acids, by showing what they called "sluggish growth" in the presence of this substrate (1.6-9%). However, other authors such as Williams *et al.*, (1976) and Keller *et al.*, (1982) found no evidence that this amino acid was a poor substrate for bacteria growth.

Another explanation that could explain the low retention of amino acid-C in the particulate matter in the present experiments, is that this compound was also being used as an energy source (as suggested before) in the low C:N ratio situations. If so, extensive release of ammonium would be expected in these incubations. In fact, in the 1996-Experiments<sup>5.3</sup>,

<sup>&</sup>lt;sup>5.3</sup> No inorganic nitrogen determinations were performed during the 1995 experimental work.

production of ammonium was detected at low C:N ratios (but not at high) which supports this interpretation, as will be shown and more thoroughly discussed in section 5.3.2.

It could also be the case that the calculated net C assimilation into the particulate matter was underestimated due to the simultaneous uptake of unlabelled forms of natural organic compounds, that would result in the dilution of the <sup>13</sup>C-label assimilated into the particulate matter. Contrary to many other experiments where the bacterial fraction was isolated from the whole sample and then tested (for instance, in Goldman *et al.*, 1987; Caron *et al.*, 1988; Kirchman *et al.*, 1990; Kuparinen & Heinanen, 1993) in the study under discussion, the whole sample was experimented upon and the bacterial fractions isolated at the end of certain incubations times. This procedure, more faithful in terms of the true conditions in the marine environment where different communities co-exist, meant that during the incubation period, different sources of unlabelled C could have been made available, by phytoplanktonic excretion and/or by the grazers "sloppy feeding", and utilised by the bacteria, particularly in those cases where C was deficient in the added substrate, leading to the dilution of the <sup>13</sup>C taken up and consequently to apparently low C retention percentages.

In conclusion, data from the experimental work undertaken and from reports in the literature on the uptake and metabolism of DOC, appear to support the view that an increase in the C:N ratio of the available substrate results in lower C conservation in the microbial particulate matter, in accordance with the proposed Hypothesis  $\Pi$ .

## 5.3.2. Bacteria as Users of Inorganic Nitrogen

#### **Expected Results According to the Two Alternative Hypotheses**

Hypothesis II (Figure 5.1b) predicts no utilisation of nitrate or ammonium by the "bacterial" size fractions at any substrate C:N ratio whereas Hypothesis I (Figure 5.1a), leads to the expectation of increased inorganic nitrogen utilisation at high C:N ratio substrates but not at low. Consequently, a higher <sup>15</sup>N uptake and its conservation in the fractionated particulate matter in the former than in the latter case would be anticipated.

#### **Results and Discussion**

Figures 5.5 a) to e) show the temporal variation of <sup>15</sup>N assimilated into the unfractionated and "bacterial" particulate matter in the presence of organic substrates of high, low and "balanced" C:N ratios. This <sup>15</sup>N assimilated into the particulate organic nitrogen was calculated according to the following expression:

$$\Delta \text{ PO}^{15}\text{N}(\mu\text{mol dm}^{-3}) = \frac{(^{15}\text{N}\text{-}\text{atom}\%_{tx} - ^{15}\text{N}\text{-}\text{atom}\%_{t0}) \times \text{PON}_{tx}}{100} 5.5$$

where  $\Delta PO^{15}N$  is the variation of <sup>15</sup>N-particulate organic nitrogen; PON<sub>tx</sub> is the PON ( $\mu M$ ) at a time (t<sub>x</sub>) of the incubations; <sup>15</sup>N-atom% t<sub>x</sub> and <sup>15</sup>N-atom% t<sub>0</sub> are the <sup>15</sup>N atomic composition of the particulate matter at a time (t<sub>x</sub>) of the incubation and at the start of the experiment (t<sub>0</sub>), prior to the addition of the labelled material, respectively.

It is noteworthy at this point to recall that the term "bacterial" stands for the  $<2\mu$ m size fraction in 1995 and for the  $<0.8\mu$ m in the 1996 Experiments. Whereas in the latter case the utilisation of inorganic nitrogen could be considered to be solely by the microheterotrophs (as the cholorophyll content of this size fraction was  $\leq 0.7\%$  of the unfractionated samples), the same cannot be said about the N uptake by the  $<2\mu$ m fraction, as picoplanktonic chlorophyll constituted between 7.8 and 35.7% of the whole community's at the start of the experiments. This difficulty in isolating the microheterotrophic processes which resulted in some uncertainty regarding the character of the N uptake in the 1995 Experiments was the reason to study a smaller size fraction in 1996, from which resulted a better separation between heterotrophic (bacterial) and autotrophic (phytoplanktonic) uptake.

The temporal profiles presented in Figures 5.5 were generally divided into two phases: phase I ("surge uptake" phase - 1 to 7 hours) and phase II ("regeneration" phase - remainder of the incubation). The uptake rates of total ammonium and nitrate during phase I are presented in Table 5.4 and they were calculated according to the expressions given in Chapter III.





Figure 5.5. Uptake of inorganic nitrogen by the whole community and "bacterial" fractions in substrates of high, low and "balanced" C:N ratio. Results from the 1995 [a) to c)] and 1996 [d) and e)] Experiments.



Figure 5.5. Continuation

## PHASE I

As indicated by its name, this phase was characterised by rapid uptake of both nitrate and ammonium, for a period between 1 and 6 hours. This rapid uptake which was much shorter than the time scale of maximum C uptake ( $\geq 24$  hours), at first, could be seen to be not associated with the organic addition but rather an expression of the nutrient depletion status of the populations. However, and as will be discussed below, it is possible that the fast inorganic N uptake recorded during this phase was due to both the nutrient starved condition of the planktonic community and, in some cases, to the presence of an organic substrate.

#### Nitrogen Uptake due to Nutrient Depleted Microbial Populations

Data from true "control" incubations (no organic additions performed) made in the 1996 set of Experiments confirm the N starved status of both the phytoplanktonic and bacterial populations (Figures 5.5 d and e), by showing surge uptake of both ammonium and nitrate during the initial stages of the incubations.

In 1995, "control" incubations (Experiment IV) were also carried out and the results (Figure 5.6) do not show surge but rather slow, linear <sup>15</sup>N-ammonium uptake for the first 38 hours of the incubation followed by its retention in the particulate matter, whilst nitrate did not appear to have been utilised or very little of it was incorporated. However, these cannot be considered to be true "control" experiments since, contrary to those in 1996, they were not run concurrently with the other incubations, as described in section 5.2. For instance in 1995, Experiment I and IV were separated in time by a period of a month, therefore different planktonic communities and/or different stages of growth or senescence of the same community could be under consideration. This is illustrated by the gross community oxygen production measurements, which on the occasion of Experiment I gave a result of 47.6  $\mu$ M O<sub>2</sub> d<sup>-1</sup> whereas by the time the "control" Experiment took place, rates had decreased to 3.8 µM O<sub>2</sub> d<sup>-1</sup>. Consequently, different behaviour of the planktonic community would be expect. Ideally, the "control" incubations would have been run concomitantly with the other assays as in 1996, but due to limitations in the available resources at the time, this was not feasible, constituting the main drawback of the 1995 set of Experiments.

							/		
Experiment	Duration of surge		Whole cor	mmunity			'Bacte	rial"	
	uptake pnase (nour)	High	Balanced	Low	Control	High	Balanced	Low	Control
		0.0041		0.0037		0.0016		0.0016	
95 Experiment I	3.5	± 0.00015	I	± 0.00006	ı	± 0.00002	·	± 0.00005	I
)5 Experiment III	3.5	-	0.0076 ± 0.00007	) 1		I	0.0013 ± 0.00004	ı	ı
96 Experiment I	1.0	0.0394 ± 0.00401	•	0.0357 ± 0.00136	0.0352 ± 0.00160	0.0210 ± 0.00050	•	0.0158 ± 0.00095	0.0163 ± 0.00008
				Nit	rate uptake	(Jumol dm <sup>-3</sup> h	ır <sup>-1</sup> )		
		0.0068		0.0070		0.0023		0.0020	
95 Experiment II	3.5	± 0.00023	•	± 0.00008	ı	± 0.00013	·	± 0.00013	ı
95 Experiment III	3.5	I	0.0038 ± 0.00020	·		·	bdl		ı
96 Experiment II	6.0	0.0064 ±	1	0.0062 ±	0.0062 ± 0.00001	0.0008 + 10000	ı	0.0006 ± 0.0000	0.00056 ± 0.00000

Laboratory Experiments

Chapter V

224



**Figure 5.6.** Uptake of ammonium and nitrate by the whole community (wc) and the  $<2\mu$ m fraction in 1995 "control" Experiment. Temporal variation of the <sup>15</sup>N-atom% in the organic particulate nitrogen. - Dark periods of the incubation. Bars represent standard errors.

The non-utilisation of nitrate in the 1995- "control" Experiments could be due to the fact that at this time of the seasonal cycle, bacteria were predominant in the planktonic community (see CHAPTER IV) and these organisms prefer more reduced forms of nitrogen (Painter, 1970). At the time of this assay, ambient ammonium had risen from below detection levels to 0.094  $\mu$ M, being therefore more abundant than nitrate (not detectable) and more readily available. Consequently, a preferential use of the former than the latter would be expected. Furthermore, these results are in agreement with Kirchman's (1994) view that the heterotrophic NO<sub>3</sub><sup>-</sup> uptake is mostly associated with the utilisation of organic carbon when there are no other sources of inorganic nitrogen available or when the concentrations of this nutrient largely exceed those of the other inorganic nitrogenous nutrients. In the present experiment, the non-utilisation of nitrate could, therefore, be interpreted as being associated with the absence of an organic substrate. Any bacterial inorganic N requirements arising from the uptake of organic compounds naturally present in the sea water sample could have been met through the uptake of ambient (and added) ammonium.

#### Nitrogen Uptake Due to High C:N Ratio Substrate

Even though the planktonic community appeared to be N starved as shown above, this alone cannot be responsible for the observed surge uptake of ammonium and nitrate as the C:N composition of the organic substrate seemed, in some cases, (1995-Experiment II; 1996-Experiment I and II), to have had a significant effect on the uptake of inorganic N from which resulted generally lower uptake rates at low C:N ratios than at high, as shown in Table 5.4. Thus, it needs to be explored whether during the short period when the bulk of N uptake took place (phase I), there was sufficient C assimilation at high C:N ratio to give rise to the observed extra N assimilated, in relation to that observed at low C:N. Since, we lack the essential piece of information- the rate of uptake of glucose- all that can be done is to undertake a speculative calculation in order to anticipate the N demanded from such C uptake. Because glucose may be expected to be a preferred energy source, it may be anticipated that it would be taken up as fast as, if not faster than, glycine. The molar ratio of the added glucose:glycine mixture in the high C:N substrate was of 3:1, resulting in a carbon ratio of 9:1. Accepting the above, the projected minimum glucose-C uptake rates would be at least 9 times greater than those of Glycine-<sup>13</sup>C. Table 5.5 shows the projected glucose uptake rates and the calculated demand for inorganic nitrogen, assuming a bacterial growth yield of 40% and C:N ratio of 4.

Exper.	Duration of phase I (hr)	<sup>13</sup> C- Glycine Taken up (μmol dm <sup>-3</sup> x1000)	Projected glucose uptake (µmol dm <sup>-3</sup> x1000)	Calculated N-demand (µmol dm <sup>-3</sup> x1000)	N taken up (µmol dm <sup>-3</sup> x1000)	N available (µmol dm <sup>-3</sup> x1000)
1995- I	3.5	41.2	370.8	37.1	5.6	30 <sup>a</sup> +20 <sup>b</sup>
1995-II	3.5	47.4	426.6	42.7	8.0	$25^{a} + 10^{b}$
1996- I	1	18.3	164.7	16.5	21	30°+25°
1996 <b>-</b> ∏	6	80.6	725.4	72.5	4.8	25°+25°

**Table 5.5.** "Bacterial" uptake of organic C in high C:N ratio substrates and corresponding inorganic N demand, actual N availability and utilisation.

Exper. - Experiments (I and II) carried out in 1995 and 1996; The available N was the ambient concentrations assumed to be at the lower limit of detection (a) plus that added (b).

Given the assumptions made, the calculations demonstrate from the relatively high N demand that there could have been sufficient glucose uptake to allow for the effect of the C:N ratio to be seen as a difference in the rate of inorganic N uptake, (observed in both nitrate and ammonium incubations), which is in accordance with the predictions of Hypothesis I.

An increased utilisation of ammonium in the presence of high C:N ratio substrates such as glucose, in relation to "control" or low C:N ratio substrates such as glycine, as in the present experiments, has been reported by others, using a range of different methods: for instances, Keil & Kirchman (1991) report a higher rate (10 to 500% more) of ammonium utilisation in incubations with added glucose in relation to "controls", with no net N regeneration detected in the former case. In 1989, Kirchman et al., have also observed significant inhibition of ammonium uptake in bacterial populations of the Subarctic Pacific by adding free amino acids to the growth medium. Later, Kirchman et al., (1990) observed that ammonium uptake by the <0.8µm size fraction increased almost four fold in the presence of a substrate containing amino acids and glucose. In substrates with amino acids only, there was an initial uptake of ammonium (although less than in the former case) but ammonium regeneration predominated. The results of Goldman & Dennet (1991) also agree with the observations of Kirchman and co-workers and with those regarding the current work, by showing that ammonium uptake increased with the C:N ratio of the added organic substrate: when ammonium was the sole nitrogen source, it was used stoichiometrically with glucose, varying from ca. 50% usage when the C:N ratio of the substrate was 3.6, up to 100% when the C:N ratio was 10. Furthermore, a very tight coupling between ammonium uptake and the availability of a suitable C substrate, even in the presence of amino acids was observed; even though amino acid catabolism and concomitant NH4<sup>+</sup> regeneration occurred for all the amino acids studied by those authors, NH4<sup>+</sup> "net regeneration" only occurred until after the glucose had been exhausted.

The work of Heinanen & Kuparinen (1992) and Kuparinen & Heinanen (1993) is also of relevance to the present discussion: by studying natural microbial populations at different stages of the seasonal primary production cycle, these authors have observed that the addition of C (sucrose) only enhanced bacterial growth in pre-bloom conditions, when there was ample availability of inorganic nitrogen. During the late summer period the addition of sucrose reduced the rate of incorporation of tritiated thymidine which was attributed to the exhaustion of inorganic nitrogenous nutrients needed to balance the excess of C. These experiments also demonstrated that the utilisation of the refractory DOM pool can be stimulated by inorganic nutrient additions, therefore, these authors concluded that correct C:N ratios are needed for the metabolism of such high C:N ratio substrates to occur.

Caron *et al.*, (1988) observed that in bacterial cultures enriched with glycine there was not a pronounced increased in the PON and ammonium was produced whereas in cultures with glucose and ammonium there was uptake of ammonium accompanied by production of PON. Also, high C assimilation efficiency (>50%) and low N assimilation efficiency (<20%) were observed for glycine additions, contrasting with low C assimilation efficiency (<30%) and high N assimilation efficiency (100%) for the glucose additions. The work of Cherrier *et al.*, (1996) relates closely to Caron's *et al.*, (1988) by showing stimulation of ammonium utilisation in glucose and ammonium amended cultures with natural populations.

Therefore, it appears that there are sufficient evidence in the literature consistent with the model of C and N mass balance described by the proposed Hypothesis I, by showing the stimulation of ammonium uptake by a high C:N ratio substrates and its inhibition in the opposite circumstances. Most of the work has been performed using ammonium as the source of inorganic N, but the results expressed in this section regarding the 1995 and 1996 Experiments revealed that bacteria will also take up nitrate (although at lower rates) when no other sources of N are available, despite the higher energetic cost to the cell. To the best of my knowledge, no other experimental work has been carried out where this was directly demonstrated although Kirchman *et al.*, (1991) report bacterial uptake of nitrate concomitant with a decrease in the ambient DOC.

In relation to the results presented in Table 5.5 and whether or not it is considered that the available inorganic N was solely provenient from the <sup>15</sup>N additions or that there was some ambient N at the lower detection limit levels, it could be seen that in 50% or less of the cases studied, the N demand caused by the metabolism of organic C could not have been met. In reality, the calculated rates of total uptake of nitrate and ammonium show that only in one case (1996-Experiment I) the N taken up met the calculated demand. In all other cases, the uptake of N was not only apparently much lower than that theoretically required but also it constituted a small proportion of that assumed available (9.6-38%) which is unexpected given that the microbial populations appeared to be N depleted. There appears to be a discrepancy between added inorganic N and its non- recovery in the particulate matter which has also been addressed in the literature (*p.e.* Glibert *et al.*, 1982c; Laws, 1984; Ward *et al.*, 1989). It has been investigated in particular by Bronk & Glibert (Bronk & Glibert, 1991; Bronk *et al.*, 1994; Bronk & Glibert, 1994) and referred to as "the mystery

of the vanishing <sup>15</sup>N", since the common observation is that in <sup>15</sup>N experiments not all the <sup>15</sup>N-label added at the start of the incubations is accounted for at the end. The potential pathways which have been recognised to lead to the loss of <sup>15</sup>N are nitrification (Glibert *et al.*, 1982c); loss of small cells through the GF/F filters used to retain particulate matter for further isotopic analysis and cellular break up during size fractionation due to filtration stress (Laws, 1984; Ward *et al.*, 1989); and finally, phytoplanktonic cellular release of DO<sup>15</sup>N, which may constitute between 25 to 41% of the total DIN taken up (Bronk & Glibert, 1994; Bronk *et al.*, 1994).

In Table 5.6, the percentage of <sup>15</sup>N-nitrogen recovered in the particulate matter at the end of phase I, in relation to that added is shown for the 1995 and 1996 Experiments. From the data presented it can be seen that the <sup>15</sup>N-nitrogen recovered in the particulate matter varied widely from percentages as low as 6.6% to a highest of 73%, taking into account both fractions studied, but in the whole community, percentages of 50-70% were more frequently found whereas in the "bacterial" percentages were generally lower than 30%. This percent recovery was, in the majority of the cases higher for the incubations involving the uptake of nitrate than ammonium, reflecting, perhaps the slower turnover rates of the less reduced form of N.

The lowest values were, however, associated with the uptake of nitrate by the "bacterial" fraction during 1996-Experiment II, possibly as a consequence of the lower affinity of these organisms for this compound (Probyn & Painting, 1985; Wheeler & Kirchman, 1986). An identical trend was observed in 1995-Experiment III, when the same microbial population was incubated with <sup>15</sup>N-ammonium and -nitrate, given on separate incubations. A retention of 27% of <sup>15</sup>N in the particulate matter in the former case in relation to only 7% in the latter was observed. In this particular case, the results agree with Bronk & Glibert's (1994) findings that when ammonium was the substrate, a larger percentage of the label appeared in the bacterial pool, then when nitrate was used. Furthermore, the same authors (1991) have also reported that only 6% of the nitrate added was transformed into particulate organic nitrogen during a 6 hour incubation which is in accordance with some of the data presented here. However, Bronk & Glibert (1991) found an ammonium-N recovery in the bacterial biomass of 80% which, in the present case amounted to a maximum of 50% (1995-"control" incubations).

		Whole com	munity			"bacterial"	fraction	
Exper.	high	"balanced"	low	control	high	"balanced"	low	control
1995 <b>-</b>	28.3		26.0		1 <b>4.8</b>		14.8	
Exp I*	±	-	±	-	±	-	±	-
-	1.05		0.20		0.23		0.30	
1995 -	67.2		68.9		32.9		33.9	
Exp II**	±	-	±	-	±	-	±	-
-	2.30		0.85		5.72		1.47	
1995 -		52.8±0.51*				27.1±0.17*		
Exp III	-		-	-	-		-	-
* **		37.8±1.98**				6. <del>9±</del> 0.38**		
1995 -				69.1				50.3
Exp IV*	-	-	-	±	-	-	-	±
				0.96				0.26
1996 -	70.9		64.3	63.2 ·	38.3		28.5	29.2
Exp I*	±	-	±	±	±	-	±	±
	7.21		2.45	2.80	1.27		1.71	0.20
1996 -	73.2		73.1	73.0	9.8		9.1	6.6
Exp II**	±	-	±	±	±	-	±	±
-	0.97		1.52	0.20	0.18		2.35	0.20

**Table 5.6.** Percentage of <sup>15</sup>N recovered in the PON at the end of the rapid uptake phase, in relation to that added (mean  $\pm$  se).

\*- <sup>15</sup>N- ammonium incubations; \*\*-<sup>15</sup>N- nitrate incubations; High, Balanced and Low stand for incubations in substrates of high, "balanced" and low C:N ratio respectively, and C for control incubations. Exper. refer to the 1995 and 1996 laboratory experiments.

In the experiments under consideration, it is possible that, as suggested by Laws (1984) and Ward *et al.*, (1989), there were some losses associated with sample processing, although every effort had been made to prevent artifactual release of labelled material from the cells, by using low filtration pressures (<125mmHg) and gentle size fractionation. However, the loss of <sup>15</sup>N through phytoplankton release of DO<sup>15</sup>N has to be one of the strongest possibilities to consider as the fate of the <sup>15</sup>N- nitrogen taken up, as stressed by Bronk & Glibert (1994). These authors report dramatically increased DO<sup>15</sup>N release in incubations longer than 0.5-1 hour, especially in oligotrophic environments. A different proportion of the added <sup>15</sup>N-label could be found in the DON pool and this varied with the trophic status of the environment, the substrate used, the length of the incubation and the period of time a sample was contained before the <sup>15</sup>N uptake incubations (Bronk & Glibert, 1991, 1993a,b; 1994).

In the light of these findings, it can be seen that the incubation conditions used in the present experimental work favoured cellular DON release by being rather long thus allowing prolonged inorganic N uptake and metabolism; the planktonic community was also nutrient stressed although the Menai Strait waters are not considered to be oligotrophic. However, as shown in Table 5.7, the percent retention of <sup>15</sup>N in the particulate matter after only one hour of incubation compare well with values reported by Bronk & Glibert (1994) for oligotrophic environments with samples incubated for the same period of time, where 13-27% of the <sup>15</sup>N was found in the unfractionated particulate matter; 6-10% in the DON plus bacteria pool and 29-55% in the bacteria pool alone.

**Table 5.7.** Percent recovery of <sup>15</sup>N in the particulate matter in relation to that added, after one hour incubation (mean  $\pm$  se).

		Whole com	munity		"bacterial" fraction			
Exper.	high	"balanced"	low	control	high	"balanced"	low	control
1995 -	14.6		11.7		7.4		6.4	
Exp I*	±	-	±	-	±	-	±	-
•	0.16		0.65		0.19		0.08	
1995 -	48.0		51.8		21.6		26.6	
Exp II**	±	-	±	-	±	-	±	-
•	2.24		5.68		3.28		1.12	
1995 -		23.0±1.97*				14.3±0.31*		
Exp III	-		-	-	-		-	-
*,**		4.5±0.76**				1.0±0.02**		
1996 -	70.9		64.3	63.2	38.3		28.5	29.4
Exp I*	±	-	±	±	±	-	±	Ŧ
-	7.21		2.45	2.80	1.27		1.71	0.20
1996 -	34.5		35.1	29.2	5.4		2.8	3.4
Exp II**	±	-	±	±	±	-	±	±
•	10.17		7.50	5.60	0.35		0.04	0.20

\*- <sup>15</sup>N- ammonium incubations; \*\*-<sup>15</sup>N- nitrate incubations; High, Balanced and Low stand for incubations in substrates of high, "balanced" and low C:N ratio respectively, and C for control incubations. Exper. refer to the 1995 and 1996 laboratory experiments.

#### Nitrogen Uptake in a "Balanced" C:N Ratio Substrate and in the 1996 -"Control" Incubations in Relation to that at Low and High C:N Ratios.

From the calculated total uptake of ammonium and nitrate (Table 5.4), it was shown that in the "balanced" C:N ratio substrate, the "bacterial" uptake rates of ammonium were among the lowest measured whereas virtually no uptake of nitrate could be detected. This is perhaps not surprising as theoretically bacteria were provided with C and N in the "right" proportions in the added organic substrate, not having the need to utilise inorganic N from other sources.

Moreover, it could be appreciated that the uptake of inorganic N in the 1996-"control" incubations<sup>5.4</sup> was closer to that measured at low than at high C:N ratios. This could indicate that the quality of the naturally occurring DOM was more akin to that of the manipulated low C:N ratio substrate than that of the high, *i.e.*, at this time of the seasonal cycle, compounds such as amino acids, peptides, proteins and perhaps nucleic acids (low C:N ratio) might predominated over those rich in C such as glucose and other carbohydrates. Little is known of the composition and seasonal variation of these compounds in the waters of the Menai Strait, as described in CHAPTER II, but the above suggestion is supported by Al-Hasan et al., (1975) results indicating that phytoplanktonreleased products such as glycollate (high C:N ratio) were at minimum concentrations during May, the month when the 1996 experiments were carried out. On the other hand, these experiments coincided with the early stages of the annual Phaeocystis dominated spring bloom, when phytoplankton exsudates are thought to be of less significance than at the later stages when large amounts of carbohydrates (>60% of the combined fraction being glucose) are released by cells under stress induced by nutrient depletion and from lysing cells and phytoplanktonic debris (Ittekkot et al., 1981). Although no data is available on the levels of amino acids in the water body under consideration, it is possible that these compounds could be fairly abundant since extensive beds of the mussel Mytilus edulis are located along the Strait, close to the sampling area, and these organisms have been shown to release large amounts of these compounds (Bayne & Scullard, 1977). For instances, Tupas & Koike (1990) have found that 69-99% of the total N released by mussels was in the form of amino acids, confirming the importance of these organisms as sources of organic nitrogenous nutrients in shallow coastal waters.

If it is accepted that amino acids were available in the waters under study, then, a concomitant uptake of organic and inorganic nitrogen was occurring. This has been observed by many researchers, namely Hagstrom *et al.*, (1984) and Wheeler & Kirchman (1986) who concluded that this co-uptake was due to the fact that the availability of low C:N ratio substrates is often not sufficient to fulfill bacteria N requirements. On the other hand, Tupas & Koike (1990), reported ammonium and amino acids uptake simultaneous with ammonium regeneration, contradicting the idea that ammonium utilisation only occurs

<sup>&</sup>lt;sup>5.4</sup> No comparisons are established between the "control" incubations and the others in 1995 for the reasons given before.

when organic N is limiting. Clearly, the relationships between DON and ammonium uptake and ammonium excretion are complex and far from totally understood as pointed out by Kirchman *et al.*, (1989) who concluded that heterotrophic bacteria preferred amino acids over ammonium and that the relative contribution of amino acids to the nitrogen required for bacterial growth controlled whether bacteria assimilated or excreted ammonium. When amino acids contributed with less that 80% of the N demand, the heterotrophic bacteria began to assimilate ammonium. When this contribution was greater than 80%, ammonium was regenerated.

## PHASE II

If the uptake rates of total N given in Table 5.4 are accepted, then, in most cases the inorganic nitrogen assumed available would have been consumed in its totality during PHASE I. Therefore the results obtained during PHASE II probably will not shed any more light on the discussion of the two alternative Hypotheses but they will contribute with further information on the subsequent fate of the ammonium and nitrate taken up in the previous phase of the incubation. From Figures 5.5a, b, and d), it can be observed that the second phase was characterised by a general tendency for a greater N conservation at high C:N ratios than at low, an outcome predicted by both Hypotheses<sup>5.5</sup>. At high C:N ratios, the <sup>15</sup>N was either retained in the PON or, if regenerated, rapidly reassimilated. The reassimilated <sup>15</sup>N could be in the form of DO<sup>15</sup>N produced by phytoplankton (Bronk & Glibert, 1991; 1993a,b; 1994). As seen in the previous sections, the incubation conditions provided favoured algal cellular DON release, which could have been subsequently utilised by the bacteria. It could also be the case that <sup>15</sup>NH4<sup>+</sup> was released by grazers after ingestion of <sup>15</sup>N-labelled bacteria, making it available for re-uptake.

The observed isotopic dilution at low C:N ratios could be attributed to the uptake of an unlabelled form of N such as that in the added glycine, ten times more concentrated in these substrates than in those with a high C:N ratio. In section 5.3.1., it was suggested that at low C:N ratios, glycine was used for both energetic and biosynthetic purposes, therefore, part of the glycine-N could have been incorporated in the PON, resulting in the dilution of the <sup>15</sup>N taken up previously, as <sup>15</sup>N-ammonium and -nitrate. The results regarding the

<sup>&</sup>lt;sup>5.5</sup> In Figure 5.5.e), this pattern was not so evident.

incorporation of  ${}^{13}$ C appear to agree with this suggestion by showing that the times of the incubation characterised by highest  ${}^{15}$ N isotopic dilution corresponded to those times when the uptake of  ${}^{13}$ C-glycine was also largest (compare Figures 5.5 with Figure 5.2).

Conservation of N at high C:N ratios and regeneration in substrates rich in N can also be seen from the measurements of nitrate, nitrite and ammonium production, made during the course of the 1996- Experiments<sup>5.6</sup> (Figure 5.7). In itself, N mineralisation does not constitute a test for deciding between either Hypotheses (Table 5.2) being part of both; nevertheless the results from these measurements are reported here.



Figure 5.7. a) Production and b) uptake of ammonium in substrates of low and high C:N ratio.

<sup>&</sup>lt;sup>5.6</sup> Inorganic N measurements during 1995 were not made, as previously indicated.

During the 1996 - Experiments, no production of nitrate or nitrite was measurable (data not shown) in any of the incubations performed. This is similar to the results of Cherrier *et al.*, (1996) and Tezuka (1990) who report no nitrification in comparable conditions.

Regeneration of ammonium was detected in the incubations with an added organic substrates of low C:N ratio (Figure 5.7a), whilst at high C:N ratios there was no production of this nutrient; instead, any ammonium available was consumed within 24 hours or less (Figure 5.7b). This could only be seen in Experiment III (2.5  $\mu$ M of NH<sub>4</sub><sup>+</sup> added at the start of the experiment), since in all other cases concentrations remained below detection levels (data not plotted). In the control incubations where no organic additions had been made, no accumulation of ammonium was detectable.

It has been suggested before that the quality of the ambient DON resembled that of the added low C:N ratio substrate; if this was the case some  $NH_4^+$  accumulation would be expected in the control incubations. One reason why this was not observed could be associated with its rapid uptake by the phytoplankton.

These results regarding variable N regeneration according to the C:N ratio of the available organic substrate are supported by and in agreement with many studies reported in the literature. In 1984, and based on simple mass balance considerations, Billen (1984) argued that whether bacteria assimilated or regenerated ammonium depended on the C:N ratio of the substrate and of the bacteria cell, after C respiration needs had been satisfied. Based on the results of Hollibaugh (1978) and Billen (unpublished data), Lancelot & Billen (1985) concluded that net uptake instead of mineral N occurs during degradation of organic matter with a C:N ratio higher than 10. Later, Goldman et al., (1987) found that indeed ammonium regeneration by enriched bacterial assemblages did depend on the C:N composition of the DOM, as predicted. The observations of these authors support the predictions of Hopkinson et al., (1989) based on a static model of bacterial N and C flows, that the relative importance of bacteria as agents of N remineralisation increases as the C:N ratio of the substrate declines. Their model showed that at C:N ratios above 18, bacteria will potentially be net immobilisers of dissolved inorganic N, with the bacterial grazers as N remineralisers increasing in importance, as the C:N ratio of the substrate increases. This immobilisation of N in the bacterial biomass at high C:N ratios was seen in the present experiments as shown before (Figures 5.5a, b, d, e and 5.7b), as well as the N mineralisation at low C:N ratios (Figure 5.7a). The present observations are also supported by the work of Caron *et al.*, (1988) who report that bacteria were major remineralisers of N in cultures supplemented with glycine, which did not happen in cultures supplemented with glucose, where  $NH_4^+$  uptake took place.

The efficiency of conversion of the added organic substrate to ammonia by amino acid catabolism in the present experiments amounted to a mean of 30% which compares well with some of the values obtained by Hollibaugh (1978) using different amino acids; however, for cultures amended with glycine, the percentage found by this author was in excess of 100%. Goldman *et al.*, (1987) report much higher regeneration efficiencies (83%) for an identical substrate (Glycine; C:N =2). These researchers report a direct relationship between NH<sub>4</sub><sup>+</sup> regeneration and the C:N ratio of the substrate', with percentages decreasing with increasing this C:N ratio: 86% for a C:N of 1.5, down to 31% when C:N was 9. Tezuka (1990) also found regeneration of NH<sub>4</sub><sup>+</sup> for substrates of C:N ratios  $\leq 10$ , with regeneration efficiencies between 60 and 80% for substrates C:N ratio of 5 and decreasing quite rapidly for a C:N of 10 (majority of values  $\leq 20\%$ ).

A reason for the lower NH4<sup>+</sup> regeneration obtained in the 1996-Experimental work and also reported by Hollibaugh (1978) (who failed to find a relationship between substrate C:N ratio and percent regeneration) could be that the experiments under discussion and those described by Hollibaugh (1978) were performed using whole samples in contrast with the studies of the other scientists referred to above, who isolated the bacterial fraction prior to the incubations. Therefore, in the former cases the regenerated ammonium could be rapidly assimilated by other organisms such as the phytoplankton, in contrast to the latter situations, where only bacteria existed, hence the lower regeneration ratios in the former than in the latter situations.

## 5.3.3. Evidence for Competition

### **Expected Results According to the Two Alternative Hypotheses**

From Table 5.1 it can be recalled that a situation of competition between bacteria and phytoplankton would only occur at high C:N ratios, in the conditions defined by

Hypothesis I, where bacteria would assimilate inorganic N to achieve their required C:N quota. In such circumstances, it is expected that the biomass of these two populations would be affected negatively. Thus, evidence for competition was sought by looking at differences in chlorophyll concentration and bacteria abundance between substrates of high and low C:N ratios.

#### **Results and Discussion**

Figures 5.8.a) to e), illustrate the temporal variation of the chlorophyll concentration in the Experiments carried out. Despite some fluctuations during the course of the incubation, the general trend appeared to be that of decreased phytoplanktonic biomass at high C:N ratios in relation to that at low. These observations are compatible with the existence of competition in the former case, following, therefore the predictions of Hypothesis I. This evidence for competition can, however, be considered to be weak, as differences in concentrations between substrates of differing C:N ratios were not very marked. Since replicate measurements at each harvesting occasion were not feasible, it is not possible the attribution of a level of significance to those differences. All that can be said is that at the end of the incubations, phytoplanktonic biomass at high C:N ratios was consistently lower than that at low C:N ratios. It could also be observed that in a number of cases, the chlorophyll concentrations decreased during the course of the incubations in both high and low C:N ratio substrates. This could be due to natural reasons such as grazing of the phytoplankters but could also be attributed to causes resulting from the experimental conditions: for instances, bleaching of the pigments could have resulted from higher light intensity "in vitro" than "in situ", which agrees with the initial (but not sustained) adaptive decrease observed in Figures 5.8a), b) and c). Furthermore, some sedimentation of the suspended matter occurred, despite the effort made to ensure continuous, thorough mixing of the cultures. Although cultures were vigorously shaken prior to sample harvesting, the heterogeneous distribution of the algae could have given rise to some of the observed fluctuations in the chlorophyll concentrations.

In the "control" incubations made during the 1996-Experiments, chlorophyll concentrations were generally closer to those observed at high C:N ratios than at low and in Experiments I and II there was a sustained decrease throughout the incubation. Despite the absence of an organic addition in these incubations, the microbial populations were N depleted (as



Figure 5.8. Temporal variation of the unfractionated chlorophyll concentrations in high and low C:N ratio substrates and in "control" incubations carried out during the 1995 and 1996 experiments. - Dark periods of the incubation.

discussed before). Therefore, it is possible to envisage that bacteria would be competing with the phytoplankton for the little available ammonium and nitrate, thus limiting the algae growth. The results from the 1996- Experiment III support this explanation by showing that when extra inorganic N was added (as 2.5  $\mu$ M ammonium), the decrease in the chlorophyll concentrations which was observed in all other cases did not occur; in all three situations (high and low C:N ratios and "control" incubations) chlorophyll concentrations not only remained fairly constant throughout the incubation period but were similar to each other. As in the other experiments, at the end of the incubation time, the phytoplanktonic biomass was somewhat higher at low C:N ratios than at high or in the control incubations, since at low C:N no competition for inorganic N was taking place and phytoplankton were possibly using the remineralised ammonium resulting from the bacterial amino acid catabolism, as discussed before.

The results obtained for the bacterial abundance during this experiment (1996-Experiment III) are also of relevance regarding the bacteria abundance estimates, since this was the only occasion where a growth in the bacterial population could be perceived and the effect of the substrate C:N ratio was clear. As it can be seen from Figure 5.3c), whilst at low C:N ratios cell numbers increased with time, at high C:N ratios the bacterial abundance remained fairly constant after an initial but slow increase. This decreased bacterial growth can possibly be a consequence of inorganic N limitation induced by the competition with the autotrophs. In all other cases where extra ammonium was not added, differences of bacterial abundance between substrates were not so marked and, in some cases (Figure 5.3.b) after a small initial increase, numbers fell throughout the rest of the incubation period. Nevertheless and similarly to the chlorophyll concentrations, lower values were generally obtained at high than at low C:N ratios at the end of the incubations<sup>5.7</sup>. The high uncertainty associated with these bacteria estimates for the reasons already given, does not allow, however, the calculation of a level of significance for these differences.

Another reason why differences in bacterial abundance between substrates were not very marked, or as marked as it would be expected in such circumstances, could be that grazing

<sup>&</sup>lt;sup>5.7</sup> See exception in Figure 5.3.d) where at the end of the incubation, higher abundance was found at high rather than at low C:N ratios. However, differences were small and can possibly be accounted for by experimental errors.

by the bacterivorous protozoa had a great impact on the bacteria population, levelling cell numbers to approximate values independently on the incubation conditions.

Therefore, from the chlorophyll and bacteria abundance results it appears that at high C:N ratios both populations were affected by the limiting concentrations of inorganic N.

To the best of my knowledge, direct experimental evidence of competition between algae and bacteria in the presence of a high C:N ratio substrate and using natural assemblages has not been often reported in the literature although the ability of bacteria to compete successfully with algae for a growth-limiting nutrient has been demonstrated in laboratorybased studies with cultured species of bacteria and phytoplankton (Rhee, 1972; Mayfield & Innis, 1978; Currie & Kalf, 1984a,b,c; Gude, 1985; Bratbak & Thingstad, 1985). The reported experimental work, however, regards specially the interactions between bacteria and phytoplankton in the uptake of phosphorus, with the competition for inorganic N less addressed but believed to take place under limiting conditions.

The work of Caron *et al.*, (1988) is among the few to consider experimentally the relationships between algae, bacteria and bacterivorous protozoa and the metabolism (uptake and regeneration) of inorganic N (ammonium). These authors observed that the presence of a natural bacterial assemblage in cultures of *Phaeodatylum tricornutum* had a significant negative effect on the growth of the diatom only when organic C was added to the culture, whereas the growth of the algae in the bacterised culture where ammonium was the only source of N and no organic C was added, was indistinguishable from the growth in the axenic cultures. These results clearly suggest the existence of competition between the diatom and bacteria, with bacteria outcompeting the autotrophs at high C:N ratios. This situation was only alleviated by the introduction of grazers which reduced the density of the bacterial assemblage and supported higher (and faster) diatom growth than in the ungrazed system, by remineralising some of the N tied up in the bacterial biomass.

In a similar fashion, the presence of grazers in the present study could be one of the reasons why the effect of an organic substrate of varying C:N ratio was not more clearly expressed in the phytoplanktonic biomass. At high C:N ratios the variations in the chlorophyll concentrations did not appear to be markedly different form those at low C:N ratios perhaps because protozoan remineralisation was taking place in the former, relieving the nutrient limiting conditions initially provided by the experimental set up.

In conclusion, results regarding the quantification of autotrophic and micro-heterotrophic abundance in the study under consideration did not constitute strong evidence for the existence of competition at high C:N ratio substrates but this was possibly due to the experimental conditions provided. However, consistent observations of lower concentrations at high C:N ratios than at low, together with results from the literature (particularly those of Caron *et al.*, 1988) and suggestive of a detrimental factor present the former case, can be reconciled with the predictions of Hypothesis I.

# **5.3.4.** Variation of the Bacterial C:N Ratio with the Composition of the Substrate.

The primary assumption on which these experiments were based - that when mineralising organic matter heterotrophic bacteria endeavour to maintain a constant C:N quota - appear to have been verified. Even though the C:N ratio of the substrate varied 10 fold from conditions of severe C limitation (C:N=2) to those of N limitation (C:N=20), the C:N composition of the bacterial fraction did not seem to have varied markedly with the C:N ratio of the substrate (Figure 5.9), although some temporal fluctuations during the incubation could be observed. With the exception of the 1996- Experiment II (Figure 5.9b) at the end of the incubation the C:N ratio of the bacterial particulate matter incubated in the presence of substrates of high and low C:N ratios was very close, not reflecting the wide range of substrate C:N composition tested. In the control incubations made in 1996, bacterial C:N ratios appeared to increase for the first 24-48 hours of the incubating period, decreasing subsequently, reaching in general higher values than those encountered in the amended cultures, for which an explanation is not obvious. In the presence of a "balanced" C:N ratio substrate (Figure 5.9e), the C:N ratio of the bacterial biomass showed to oscillate little during the incubation period, in contrast with the other amended cultures where temporal fluctuations were, at times, quite wide (e.g. Figure 5.9c). In Table 5.8, the average bacterial C:N ratios measured during each of the incubations carried out is presented:



Figure 5.9. Temporal variation of the C:N ratio of the "bacterial" particulate matter in 1995 and 1996 Experiments, in substrates of high (a to d), low (a to d) and "balanced" (e) C:N ratio. - Dark periods of incubation. Bars represent the standard error of the POC/PON ratio.

values within the same set of experiments were very close, despite the differing C:N ratios of the substrates.

The ratios obtained are higher than the range generally described for natural assemblages of bacteria; for instances, Nagata (1986) report values between 3.3 and 7.4 and Bratbak (1985) between 4.8 and 6.7. Goldman *et al.*, (1987) have also obtained low estimates (4.8-6.1) for organisms grown in substrates of varying C:N ratio (among them several amino acids) but for bacteria grown in cultures amended with glycine these authors obtained a C:N ratio of  $8.9 \pm 1.15$ , which compares well with the estimates obtained in the present work.

Table 5.8. Average C:N composition of the "bacterial" particulate matter at each Experiment carried out.

Experiment	High	Low	Control	Balanced
1996-Experiment I	8.5	9.3	10.4	_
1996-Experiment II	9.5	8.3	9.4	-
1995-Experiment I	7.3	7.4	-	-
1995-Experiment II	6.4	6.4	-	-
1995-Experiment III	-	-	-	6.4

"High", "low" and "balanced" refer to the C:N ratio of the substrate available for bacterial growth and "control" to those cultures where no organic additions were performed.

Goldman *et al.*, (1987) attributed the higher ratio obtained in the glycine amended cultures to the sluggish growth exhibited by bacteria under those conditions, compared to that in the presence of other substrates. However, in the present case in the "control" incubations where no additions were made, the bacterial C:N ratios were equally high.

The results of Goldman *et al.*, (1987) also show that the C:N ratio of bacteria growing in substrates of varying C:N ratio (1.5 - 10) did not change with the C:N ratio of the substrate. This is similar to our findings but contrast with the results of Tezuka (1990) who report wide changes in the bacterial C:N ratio depending on the substrate C:N composition, as shown in Figure 5.10. Tezuka (1990) attribute the constancy of the Goldman's *et al.*, (1987) results to N:P ratio which was kept constant and to the C:P ratio which varied

between 15 to 100, and which might have brought about a constant C:N:P ratio of the bacterial biomass.



Figure 5.10. Variation of the bacteria C:N ratio with the C:N composition of the available substrate. From Tezuka (1990).

### 5.3.5. Summary and Conclusions

Over the past 10 years, it has been established that bacteria can be responsible for an important part of the total inorganic nitrogen uptake in the marine environment, with the percentage of ammonium uptake attributed to bacteria in the range of 5-75% (median of 30%) and that of nitrate constituting <10% (Kirchman, 1994). However, the factors controlling the heterotrophic uptake and its effect on the planktonic community are currently under discussion.

The experimental work described in this chapter attempted to shed some light on the effect of the C and N quality of the DOM on the uptake of ammonium and nitrate by bacteria, in particular on the strategies used by these organisms to metabolise high and low C:N ratio organic substrates whilst endeavouring to maintain a constant C:N cell quota.

Classically there have been two views on how bacteria would achieve this, one involving C conservation and inorganic N uptake or remineralisation (Figure 5.1a; Hypothesis I) depending on the C:N ratio of the substrate; the other, involving conservation of a constant proportion of N and mineralisation or conservation of C according to the C:N composition of the DOM (Figure 5.1b; Hypothesis II). These two models were examined in the present

study and although they are not mutually exclusive there was the need to assume so in order that they could be tested.

In the experimental work undertaken, the coupling of N and C metabolism was addressed using  $^{15}$ N- and  $^{13}$ C-labelled compounds and natural assemblages of bacteria and phytoplankton. The use of heavy isotopes allowed the analysis of both C and N metabolism in the same sample, greatly reducing errors due to inter-sample variability. Furthermore, it made possible to follow the fate of the compounds of interest into the bacterial particulate fraction without having to size fractionate the samples prior to the incubations; therefore, throughout the experiments the whole planktonic community co-existed, a more realistic situation than in the numerous studies reported in the literature where the microheterotrophic community was isolated *a priori*. The basic assumption on which these experiments were based - that when mineralising organic matter heterotrophic bacteria endeavour to maintain a constant C:N quota - appeared to have been verified, with the bacterial C:N ratio remaining very approximate in the four incubating situations: high, low and balanced C:N ratio substrates and in "control" conditions.

The results have shown that the two models cannot be considered as two alternative strategies and that both will operate depending on the availability of inorganic nitrogen. At low C:N ratios, bacteria will dispose of excess N increasing at the same time C conservation. At high C:N ratios, bacteria will take up inorganic N when available. No marked differences were detected between the incubations performed with ammonium and nitrate. When no inorganic N is available, bacteria will dispose of excess C by mineralisation to  $CO_2$ . These observations are in agreement with data from the literature and they suggest that the question should be perhaps best posed not as which strategy bacteria adopt but in which circumstances they are employed.

It is now, and more than ever, apparent that the N and C microbial cycles should not be considered separately and that it is irrelevant to speak about N limitation without considering the C dynamics of the system under study, and *vice-versa*. As it will be discussed in CHAPER VI, the consequences of the recent findings on the role of bacteria in the C and N cycling are far reaching.

"Truth is the daughter of time"

# CHAPTER VI General Discussion and Conclusions

The primary aim of this work was to study the interaction between bacterial and phytoplanktonic inorganic nitrogenous nutrition. Whilst the utilisation of inorganic N by phytoplankton has been extensively dealt with in the past that of bacteria remains far less well documented. The present work has led to the conclusion that bacteria can contribute substantially to the total inorganic N uptake (nitrate and ammonium) in marine coastal waters; it has also confirmed the observations reported in the literature regarding the phytoplankton dynamics but this work did not intend to contribute with novel information to this. The main thrust of this study has been rather to deal with the association between the bacterial and the phytoplanktonic utilisation of inorganic nitrogen, their dynamics and controls in the Menai Strait planktonic ecosystem.

The acknowledgement that natural assemblages of planktonic bacteria may acquire a significant fraction of their N *via* the uptake of dissolved inorganic nitrogenous nutrients has not only changed the traditional view of these micro-organisms as nutrient remineralisers in plankton communities but has led to the conclusion that, in N depleted

conditions, this heterotrophic uptake may place bacteria and phytoplankton in competition for growth-limiting nutrients instead of their classical roles as "source" and "sink", respectively, in the marine environment.

Of the many questions that this relatively new concept of competition between bacteria and phytoplankton arise, the following are of particular interest in the domain of this work:

- (1) What are the dynamics of the bacterial N metabolism in the coastal marine environment relative to those of the algae?
- (2) What mechanisms control the switching between bacterial inorganic N uptake and remineralisation, *i.e.*, what environmental and physiological conditions determine the role of bacteria, respectively as "sinks" or "sources" of inorganic nutrients and consequently the existence (or not) of competition between these heterotrophs and the algae?
- (3) What are the ecological consequences of the bacterial roles of N remineralisers and consumers?

The Menai Strait seasonal study and the experimental laboratory work carried out provided information which directly, in some cases, and indirectly, in others, contributed to the formulation of temptative answers to those questions in relation to the marine environment in question, as will be discussed below.

## 6.1. DYNAMICS OF THE BACTERIAL AND ALGAL INORGANIC NITROGEN METABOLISM IN THE MENAI STRAIT.

In relation to the bacterial N dynamics in this marine ecosystem, three questions arose:

- (i) the periods of the seasonal cycle when bacteria assimilate inorganic N and the forms of this nutrient utilised;
- (ii) the periods when bacteria release ammonium;

(iii) the periods when there appears to be competition between bacteria and phytoplankton for ammonium and nitrate.

The field work carried out in the Menai Strait allowed the attribution of a time scale for nitrate and ammonium utilisation by the bacteria. During February and March, nitrate was predominantly used, from April through to May ammonium and nitrate were comparable in their importance to the bacteria. Following the reduction in ambient nitrate after the bloom, ammonium became the predominant, but not exclusive, form of N taken up (~ 60% of the total bacterial uptake). In the period from July until November, ammonium was essentially the exclusive N form utilised by these organisms. Therefore, in the case of bacteria there is a progressive drift from nitrate being the dominant source, to ammonium being the dominant source through the year. A similar pattern but less biased towards ammonium was seen in the case of the algae.

In general, periods of high bacterial ammonium uptake corresponded to peaks of algal uptake of this nutrient, in the early part of the year, in particular at times of increased primary production. After August, however, this pattern was broken and peaks of bacterial ammonium uptake were not synchronised with those of algal assimilation of that nutrient. For nitrate, the algal and bacterial maxima uptake never coincided (Figure 6.1). These observations appear to suggest that the phytoplanktonic and bacterial uptake is only synchronous when the availability of the nutrients in question is ample; otherwise, there appears to be a mutual exclusivity of these two processes with the bacterial inorganic N uptake lagging behind that of the algae. The absolute duration of this delay was not possible to ascertain in the present field study because of the infrequency of sampling ( $\geq 15$  days). The reasons for its existence appear linked to the quality of the DOM available at the various stages of the primary production cycle, with origin in the algal metabolism.

In relation to question ii), no information on ammonium production was available; however it was thought that this could be inferred in two ways:

 by considering that ammonium production was directly related to bacterial respiration. A number of assumptions would have to be made, such as that the C and N metabolism are synchronous and that the release of ammonium by bacteria is continuous, which is against the observations that at times, bacteria take up inorganic N instead of releasing 2) By assuming that bacteria are the principal producers of the ammonium utilised by algae, thus the rate of algal assimilation would be a measure of bacterial ammonium production.





Figure 6.1. Seasonal variation of the algal and bacterial uptake of inorganic N. Bact. and algal ammon. and nitra. uptk. stands for bacterial and algal ammonium and nitrate uptake, respectively.

This assumption requires a very tight coupling between the bacterial production of ammonium and its algal utilisation. Estimates of ammonium turnover rates which were in the order of hours (minimum of 13) to days (maximum of 13.6), indicate that this coupling was lose, however, (Figure 6.2) for the great majority of the year. But for the period between early May and the end of July (days 131 to 208), the ammonium turnover time was  $\sim 1$  day, therefore, it seems legitimate to consider

that at these times the rates of ammonium production by bacteria could be assumed to be equivalent to those of ammonium uptake by the algae (Table 6.1).

The balance between inferred ammonium production and measured utilisation is shown in Figure 6.3. The inferred ammonium production by bacteria was highest during July (day 188) and lowest in June, when the uptake of this nutrient predominated. High ammonium production rates were also obtained on day 131, suggesting that on these occasions organic matter of high N content was utilised, as corroborated by the high oxygen dark respiration rates registered (Table 6.1) and ambient accumulation of ammonium (Figure 4.2c).

In relation to question iii), regarding the time of the year when there appeared to be competition between algae and bacteria for the nitrogenous nutrients under study, the only period of the seasonal cycle when it was evidently more likely to have been present was after the main phytoplankton spring bloom.



Figure 6.2. Seasonal variation of ammonium and nitrate turnover rates.

During the month that followed (June), both nitrate and ammonium concentrations reached below detection levels, with nitrate having just been consumed to the point of virtual exhaustion and ammonium not remineralised being yet sufficiently to be accumulated in the environment, a situation that only became more apparent from July onwards.

day of the year	NH₄ <sup>+</sup> turnover rate (days)	Bact. NH₄ <sup>+</sup> uptake (µmol dm <sup>-3</sup> d <sup>-1</sup> )	Inferred bact. NH₄ <sup>+</sup> prod. (µmol dm <sup>-3</sup> d <sup>-1</sup> )	Bact. resp. (μmol O <sub>2</sub> dm <sup>-3</sup> d <sup>-1</sup> )
131	1.26	0.1002	0.5550	5.30
143	0.58	0.0174	0.0521	7.42
158	0.60	0.0375	0.0550	7.30
173	1.06	0.0154	0.0282	5.55
188	1.05	0.0076	0.0896	1.80
208	1.04	0.1805	0.6366	6.24

Table 6.1. Bacterial ammonium and oxygen metabolism in the Menai Strait from May until July.

Bact.resp. and prod. are bacterial respiration and production, respectively, where the inferred bacterial ammonium production was assumed to be equal to the algal uptake of that nutrient at this time of the year.

A peak of nitrate uptake by the bacteria at the end of May and another of ammonium in the beginning of June, concomitant with a sharp decrease in the autotrophic assimilation of these nutrients suggest that the increased bacterial N demand may have resulted in the observed rapid decline of the phytoplankton bloom, as the latter organisms are less able of exploiting the nutrient stock at very low concentrations than the former. It can be argued that every ammonium molecule assimilated by bacteria is one less for the phytoplankton

which means that the algae would have to assimilate a nitrate molecule to maintain the same growth, in a non-steady state system (Kirchman, 1994).



Figure 6.3. Uptake and inferred production of ammonium by bacteria, from May until July.

In the conditions present in the Menai Strait at the end of May, beginning of June, with bacteria assimilating from both the depleted stocks of ammonium and nitrate. phytoplankton did not appear to be capable of sustaining its growth, contrary to the micro-heterotrophs which abundance increased markedly at this time of the seasonal cycle.

Remineralisation of DOM and grazing on bacteria regenerates inorganic N, which may contribute to the amelioration of the impact of bacterial N uptake on the phytoplankton population. Such processes are likely to have been present at different times during summer and early autumn, in the waters under study, contributing to the observed increased levels of ambient ammonium and the general phytoplankton non-nutrient limitation, discussed before and which may be seen as indicative of reduced competition for inorganic N between the autotrophs and the heterotrophs at these times of the year.

## 6.2. ENVIRONMENTAL AND PHYSIOLOGICAL CONTROLS OF BACTERIAL UPTAKE AND REMINERALISATION OF NITROGEN.

Whether bacteria take up or remineralise inorganic N will depend ultimately on the stoichiometric ratios of the elements in the bacteria and the substrates that are used for

growth. It is now apparent that the bacterial utilisation of inorganic N is dependent on the availability and quality of organic matter but the regulations of this relationship are yet to be fully understood. In CHAPTER V, two alternative strategies were envisaged to describe the bacterial use of DOM whilst endeavouring to maintain a constant C:N quota:

- Hypothesis I Bacteria toggle between inorganic N utilisation and release driven by a combination of the C:N cell quota and a fixed C growth yield (Lancelot & Billen, 1985; Anderson, 1992).
- **Hypothesis II C** will be respired until the appropriate C:N is achieved, whilst N will be conserved; this strategy assumes that there is DOM available.

From a series of manipulation experiments and from data available in the literature discussed in CHAPTER V, the conclusions were that the C:N ratio of the DOM used by bacteria sets the boundary on the role of heterotrophic bacteria as inorganic N remineralisers in a manner that is compatible with the predictions of Hypothesis I, *i.e.* at low C:N ratios ( $\leq 10$ ; Lancelot & Billen, 1985; Goldman *et al.*, 1987) and when DON is the major N source promoting growth, bacteria will excrete ammonium as a way of compensating for the excess ambient N, whereas at high C:N ratios, bacteria will take up and conserve N. When the required inorganic N is limiting and not available for assimilation, which would be typical of a high C:N ratio situation, bacterial respiratory losses will increase in order to dispose of the excess C, whereas at low C:N ratios C conservation is likely to occur, in accordance with the predictions of Hypothesis II.

If the release of DOM by the algae is the prime source of organic material for the bacteria, then the switching of the inorganic N metabolism of the bacteria, controlled by the organic N content of the DOM, will be driven by the algae. In nutrient rich conditions such as those observed in the Menai Strait during the early stages of the main spring phytoplankton bloom, the photosynthetic products will be naturally N replete (low C:N ratio and molecular weight compounds) and in these conditions bacteria are presumed to more than fulfil their N requirements from the utilisation of such organic products which, due to their composition, would not require further assimilation of inorganic N from the environment. With the declining stages of the bloom which accompanies ambient nutrient depletion, the production of organic materials of high C:N ratio and molecular weight is to be expected. Subsequent utilisation of these organic materials by the bacteria would create an
intracellular nitrogen demand thereby stimulating uptake of dissolved inorganic nitrogen by these organisms. In conditions of severe nutrient limitation, bacterial growth could be impaired due to impossibility of utilising organic matter. Under these conditions, Ndepleted compounds would accumulate in the water (Williams, 1995). This is in contradiction with the more classical view that bacterial are always capable of utilising inorganic nutrients to meet all their growth requirements, being ultimately energy limited (Kirchman, 1994). Certainly this could be the case at the early stages of the seasonal cycle, when nutrients are amply available, prior to phytoplankton development and before photosynthetic products become available (although early in the year bacterial metabolism would also be temperature inhibited); however, during the productive season DOM is believed to be in sufficiently high concentrations to sustain bacterial growth and in the present context one of the reasons why it would not, would be due to inorganic nutrient limitation. There remains the further hypothesis that accumulation is a kinetic phenomenon, where the accumulation results from lack of planktonic heterotrophic metabolism capacity due to 'top down' control of the bacteria by microzooplankoon, exacerbated, in the case of N- deficient compounds, by the need to compete for inorganic nitrogen (Thingstad et al., 1997). In these ideas may lie possible answers for the low turnover of the DOM pool, one of the largest C pools on Earth.

All the above considerations are made based on assumptions regarding the chemical nature of the organic compounds utilised by the bacteria but until the characterisation of these materials is made, it can only be presumed. It was thought, in this study, that by looking at the ratio between bacterial oxygen dark respiration and ammonium production (inferred from algal ammonium uptake rates), an estimate of the extent of organic N respiration could be obtained and thus the chemical nature of the organic materials utilised by the bacteria deduced, with high ratios characteristic of N poor compounds, such as carbohydrates and lipids, and low ratios typical of proteins and nucleic acids. For the reasons already given, such calculations were only possible to carry out for the period between May and July. Given the mean composition of the primary metabolites of marine phytoplankton (Anderson, 1995) presented in Table 6.2., the number of moles of oxygen required to respire one mole of such compounds can be estimated, as well as their characteristic O/N. For instances, in the case of protein, approximately 4 moles of oxygen are needed to respire a mole of this compound (equation 6.1) with an O:N ratio of four.

 $C_{3.8} H_{6.1} O_{1.3} N + 3.925 O_2 \longrightarrow 3.8 CO_2 + NH_3 + 1.55 H_2O$  (6.1)

**Table 6.2.** Mean composition of the primary metabolites of marine phytoplankton estimated by Anderson (1995).

Metabolite	Composition			
Carbohydrate Lipid	C <sub>6</sub> H <sub>10</sub> O <sub>5</sub> C <sub>40</sub> H <sub>74</sub> O <sub>5</sub>			
Protein Nucleic acid	$\begin{array}{c} C_{3.8}H_{6.05}O_{1.25}N\\ C_{9.63}H_{12}O_{6.5}N_{3.75}P\end{array}$			

From Table 6.1, it can be estimated that the O/N ratios for the Menai Strait between May and July, varied widely from 9.5 to ~200, suggesting that bacteria were predominantly utilising N impoverished compounds such as polymeric carbohydrates rather than amino acids, proteins or nucleic acids whose metabolism would result in much lower O/N ratios than those observed.

On days 131 and 208, it may have been possible that N-richer compounds were present, since the O:N ratios observed were much lower than those obtained on the remaining occasions. Accordingly, the ammonium turnover rates were low (~1 day) and relatively more ammonium was released than taken up (Figure 6.3). Considerable availability of these compounds may have been possible, as these data points coincided with times of high phytoplanktonic photosynthetic activity (Figure 4.6a), a source of dissolved free amino acids (Eberlein *et al.*, 1985; Amon & Benner, 1996; Biddanda & Benner, 1997). This contrasts with the situation observed during June, when the highest O/N ratios were seen and relatively more ammonium was assimilated than released by the bacteria (Figure 6.3). These inflated O:N quotients in June are in agreement with the previously assumed high availability of C-rich compounds in the Menai Strait at this time of the seasonal cycle, with origin in the decline of the main spring phytoplankton bloom, as discussed before.

Because ammonium may also come from the local sediments and sewage discharges, as well as from zooplankton excretion, the estimates of bacterial ammonium production are maxima. Therefore, the O:N ratios calculated are minimum and the presumed availability of organic compounds rich in N, even smaller. Conservatively, the organic source used for respiration was, therefore, predominantly non-nitrogenous throughout most of this period of the seasonal cycle and bacteria may be regarded as predominantly "sinks" for the inorganic nutrients. This is partially in contradiction with the data plotted in Figure 6.3 where it can be seen that during May and July bacterial production of ammonium predominated over its uptake. However, it has to be remembered that the ammonium production data was inferred from algal ammonium uptake and did not constitute direct measurements.

### 6.3. ECOLOGICAL CONSEQUENCES OF THE CONTROL OF THE BACTERIAL ROLES IN THE PLANKTONIC COMMUNITY

From what has been said so far, it can be concluded that the ecological significance of the control of the substrate C:N ratio on the bacterial N and C metabolism has several aspects:

1. At low C:N ratios ( $\leq 10$ ; Lancelot & Billen, 1985; Goldman *et al.*, 1987), bacteria will act primarily as mineralisers of organic matter constituting a "link" in the flows of N through the microbial community by providing inorganic nutrients for phytoplankton growth. The bacteria fixation of inorganic N in these substrates is, therefore, low and protozoan nutrient regeneration is insignificant by comparison with that by bacteria (Caron *et al.*, 1988; Hopkinson *et al.*, 1989). As the N quality of the DOM improves, C assimilation efficiency increases with less losses by respiration taking place; thus bacteria act as C 'sinks' in these circumstances. Therefore, more C can be transferred to higher trophic levels.

It has been frequently observed over the past years that free amino acids do not exist in the marine environment in quantities high enough to satisfy bacterial N demands and that these demands have to be fulfilled by the bacterial uptake of inorganic N (Kirchman *et al.*, 1986; Wheeler & Kirchman, 1986; Goldman & Dennet, 1991; Keil & Kirchman, 1991; Jorgensen

et al., 1993, 1994). It is then apparent that the C:N ratio of the available organic substrates in the marine environment is frequently >10 and consequently bacteria are less important remineralisers of organic matter than previously thought, as pointed out by Goldman *et al.*, (1987).

2. At high C:N ratios (>10; Lancelot & Billen, 1985; Goldman et al., 1987), bacteria will act as sinks for N as they will immobilise additional nitrogen from the pool of inorganic nutrients (NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>) in the surrounding medium. By grazing the increased bacterial biomass, the role of bacterivorous protozoa as remineralisers of a growth-limiting nutrient is maximal (Caron et al., 1988; Hopkinson et al., 1989) and also in determining the partitioning of nitrogen between bacteria and phytoplankton and ultimately the relationships between the autotrophs and the heterotrophs. At the same time, C losses through respiration will increase until the required C:N stoichiometry is achieved. In conditions when no inorganic N is available, carbon assimilation efficiency decreases to a minimum; thus one of the net effects of high C:N ratio substrates in nutrient limiting circumstances is a lower transfer of C to higher trophic levels, such as the protistan and metazoan grazers, than it would occur under conditions of C limitation (low C:N ratios). At each step in this trophic transfer there would be a significant (nominally 40-50%) loss of C through mineralisation to CO<sub>2</sub> (Jahnke & Craven, 1995); thus, when bacterial growth is limited by inorganic nutrients, the quantity of DOC that does not get to be taken up by bacteria, and of POC, both of which would be later remineralised, is exported to the depth of the sea in a greater extent than would occur if DOC was first incorporated into bacterial biomass (Thingstad & Rassoulzadegan, 1995; Rivkin & Anderson, 1997; Thingstad et al., 1997). Inorganic nutrient limitation of bacteria could, therefore, directly control the uptake, accumulation and stock of DOC thus ultimately influencing the sequestration of atmospheric CO<sub>2</sub> into the ocean (Rivkin & Anderson, 1997). There are, however, problems which have been ignored in this view, stemming from the fact that as DOC diffuses downwards it would be mixed with dissolved inorganic nitrogen from deep water and thus the DOC export by the proposed mechanism, questionable (P. leB Williams, pers. commun.)

**3.** In N-limited systems, an increase in uptake of ammonium or nitrate by bacteria caused by increased C supply (high C:N ratio situation) would mean that they would be competing

with the primary producers for these growth-limiting nutrients, with the possibility of even inhibiting or severely restricting the growth of the autotrophs. Furthermore, Bratbak & Thingstad (1985), have observed that when competing with bacteria for a growth-limiting nutrient, phytoplankton release organic compounds that would increase bacterial N demand if utilised by these organisms, leading to an even more severe inhibition of phytoplankton growth. With the recognition that grazing activities by bacterivorous protozoa are instrumental in returning remineralised nutrients to the medium, thus relieving the nutrient stress for the primary producers, this situation of bacteria outcompeting their ultimate source of assimilable C products appears less paradoxical. A recent conceptual model by Thingstad *et al.*, (1997) suggest another solution to the paradox of nutrient-limited phytoplankton stimulating their bacterial competitors by DOC excretion: if mineral nutrient limitation of bacteria is the rule rather than the exception, bacteria will simply not be stimulated by increased DOC supply therefore not being in a position to severely limit phytoplankton growth through accentuated competition for the inorganic nutrients.

4. In situations where bacterial uptake of inorganic N is significant, estimates of "new" and "regenerated" production, as defined by Dugdale & Goering (1967) would be expected to be affected. However, it appears that the steady state mass balance of N and C in the upper ocean would not be affected by bacterial nitrate uptake (Kirchman *et al.*, 1992). Nitrate uptake by bacteria would still need to be balanced by export (over some time scale) and would have to be accompanied by the same net C fixation as if phytoplankton were assimilating the nitrate (Figure 6. 4). Kirchman *et al.*, (1992) pointed out, however, that N uptake by the microheterotrophs and other small organisms, may have a large impact on the coupling between nitrate uptake and particle export from the upper ocean and on other relationships among "new", "regenerated" and export production. With regard to ammonium uptake, Kirchman *et al.*, (1992) think that the estimates of regenerated production should include only net ammonium uptake by bacteria, not total ammonium uptake (net uptake = total uptake - regeneration).

If the total ammonium bacterial uptake was included, N would be counted twice: once when it was assimilated by phytoplankton to synthesise DON and again when the ammonium regenerated from DON catabolism is assimilated by either algae or bacteria.



Figure 6.4. Schematic diagram to illustrate relationship among nitrate uptake by heterotrophic bacteria, new production and N export. Even though heterotrophic bacteria assimilate all nitrate in a) the balance between nitrate uptake and N export has to be the same as in b) where only phytoplankton assimilate nitrate. The only difference between the two cases is which organism synthesises the organic N. From Kirchman *et al.*, (1992).

**5.** In environments with high nitrate concentration, such as the Subarctic Pacific, primary production would not be affected by bacterial ammonium uptake because the algae could switch to the use of nitrate. However, a shift from ammonium to nitrate use by the algae could still have system-level effects. Increased use of ammonium by bacteria could result in large phytoplankton species outcompeting small cell algal communities. Ammonium uptake is predominantly by the small size fractions (Harrison & Wood, 1988) and there is a correlation between the abundance of large phytoplankton species and high nitrate concentrations (Malone, 1980).

Finally, it is now, and more than ever, apparent that the N and C microbial cycles cannot be considered separately and that it is irrelevant to speak about N limitation without considering the C dynamics of the system under study, and *vice versa*. The consequences of the recent findings on the role of bacteria in the C and N cycling are expected to be far reaching and of practical importance since many marine pollution situations are dealt with by the addition of mineral nutrients and /or organic carbon to the ecosystem.

At the end of this study it can be considered that its main aim was accomplished and that results from this and other research work has brought closer the understanding of the relationships between the autotrophic and the microheterotrophic community of the plankton and consequently of the functioning of the "microbial loop". In their concluding remarks of their publication on the ecological role of water-column microbes, Azam et al., (1983) state that more information on the coupling between the C and N microbial metabolism is needed so that a deeper understanding of the dynamical behaviour of the microbial loop can be achieved. Fifteen years after the publication of that paper, the intrinsic nature of such relationship is emerging. It is perhaps time to assemble all the information now available in the literature, in a revised paradigm of the role of the microbial community in the oceans and its extensive consequences in the cycling of N and C in the worlds' ocean ecosystem. One of the main areas that needs exploring and which importance has become increasingly more and more relevant is the chemical characterisation of the DOM pool and the dynamics of its utilisation by bacteria. Some of the concepts on which many classical ideas were based, such as that these materials were predominantly refractory to the bacterial use, are now being questioned and a more sophisticated picture of the dynamics of this pools needs to be constructed, based on what it is now known about the close coupling between the organic C and inorganic N utilisation by these organisms.

## APPENDIX

# Experimental Study of the Retentiveness of Glass Fibre Filters (WHATMAN GF/F)

The nominal porosity of the Whatman glass fibre filters GF/F is reported to be 0.7  $\mu$ m (Philip Harris, Ltd. General catalogue, 1995). As this type of filters was used to collect the particulate matter contained not only in the unfractionated samples but also in the smaller size fractions (<0.8 $\mu$ m and <2.0  $\mu$ m), an experiment was carried out in order to ascertain their efficacy to retain bacteria cells. Different, experimentally relevant volumes of unfractionated and fractionated samples (ranging from 25 to 750cm<sup>3</sup>) were filtered onto 25mm diameter GF/F filters, under gentle vacuum (<100 mmHg) and the number of bacteria in the pre-filtered subsamples and filtrates determined by means of the DAPI staining technique (Porter & Feig, 1980). The difference between the number of bacteria in the pre-filtered subsamples and corresponding filtrates is equivalent to the number of cells retained by the filter and it is here presented as a percentage of the bacteria abundance in the pre-filtered subsamples (Table I).

**Table I.** Effectiveness of GF/F filters in retaining bacteria from unfractionated (Whole community) and fractionated seawater subsamples of varying volumes, given as the % of cells trapped in the filter in relation to the number of cells in the pre-filtered seawater.

	Volume of subsample filtered onto the GF/F filter (cm <sup>3</sup> )					
	25	50	100	250	500	750
Whole Community	83	78	92	98		
<2µm	-	-	86	90	84	89
<0.8µm	-	-	89	94	90	83

In the majority of the cases, the GF/F filters were capable of retaining more than 80% of the bacteria cells present in both unfractionated and fractionated samples and appear, therefore, to be suitable for the collection of particulate matter of a wide range of sizes.

250

According to this experiment, the volumes of unfractionated and fractionated samples which were normally filtered for the measurement of particulate nitrogen and carbon and corresponding isotopic composition, yield a retention percentage above 90%. During the productive season this percentage is likely to be higher as a result of the clogging of the filter by the increased suspended materials in the water. This is particularly the case during a *Phaeocystis* bloom (characteristic of the environment under study) when the mucillage covers the surface of the filter turning water filtration into a near impossible task. This situation can be partially avoided by decreasing the volume of the sample without detrimental effects on the percentage of small particles retained. However, care must be taken to ensure that sufficient particulate matter is collected to produce a measurable signal when analysed by mass spectrometry. Contrary to what might have been expected, there was not an increase in the percentage of cells retained with increasing the volume of sample filtered as a result of progressive clogging of the filter. This may have been due the fact that this experiment was performed during winter (January) when suspended particulate matter of a biological origin is low in the waters of the Menai, Strait, and consequently, filter clogging less likely to occur.

The results obtained in this experiment are in agreement with Li *et al.*, (1983) who demonstrated that Whatman GF/F filters were adequate for sampling picoplankton in oligotrophic open waters, by reporting that 94% of the <sup>14</sup>CO<sub>2</sub> fixation measured on a 0.2 $\mu$ m Nuclepore filter was retained on a GF/F. Taguchi & Laws (1988) have also found that the chlorophyll content of a picoplanktonic size fraction (<2 $\mu$ m) filtered consecutively through a GF/F and a 0.2 $\mu$ m, contained only *ca*. 10% of that of the pre-filtered picoplankton sample. The authors report no difference of retentiveness between GF/F filters and 0.2 $\mu$ m Nuclepore membranes, indicating that although the nominal pore size of the former is 0.7 $\mu$ m, due to their structure these filters are, in reality, capable of retaining particles of a much smaller size. According to Logan (1993), the median particle passing through a GF/F is in the range of 0.2-0.3 $\mu$ m, with larger particles than that being efficiently removed. Taguchi & Laws (1988) further consider that small cells have "a low but finite probability" of passing through either type of filter and that consecutive filtrations are the only way to ensure that all cells are trapped.

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976

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277

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279

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### Phasing of autotrophic and heterotrophic plankton metabolism in a temperate coastal ecosystem

Stephen P. Blight, Tracy L. Bentley, Dominique Lefevre, Carol Robinson, Rubina Rodrigues, John Rowlands, Peter J. leB. Williams<sup>\*,\*\*</sup>

University of Wales, Bangor, School of Ocean Sciences, Menai Bridge, Gwynedd LL59 5EY, United Kingdom

ABSTRACT: Plankton abundances, bacterial production, and the size distribution of oxygen metabolism and chlorophyll a concentration were determined through 3 seasonal cycles in the Menai Strait (North Wales, UK). Spring blooms were comprised of a diatom to *Phaeocystis* succession. Meso- and microphytoplankton dominated phytoplankton production and biomass during diatom blooms, and nanophytoplankton predominated during summer, when activity and biomass were low. Correlation analysis showed temperature to be the best predictor for chlorophyll a-specific gross community production. Bacterioplankton were implied to be the major respirers. Consequently the phasing of respiration in relation to photosynthesis was strongly influenced by bacterioplankton metabolism and abundance changes. The respiration maximum occurred 1 to 2 wk after the *Phaeocystis* abundance maximum. An explanation for this temporal lag was sought by considering the time scales of flow of organic material between the phytoplankton and the bacterioplankton. The observations were consistent with routes via a slowly cycling pool, such as polymeric organic material. This pool would function as a reservoir and result in microheterotrophic respiration persisting after the decline of photosynthesis, and causing a positive to negative temporal sequence in net community production.

KEY WORDS: Plankton  $\cdot$  Autotrophic  $\cdot$  Heterotrophic  $\cdot$  Photosynthesis  $\cdot$  Respiration  $\cdot$  Phasing  $\cdot$  Bacteria  $\cdot$  Diatom  $\cdot$  Phaeocystis

### INTRODUCTION

The coastal water regions are areas of high primary production in the ocean. Although these waters constitute less than 10% of global ocean area, they account for 25% of the total oceanic primary production (Berger et al. 1989). The effect of this elevated primary production on the process of net deposition is magnified by its pulsed nature (e.g. the spring bloom in temperate coastal waters) and the proximity of the benthos (Berger et al. 1989). Consequently, coastal sediments are an important sink for carbon and the magnitude of this sink may be increasing as eutrophication of coastal waters continues (Kempe & Pegler 1991). Therefore, characterization of the production and loss processes for coastal plankton is important if we are to understand the role of coastal waters in the global carbon cycle.

The most ubiquitous loss process is respiration. Size fractionation studies have shown this process to be dominated by the smaller size fractions, especially the heterotrophic bacterioplankton (e.g. Williams 1981, Harrison 1986). Consequently, the phasing of photosynthesis and respiration will be strongly influenced by the nature of the links between the phytoplankton and the bacterioplankton. One link occurs via a dissolved organic matter (DOM) pool that can be considered as comprised of 2 pools. One, utilizable monomers and oligomers, i.e. low molecular weight material (LMW), is rapidly turned over by the bacterioplankton (e.g. Fuhrman 1987). The other is comprised of polymers, i.e. high molecular weight material (HMW) and must be hydrolysed before utilization by the bacterioplankton (Chróst 1990a); the hydrolytic step is believed to be rate limiting (Chróst 1990b) and so this

<sup>\*</sup>E-mail: oss074@bangor.ac.uk

<sup>\*\*</sup>All authors other than the first are listed alphabetically

pool turns over more slowly (e.g. Billen 1990). Thus, pathways via the HMW pool give rise to a looser coupling between photosynthesis and bacterial respiration, with the respiration response more protracted. This would allow for the temporal extension of bacterial respiration beyond the decline of photosynthesis.

The aim of this study was to determine the phasing of community photosynthesis and respiration during the temporal evolution of phytoplankton blooms and in the analysis of the data to address the basis of this phasing.

#### METHODS

Sampling. Water was collected from St. George's pier, Menai Bridge (North Wales, UK; Fig. 1) over the period of January 1992 to June 1994. During 1992, 5 dm<sup>3</sup> water samples were collected in the morning, irrespective of tidal state, from a depth of 1 m using a Niskin bottle, at 30 to 35 d intervals during autumn and winter months and at 5 to 8 d intervals during the more productive spring and summer months. In 1993 and 1994, 100 to 150 dm<sup>3</sup> of water was drawn through a 200 µm mesh from a depth of 1 m using a hand bilge pump (5 dm<sup>3</sup> min<sup>-1</sup>) and collected in 50 dm<sup>3</sup> opalescent polypropylene aspirators. During 1993, sampling was undertaken before dawn and within 2 h of high water at 14 d intervals during spring and summer and at 28 to 42 d intervals during autumn and winter. In 1994, samples were collected before noon and within 2 h of high water every 6 to 13 d. Additionally, in 1994 unfractionated samples were obtained by immersing and filling a 10 dm<sup>3</sup> opalescent polypropylene aspirator, avoiding the surface microfilm. Air temperature was obtained from the nearest meteorological station (Valley, Fig. 1).

In 1994, a Secchi disc was used to estimate the vertical attenuation coefficient for downward irradiance  $(k_d)$  and the relationship  $k_d Z_s = 1.45$ , where  $Z_s$  denotes Secchi depth used to calculate  $k_d$  (Walker 1980).

Size fractionation. Custom made, gravity driven, large diameter (142 mm) reverse flow filtration systems were used in parallel to perform most of the 1993 and all of the 1994 fractionations (<200, <53, <20, and <0.8 µm). During the 1993 *Phaeocystis* bloom, a crossflow filtration unit (Amicon, USA) was used to prepare the <0.8 µm size fraction. Nylon meshes were used for separating the <200, <53 and <20 µm fractions; polycarbonate filters (Poretics Inc., Livermore, CA, USA) for the <0.8 µm fraction. All fractionations were performed outdoors, shielded from direct sunlight and completed within 4 h of sampling.

Oxygen flux measurements. Dissolved oxygen concentrations were determined by Winkler titrations using a PC-based system with a photometric endpoint detector (Williams & Jenkinson 1982). Borosilicate glass bottles (50 to 150 cm<sup>3</sup>, 3 to 6 replicates) were used to perform light and dark bottle incubations for  $8 \pm$ 0.5 h (1992) and 24 ± 0.5 h (1993 and 1994) in outdoor water cooled light and dark incubators; the temperature was not greater than 3°C above in situ water temperature. For all incubations the oxygen concentration was measured at the start and end of the incubation. In addition, intermediate time points (every 2 to 4 h) were measured on 2 separate occasions to follow the time course of respiration, In 1993 and 1994, the incubation light intensity was attenuated to 60% of total incident solar radiation by a neutral density mesh.

Tritiated thymidine incorporation (TTI). The incorporation of <sup>3</sup>H-methyl thymidine into ice-cold trichloroacetic acid (TCA) insoluble material was used to estimate heterotrophic bacterioplankton production



Fig. 1. Map of sample area

(Fuhrman & Azam 1980). <sup>3</sup>H-methyl thymidine (Amersham, specific activity 84 Ci mmol<sup>-1</sup>, final concentration 5 nM) was added to subsamples in sterile tissue culture tubes and incubated for 1 h. Two replicates  $(10 \text{ cm}^3)$  were made along with a formalin-killed blank. Additional time course experiments were occasionally performed and thymidine incorporation was always found to be linear over at least 2 h. Thymidine incorporation was halted by the addition of formaldehyde  $(0.5 \text{ cm}^3, 1\% \text{ final concentration})$  and within 12 h macromolecular material was precipitated by adding 1  $cm^3$  of ice-cold 20% TCA and leaving the samples to stand on ice for 15 min (Wicks & Robarts 1987). The precipitates were then collected on 0.2 µm polycarbonate filters (Ducklow et al. 1992; Poretics Inc.), washed twice with 1  $cm^3$  of ice-cold 5% TCA and once with 5  $cm^3$  of ice-cold 80% ethanol to remove unincorporated label. The filters were dissolved in 4 cm<sup>3</sup> of Optiphase scintillation cocktail in minivials and counted with a Packard Tricarb series scintillation counter. The obtained counts were converted to disintegrations by the external standard method.

A conversion factor experiment was performed on Day 130 of 1994. The <0.8  $\mu$ m fraction was diluted approximately 5-fold with seawater filtered through a 0.2  $\mu$ m membrane disc (Anotec, UK). This diluted sample was incubated in a 2.5 dm<sup>3</sup> polycarbonate bottle within 1°C of *in situ* water temperature. Subsamples were taken every 4 h over 36 h for the determination of the thymidine incorporation rate and bacterial numbers. The conversion factor was derived by dividing the increase in bacterial numbers over the incubation period by the integrated thymidine incorporation, as recommended by Bell (1988).

Micro- and nanoplankton (Sieburth et al. 1978) counts and biovolume estimates. Subsamples were collected in glass medical bottles (50 to 100 cm<sup>3</sup>) into which 4 to 8 drops of acid Lugols (Throndsen 1978) had been pipetted and the bottles stored at 4°C. Aliquots (10 to 50 cm<sup>3</sup>) were allowed to settle overnight in sedimentation chambers before counting the plankton by inverted microscopy (Utermöhl 1958). Cell volumes were estimated according to basic steriometrical formulas (Edler 1979).

Bacterial numbers. Subsamples (50 cm<sup>3</sup>) were fixed with 50% glutaraldehyde solution (final conc. 0.5%) and stored at 4°C. Within 24 h, 3 to 5 cm<sup>3</sup> of the subsamples were stained for 6 min with the DNA stain DAPI (Porter & Feig 1980; final conc. 1.25  $\mu$ g cm<sup>-3</sup>, Sigma, USA), filtered down onto 0.2  $\mu$ m polycarbonate filters (Poretics Inc.) and stored at -20°C until counted. Measurements of bacterial numbers (minimum of 400 cells or 30 grids) were made with a Leitz Orthoplan fluorescence microscope at ×1250 magnification. Rates of bacterial growth and net growth from changes in cell numbers. In 1994, bacterial numbers were determined for unfractionated and <0.8 µm fraction samples at both the beginning and the end of the oxygen flux incubations. Additional intermediate counts at intervals of 4 h were performed on Day 159 during the respiration time course experiment. For all incubations, subsamples for bacterial counts were taken from dark bottles. Daily growth rates ( $\mu$ ) were estimated using a linear ( $\mu = N_t - N_0$ ) growth model (Wright & Coffin 1984) where ( $N_0$ ) and ( $N_t$ ) are the bacterial densities after 0 and 24 h respectively. For Days 130 and 159, daily growth rates were estimated by least-squares linear regression of bacterial density versus time.

Chlorophyll a. Subsamples (250 to 750 cm<sup>3</sup>) were filtered onto 47 mm Whatman GF/F filters and the phytopigments extracted in 10 cm<sup>3</sup> (1992) and 8 cm<sup>3</sup> (1993 and 1994) of neutralised 90% acetone solution for 24 to 48 h. In 1992 phytopigment concentrations were determined using a diode-array spectrophotometer (Hewlett-Packard, model 8452A) following the procedure of Parsons et al. (1984). During 1993 and 1994, phytopigment concentrations were estimated using a Turner 10 Designs fluorometer in accordance with the recommendations of Tett (1987).

Particulate organic carbon and nitrogen. Subsamples (0.01 to 1 dm<sup>3</sup>) were filtered onto 25 mm pre-combusted (500°C for 3 h) GF/F filters. The filters were oven dried overnight at 40°C then wrapped in pre-combusted aluminium discs. The prepared filters were analysed using a Europa Scientific Robo-prep CN analyser attached to a tracermass stable isotope analyser.

Nitrate. The samples for nitrate concentration determination were processed immediately after collection according to the method of Parsons et al. (1984).

#### RESULTS

The water temperatures in both 1993 and 1994 were consistently higher than the 1992 values (Fig. 2A). This difference was particularly marked for the first sampling point in 1994 when the concurrent air temperature showed a decrease (Fig. 2B). A similar fall in air temperature also occurred at about the same time in 1993 (Fig. 2B).

#### 1992 seasonal pattern

The patterns for unfractionated chlorophyll a (chl a) (Fig. 3A) and gross community production (GCP, Fig. 3B) were similar, with 2 distinct peaks in the


Fig. 2. Spring and early summer trends for (A) water temperature in the Menai Strait (North Wales, UK) and (B) air temperature at Valley (Anglesey, North Wales) during 1992, 1993 and 1994. The mean monthly temperatures for both parameters between the years 1955 and 1968 (Harvey 1972) are also shown for comparison

spring to early summer period. For both parameters the recorded annual maximum was on Day 128. Unfractionated dark community respiration (DCR) was also characterized by 2 peaks (Fig. 3C) over the spring and early summer period, with the annual maximum (Day 142) recorded 2 wk after the chl a and GCP maximum. Net community production (NCP, Fig. 3D) exhibited 2 peaks in phase with those for chl a and GCP. Its annual maximum (Day 128) was followed by a net heterotrophic phase lasting approximately 10 d.

## 1993 seasonal pattern

The <200 µm fraction chl a (Fig. 4A) and particulate organic carbon (POC, Fig. 4B) exhibited 2 peaks over the spring period. The first peak (diatomaceous) was recorded on Day 117. The second, constituting the annual maximum, was measured on Day 146 and was comprised predominantly of *Phaeocystis* cells. During the decline of this bloom the annual particulate organic nitrogen (PON) maximum was recorded (Day 160, Fig. 4B). Chl a concentrations then remained low, near 1 mg m<sup>-3</sup>, through July and August until a late summer diatom bloom (*Guinardia flaccida*, 3.4 ×  $10^9$  µm<sup>3</sup> dm<sup>-3</sup>) was observed on Day 237. This bloom declined over the course of 2 wk and chl a remained low, approximately 1 mg m<sup>-3</sup>, for the rest of the year. During the diatom blooms, the contribution of the



Fig. 3. The 1992 seasonal patterns in the Menai Strait for the unfractionated plankton assemblage. Parameters shown are (A) chlorophyll a (chl a); (B) gross community O<sub>2</sub> production (GCP); (C) dark community O<sub>2</sub> respiration (DCR); and (D) net community O<sub>2</sub> production (NCP)

<20  $\mu$ m fraction of chl *a* relative to the <200  $\mu$ m fraction was 20 to 25%. During the *Phaeocystis* bloom, this contribution increased to approximately 50% and in July, when phytoplankton biomass was low, roughly 70% of the measured chl *a* was attributable to the <20  $\mu$ m fraction.

The seasonal pattern of GCP and its size distribution was similar to that exhibited by the chl a concentration (Fig. 4C). The <200  $\mu$ m fraction DCR rate was characterized by a smooth curve peaking on Day 160 (Fig. 4D). Maximal rates were exhibited for all size fractions (except for the <0.8  $\mu$ m fraction) on Day 160. The respiration rate of the <0.8  $\mu$ m size fraction appeared to peak a fortnight earlier (Day 146), when the <0.8  $\mu$ m rate was significantly greater than the



Fig. 4. The 1993 seasonal patterns in the Menai Strait for the <200  $\mu$ m fraction and where available the <53, <20 and <0.8  $\mu$ m fractions. Parameters shown are (A) chlorophyll a; (B) particulate organic carbon (POC) and particulate organic nitrogen (PON); (C) gross community O<sub>2</sub> production (GCP); (D) dark community O<sub>2</sub> respiration (DCR); (E) net community O<sub>2</sub> production (NCP); (F) nitrate concentration; and (G) tritiated thymidine incorporation (TTI) rate

<200  $\mu$ m DCR rate (Student's sampled paired *t*-test, p < 0.01, n = 3). The <0.8  $\mu$ m fraction on average accounted for 69% (range 28 to 131, n = 10) of the <200  $\mu$ m fraction DCR rate and this contribution was smallest on Day 178. The <200  $\mu$ m fraction net community production exhibited 3 peaks during the year (Fig. 4E). Each of these peaks coincided with a phytoplankton bloom. Following the *Phaeocystis* bloom, the net autotrophic community was succeeded by a net heterotrophic one, and this phase lasted for approximately 1 mo.

The nitrate concentration declined steadily prior to and during the spring diatom blooms period (Fig. 4F). The *Phaeocystis* bloom reduced the nitrate concentration to below the limit of detection (0.05  $\mu$ M). Nitrate then remained low (<1  $\mu$ M) for approximately 4 mo before increasing again in late autumn. The tritiated thymidine incorporation rate exhibited a number of peaks over the course of the year, with the annual maximum occurring on Day 146 (Fig. 4G).

# 1994 spring bloom pattern

The spring bloom was characterized by 3 chl a peaks (Fig. 5A). The first peak was comprised of mixed diatoms (Day 116) with Asterionella glacialis and Ditylum brightwelli dominating numbers and biovolume respectively. These cells and most of the detritus largely disappeared from the water column by Day 123. The second chl a peak (Day 130) was again pre-.dominantly diatomaceous with Rhizosolenia delicatula  $(3.5 \times 10^5 \text{ cells dm}^{-3})$  as the major diatom, although Phaeocystis bladders (first observed on Day 129) were also present. The third peak occurred on Day 145 when R. delicatula  $(4.5 \times 10^5 \text{ cells } \text{dm}^{-3})$  was still abundant and the measured Phaeocystis maximum occurred (approximately  $1 \times 10^3$  bladders dm<sup>-3</sup> and  $5 \times$ 10<sup>6</sup> cells dm<sup>-3</sup>, Fig. 5B). The *Phaeocystis* cell counts had decreased dramatically by Day 159, whereas high numbers of R. delicatula persisted until Day 168. Both R. delicatula and Phaeocystis bladders were absent from the water column by Day 181. Size fractionation of the chl a during the diatom blooms showed only 20% of the unfractionated chl a to reside in the <20  $\mu$ m fraction. This contribution increased to 34-44 % during the Phaeocystis-Rhizosolenia bloom and after the decline of this bloom increased to 87%.

Three peaks in ciliate numbers and biovolume were observed over the phytoplankton blooms period (Fig. 5B, C). The first 2 of these coincided with the first 2 chl a peaks. The third and largest ciliate peak occurred approximately 1 wk after the *Phaeocystis* maximum when aloricate choreotrichs approximately 20 µm in diameter predominated. Dinoflagellates (pre66



dominantly *Gyrodinium* spp.) also increased during the decline of the *Phaeocystis* with the maximal abundance and biovolume occurring on Day 169 (Fig. 5B, C). Mesozooplankton were not effectively sampled in this study, but their larvae were present in the sample bottles in small numbers (maximal abundance very approximately 100 larvae dm<sup>-3</sup>).

Bacteria numbers showed a gradual increase from  $3 \times 10^5$  cells cm<sup>-3</sup> on Day 52 to  $4.5 \times 10^6$  cells cm<sup>-3</sup> on Day 137 (Fig. 5D). A second more protracted peak (> $3 \times 10^6$  cells cm<sup>-3</sup>) occurred between Days 153 and 168 and numbers then declined to  $1.5-2 \times 10^6$  cells cm<sup>-3</sup>. On average 61% (45 to 92%, n = 14) of the unfractionated bacterioplankton assemblage was isolated in the <0.8 µm fraction and this fraction had no significant eucaryote abundances. Although bacterial cell sizing was not undertaken, those that were sized were mostly 0.4 to 0.5 µm diameter cocci.

The thymidine conversion factor experiment yielded a factor of  $2.76 \times 10^{18}$  cells mol<sup>-1</sup>, which lies within the range reported in the literature (median  $2 \times 10^{18}$ , range  $1 \times 10^{17}$  to  $60 \times 10^{18}$  cells mol<sup>-1</sup>; Ducklow & Carlson 1992). Using this factor the estimates of growth from TTI and changes in cell numbers in the <0.8 µm fraction were similar, with no significant growth before Day 130 (Fig. 5D). The estimates of net growth, from changes in cell numbers in the unfractionated sample, similarly showed no significant net growth before Day 130 (Fig. 5D). There were 2 peaks in positive net growth, Days 130 and 145, and 2 periods of negative net growth, Day 137 and Days 153 to 168.

Unfractionated GCP followed the same trend as the chl *a* concentration although the second 2 peaks were less well defined (Fig. 5E). Size fractionation showed that during the mixed diatom and *Rhizosolenia delicatula* blooms, approximately 20% of the unfractionated GCP was accounted for by the <20  $\mu$ m fraction. This percentage contribution continued to remain low, at 16 to 20% of the unfraction-

Fig. 5. The 1994 spring and early summer patterns in the Menai Strait for the unfractionated plankton assemblage (Unfractionated), and where available, the <200, <53, <20, and <0.8 µm fractions. Parameters shown are (A) chlorophyll a; (B) diatom, *Phaeocystis* (Phaeo.), ciliate and dinoflagellate (dino.) cell counts; (C) diatom, ciliate and dinoflagellate (dino.) biovolumes; (D) bacterial abundance (Bact. No.), bacterial net growth rate (Net growth), bacterial growth rate (Growth), and tritiated thymidine incorporation rate (TTI) using a conversion factor of 2.76 × 10<sup>18</sup> cells mol<sup>-1</sup>; (E) gross community O<sub>2</sub> production (GCP); (F) dark community O<sub>2</sub> respiration (DCR); (G) net community O<sub>2</sub> production (NCP); and (H) nitrate concentration

ated value, during the *Phaeocystis–Rhizosolenia* bloom. Two peaks were observed in DCR (Fig. 5F) over the spring period. Respiration was largely attributable to the smallest size fractions. The <0.8  $\mu$ m fraction on average accounted for 49% (range 21 to 101%, n = 13) of unfractionated DCR. This contribution was highest (>70%) during the early phases of the *Rhizosolenia* and the *Phaeocystis* blooms. Unfractionated NCP exhibited 2 peaks (Fig. 5G). The second of these, the *Phaeocystis–Rhizosolenia* bloom, was succeeded by a net heterotrophic phase lasting approximately 12 d.

A 2 phase decrease in nitrate concentration was observed (Fig. 5H). The second, smaller phase (Days 123 to 137), coincided with the rise of the *Phaeocystis* bloom. This bloom reduced the nitrate concentration to below the limit of detection.

An area budget for NCP over the bloom period was estimated for a representative  $1 \text{ m}^2$ cross-section of the Menai Strait, North Wales. Secchi disc depths were used to estimate  $k_d$ , and assumptions made that GCP was proportional to light and DCR constant with depth. NCP for the cross-section equals the numerically integrated GCP minus the integrated DCR. This value was divided by the cross-sectional width to derive the average NCP per unit area (Fig. 6).





## Time course experiments of respiration

Time course experiments were undertaken to determine if major artifacts occurred in the <0.8  $\mu$ m fraction due to removal of predator pressure. Respiration was linear with time during the 2 separate experiments. Bacterial counts were measured during the second time course experiment (Day 160 of 1994, Fig. 7). These showed no obvious increase in bacterial abundance in the unfractionated sample, but a marked increase in abundance in the <0.8  $\mu$ m fraction.



Fig. 7. Oxygen concentrations and bacterial abundances during the time course experiment for dark community respiration on Day 153 in 1994. Upper curves show the least squares linear regressions for the oxygen replicates; lower curves show the bacterial abundances  $\pm 1$  SD

#### DISCUSSION

The Menai Strait is a shallow turbulent stretch of water separating the island of Anglesey from the mainland of North Wales. The estimated magnitude and direction of residual flow (Harvey 1968) suggest that plankton sampled from Menai Bridge largely originates from the adjacent coastal waters of Liverpool Bay, with the advected plankton residing in the Strait for approximately 2 d. A density discontinuity isolates Liverpool Bay and fluvial inputs lead to nutrient buildup during winter months (Foster et al. 1982). The nitrate concentrations measured in the present study compare well with previous observations (Ewins & Spencer 1967).

The general patterns of chl *a* concentrations during the spring bloom periods of the 3 yr of this study conformed to the succession typical for the area: a mixed diatom bloom in March and April followed by blooms of diatoms and *Phaeocystis* in May and June (Jones & Haq 1963, Jones & Spencer 1970, Al-Hasan et al. 1975). For each of these years, the annual chl *a* maximum coincided with the presence of *Phaeocystis* in the Strait.

In 1994, the measurement of chl a was supplemented with phytoplankton and microheterotroph cell counts and biovolume estimates. These measurements are difficult to make without error: the cell abundances estimated by the inverted technique are probably underestimates because of incomplete settling and shielding by detritus. Furthermore, there is now evidence that the interpretation of the standard DAPI count for bacteria is not straightforward (Suzuki et al. 1993, Zweifel & Hagström 1995).

During the first chl a maximum (Days 116 to 123), the contribution of small colonial diatoms to total diatom numbers was greater than 80 %. Previous studies in the Strait have also recorded these small diatoms, especially Skeletonema costatum, to predominate in the early spring period. These diatoms possess high surface area: volume ratios and maximal growth rates and characterize the first stage of the typical succession (Margalef 1958), However, the biovolume contribution of these small diatoms was only 40%, with the larger diatom Ditylum brightwelli accounting for a further 35 to 40%. The D. brightwelli, the small diatoms and the obvious detrital loading largely disappeared from the water column over the course of a week (Days 123 to 130), suggesting advection or sinking losses to be important. This is consistent with previous studies of coastal diatom blooms (Kiørboe 1993).

The second chl a maximum (Day 130) corresponded to a Rhizosolenia delicatula (a medium-sized stage 2 diatom; Margalef 1958) bloom in its mid-late exponential phase. The increases in bacterioplankton and ciliate numbers associated with this exponential phase is in keeping with a very close coupling between the autotrophs and microheterotrophs. This Rhizosolenia bloom persisted for a further 30 d, overlapping with the Phaeocystis bloom that reached its maximum abundance on Day 145, the day of the third chl a maximum. The morphology of unfixed bladders best fitted Phaeocystis globosa characteristics as described by Baumann et al. (1994), and the maximum Phaeocystis cell count of approximately  $5 \times 10^6$  cells dm<sup>-3</sup> falls at the lower end of the range previously recorded for the Strait (1.4 to  $100 \times 10^6$  cells dm<sup>-3</sup>; Jones 1968, Tyler 1977, Lennox 1979). For this bloom, the autotroph-heterotroph coupling appeared looser, with a delay of approximately 1 wk between the Phaeocystis maximum and the following bacterioplankton and ciliate increase; similar delays have been documented for Phaeocystis blooms in the coastal waters of the German Bight (Laanbroek et al. 1985, Billen & Fontigny 1987, van Boekel et al. 1992). The dinoflagellate assemblage also exhibited a distinct maximum (Day 168) after the Phaeocystis bloom when chl a and gross community production were very low, suggesting this assemblage to have an important heterotrophic component. The spring bloom succession finished with an autotroph assemblage dominated in numbers by small cryptophytes.

After the spring blooms in 1992 and 1993, phytoplankton biomass remained low, with intermittent small, probably diatomaceous blooms, in agreement with previous observations in the Strait (Jones" & Spencer 1970). The 1993 late summer diatom bloom was essentially monospecific; this is also in accordance with Jones & Spencer (1970). In Liverpool Bay, the breakdown of the density discontinuity in late summer or early autumn influenced the timing of later diatom blooms (Voltalina et al. 1986) and blooms in the Strait may be similarly influenced.

The main mixed *Phaeocystis* and diatom bloom in 1992 occurred approximately 20 d earlier than in 1993 and 1994. Similar variability was observed previously in the Strait by Jones & Haq (1963), who suggested that water temperature in early spring influenced the timing of the bloom. Our limited observations of water temperature and air temperature readings offer support for this contention; although other factors such as detrital loading (Jones & Spencer 1970) may also be important.

The general patterns and scales of GCP are similar for all 3 yr and are in broad agreement with previous <sup>14</sup>C studies in the Menai Strait (Al-Hasan 1976, Bajpai 1980). They are also comparable to those reported for Liverpool Bay in the spring (4 to 141 mmol C m<sup>-2</sup> d<sup>-1</sup>; Savidge & Kain 1990). Respiration has not been measured previously in the area, but our measurements are comparable to those reported during the spring bloom period in the German Bight (Laanbroek et al. 1985).

The correlation analysis showed DCR to be highly correlated to GCP and not significantly related to temperature (Table 1). This contrasts with temperate estuarine studies that usually report a better correlation between DCR and temperature (e.g. Smith & Kemp 1995) and is consistent with an ecosystem whose pelagic metabolism is based on phytoplankton production rather than allochthonous inputs (Hopkinson 1985). However, temperature was the best predictor for chl a-specific GCP in accordance with previous observations in the Strait and other temperate waters (Fogg & Bajpai 1988). Harrison (1986) reported that chl a-specific GCP increases with decreasing cell size. Our size fractionation data for 1994 (Fig. 8) generally does not show this relationship and so a shift in the size structure of the phytoplankton community towards smaller forms cannot account for the correlation with temperature; this agrees with Fogg & Bajpai (1988). These authors discussed other possible explanations, including shade adaptation and ribulose bisphosphate carboxylase (RUBISCO) activation.

There is a potential error involved in measurement of size fractionated metabolism due to the increase of fast growing organisms as a result of the removal of predators. With this in mind, we undertook time course measurements of DCR. During the time course experiment for DCR on Day 159 the increase in bacterial numbers was not accompanied by an increase in DCR; this is in accordance with the observations of Pomeroy et al. (1994). Although size fractionation is reported as sometimes producing enhanced respiration rates (e.g. Hopkinson et al. 1989), the only data point in our stud-

Table 1. Correlation coefficients between temperature, chlorophyll a, bacterial abundance, ciliate abundance, gross community production (GCP), dark community respiration (DCR), tritiated thymidine incorporation rate (TTI), <0.8  $\mu$ m fraction respiration rate (< 0.8  $\mu$ m DCR), bacterial cell-specific tritiated thymidine incorporation rate (TTI cell<sup>-1</sup>), <0.8  $\mu$ m fraction cell-specific respiration rate (<0.8  $\mu$ m DCR cell<sup>-1</sup>), and chlorophyll *a*-specific gross community production (GCP chl *a*<sup>-1</sup>) for the Menai Strait in 1994 (n = 13). Significant at \*p < 0.05, \*\*p > 0.01, \*\*\*p < 0.001

	Temp.	Chl a	Bacteria numbers	Ciliate numbers	GCP	DCR	TTI	<0.8 µm DCR	TTI . cell <sup>-1</sup>	<0.8 µm DCR cell <sup>-1</sup>	GCP chl a <sup>-1</sup>
Temperature Chlorophyll a Bacteria numbers Ciliate numbers GCP DCR TTI <0.8 µm DCR TTI cell <sup>-1</sup> <0.8 µm DCR cell GCP chl a <sup>-1</sup>		0.04	0.73** 0.21 -	0.47 0.45 0.64*	0.11 0.95••• 0.42 0.59•	0.51 0.70• 0.53• 0.81•• 0.72••	0.67* 0.72* 0.78** 0.44 0.75* 0.56 -	0.67 - 0.51 0.71 - 0.82 0.60 - 0.94	0.40 0.71 0.30 0.12 0.60 0.47 0.80 0.47	0.32 0.62* 0.71** 0.41 0.61* 0.71** 0.49 0.69** 0.51	0.65* -0.34 0.41 0.05 -0.21 -0.10 0.28 0.16 -0.14 0.07 -



Fig. 8. Chlorophyll a-specific gross community  $O_2$  production (GCP chl  $a^{-1}$ ) during the spring blooms period in the Menai Strait in 1994 for the unfractionated plankton assemblage (Unfractionated), and <200, <53 and <20 µm fractions

ies consistent with such an effect is the Day 146 sampling of 1993. We used a cross-flow filtration system for the first time for the preparation of this sample and are inclined to attribute the anomalous result to our inexperience with this technique.

During the spring bloom period of 1994, the fractionations revealed micro- and mesophytoplankton to dominate autotroph production and biomass, which is typical for temperate coastal spring blooms (Kiørboe 1993). However, although the nanophytoplankton contribution to total GCP during this bloom period was small (20%), respiration by the organisms in this size range was substantial, accounting for approximately 70% of the total DCR. This differential effect of size fractionation on GCP and DCR has been reported previously for diatom bloom communities (e.g. Williams 1981, Harrison 1986, Smith et al. 1986). The results of the 0.8 µm fractionations showed this effect to be largely attributable to the activity of the bacterioplankton in accordance with earlier studies (Williams 1981, Harrison 1986).

We have combined the <0.8  $\mu$ m fraction DCR and the DAPI bacterial counts in order to estimate the cellspecific respiration rates (Fig. 9A). There are clearly uncertainties to these calculations — the DAPI count may potentially grossly overestimate the number of metabolically active cells (Zweifel & Hagström 1995), thus the rate may be proportionately underestimated. The range (0.4 to 6.8 fmol O<sub>2</sub> cell<sup>-1</sup> d<sup>-1</sup>) is similar to that



Fig. 9. Bacterioplankton metabolism during the spring blooms period in the Menai Strait in 1994. Showing (A) cell-specific respiration (DCR) and tritiated thymidine incorporation (TTI) rates, and (B) the bacterial contribution (bacteria) to unfractionated plankton assemblage (Unfractionated) respiration. This contribution is estimated by dividing the <0.8  $\mu$ m fraction respiration rate by the fraction of the unfractionated bacmetabolism bloom is also as the second se

terial assemblage isolated in the <0.8 µm fraction

estimated by Biddanda et al. (1994) for the <1  $\mu$ m fraction in Louisiana (USA) shelf waters: 2.4 to 8.7 fmol O<sub>2</sub> cell<sup>-1</sup> d<sup>-1</sup>.

The 3 distinct peaks in the cell-specific respiration rates, coincident with the 3 phytoplankton blooms (Fig. 9A), are consistent with a very close coupling between bacterial cell respiration and phytoplankton production and biomass. The bacterial cell specific TTI rates showed a similar trend (Fig. 9A). As the percentage of the total bacterioplankton assemblage isolated in the <0.8 µm fraction varied considerably, multiplication of the cell-specific respiration rates by the total bacterial abundance gives a better assessment of the bacterial contribution to unfractionated DCR (Fig. 9B). This shows the dominant contribution of the bacteria to unfractionated metabolism, but also identifies 4 sampling points (Days 116, 130, 145 and 153) as having substantial non-bacterial components. The Days 116, 130 and 145 correspond to the 3 chl a maxima, suggesting that autotroph dark respiration may have been significant. This is in agreement with previous studies (e.g. Iriarte et al. 1991). The sampling point on Day 153 corresponds to the distinct maximum in ciliate abundance concomitant with the declining Phaeocystis, which suggests that these microheterotrophs may be significant respirers. However, an upper limit on the ciliate respiration contribution for Day 153 can be estimated from the ciliate respiration rates reported in Fenchel & Finlay (1983). This suggests ciliates could have accounted for a maximum of 25% of the nonbacterial respiration. Other major microhetereotrophic respirers may have included nano- and picoflagellates, a group that unfortunately were not enumerated in this study, and mesozooplankton larvae. The biomass and metabolism parameters for the <53  $\mu$ m fraction (Fig. 10) also show that an increase in the dark respiration of Phaeocystis cannot be excluded. Indeed Phaeocystis cells face new energetic demands during the blooms' senescent phase, e.g. flagella synthesis (Rousseau et al. 1994), and the reassimilation of colonial matrix components (Lancelot & Mathot 1985,



Fig. 10. Parameters for the <53 µm fraction on Day 153 of 1994 is percentages of the unfractionated plankton assemblage parameters

Veldhuis & Admiraal 1985) may be able to support increased respiration as photosynthesis declines.

Correlation analysis of the 1994 data set (Table 1) showed ciliate abundance to be the best biomassrelated predictor of unfractionated DCR. However, because we did not enumerate nano- and picoflagellates, we cannot rule out the possibility that the ciliates were simply a proxy for these protozoans. The analysis also showed the TTI and  $<0.8 \mu m$  fraction respiration rates to be correlated with temperature, and more closely correlated with GCP and chl a than bacterial abundance. Analysis of bacterial cell-specific metabolic rates (TTI cell<sup>-1</sup> and <0.8 µm DCR cell<sup>-1</sup>) revealed significant correlations with GCP and chl a, but no close relationship with temperature. This indicates that biological parameters (e.g. substrate quality and quantity) limit bacterial metabolism rather than temperature, contrasting with the observations of Shiah & Ducklow (1994a, b) for non-summer conditions in a temperate estuary.

The phasing of GCP and DCR can be considered by plotting the 2 parameters against one another, as in Fig. 11. The separation of GCP and DCR maxima in time for the Phaeocystis bloom is similar to the pattern observed in space for an autotroph-heterotroph succession along a river length (Odum 1956). In our study, the phasing of GCP and DCR consisted of periods of positive production relaxing into negative production, a temporal succession similar to the longitudinal (i.e. time-dependent) succession reported for the Peruvian upwelling zone (Vinogradov & Shushkina 1978). Simple calculations of volume-based rates of NCP are misleading because of the difference in the depth distribution of GCP and NCP in mixed water. Areanormalised rates are more meaningful. Our estimates of area NCP (using Secchi depths to estimate  $k_d$ ) integrated for the bloom period in 1994 suggest that metabolism within the water column approximately balanced. Although use of the Secchi depth to estimate k<sub>d</sub> is not ideal (Kirk 1994), previous work in the Strait has shown this depth is well correlated with  $k_d$  (D. Bowers pers. comm.). The phasing of GCP and DCR for the diatom blooms was different from that for Phaeocystis, i.e. the absence of an obvious DCR maxima in association with the mixed diatom blooms, and the distinct DCR maxima in phase with the rise of the Rhizosolenia delicatula in 1994.

The aim of this study was to address the basis of this phasing and to consider the mechanisms involved. We have identified heterotrophic bacterioplankton as major respirers; thus the time scales of the different routes by which autotroph organic matter becomes available to the bacterioplankton are critical in explaining the phasing of GCP and DCR over the bloom periods. We now consider these routes in an



Fig. 11. Phase plots of photosynthesis versus respiration in the Menai Strait for (A) 1992, (B) 1993 and (C) 1994. Arrows indicate the temporal sequence, D denotes a diatom maximum,

and P denotes a Phaeocystis maximum

effort to explain the different DCR responses for the 3 bloom types: the mixed diatom blooms, the *Rhizosole-nia delicatula* bloom, and the *Phaeocystis* blooms.

There are a number of possible pathways of organic matter transfer between phytoplankton and bacterioplankton. The following section is an attempt to ascribe broad time scales (e.g. 1 d, 1 wk, 1 mo) to these various steps, using data, where possible, from similar ecosystems and similar times of the year. The principal routes linking microbial photosynthetic and respiratory activity are shown in a simplified diagram (Fig. 12). The key assumptions in this scheme are as follows:

(1) Phytoplankton exudation is closely associated with photosynthesis and biomass and largely produces LMW material (Bjørnsen 1988) (Pathway I).

(2) The pool of LMW material is readily assimilable by bacteria and is turned over on a time scale of  $\leq 1$  d (e.g. Fuhrman 1987) (Pathway II).

(3) LMW material constitutes a small amount of the total organic material present in a phytoplankton cell (e.g. Smith & Geider 1985), so cell lysis (self or viral) and breakage (sloppy feeding) largely introduce high molecular weight (HMW) material into the water column (Pathway III).

(4) The HMW material arising from these sources must be hydrolysed by ectoenzymes before it can be assimilated by the bacterioplankton (Chróst 1990a). The derepression and induction of these enzymes is a rapid process (<1 d, Chróst 1990b), and their activity results in the HMW pool turning over every few days to a week (e.g. Billen 1990) (Pathway IV).

(5) Nano- and microzooplankton maximal generation times (<1 d) are similar to those of the phytoplankton and their potential grazing activity is proportional to their biomass. A significant fraction of their organic intake is egested concurrently, with the assimilation

Fig. 12. Pathways linking gross community production (GCP) and dark community respiration (DCR) within a microbial foodweb. LMW: low molecular weight organic matter, HMW: high molecular weight organic matter; I: phytoplankton exudation; II; bacterial uptake; III: phytoplankton lysis; IV: ectoenzyme catalysed hydrolysis; V: microzooplankton grazing; VI: mesozooplankton grazing, VIIa: phytoplankton sedimentation, VIIb: detritus resuspension; VIII: release of hydrolysate derived from epiphytic bacterial activity. Numbers in parentheses indicate secondary pathways. Values in parentheses within the boxes are estimates of (biomass) doubling times



efficiency inversely proportional to grazing rate (Nagata & Kirchman 1992) (Pathway V).

(6) Mesozooplankton generation times ( $\geq 1$  mo, e.g. Klein Breteler et al. 1982) are much longer than those of the nano- and microzooplankton, so they show no numerical response during a phytoplankton bloom; however, their larvae can exhibit abundance pulses during a bloom. Mesozooplankton potential grazing activity is proportional to their biomass and a significant proportion of their organic intake is egested and excreted concurrently (Jumars et al. 1989, Kiørboe 1993) (Pathway VI).

For the first bloom type, the mixed diatom blooms, an increase in bacterial cell-specific respiration was observed in phase with GCP in 1994, but low bacterial biomass meant this was not paralleled by an obvious unfractionated sample DCR response. Bacterial growth rate estimates (TTI and changes in cell numbers over 24 h) also indicated low bacterial activity. These observations suggest that the passage of organic matter from the diatoms to the bacterioplankton was minimal and only sufficient for maintenance of the bacterioplankton community; although the substrate concentration required for bacterial cell growth may have been higher during this phytoplankton bloom than during the following ones because of the lower water temperature (Wiebe et al. 1993). Given the high GCP during the bloom, it is evident that much of the production was lost elsewhere. If mesozooplankton grazing was the predominant loss factor, we would expect (see point 6) that the flux of organic matter to the bacterioplankton should have been more substantial. By elimination this suggests physical losses (e.g. pathway VIIa, phytoplankton sedimentation, with no significant immediate resuspension) most likely predominated, which is in agreement with previous studies for coastal spring diatom blooms (Kiørboe 1993).

The second bloom type, the exponential phase of the Rhizosolenia delicatula bloom (e.g. ca Day 130), was characterized by a large concurrent increase in bacterial cell-specific respiration and growth rates, increasing bacterial abundance and a distinct ciliate biovolume maximum. Community bacterial growth rate estimates (TTI and changes in cell numbers over 24 h) also indicated high bacterial activity and net growth. The bacterial abundance maximum was observed the following week (Day 137) when both cell-specific respiration and growth rates had decreased. Consideration of the most probable pathways of organic matter transfer to the bacteria, given this very close coupling, suggests the main routes to be phytoplankton exudation (I) and the LMW forks of the grazing pathways (V and VI). Significant flux to the bacterioplankton via phytoplankton exudation during the exponential

growth phase of phytoplankton blooms has been reported previously (e.g. Chróst 1990b). Indeed, flux through the phytoplankton exudation pathway, whether this pathway is an overflow response (Fogg 1983), or a passive loss process (Bjørnsen 1988), would be more substantial during this bloom than during the earlier mixed diatom bloom.

Finally, the third bloom type was the Phaeocystis bloom maximum (e.g. ca Day 145), characterized by large concurrent increases in bacterial cell-specific respiration and growth rates and increasing bacterial and ciliate abundance. Community bacterial growth rate estimates (TTI and changes in cell numbers over 24 h) also indicated high bacterial activity and net growth. Maximal bacterial and ciliate abundances were observed the following week (Day 153) when the bacterial cell-specific respiration rate was still elevated. This continuation of elevated bacterial cellspecific respiration rates contrasts with the Rhizosolenia delicatula bloom observations and is the major explanation for the delay between the GCP and DCR maxima. A possible explanation for this persistence of bacterial cell respiration is a change in the major pathway of organic matter transfer to the bacterioplankton from those directly feeding into the LMW pool to those producing HMW material (e.g. phytoplankton lysis (IV) and the HMW forks of the grazing pathways (V and VI)). Our observations of an elevated rate of cell-specific respiration persisting, whilst the bacterial cell-specific tritiated thymidine incorporation rate decreased (i.e. growth efficiency decreased), are consistent with increased bacterial utilization of nitrogen deficient organic material (e.g. carbohydrates). Indeed, very high concentrations of polysaccharides have been reported during Phaeocystis blooms (e.g. Eberlain et al. 1985). Further, the Day 159 time course experiment observations for the < 0.8 µm fraction of an increase in bacterial numbers, but linearity in oxygen consumption, are consistent with substrate limitation of the bacterioplankton, with hydrolysis of HMW material constituting the rate limiting step. Bacterial colonization of Phaeocystis bladders was also observed in the sample taken on Day 159. Similar colonization has been reported previously (Putt et al. 1994). Epiphytic bacteria are reported as net releasers of hydrolysate during the decline of a diatom bloom (Middelboe et al. 1995) and thus their activity may have contributed to the LMW pool. Significant flux via an HMW pool during a Phaeocystis bloom was previously inferred by Billen & Fontigny (1987). The flux of organic matter through the grazing activity of the mesozooplankton during the Phaeocystis bloom was probably small, as only low copepod grazing rates (<1.5% d<sup>-1</sup>) on Phaeocystis have been reported for Liverpool Bay (Claustre et al.

1990) and the Southern Bight (Hansen & van Boekel . 1991).

In summary, our observations over the 1994 spring bloom period are consistent with 3 distinct phases. An initial mixed diatom bloom with limited transfer of organic material to the bacteria, a *Rhizosolenia delicatula* bloom with flux of material to the bacteria via an LMW pool, and finally, a *Phaeocystis* bloom with transfer of material predominantly via an HMW pool. This HMW pool and other temporary storages of non-living organic material (Williams 1995) may support microheterotroph metabolism after the decline of photosynthesis resulting in a temporal positive-negative sequence of net community production.

Our analysis of the basis of the phasing differences in GCP and DCR involved a number of speculations. It nonetheless highlights areas of incomplete understanding (e.g. the partitioning of the DOM pool and the identification of the non-bacterial respirers) and creates an initial framework for modelling and other experimental studies.

Our conclusions demonstrate that the annual microbial cycle for the Menai Strait is comprised of a mixed diatom bloom in late March or April followed by blooms of diatoms and Phaeocystis in May and June. This succession is followed by low phytoplankton biomass and production with intermittent smaller blooms. Size fractionation showed that meso- and microphytoplankton dominated phytoplankton production and biomass over the spring bloom period and nanophytoplankton dominated during summer when activity and biomass were low. Throughout the year, bacterioplankton were shown to be the major respirers and so the phasing of GCP and DCR was strongly influenced by bacterioplankton metabolism and abundance changes. Correlation analysis showed temperature to be the best predictor for chl a-specific GCP. Conversely, bacterial cell-specific metabolic rate parameters were not significantly correlated with temperature. We argue that this is consistent with biological parameters limiting bacterial metabolism rather than temperature. For the mixed diatom bloom in all 3 yr, no obvious DCR response was observed. We believe the most likely explanation to be limited transfer of organic material to the bacteria because physical phytoplankton losses predominated. For the exponential phase of the Rhizosolenia delicatula bloom in 1994, unfractionated GCP and DCR maxima occurred in phase; this very close coupling was attributed to the link between the phytoplankton and the bacterioplankton occurring largely via the LMW pool. For the Phaeocystis bloom in all 3 yr, there was a delay of 1 to 2 wk between GCP and DCR maxima. Consideration of the 1994 observations attributed this delay to the link between

the phytoplankton and the bacterioplankton occurring predominantly via an HMW pool. The consequence of this shift in the partitioning of the DOM pool was that microheterotroph respiration persisted after the decline of photosynthesis and this caused net community production to exhibit a positive-negative temporal sequence.

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