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THE STUDY OF THE DISSOLVED NITROGEN CONTAINING MACROMOLECULES IN THE MARINE ENVIRONMENT

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CONTENTS.

CHAPTER 5.

SEASONAL LEVELS AND COMPOSITION OF DISSOLVED FREE AND DISSOLVED COMBINED AMINO ACIDS IN LOCAL COASTAL WATERS.

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by

Jasmin Chapman.

ABSTRACT.

A method was developed for the direct determination of the dissolved combined amino acid (DCAA) content of seawater involving the acid hydrolysis of seawater under specified conditions, followed by the determination of the amino acids by reverse phase liquid chromatography (RPLC). No preconcentration or co-precipitation steps were required. Under optimal conditions approximately sixty percent of the total amino acids in the macromolecular fraction are probably recoverable by this procedure.

The commonly used reflux method for DCAA detection was shown to be inaccurate due to a combination of degradative losses and contamination.

The technique of DCAA determination, developed in this study, was not suitable for estuarine or other waters containing high nitrate levels, due to the occurrence of oxidative losses.

The seasonal relationship between the dissolved combined and the dissolved free amino acid levels and compositions, both at surface and at depth in the coastal waters of Plymouth UK, were studied.

Levels of dissolved combined amino acids were shown to be between 0.246 and 6.741 uM, with a mean of 1.7 uM, while the levels of dissolved free amino acids (DFAA) ranged from below the limits of detection of the analytical technique, to 9.372 uM, with a mean of 0.403 uM. The DFAA fraction showed large fluctuations in levels and composition. The ratio of the DCAA to the DFAA varied between 10:1 and 30:1 depending on season. The major acids of the combined fraction were glycine, histidine, aspartate, glutamate, alanine and valine. Whereas the dominant acids in the free fraction were aspartate, glutamate, serine, glycine and alanine. Evidence is presented that certain specific amino acids may used preferentially by the biota.

Fractionation studies, using ultrafiltration techniques, showed that the bulk of the dissolved combined fraction was of a small size with molecular weight less than 25,000.

Samples obtained from coastal waters, containing large numbers of phytoplankton, were shown to contain, in addition to the normal spectrum of free amino acids, certain compounds not normally found in seawater samples. These compounds were tentatively identified as glutamine, ethanolamine, taurine and glucosamine, from their RPLC retention times.

The results of this study emphasise that DCAA have a complex role in the nitrogen cycle of the marine environment.

CHAPTER 1.

INTRODUCTION.

1.1 Primary Productivity.

Phytoplankton are very small aquatic plants which are of central importance to the ecology of their environment as they form the base of the food chain (Clegg 1980).

Photosynthesis carried out by the phytoplankton converts light energy to chemical energy which is stored in the form of organic compounds such as carbohydrates, lipids and proteins. The amount of new organic matter produced by autotrophs (the phytoplankton and photsynthetic bacteria) is called primary production, while the amount produced per time unit (on a volume or area basis) is called primary productivity.

The equation,

$$6\ CO_2 + 6\ H_2O \xrightarrow[\text{chlorophyll}]{\text{light}} C_6H_{12}O_6 + 6\ O_2$$

is a summary of the photosynthetic reaction. The rates of carbon dioxide consumption and oxygen evolution are used to measure primary productivity. Darley (1982) discusses the practical methods used to determine the primary productivity and the limitations of these techniques. The most widely used method is the incorporation of

^{14}C-labelled bicarbonate. The radioactive bicarbonate is added to water samples in light and dark bottles which are incubated in situ, or in incubators, for 1 to 6 hours. After this the cells (and other particulate matter) are recovered by filtration and the radioactive count determined. In order to be able to convert the radioactive count to mgC assimilated it is necessary to known the total counts added, the total amount of inorganic carbon added and the counting effiency of the system being used.

The limitations of the labelled bicarbonate method are as follows. The containment of the water in bottles can lead to the growth of bacteria which will increase the respiration and lead to an inaccuracy in the determination of the figures for primary productivity. Containment may also effect the physiology of the phytoplankton cells. Species composition may vary due to the differences in the abilites of various species to adapt to the modified environment inside the bottle. Loss of the assimilated ^{14}C from cells may be caused by filtration and fixation processes and the release of dissolved organic carbon, in the form of extracellular products, by the cells. Darley (1982) concludes that "one must simply keep its, [the ^{14}C method], limitations in mind when interpreting data".

These possible changes in the nature of the samples are a fundamental difficulty encountered in the analysis of environmental samples.

Two theories have been put forward as to the factor controlling primary productivity (Flynn & Butler 1986).

The first can be described as the agricultural model being based on the growth of terrestrial plants. This leads to the concept of plant growth being dependent on the presence of the nutrients, nitrate and phosphate, in the soil. This concept of nutrient limitation was applied to the phytoplankton of the seas. However two difficulties were experienced when applying this agricultural model to the oceans. Firstly it cannot account for the species succession of phytoplankton seen in the natural enviroment. Secondly in temperate waters the growth of phytoplankton continues in the absence of nitrate during the summer months. McCarthy (1980) coined the phrase the "something for nothing" paradox to highlight this anomaly.

The second theory as to the control of primary productivity is the predator/prey model which considers that grazing by heterotrophs limits productivity.

1.2 Dissolved Organic Nitrogen (DON).

Duursma (1965) recognized 4 main groups of dissolved organic compounds. These are;

1. Nitrogen free compounds,
2. Nitrogenous compounds including amino acids and peptides,
3. Fat like substances,
4. Complex substances such as Humics that are derived from groups 1 and 2.

The external sources of dissolved organic material of which DON is a part are the atmosphere, rivers and sediments. While the internal sources of organic material are phytoplankton exudation, zooplankton excretion, sloppy feeding by zooplankton, cell lysis and decay. (Johannes & Webb 1970, Menzel & Ryther 1970, Sharp 1975, Williams 1975, Gagosian & Lee 1981, Eppley et al. 1982, Dagg et al. 1982, Bidigare 1983, Fogg 1983, Laanbroek et al. 1985, Mopper & Zika 1987). Fig. 1.1 taken from Parsons et al. (1984) illustrates the origins of dissolved organic matter in the oceans.

There is an inverse relationship between nitrate and DON levels. Therefore as nitrate declines in the summer the DON levels increase and as the DON falls in the winter nitrate levels rise. This can be seen pictorially in Fig. 1.2 taken from Butler et al. (1979).

The fundamental question as stated by the Royal Society Study Group (1983) "is whether during the summer months when inorganic nitrogen concentrations in the euphotic zone are low, the phytoplankton utilize as a source of nitrogen at least part of the large quantities of DON which are present". If components of the DON are used by the phytoplankton as a nutrient source to support growth then primary productivtiy is more likely to be controlled by the predation of heterotrophs.

Without some knowledge of the constituents of the DON it is difficult to determine if it can be utilized as a nutrient source. Braven et al. (1984) state that "the amount of unidentified forms of nitrogen remaining in the

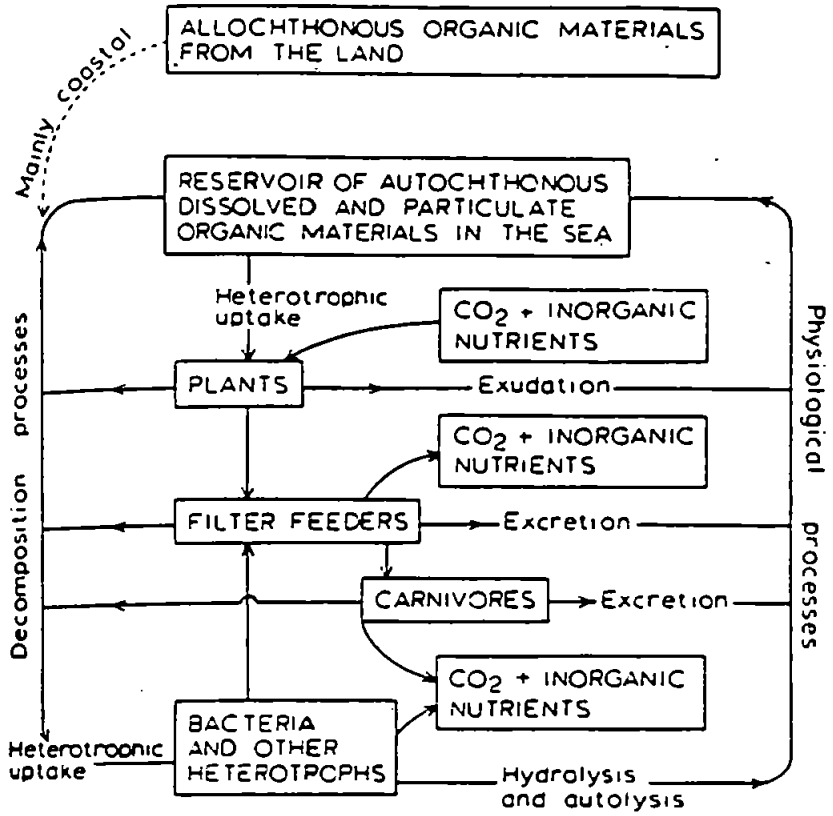


Fig 1.1 Pathways Of Transfer And Regeneration Of Organic Substrates In An Aquatic Ecosystem, (from Parsons et al. 1984).

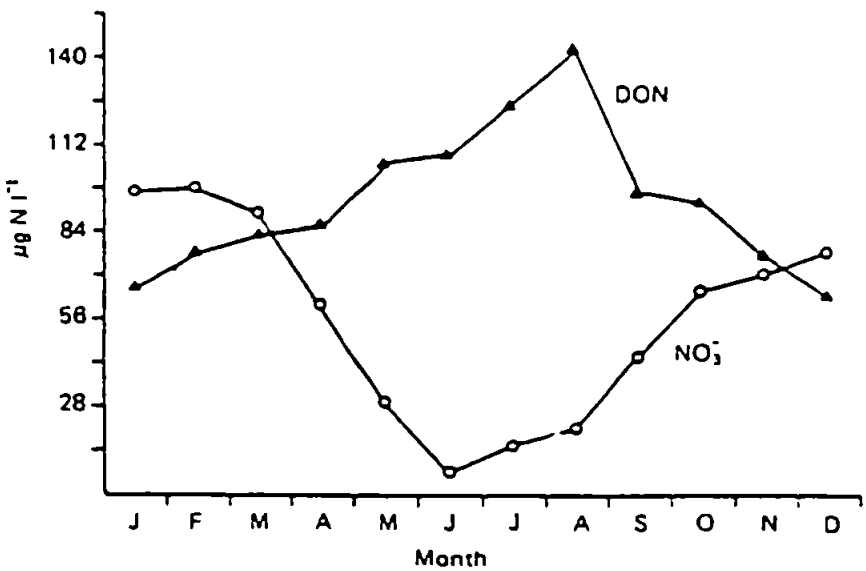


Fig 1.2 Diagram Showing The Inverse Relationship Between Dissolved Organic Nitrogen And Nitrate Levels Over The Season. (from Butler et al.1979).

upper layers of the sea is relatively large". Figures 1.3 a+b, taken from Braven et al. (1984) shows that in the summer months approximately 50 to 70 percent of the total dissoved nitrogen is unidentified. The authors were able to identify a significant fraction of the DON as dissolved free amino acids. Also they concluded that much of the remaining unidentified fraction present in the spring and summer was high molecular weight material and probably protein. Paul (1983) believes that "perhaps the most complex and least understood interactions in the nitrogen cycle in the marine environment involved dissolved organic nitrogen".

1.3 Dissolved Combined Amino Acids.

Hammer & Katner (1986) state that phytoplankton contain 1 to 3 percent nitrogen of which 15 to 20 % is dissolved free amino acids and 70 to 90% protein. Both the dissolved combined and dissolved free amino acids are derived from the metabolism and degradation of the particulate organic matter. The breakdown of the particulate material is thought to be due to an enzymatic hydrolysis by bacteria. [Daumas 1976, Bada & Lee 1977, Amano et al. 1982, Hollibaugh & Azam 1983, Azam et al. 1983, Lancelot & Billen 1984, Hagstrom et al. 1984, Bauerfund 1985, Furhman & Fergeson 1986, Kircham et al. 1986]. Fig. 1.4 redrawn from Daumas 1976 illustrates the sources and sinks of the dissolved combined amino acids in the marine environment. While fig. 1.5 redrawn from Duursma 1965 shows the breakdown products of proteins.

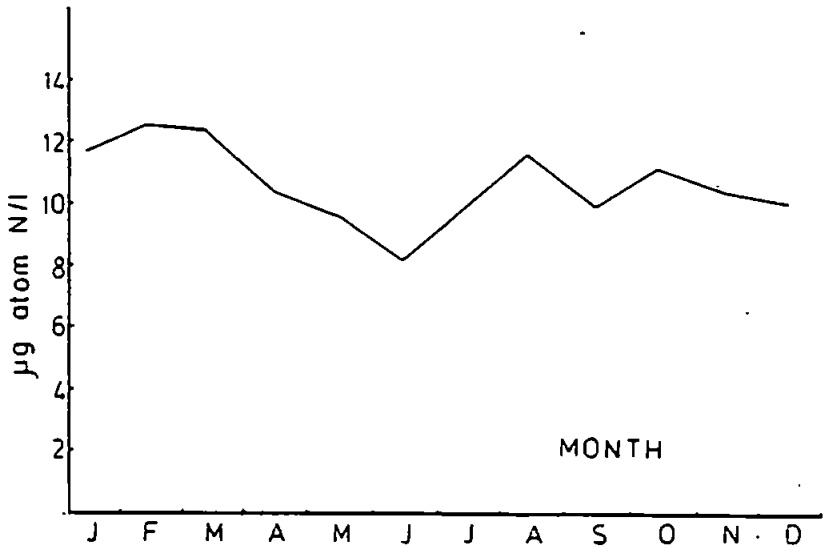


Fig 1.3a Total Dissolved Nitrogen Compounds In The Waters Of The Western English Channel. Average Of Values Over 8 Years. (from Butler et al.1979).

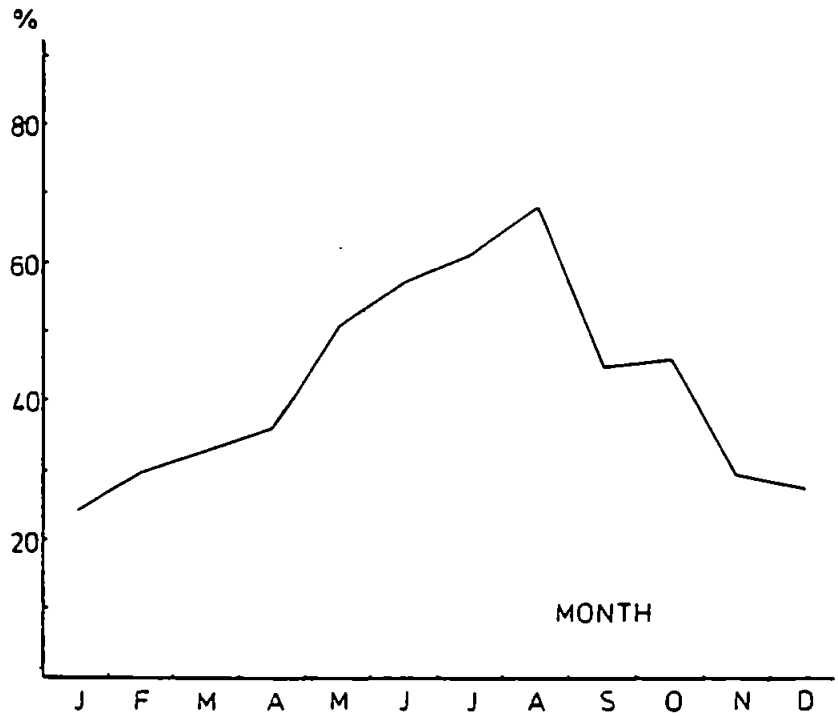


Fig 1.3b Unidentified Fraction Of The Total Dissolved Nitrogen Shown In Fig 1.3a After Allowing For Average Concentrations Of Ammonia, Nitrate And Urea, Expressed As A Percentage Of Total Nitrogen. (from Parsons et al. 1984).

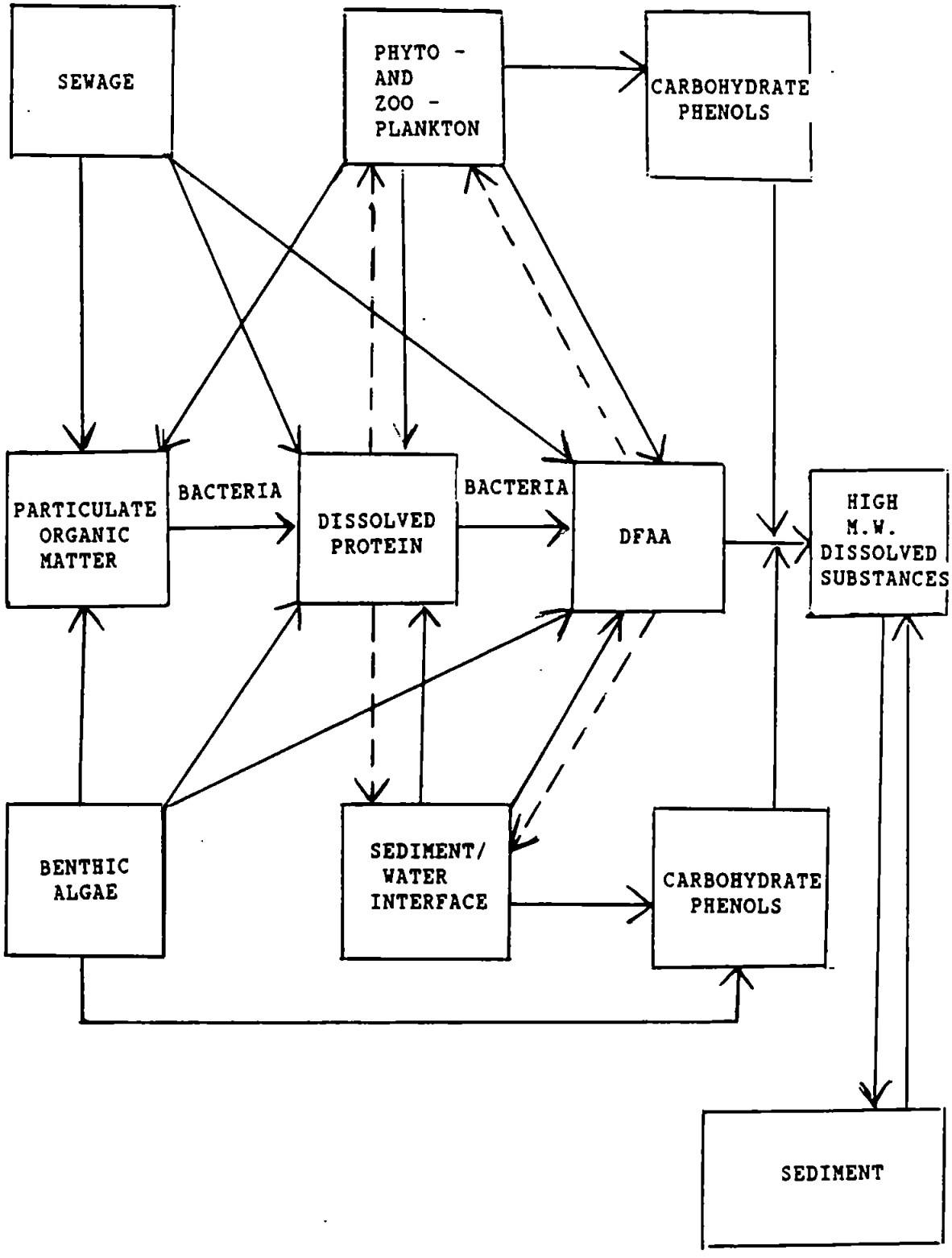


Fig 1.4 The Sources And Sinks For DCAA. (redrawn from Daumas 1976).

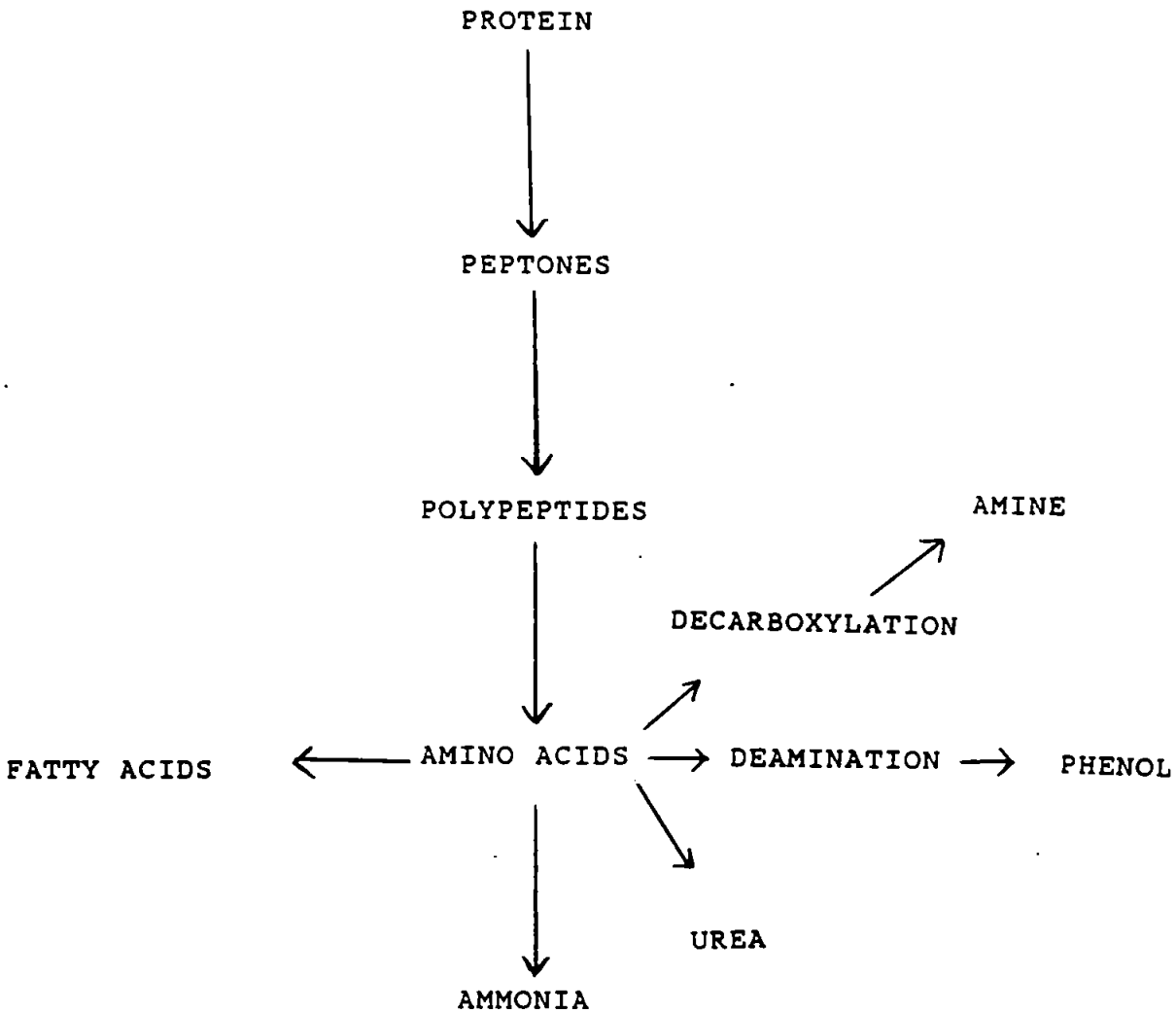


Fig 1.5 The Breakdown Products Of Proteins, (redrawn from Duursma 1965).

Hollibaugh & Azam (1983) added a known protein, bovine serum albumin which has a molecular weight of approximately 68,000, to seawater. The protein was degraded at a rate of 4% per hour with a maximum rate of 15% per hour at nine hours. Using gel filtration chromatography the authors showed that peptides of intermediate size between 68,000 and 700 molecular weight did not accumulate in the medium. It was also concluded that the dissolved combined and dissolved free amino acids derived from protein hydrolysis follow indepedent pathways.

The dissolved combined amino acids present in the marine environment is envisaged as containing proteins peptides, and nitrogen compounds bound in other forms. Lee (1988).

Dissolved combined amino acids composition and levels in seawater have been determined since the 1960's. [Parks et al. 1963, Degens et al. 1964, Seigel & Degens 1966, Riley & Segar 1970, Lee & Bada 1975, Daumas 1976, Lee & Bada 1977, Bada et al. 1982, Bolter & Dawson 1982, Henrichs & Williams 1985, Thurman 1986, Mopper & Zika 1987]. The most common hydrolysis method employed has been to reflux seawater with 6M hydrochloric acid at atmospheric pressure. All methods used a concentration step. The more stages there are to an analysis the more change there is of introducing a contaminant. Most analyses employed the ion or ligand exchange techniques discussed in section 1.4 to identify the free acids. The levels for the dissolved combined amino acids found by previous workers range from 0.40 to 4.534 uM.

1.4 Dissolved Free Amino Acids.

Flynn & Butler (1986) state that "the dissolved free amino acids are products of protein hydrolysis by bacterial proteases and may also be released by healthy as well as senescent phytoplankton.....There is also release of amino acids from invertebrates". Keller et al. (1982) gives the sources of dissolved free amino acids as phytoplankton release, zooplankton and macroalgae. While Lee (1988) discussing particulate amino acids considers the major source to be "the formation of phytoplankton biomass by photosynthetic processes in the euphotic zone". Fig. 1.6 taken from Doolittle (1985) gives the structures, size, polarities and commonly used abbreviations for the 20 amino acids occuring in proteins. Marine chemists are also interested in non-protein amino acids such as ornithine (Orn) which is a decomposition product of arginine.

Lee 1988 states that "in both seawater and sediments the major mechanism for the loss of amino compounds is heterotrophic decomposition by microbes, zooplankton or benthic macro-organisms". While Dring (1982) writes that "recent studies have shown amino acids to support the growth of most marine algae tested". Therefore the uptake of the dissolved free amino acids, which are part of the dissolved organic nitrogen, by phytoplankton and heterotrophs is well documented. [Hellebust & Guillard 1967, Williams & Gray 1970, Williams 1970, Andrews & Williams 1971, Crawford et al. 1974, Liu & Heelebust

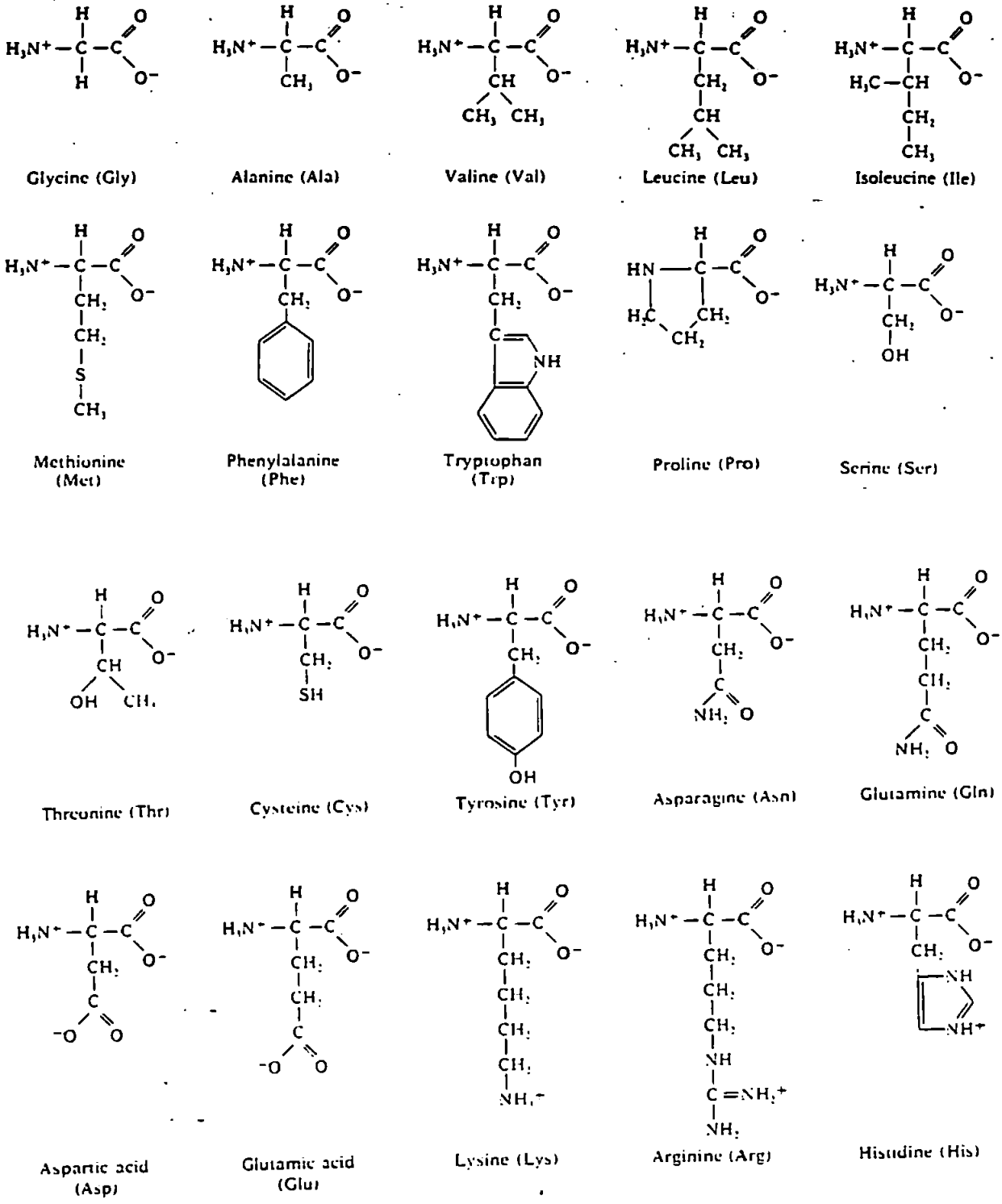


Fig 1.6. The Twenty "Protein" Amino Acids.

1974, Schell 1974, Wheeler et al. 1974, McCarthy et al. 1977, Fisher & Cawdell 1982, Lu & Stephens 1984, Admiraal et al. 1984, Ming & Stephens, 1985, Price et al. 1985, Admiraal et al. 1986, Carlucci et al. 1986 a+b, Flynn et al. 1986 a+b, Syrett et al. 1986, Vieira & Klaveness 1986, Wheeler & Kircham 1986].

Dissolved free amino acid analyses of marine samples have been carried out since the 1960's. [Tatsumoto et al. 1961, Parks et al. 1963, Degens et al. 1964, Chau & Riley 1966, Siegel & Degens 1966, Riley & Segar 1970, Bohling 1970, Andrews and Williams 1971, Kawahara & Maita 1971, North 1975, Daumas 1976, Josefsson et al. 1977, Lee & Bada 1977]. The early methods of dissolved free amino acid analysis required a concentration step to the ensure that the analyte was present in a sufficient quantity to produce a visible colour with the ninhydrin used to detect the compounds. The desalting technique used by early workers was a very lengthy process taking approximately two days; however, due to the labile nature of dissolved free amino acids, a short analysis time is desirable. Therefore the accuracy of some of the earlier data has been questioned due to the work of Dawson & Gocke (1978), Dawson & Mopper (1978), and Garrasi et al.(1979). These workers demonstrated that the ion exchange technique gave an over estimation of the dissolved free amino acids while the ligand exchange method gave an underestimation.

Another factor critical in the analysis of trace organics in seawater is to preclude the possibility of sample contamination from such sources as finger prints

and dust. (Hamilton 1965).

The problems of the analytical technique were largely overcome by the work of Lindroth & Mopper 1979, which used a fluorescent amino acid derivative with much lower limits of detection than the ninhydrin method. This meant that there was no longer any need to concentrate the environmental samples. The derivatives were separated using reverse phase liquid chromatography. Some of the workers who have published data for the dissolved free amino acid levels using techniques based on Lindroth and Mopper are Bolter & Dawson (1982), Keller et al. (1982), Ittekot (1982), Mopper & Lindroth (1982), Poulet et al. (1984), Braven et al. (1984), Jorgenson & Sondergaard (1984), Henrichs & Williams (1985), Eberlein et al. (1985), Liebezeit (1985), Hammer & Kattir (1986), Furham & Ferguson (1986), William & Poulet (1986). These workers report dissolved free amino acid values ranging from below the limits of detection of the method to 3.7 uM.

These analyses showed that many of the free acids were not present in the seawater. However, the ambient concentration of a compound cannot be taken as an indication of its importance. (Flynn & Butler 1986). A compound absent from the water or present at a low concentration supports two contrasting arguments. Dissolved constituents may be present at low concentrations because of the uptake by the biota, or, alternatively, they occur naturally at the low concentrations. It is therefore not the ambient concentration, but the flux which is an indicator of a compounds importance as a nutrient source. Amano et al.

(1982) point out that the main factor that determines the dissolved free amino acid composition of seawater has not yet been explained.

1.5 Aims of the Research.

The controversy surrounding the control of primary productivity in the oceans has been discussed earlier (section 1.1). Braven et al. (1984) shows that there is still a large fraction of the DON which is unidentified and which is of a large molecular weight. Without a knowledge of the nature of this material it is difficult to quantify the use of DON as a potential nutrient source. Determination of the dissolved combined and dissolved free amino acids in seawater would allow a comparison of both the levels and composition of these fractions which may answer the key question as to the flux of the acids and therefore their importance as a nutrient source in the natural marine environment.

Therefore the aim of the research project is to develop analytical methods for the reliable detection and determination of dissolved nitrogen containing macromolecules in seawater with particular reference to proteins. The investigation of the nature of the nitrogen containing macromolecules, (i.e. the dissolved combined amino acids), will include a determination of the levels and composition of the combined and free amino acid fraction to allow the flux of individual acids to be determined. The size of the macromolecular species will be investigated. Large molecules are unlikely to be used

directly, however small peptides may be taken up. Therefore a knowledge of the size of the nitrogen containing species may indicate how easily it can be used as a nutrient source. The relationship of the total dissolved amino acids i.e. combined plus free to the total dissolved nitrogen and dissolved organic nitrogen will be investigated.

Therefore the methods developed in this study, together with established methods, will be used to undertake a seasonal study of the coastal waters of the English Channel in order to increase the knowledge of the nature and role of dissolved organic nitrogen compounds in seawater.

CHAPTER 2.

AMINO ACID ANALYSIS.

2.1 Introduction.

The analysis of amino acids in sea water has been carried out since the 1960's, following the development of the amino acid analyser by Spackman et al.(1958). This method used ion exchange chromatography for separation of the amino acids, followed by post column reaction with ninhydrin for detection. Due to the lack of sensitivity of the ninhydrin detection method, at the very low concentrations of these compounds in sea water, early methods required a concentration step to enable the analyte to be measured. Tatsomoto et al. (1961), Parks et al. (1963), and Chau & Riley (1966) used co-precipitation of organic compounds with ferric hydroxide to preconcentrate. Later workers e.g. Riley & Segar (1970), Bohling (1970), Daumas (1976), Lee & Bada (1977), Dawson & Pritchard (1978) used ion or ligand exchange techniques for isolation of amino acids, i.e. desalting and enrichment, prior to analysis.

The accuracy of some of this earlier data, is however, in some doubt. Thus Dawson & Gocke (1978), Dawson & Mopper (1978) and Garrasi (1979) demonstrated that the ion exchange technique leads to an over estimation of free amino acids; while the ligand exchange technique of desalting and enrichment produces an underestimation of the dissolved free amino acids.

The work of Hamilton (1965) emphasizes the problems of contamination of the sample in amino acid analysis from such sources as fingerprints.

The problem of losses and contamination outlined above was largely overcome by Evens et al. (1982) using an HPLC method based on the work of Lindroth and Mopper (1979).

The present investigation was based upon the amino acid analysis method of Evens et al. (1982) which consists of the following steps;

1. Precolumn derivatization of an amino acid mixture with ortho-phthaldialdehyde (OPA) and 2-mercaptoethanol (MCE) in borate buffer to produce a highly fluorescent isoindole derivative.

2. Separation of the derivatives using gradient elution reverse phase high performance liquid chromatography (phosphate buffer and acetonitrile as mobile phases).

3. Fluorescence detection.

The following section gives a brief outline of the theory behind the analysis e.g. the derivatization of the amino acids, minor modifications made to the method during the current study, the criteria defining the analytical method such as linearity, limits of detection, precision and accuracy. Finally the storage and filtration of environmental samples are discussed.

2.1.1 The Derivatization Process.

Ortho-phthaldialdehyde derivatisation is based on the work of Roth (1971) and Roth & Hampai (1973). The amino acids are derivatized with ortho-phthaldiadehyde in the presence of the reducing agent, 2-mercaptoethanol, forming a fluorescent product. The reaction details and the nature of the adduct formed were determined by Simons & Johnson (1973). The reaction scheme are given in Fig. 2.1

2.1.2 Separation Technique.

The separation of the mixture of derivatized amino acids is accomplished using gradient elution, reverse phase liquid chromatography. The chromatographic column consists of a C-18 hydrocarbon covalently bonded to a silica support. The mobile eluting phase consists of a mixture of an organic solvent and an aqueous buffer. Different amino acids are separated according to their distributions between the mobile eluting phase and the stationary phase. If the fluorescent adduct is distributed predominantly in the mobile phase it will be rapidly eluted, molecules distributed mainly in the stationary phase will have longer elution times. The length of time spent on the column, for a strongly held amino acid derivative, can be decreased by a process known as gradient elution. Here, the percentage polar buffer content is reduced over time and is replaced with

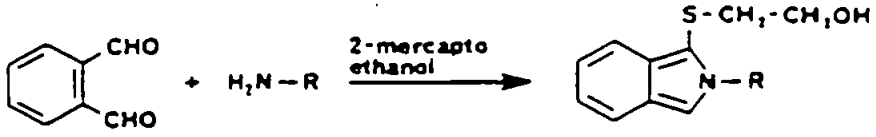
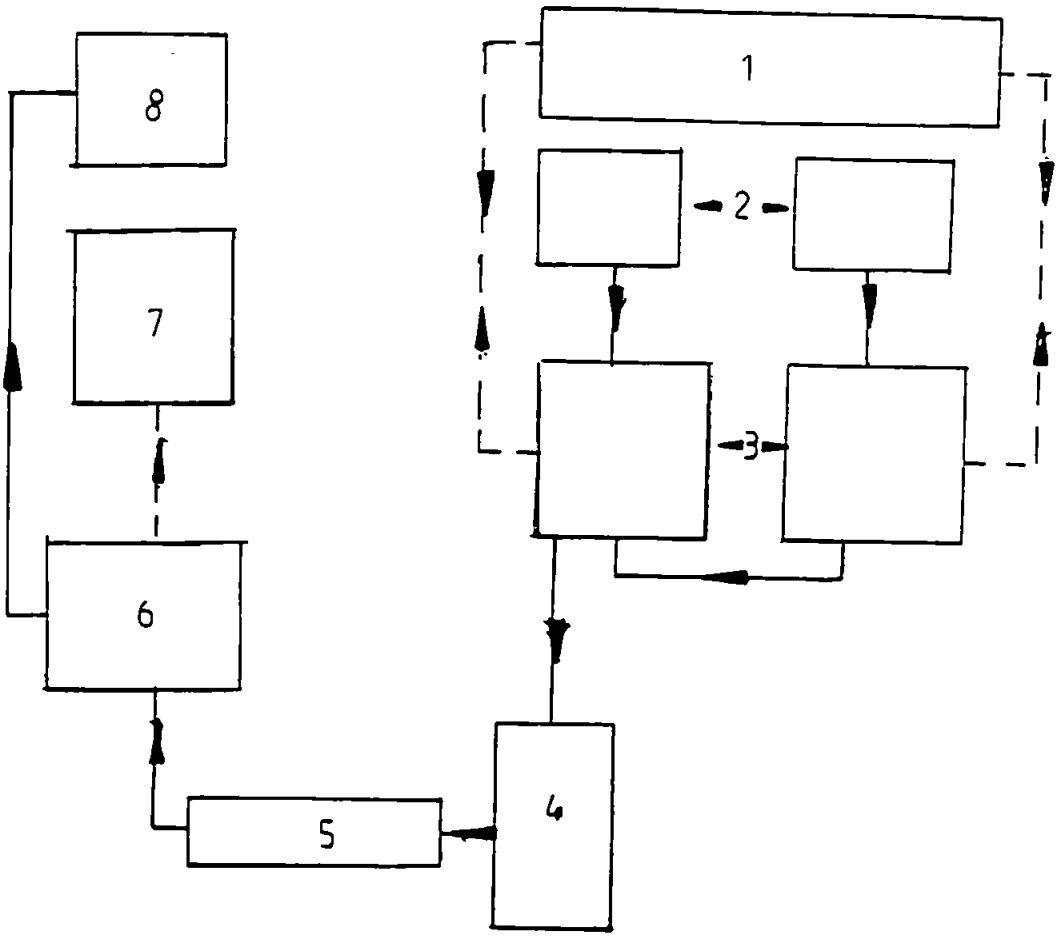


Fig 2.1 The Reaction Pathway For The Derivatization Of Free Amino Acids. (from Evens 1986).



Solvent flow

Electrical connections

1 Waters gradient controller
2 Solvent reservoirs
3 Waters M6000A pumps
4 Waters U6K injecter
5 Hypersil ODS column
6 Detector
7 Integrator
8 Waste vessel

Fig 2.2 A Schematic Diagram Of The HPLC System Used In This Study.

a less polar organic phase. The gradient compositions used in this study are detailed in section 2.1.3. Once the amino acid derivative has been eluted from the column it passes to a fluorescence detector. Fig. 2.2 shows a schematic diagram of the chromatographic system used.

2.1.3 Modifications to the Method of Evens et al. 1982.

The amino acids were derivatized and separated using the method of Evens et al. (1982) with the following modifications.

1. Previously, the borax solution, used in the ortho-phthaldialdehyde reagent, was prepared by titrating boric acid (0.4M) with sodium hydroxide to a pH of 9.5. During the initial stages of this study it was found that maintaining the glass electrode in a sufficiently clean condition to produce amino acid free reagent blanks was a problem. Therefore, commercially produced sodium borate was purchased and the buffer made up directly as follows:

3.81g of sodium tetraborate ($Na_2B_4O_7.10H_2O$) was dissolved and made up to 100 cm^3 with pure water. This gave a 0.1 M solution of sodium tetraborate of pH 9.6. (Britton 1955).

2. Previously, the ortho-phthaldialdhyde reagent was prepared by dissolving 135 mg of OPA in 5 cm^3 HPLC grade

methanol, adding 100 mm^3 of 2-mercaptoethanol (MCE) and making up to a final volume of 25 cm^3 with borate buffer (pH9.5). The reagent was then kept without refrigeration in glass. This process was altered as follows:

The 135 mg of OPA plus 5 cm^3 of HPLC grade methanol was made up to 25 cm^3 with borax solution (pH9.6), and the solution was then kept refrigerated. When the reagent was required 2 cm^3 of the above solution was removed and 10 mm^3 of 2-mercaptoethanol added to it. This reagent was then kept unrefrigerated and used the same day.

3. The gradient was altered from that published in Evens et al. 1982, in order to increase the resolution of glycine, threonine and arginine (Evens 1986). A table of the gradient elution system employed is shown below:

Table 2.1 The Gradient Elution Programme Used For Amino Acid Separation.

Time (min)	% 0.1M Phosphate buffer. Pump A	% Acetonitrile. Pump B.	Curve
Initial	97	3	
6	93	7	7
18	91	9	8
24	80	20	7
33	80	20	6
36	78	18	6
38	67	33	8
42	67	33	6

The slope of the change can be convex, concave or linear depending on the curve number defined in the method. An explanation of the curves and their respective numbers can be found in the Waters System Controller manual.

4. The Waters 420-C fluorescence detector was modified to the more sensitive 420-AC version by the replacement of a circuit board.

Full experimental detail of the method used for amino acid analysis during this study is detailed in the following section. The preparation of the borate buffer has already been described above. All chemicals were of the purest grade obtainable and were purchased from B.D.H. Ltd Poole. U.K. MilliQ water was used to prepare all solutions.

Phosphate buffer was prepared by dissolving 15.6g of sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$, 0.1M), and 35.8g of disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$, 0.1M), in 2 l of water, producing a buffer of pH 6.9.

The boric acid solution used to quench the derivatization reaction was prepared by dissolving 2.48g of boric acid (H_3BO_3) in 100 cm^3 of water.

Ortho-phthaldialdehyde (OPA) derivatizing reagent was prepared by dissolving 135mg of OPA in 5cm^3 of HPLC grade methanol and the solution was then made up to 25cm^3 with borate buffer (pH 9.6); this stock solution was stored in a refrigerator. When the reagent was required, 2cm^3 of the stock solution was removed and 100 mm^3 of mercaptoethanol (MCE) was added to it. The OPA plus MCE solution was discarded after one days work.

To quantify the amino acids in sea water a calibration table was created by analysing an amino acid mixture containing known amounts of amino acids. (see section 2.8). Alpha amino adipic acid, (internal standard) and the amino acid calibration mixture were prepared as $1x10^{-3}$M stock solutions. (These were made up weekly and kept refrigerated). Immediately prior to use the

millimolar stock solutions were diluted to give a $1x10^{-3}$M alpha amino adipic acid solution and a $4x10^{-6}$M amino acid calibration mixture. The procedure for the analysis of standard solutions and sea water samples was as follows:

Calibration analysis.

40 mm^3 of $1x10^{-3}$M alpha amino adipic acid was pipetted into a 5 cm^3 conical flask, to this was added 100 mm^3 of the $4x10^{-6}$M amino acid calibration mixture and then 360 mm^3 of MilliQ water were added to bring the volume up to 500 mm^3, finally 100 mm^3 of the derivatizing reagent was added to the flask. After exactly 2 minutes the reaction was quenched by adding 300 mm^3 of the boric acid solution, after which 75 mm^3 of this reacted solution was injected into the amino acid analyser.

Sea water sample analysis.

The process described above for the amino acid standards was repeated for the sea water samples, except that 20 mm^3 of alpha amino adipic acid was used instead of 40 mm^3 and 480 mm^3 of sea water replaces the 460 mm^3 of amino acid and water mixture. The injection volume for derivatized sea water was 150 ul.

The acetonitrile used in the gradient elution separation of the amino acid mixtures was degassed by vacuum filtration using a 0.45 um Millipore PTFE filter. The phosphate buffer was degassed in the same way using

0.45um Millipore HA type filter was used.

2.2 Contamination.

The reagent blanks for the analysis of amino acids, using the method given above, were consistently below the limits of detection of the method. Fig. 2.3 shows a typical reagent blank containing the internal standard, amino adipic acid (Adi).

Another source of contamination is the inadequate washing of the chromatographic column. If the column is not washed, as described below, then random peaks occur in the chromatogram which may be spuriously identified as amino acids. To wash the column after use, 100 cm^3 of water is run through the column, followed by 50 cm^3 of a 50:50 methanol/MilliQ water mix. The column is then stored overnight in methanol/ water. Before analysis begins the methanol/water is removed by flushing with 100 cm^3 of MilliQ water. Then 30 cm^3 of acetonitrile is pumped through the column followed by 50 cm^3 of water. The column is then ready for conditioning in the buffers used for analysis.

2.3 Elution Order.

The elution order of the amino acids routinely identified in seawater samples are given in Table 2.2. together with a retention time and its variability expressed as the coefficient of variation (CV). Peak identification was by coinjection with standards.

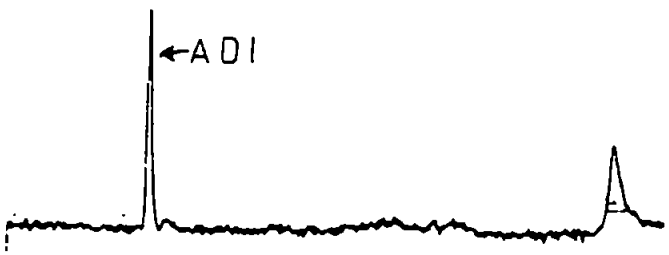


Fig 2.3 A Typical Reagent Blank, Containing The Internal Standard Alpha-aminoadipic Acid (ADI).

Table 2.2. Elution Order Of The Amino Acids Expressed As Retention Time. (The variability of the retention time is given by the co-efficient of variation, CV, n=4.)

Amino acid.	Retention Time (min).	CV.
Asp	2.79	0.5
Glu	5.37	0.7
Adi	9.06	0.4
Ser	10.22	0.4
His	11.90	0.4
Gly	16.86	0.7
Thr	18.49	0.2
Arg	21.18	0.5
Ala	23.79	0.04
Tyr	25.86	0.02
Abu	26.28	0.06
Val	28.71	0.2
Met	29.14	0.2
Trp/Ile	35.71	0.2
Phe	36.48	0.2
Leu	37.55	0.3
Orn	40.59	0.1
Lys	41.06	0.1

It should be noted that retention times of the acids will vary slightly, from column to column, and with column wear. Therefore, regular calibration is necessary to ensure accurate identification of the amino acids.

Tryptophan and Isoleucine co-elute but can be separated by raising the column temperature to approximately 30° C using a water bath and column jacket. Maintaining an easy-to-use, clean system for the jacket was a problem due to algal growth. As Tryptophan and Isoleucine were found to be minor constituents of the free and combined fraction, a decrease in the possibility of contamination was considered of more benefit than resolution of these acids. Use of the water jacket was terminated and the pair are reported together.

2.4 Limits of Detection of the Method.

Adding an oxidizing agent to sea water followed by exposure to a strong ultraviolet (UV) light, for many hours, has the effect of breaking down the organic content to inorganic forms, leaving the salt matrix unaffected. This UV-irradiated sea water was used for blanks and for preparing amino acid solutions in a series of experiments to find the limits of detection (LOD). By using irradiated seawater, as opposed to ultrapure water, the blanks etc. were more closely matched to the matrix of the environmental samples. The LOD were determined by running UV-irradiated blanks six times. Standard amounts of an amino acid mixture were then added, at increasing levels, until all the amino acids in the mixture were detected (Miller & Miller 1985). The results can be seen below in Table 2.3.

Table 2.3 Limits Of Detection (LOD) In Picomoles (p.mol) For Free Amino Acids (a.a.) In UV Irradiated Seawater.

a.a.	LOD (p.mol).	LOD (u.mol)
Asp	2	0.025
Glu	1	0.012
Ser	4	0.050
His	9	0.112
Gly	6	0.075
Thr	6	0.075
Arg	6	0.075
Ala	2	0.025
Try	1	0.012
Abu	2	0.025
Val	1	0.012
Met	4	0.050
Trp/Ile	4	0.050
Phe	6	0.075
Leu	4	0.050
Orn	10	0.125
Lys	10	0.125

2.5 Sensitivity and Linearity.

Having obtained the limits of detection for each amino acid, the next stage was to determine if the range of concentrations found in the environmental samples fell within the linear working range of the fluorescence method. Increasing amounts of each amino acid were injected on to the column and eluted. The fluorescence detector was linked to a Hewlett Packard 3390A reporting integrator which gave an area count for each peak. The data for area count versus amount on the column was fed into a statistical program (Microtab) to ascertain the correlation coefficient (r) between them. The r value was tested for statistical significance using the student t-test. All amino acids were found to have a significant correlation between area count and amount on the column. The r values can be seen below in Table 2.4.

The slope of the graph of area count versus amount on the column is a measure of the sensitivity of the method. Fig.2.4 to 2.6 shows the slope of the lines for three of the eighteen amino acids tested. Fig.2.7 illustrates the difference in sensitivity between aspartic acid and ornithine, shown by the varying slope. The reciprocal of the slope is the Response Factor (RF) programmed into the Hewlett Packard integrator for the calculation of the amount of each individual amino acid in an environmental sample. The calculations performed by the integrator to find the amount and concentration of individual amino acids in the samples is discussed further in section 2.8. A typical RF for each amino acid can be seen in Table 2.4.

Table 2.4 The Response Factors (RF) And Pearsons Correlation Coefficient (r) For The Amino Acids (a.a.).

a.a.	RF	r
Asp	5.205×10^{-5}	0.998
Glu	5.502×10^{-5}	0.999
Ser	4.815×10^{-5}	0.977
His	1.019×10^{-4}	0.986
Gly	7.452×10^{-5}	0.977
Thr	6.230×10^{-5}	0.994
Arg	4.802×10^{-5}	0.991
Ala	4.723×10^{-5}	0.994
Tyr	4.949×10^{-5}	0.999
Abu	5.222×10^{-5}	0.999
Val	4.347×10^{-5}	0.988
Met	4.308×10^{-5}	0.995
Trp/Ile	6.211×10^{-5}	0.985
Phe	5.942×10^{-5}	0.999
Leu	5.565×10^{-5}	0.984
Orn	3.405×10^{-4}	0.952
Lys	2.360×10^{-4}	0.915

Response factors change very slightly during the life of a column, and from column to column; they are

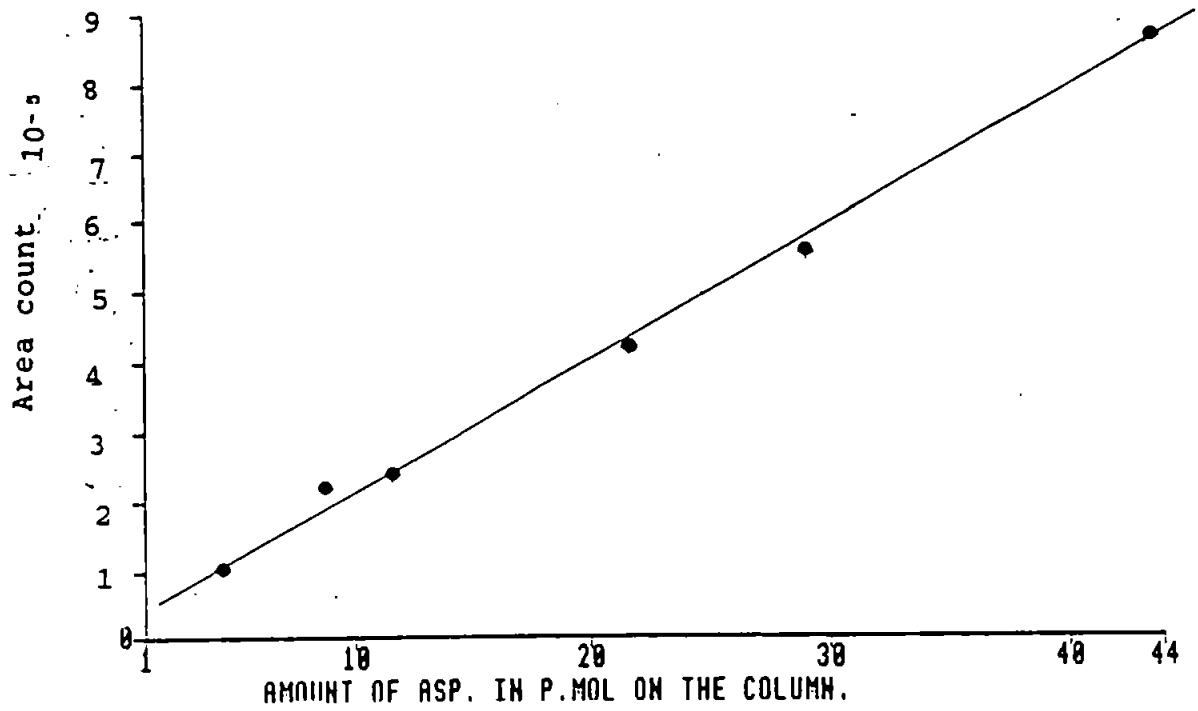


Fig 2.4 Graph Showing The Linearity Of Response For Aspartate.

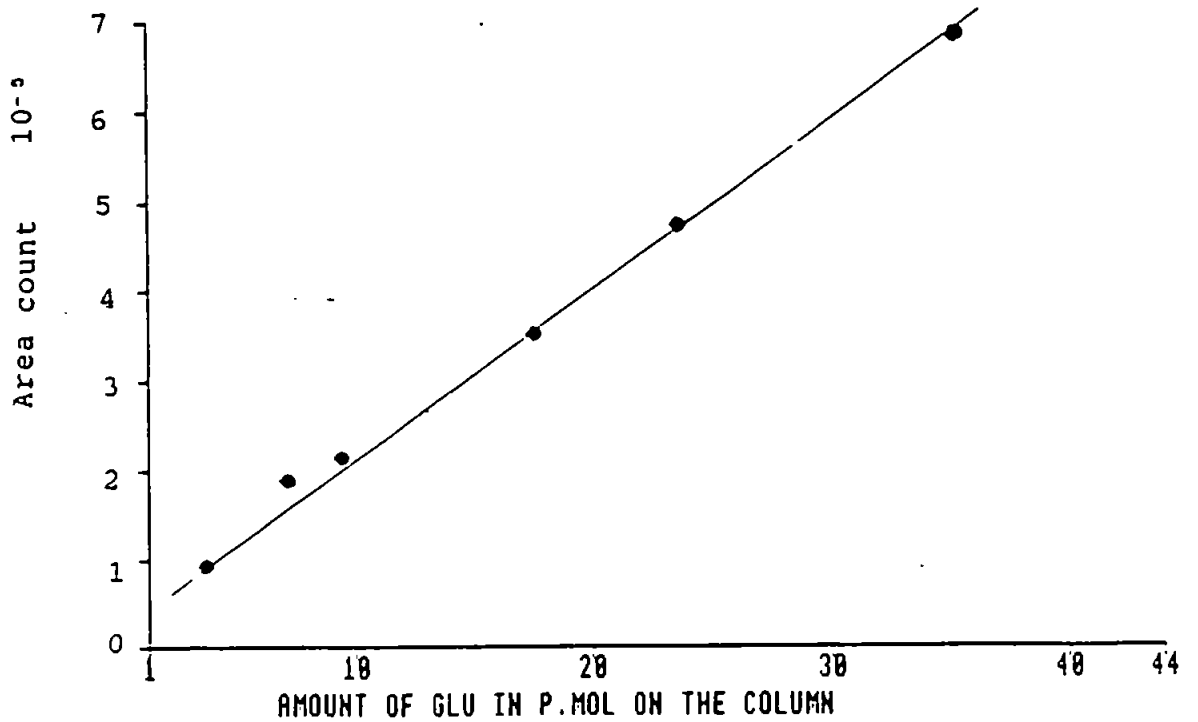


Fig 2.5 Graph Showing The Linearity Of Response For Glutamate.

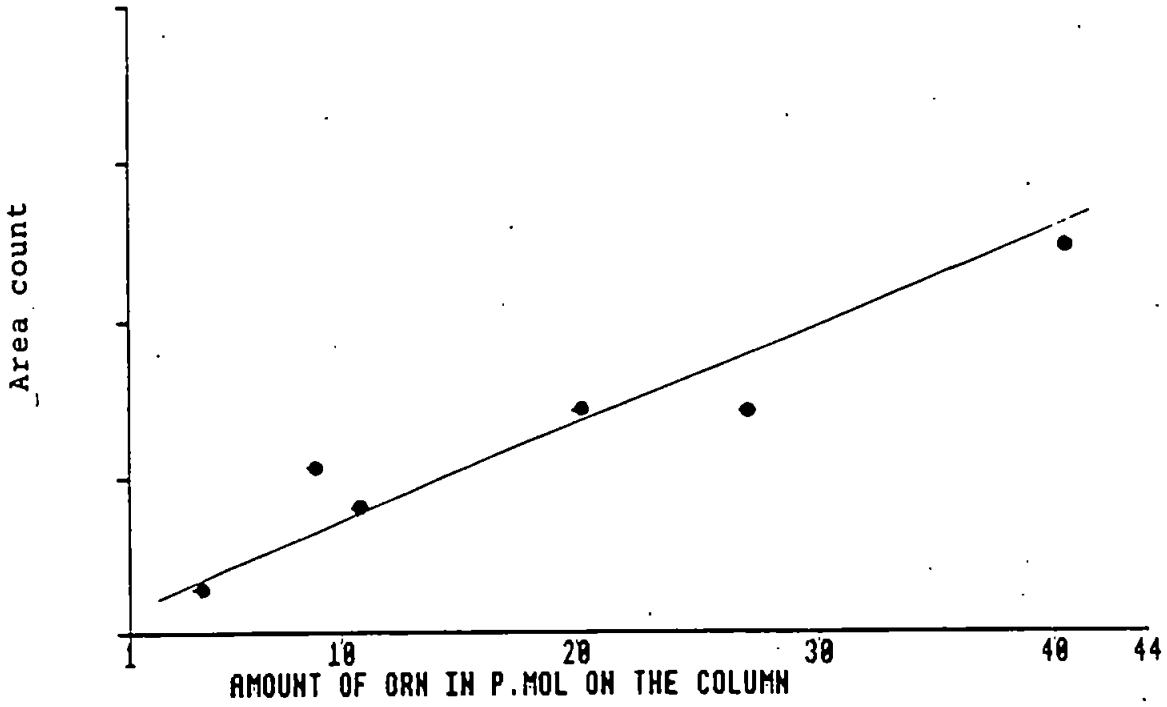


Fig 2.6 Graph Showing The Linearity Of Response For Ornithine.

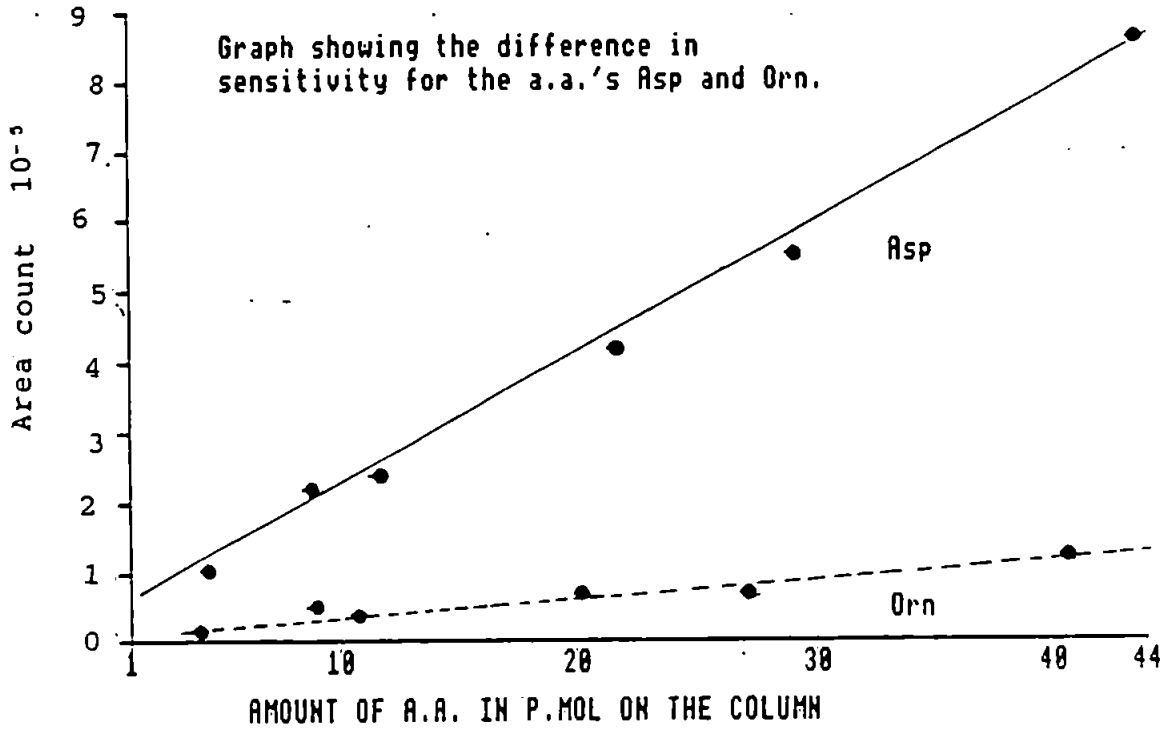


Fig 2.7 Graph Showing The Difference In Sensitivity For The Amino Acids Aspartate And Orhithine.

therefore redetermined at appropriate intervals.

2.6 Precision.

Precision is a measure of the repeatability of an analysis. The technique used to find the precision of a method is to make repeat injections of a standard and work out the variablity of peak height or area. In the present case, precision, was determined at three different concentration levels, approximately 0.038, 0.075 and 0.25 uM, for each acid. This is equivalent to 3, 6 and 20 picomoles on the column. The area counts from the Hewlett Packard integrator were used to calculate the coefficient of variation (CV). The results are given in Table 2.5.

Table 2.5. Coefficient Of Variation (CV) For Repeat Analyses At 0.038, 0.075 and 0.25 (uM) Levels (n=4 in all cases). CV= standard deviation divided by the mean multiplied by 100. BLD= below the limits of detection.

	<---------- CV---------->		
a.a.	0.038	0.075	0.25 uM.
Asp	10	10	4
Glu	12	11	13
Ser	32	17	9
His	BLD	BLD	16
Gly	200	38	35
Thr	BLD	97	3
Arg	BLD	39	11
Ala	78	4	4
Tyr	16	6	6
Abu	16	15	7
Val	21	7	4
Met	BLD	4	1
Tpr/Ile	200	110	7
Phe	200	90	9
Leu	115	94	7
Orn	BLD	BLD	5
Lys	BLD	BLD	15

2.7 Accuracy.

Accuracy is a measure of how close the observed value is to the correct value. There are several methods for the determination of accuracy, including reference standards, standard additions and blind tests. The best method is to compare the value given for a reference sample with those detected by the method under investigation. However, commercially available reference standards of amino acids are prepared for traditional ion-exchange amino acid analysers and are stabilized in acid. It was therefore not possible to use that method of establishing the accuracy of the analysis in this present study. Therefore two different methods were employed i.e. standard additions and a "blind" test. In standard additions known amounts of amino acids are added to a sample which has been previously analysed for amino acid content. The percentage recoveries of the added acids are then calculated. It is possible in the method of standard additions for the analyst to make a constant error and this is best checked with a blind test. A solution of amino acids is prepared by another worker so that the analyst has no knowledge of its composition or levels. The unknown solution is then analysed. The reported composition and levels are then compared to those of the original solution. Results for the percentage recovery of amino acids at a 20 picomole level is shown in Table 2.6 while those for the blind test appear in Table 2.7.

Table 2.6. Percentage Recovery Of Added Amino Acids At The 0.25 uM. Level. (n=4)

a.a.	% recovery.
Asp	116
Glu	102
Ser	127
His	106
Gly	104
Thr	107
Arg	105
Ala	117
Tyr	102
Abu	106
Val	101
Met	124
Trp/Ile	111
Phe	100
Leu	122
Orn	102
Lys	129

Table. 2.7. Results Of The Blind Test.

a.a.	Concentration of original solution (mM).	Concentration found by analysis (mM).
Asp	1.45	1.49
Glu	0.46	0.47
Ser	0.54	0.61
Gly	0.24	0.25
Thr	0.86	0.92
Ala	2.23	2.31
Tyr	0.45	0.49
Val	0.33	0.34
Phe	0.43	0.52
Leu	0.30	0.40
Orn	1.06	1.50
Lys	0.51	0.86

The amino acids were diluted and analysed at the picomole level, the results were then related to the concentration of the original solution. All twelve amino acids were correctly identified.

2.8. Hewlett Packard 3390A Integrator Calculations Used to Quantify Amino Acids in Environmental Samples.

To be able to determine the amount of an amino acid in an environmental sample, a calibration table must be created in the memory of the integrator. This is achieved by analysing standards of amino acids including the internal standard (ISTD) alpha amino adipic acid (Adi). Then for each acid a retention time and response factor (RF) is programmed into the memory. Retention time is used to identify the acid and RF used to calculate the amount of an acid injected onto the column and from this the concentration in the sea water. The retention time is taken from the chromatogram of standards. A retention time window is selected by the operator and is in this case the retention time plus or minus 0.5 of a minute. The RF is the amount on the column divided by the area count of that peak. The amount of a standard placed on the column is calculated as shown below:

100 mm^3 of a $1x10^{-3}M$ amino acid solution is made up to 25cm^3 with ultrapure water giving a concentration of:

$100/25,000 \times 1.10^{-3}M = 4x10^{-6}M$ solution.

100 mm^3 of this $4x10^{-6}M$ solution is added to 40 mm^3 of internal standard and 360 mm^3 of water giving a total of 500 mm^3. The derivatization procedure involves the addition of 100 mm^3 OPA reagent and 300 mm^3 of Boric acid

giving a total of 900 ul. Therefore the final strength in the reaction mixture is:

100/900 x $4x10^{-6}$M solution = $4.444x10^{-7}$M solution.

75 ul of this solution was injected into the amino acid analyser. Amount injected is given by:

$75/1x10^{6}$ X $4.444x10^{-7}$M = $3.333x10^{-11}$ moles or 33.33 picomoles.

The 33.33 picomoles would produce an area count on the chromatogram and the response factor is obtained from: RF= amount on column /area count .

For most of the amino acids the RF is of the order $5x10^{-5}$. The integrator calculates a RF for each amino acid including the internal standard. The calculation of the concentration of an amino acid in an environmental sample is shown below:

The amount of amino acid (p.mol) in an environmental sample is calculated as follows:

$$\frac{\text{RF of acid in calibration table.} \times \text{Area count of the acid in the sample.}}{\text{RF of Adi in table.} \times \text{Area count of Adi in the sample.}} \times \text{The amount of Adi added to the sample.}$$

In this way all the amino acids are normalized to the response of the internal standard (Adi). Conversion from amount of amino acid present to concentration expressed as u moles l^{-1} in the original sea water is as follows:

20 mm^3 of internal standard is added to 480 mm^3 of sea-water. This is derivatized and in the process is made up to 900 mm^3. An aliquot (150 mm^3) of this is mixture then injected i.e.

480/900 x 150 = 80 mm^3 of seawater is injected.

The amount on the column originates from 80 mm^3 of sea-water. Therefore the concentration in the sea water is given by Y p.mol in 80 mm^3. The conversion to umoles/l is as follows:

$$\frac{1\times10^{6}}{80} \times Y\times10^{-12} \text{ moles of acid} = \frac{Y\times10^{6}}{80} \text{ u molar} = Y \times 0.0125 \text{ uM.}$$

Therefore the amount of an amino acid on the column in picomoles x 0.0125 = concentration (u molar) in the original seawater sample.

2.9 Sample treatment.

2.9.1. Filtration.

Filtration of sea water samples prior to analysis is a common practice. Recently a number of texts have become available which question the use of filtration,[Casey & Walker (1983), Ridder et al. (1985), Grasshoff (1986), Mopper & Lindroth (1982) and Fuhrman & Bell (1985)]. Grasshoff (1986) states that filtration can cause contamination and may lead to cell disruption with the concomitant release of intracellular fluids and the reader is cautioned to filter only when it is unavoidable. Fuhrman & Bell (1985) give a detailed discussion of the filters commonly used and volumes filtered and conclude that glass fibre filters lead to cell rupture with volumes as low as 10 cm^3 and that they should not be used for dissolved free amino acid analysis. If filtering is used it should be with small volumes 5-10 cm^3 at low pressures and through 0.2 or 0.45 um membrane filters.

In this study the coastal and depth profile samples

for DFAA analysis were not filtered. Estuary samples due to high levels of suspended solids were filtered through a 0.45 um Millex HA type filter. Coastal samples for hydrolysis were filtered using sterile, bubble tested 0.2 um Millex GV filters, which contain a low protein binding Durapore membrane. The individual filter is attached to a glass syringe containing 10 cm^3 of seawater, and 1cm^3 of seawater is expelled through the filter by gentle pressure.

2.9.2 Sample Preservation and Storage.

Previous attempts at preserving samples have involved acidification,(Webb & Wood 1966), addition of mercuric chloride ($HgCl_2$), (Garrasi 1979, Bada et al. 1982, Ittekkot 1982, Muller et al. 1986), toluene (Josefsson et al. 1977), chloroform (Parks et al. 1963, Riley & Segar 1970), or pentachlorophenol (Dawson and Pritchard 1978). Fuhrman & Bell (1985) studied the effect of addition of $HgCl_2$ on amino acid levels and reported a fourfold increase in the treated samples compared to the untreated samples. Webb & Wood 1966 found that the acidification of samples leads to increased amino acid levels. Grasshoff 1986 reports that Thayer (1970) showed that chemical methods of preservation were unsuitable for micronutrients.

An alternative to chemical preservation has been sample storage by freezing. Morris et al.(1985) discuss the damage to cell membranes during the freezing process which again can lead to intracellular leakage of amino

acids.

Due to the difficulties outlined above none of the samples in the present study where subjected to any form of preservation or storage.

2.9.3 Sample Collection.

Samples were collected by the crew members of the Marine Biological Association ships.

Coastal samples, all of which were surface samples, were collected using standard reversing bottles. Samples were transferred to acid washed plastic bottles. Each bottle was rinsed several times with its particular sea-water sample before being capped. The bottles were placed in an insulated container to remain cool. On arrival at the laboratory the samples were transferred to a refrigerator to await analysis.

Depth samples were collected using standard plastic reversing bottles. The samples were dealt with as above. During the July 1987 cruise, depth profile samples were transferred directly to the refrigerator.

Estuary samples were collected using a clean plastic bucket rinsed several times in the estuary water. Samples from the bucket were filtered at 0.45um and transferred to the plastic bottles. Again the bottles were kept in an insulated container until arrival at the laboratory.

2.9.4 Analysis Time.

The analysis time for each sample is approximately 50 minutes. Samples were collected by the Marine Biological Association Boats and the analyses were commenced as soon as the samples were brought to the laboratory. Samples awaiting analysis were kept in a refrigerator at 4°C.

CHAPTER 3.

DEVELOPMENT OF THE METHOD FOR THE DETERMINATION OF DISSOLVED COMBINED AMINO ACIDS BY ACID HYDROLYSIS.

3.1. Introduction.

Chapter 3 deals with the development of the hydrolysis procedure used in the present study to determine the dissolved combined amino acid levels in sea water. Table 3.1 gives details of the methods used by previous workers.

Table 3.1 Hydrolysis Methods Used By Previous Workers.

Reference	Hydrolysis method.
Park et al.(1962).	Co-precipitation of organics with ferric hydroxide. Acid hydrolysis with 6M HCl.
Degens et al.(1964).	Glass ampoule sealed under Nitrogen.(No other details given).
Bishop & Louden (1965).	Co-precipitation of organics with ferric hydroxide. Acid hydrolysis with 6M HCl.
Siegel & Degens (1966).	Refluxed for 24 hours with 6M HCl.
Riley & Segar (1970).	Evaporation of sample. Addition of HCl to 6M. Hydrolysis for 10 hours at 110°C under nitrogen.
Lee & Bada (1975).	Reflux for 24 hrs with 6M HCl.
Daumas (1976).	Acid hydrolysis no details.
Siezen & Mague (1978).	6M HCl,110°C, 20 hours, glass ampoule sealed under vacuum.
Garrasi et al.(1979).	10 cm^3 of seawater placed in

	ampoule then evaporated to dryness. 1 cm^3 of 6M HCl added tube sealed under nitrogen. 22 hrs.
Naletova (1979).	6M HCl, 105°C, 24 hours.
Bolter & Dawson (1982).	Mixed 8M HCl 1:1 with sea water. 22 hours, 110°C.
Bada et al. (1982).	Evaporation of sample. 6M HCl reflux 24hours. 200 mg of Ascorbic acid added to prevent degradation of combined amino acids.
Ittekkot (1982).	6M HCl, 110°C, 22 hours.
Lee & Cronin (1982).	Acid hydrolysis no details.
Henrichs & Williams (1985).	6M HCl, 110°C, 24 hours. Glass ampoule sealed under nitrogen.
Mopper & Zika (1987).	Acid hydrolysis no details.

In our initial studies we have experienced a variety of difficulties when using the published methods. The following account outlines the series of experiments undertaken to produce a reliable hydrolysis procedure.

One of the main problems with amino acid analysis is the possibility of contamination; for free amino acids this can be checked by running a reagent blank. For the combined amino acid analysis a procedural blank was used i.e. water of a high purity which was considered free from dissolved free or combined amino acids was taken through the hydrolysis procedure. Its subsequently determined amino acid content was an indication of the level of contamination produced by the method. The most common practice for the hydrolysis of sea water has been to reflux at atmospheric pressure with 6M hydrochloric acid (HCl). Section 3.3 below will deal with the problems

of obtaining low reproducible procedural blanks (PB) using this method.

However, before a detailed discussion of the reflux method attention will be paid to the technique developed for the pH adjustment of hydrolysed samples preceeding amino acid analysis.

3.2 Hydrolysate pH Adjustment.

Initial work was directed towards finding a suitable method of removing the HCl so that the amino acid analysis could be carried out on a non-acidic sample as the derivatization technique demands. Neutralization with sodium hydroxide was unacceptable as the Hypersil ODS column packing was already near its tolerance limit for salt content with the presence of seawater (0.6M NaCl). Therefore a method had to be found that did not increase the ionic content of the sample. Rotary evaporation of 2 cm^3 of a 6M HCl solution containing amino acids, followed by storage under vacuum over potassium hydroxide pellets for 15 hours, gave a procedure which did not affect the derivatization reaction. This was determined by comparing the peak area for the internal standard, alpha amino adipic acid, in an aqueous amino acid solution with that from amino acids in 6M HCl solution which had undergone the above process of evaporation and desiccation. No difference between peak areas was observed. There was a 95 percent recovery of amino acids from the evaporated acidic solution.

3.3 An Investigation Of Seawater Hydrolysis Using The Common Reflux Methodology.

3.3.1. Problems Encountered Using Deionized Double Distilled Water (DDDW) As The Hydrolyis Procedural Blank.

The first set of hydrolyses undertaken was that of deionized double distilled water (DDDW), which had been shown to be free of dissolved amino acid. The apparatus for the experiment, a microscale reflux system under a Nitrogen atmosphere is shown in Fig. 3.1. A typical procedural blank (PB) chromatogram, showing high levels of contamination, can be seen in Fig.3.2.

The possible sources of contamination could have been; the Nitrogen gas, antibumping granules, the DDDW, the Aristar Hydrochloric acid, a proteinaceous monolayer on the glassware which was not removed by acid washing or a contaminant entering the system used for hydrolysis.

A series of experiments was undertaken to eliminate one at a time the possible causes of contamination listed above.

3.3.2 Effect of the Nitrogen Atmosphere.

An experiment consisting of 4 hydrolyses of DDDW, using 6M Hydrochloric acid (HCl) at reflux were set up. Two were under a nitrogen atmosphere and two without nitrogen. In the case of those without nitrogen a Quickfit stopper was placed in the top of the reflux

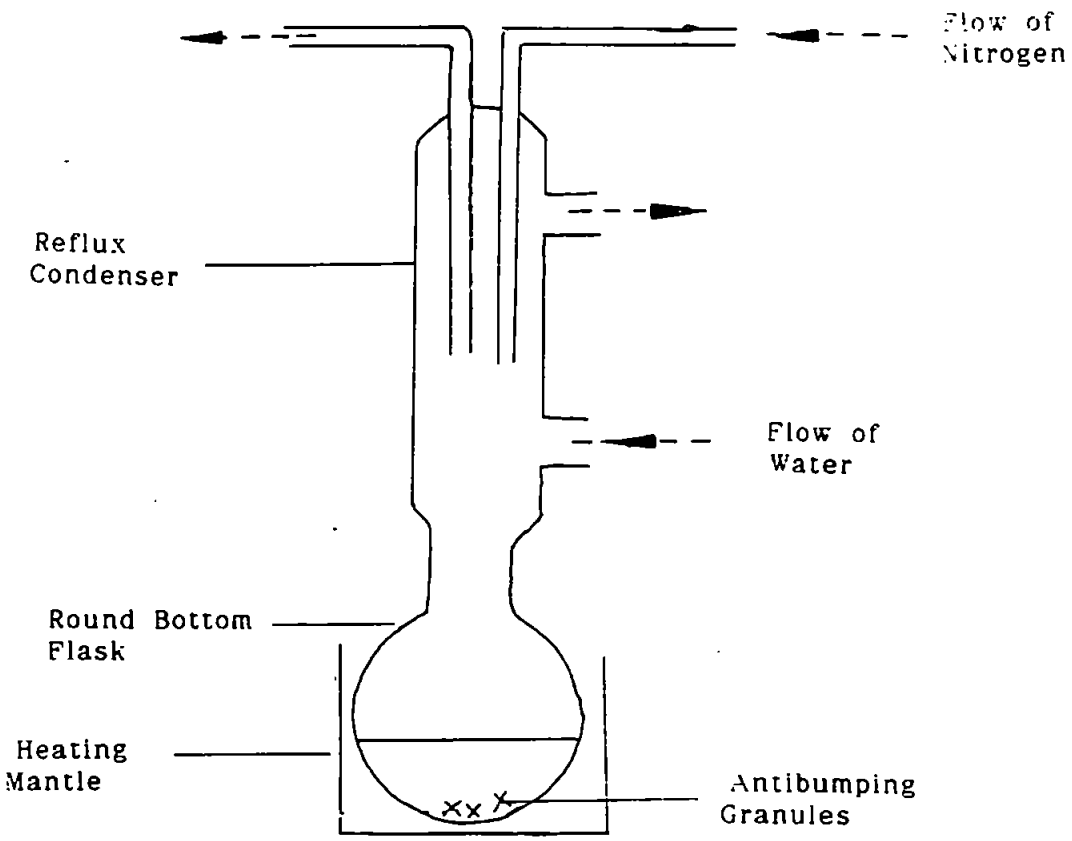


Fig 3.1 A Schematic Diagram Of The Reflux Apparatus Used For The First Hydrolysis Experiments.

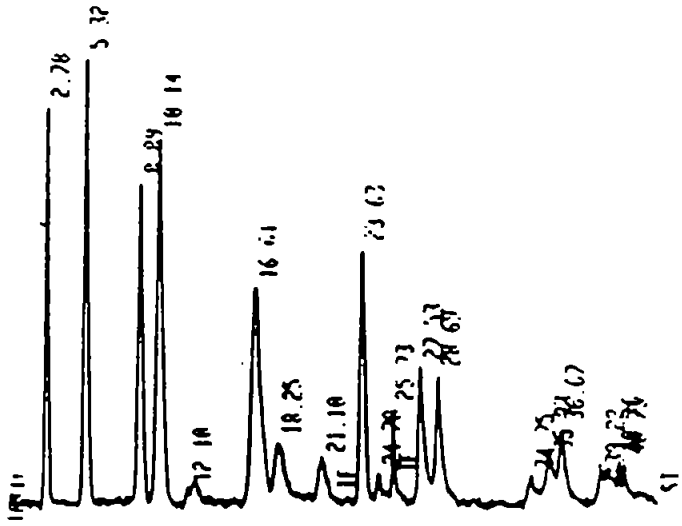


Fig 3.2 A Typical Chromatogram For The Acid Hydrolysis Of Deionised Double Distilled Water: The Amino Acid Procedural Blank Shows High Levels Of Contamination.

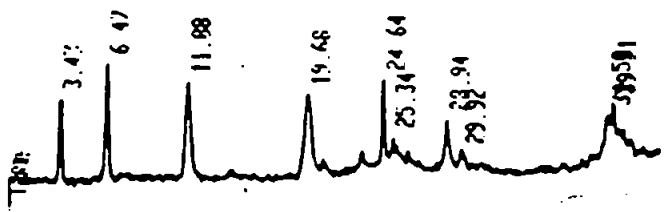


Fig 3.3 Aristar Hydrochloric Acid Taken Through The Hydrolysis Process With No Water Present, And Showing Reduced Levels Of Contamination.

condenser and a filter funnel inverted over the apparatus with approximately a quarter of an inch gap between the funnel neck and the stopper. This kept the stopper in place while allowing the air pressure to equalize, this was designated a semi-sealed system. Results showed amino acid contamination in both sets of hydrolysates. It was therefore concluded that the nitrogen gas was not the cause of contamination. However nitrogen was no longer used and all further experiments employed the semi-sealed system.

3.3.3 Antibumping Granules.

Two sets of duplicate hydrolyses of DDDW were undertaken, one set with antibumping granules, and the other set without. Again, contamination was present in both sets of results. Therefore, the antibumping granules were not the cause of the contamination, but as previously they were no longer included in the method.

3.3.4. Investigation Of The Proteinaceous Monolayer Theory.

Chromic acid washing of glassware is known to leave a monolayer of lipid attached to the glass.(Personal communication, E.I.Butler). There was a possibility that washing with 10 % hydrochloric acid left a proteinaceous layer on the glassware used for the hydrolysis which could be the source of contamination. Therefore the glass washing procedure was changed. The analar hydrochloric

acid was replaced by a 5% solution of Decon 90.(BDH).The following set of duplicate hydrolysis experiments were then undertaken. Set one, deionized double distilled water (DDDW) plus acid without heat. Set two, DDDW plus acid sonicated for 2 hours without heat. Set three DDDW plus acid refluxed for 2 hrs. The experiment without heating produced no amino acids, while those which were sonicated or heated gave amino acids. This indicates that a bond breaking occurs which gives rise to the contamination i.e. the source must be macromolecular. Also the contamination is not due to a protein monolayer. The Decon 90 cleaning method was used for the rest of the experiments on the reflux method.

3.3.5. Role Of Hydrochloric Acid In The Problem Of Contamination.

The next series of duplicate hydrolysis experiments were as follows: one, water only sonicated; two, water plus acid sonicated (2 hrs); three, water plus acid heated for a period of 2 hrs. The sonicated water without acid gave no amino acids. While both the other sets of experiments gave the usual contamination. From this result it was concluded that the acid must be present for the contamination to occur.

Two set of duplicate reflux apparatus were used for the next experiment. In the flask of each set was placed Aristar Hydrochloric acid only. Set one was left at room temperature while set two was heated for two hours. Neither set of experiments showed the typical pattern or

levels of amino acids observed in previous hydrolysates (see Fig.3.3). The result of the above experiment leads to the conclusion that while the acid must be present for the contamination to occur it is not itself the source of the contaminant.

The possibility that the DDDW was the source of the contamination was investigated as follows: two duplicate sets of reflux apparatus were prepared. One set contained only Aristar hydrochloric acid. The second set contained acid plus water. Both the acid and the acid plus water were refluxed. Acid only gave the same result as previously i.e. no typical contamination pattern. The acid and water however gave the usual pattern and levels of contamination. Therefore the problem of contamination appeared to be a macromolecular species in the unfiltered deionized water. Filtration of the DDDW at the 0.45 um level greatly reduced the level of contamination, further supporting the idea of contamination by a macromolecular species. A supporting experiment was carried out in which 10 times $2cm^3$ aliquots of DDDW were serially evaporated from the same 10 cm^3 round bottomed flask. Close inspection of the flask at the end of the experiment showed the flask to contain a white deposit. Acid hydrolysis of the deposit showed that it contained high levels of combined amino acids. This confirms that the DDDW which had been used for all work up to this point contained an impurity which had caused the typical contamination pattern observed during the procedural blank hydrolyses. A recent paper by Samata & Matsuda (1986) confirms these findings.

Subsequent work including glassware washing was carried out using 0.45 um filtered DDDW, until the purchase of a MilliQ ultrapure water system when the use of DDDW was terminated.

3.4 Evaluation Of The Generally Used Reflux Method.

At this stage the hydrolysis method development had been successful in removing hydrochloric acid prior to derivatization and producing a reflux method with a low but not insignificant blank.

It was decided at this stage to conduct a preliminary investigation of seawater samples to determine the magnitude of the dissolved combined amino acids relative to the procedural blanks. Table 3.2 shows procedural blank and seawater levels determined on a variety of occasions. The blank levels were inconsistent. By comparing the levels of dissolved free amino acids and the total amino acids in the seawater samples it was also clear that amino acid destruction was occuring in some of the samples. This meant that a fuller investigation of the hydrolysis by reflux was necessary.

Table 3.2. Duplicate Procedural Blanks (0.45 um filtered DDDW) And Sea Water Samples Hydrolysed Using The Semi-sealed Reflux Methodology. TAA=Total amino acids free plus combined.

Date	Blanks: TAA (uM).	Sea water: TAA (uM). without subtraction of the blank values.
08.01.86	2.203, 2.118	3.755, 3.291
21.01.86	1.230, 2.590	2.064, 2.957
23.01.86	0.952, 1.234	1.596, 2.041
28.01.86	0.250, 0.164	1.158, -----
03.02.86	0.066, 0.053	0.868, 0.273
11.02.86	1.251, 0.906	0.714, 0.402
25.02.86	0.125, 0.106	0.224, 0.210
06.03.86	0.645, 1.638	1.327, 2.963

3.4.1. Recovery of Free Amino Acids.

The first step in this investigation was to examine the recovery of amino acids from a solution of free amino acids subjected to reflux hydrolysis conditions. Water was spiked, with the eighteen amino acids listed in chapter 2, at three different levels i.e. 0.362, 2.000 and 4.000 uM. for each amino acid. This free amino acid mixture was subjected to acid hydrolysis conditions using the semi-sealed apparatus described above. The percentage recovery of these free amino acids are shown in Table 3.3.

Table 3.3. The Recovery Of Free Amino Acids From Water Hydrolysed Using The Semi-sealed Reflux Methodology.

	% recovery		
amino acid.	0.362	2.000	4.000 (uM).
Asp	29	23	28
Glu	24	54	59
Ser	18	52	59
His	0	0	3
Gly	40	76	80
Thr	0	39	42
Arg	0	47	64
Ala	91	107	99
Tyr	0	2	3
Abu	104	92	87
Val	70	101	97
Met	0	0	0
Trp/Ile	18	42	48
Phe	0	44	42
Leu	48	62	70
Orn	0	22	46
Lys	0	44	81

The hydrolysis at three levels was repeated but the losses were not reproducible. Such losses during the hydrolysis procedure were considered unacceptable and it was decided at this stage to abandon the reflux hydrolysis procedure and examine the procedures based upon hydrolysis under sealed tube conditions.

3.5 Sealed Tube Methodology.

The first experiment undertaken was to run several procedural blanks (PB) using filtered deionized double distilled water hydrolysed with 6M HCl in a sealed glass ampoule for 16 hrs at 110°C. Initial results were encouraging as apparently amino acid-free blanks were produced.

The experiment describe above (section 3.4.1) where

water was spiked at three levels with an amino acid mixture was repeated using the sealed tube procedure. This gave zero amino acid recoveries in all three cases. Degassing of the samples with helium or flushing with nitrogen were without affect on the a.a. recoveries.

It is well known that degradative losses occur during protein hydrolysis (Hunt 1985). In most protein analysis studies the hydrolysis is carried out at approximately 1mg of protein for 1 cm^3 of acid. It was therefore decided to examine the percentage amino acid recovery using a protein of known amino acid composition (Bovine Serum Albumin) hydrolysed by both the reflux and sealed tube method at the 1 mg/cm^3 level.

3.6. Percentage Recoveries Of Amino Acids From A Known Protein At The 1mg/cm^3 Level Using The Semi-sealed Reflux And Sealed Tube Methodologies.

Amino acid recoveries of the order of 90 percent were obtained by hydrolysing 1mg/cm^3 of Bovine Serum Albumin using either simple reflux or sealed tube procedures. The total loss of amino acids under sealed tube conditions even at 4.000 uM can therefore only be explained by an extensive degradation occurring when amino acids concentrations are several orders of magnitude below those commonly used for protein analysis work.

This experiment also shows that the irregular hydrolysis results (Table 3.2.) is an indication that, under reflux hydrolysis conditions, both extensive

degradation of amino acids ,together with contamination presumably of aerobic origin, are encountered.

3.7 Evacuated Sealed Tube Methodology.

The main difference between the reflux and sealed tube methodology is the pressure developed in the apparatus during the hydrolysis process. Therefore, determination of blanks and recoveries of free amino acids at the 0.362 uM. level, were repeated using a technique in which pressure was reduced to 0.05 mm Hg before the tubes were sealed. This was designated the Evacuated Sealed Tube method (EST). Full experimental details of this evacuated sealed tube technique are given in section 3.10. below.

The results of the blank determinations are given in Table 3.4 while those for free amino acid recoveries are presented in Table 3.5.

Table 3.4. Within Batch (Column 1) And Between Batch (Column 2) Means And Co-efficient Of Variation (CV) For Procedural Blanks Prepared By EST.

Column 1		Column 2	
amino acids detected.	mean of 3 analyses (uM).	amino acids detected.	mean of 20 analyses. (uM).
Asp	0.083	Asp	0.084
Glu	0.073	Glu	0.090
Ser	0.192	Ser	0.175
Gly	0.087	Gly	0.122
Ala	0.102	Thr	0.014
Total	0.537	Ala	0.096
CV	3.2%	Tyr	0.004
		Val	0.034
		Phe	0.002
		Leu	0.019
		Total	0.644
		CV	33%

Table 3.5. Percentage Recovery Of Added Free Amino Acids At The 29 Picomole Level Which Have Been Subjected To The Hydrolysis Conditions Of The Evacuated Sealed Tube Method.

amino acid	% recovery.
Asp	112
Glu	118
Ser	102
His	116
Gly	89
Thr	125
Arg	105
Ala	111
Tyr	89
Abu	79
Val	97
Met	0
Trp/Ile	90
Phe	68
Leu	76
Orn	57
Lys	52

It was now considered that both the blanks and free amino acid recoveries were such that further experiments

could be undertaken using this evacuated sealed tube procedure for hydrolysis.

The next stage of the investigation was therefore to evaluate the hydrolysis procedure with respect to the percentage recovery of amino acids from a sample containing known amounts of dissolved combined amino acids. This was again achieved by hydrolysing a protein of known amino acid composition (Bovine Serum Albumin, BSA) under various conditions. The results are given in Table 3.6.

Table 3.6. Percentage Recovery Of Amino Acids Obtained By Acid Hydrolysis Of BSA (at different levels), In Sealed Tubes Under Various Conditions.

Amino acid	Column 1	Column 2	Column 3	Column 4
Asp	23.0	62	57	67
Glu	31.4	51	51	65
Ser	11.3	114	43	64
His	7.2	83	54	80
Gly	6.8	142	110	62
Thr	13.7	62	48	70
Arg	11.1	53	73	84
Ala	19.6	65	63	65
Tyr	7.5	59	69	69
Val	14.2	74	77	59
Phe	11.2	44	50	61
Leu	26.0	48	50	56
Lys	24.6	51	50	66

Column 1. Theoretical level of amino acid (picomoles) obtainable from a 3x10-7g/cm3 aqueous solution of BSA (calculated from data given by Haurowitz 1963).

Column 2. % recovery of amino acids from BSA, hydrolysed in Ultrapure water at 4x10-7g/cm3, 0.05 mm Hg.

Column 3. % recovery of amino acids from BSA, hydrolysed in seawater at 3x10-7g/cm3, 0.05 mm Hg.

Column 4. % recovery of amino acids from BSA, hydrolysed in UV-irradiated seawater at 3x10-7g/cm3, 0.05 mm Hg.

An aqueous solution of BSA at a concentration of

$3x10^{-7}$ g/ml was selected for the studies since it contains dissolved combined amino acids at approximately the same order of magnitude as expected to be present in the environment.

The results in Table 3.6 were felt to be encouraging. However a series of experiments was undertaken to see if further improvements could be made. Changes in the ratio of volume of sample to volume of tube, further reduction in pressure to 0.01 mm Hg in the sealed tube, and degassing with nitrogen or helium did not yield any noticeable improvement to the data given in Table 3.6.

A comparison of the percentage recoveries of added free amino acids to a hydrolysis mixture as given in Table 3.5 with the percentage recoveries of the same amino acids when present in the combined form as protein Table 3.6, Column 2, shows that in several cases extensive degradation of individual acids occurs during the process of peptide bond breaking occuring in the hydrolysis e.g. aspartate and glutamate recoveries are reduced to approximately fifty percent.

It was decided however, that the evacuated sealed tube method would suffice for the environmental work to be undertaken and a short series of complete determinations of dissolved free and dissolved combined amino acids was undertaken to test the method.

3.8. Precision Of The Evacuated Sealed Tube Technique For The Hydrolysis Of Environmental Samples.

It was necessary to determine the precision of the evacuated sealed tube technique. This was achieved by hydrolysing duplicate environmental samples and examining the variation in dissolved combined amino acid levels obtrained. Table 3.7 details the results of these experiments and shows the variation to be at an acceptable level.

Table 3.7. Hydrolysis Of Duplicate Environmental Samples.

Date:	Duplicate DCAA analyses (uM).
5.6.86	2.395, 2.200
12.6.86	1.520, 1.367
3.9.86	2.480, 1.975 2.241, 2.180 4.346, 4.743

The possible effect of nitrate levels on amino acid recoveries was first discussed in the paper of Henrichs & Williams (1985). The level of nitrate found in natural waters can vary from below 1 uM to approximately 50 uM in estuarine waters. The waters of the English Channel (E1) have a maximum of 12 uM, though values for coastal waters may be higher (Butler et al. 1979). The high acidity of the hydrolysis medium would result in nitrate being present as the powerfully oxidizing Nitric acid species. It was felt this could cause increased

degradation of amino acid species during the hydrolysis process. Accordingly the effect of nitrate levels upon amino acid recoveries from hydrolysis experiments was investigated.

3.9. The Effect Of Nitrate Levels On The Amino Acid Recoveries From Protein.

A seawater sample was UV-irradiated in the presence of hydrogen peroxide to breakdown any organics present. In the case of nitrogen containing organic molecules these would have been degraded to nitrate. In this way the UV-irradiated seawater contained the highest levels of nitrate likely to be found in sea water. Bovine Serum Albumin (BSA) was added to the UV-irradiated seawater and hydrolysed. The percentage recovery of the amino acids are given in Column 4 of Table 3.6. Having established that amino acids recoveries were unlikely to be affected by the nitrate levels in the sea, attention was turned to the estuary. The highest levels of nitrate found in the Tamar estuary is 36 u Molar. A pure water solution was spiked with nitrate to 36 u Molar and BSA at $3x10^{-7}$g/cm3. This solution was then hydrolysed using the evacuated sealed tube technique. This gave negligible recoveries of amino acids. Consequently no hydrolyses of estuarine samples have been carried out in this study. It is important to note the limitations imposed by the presence of high nitrate values upon dissolved combined amino acid determinations by hydrolysis procedures.

3.10. Use Of The Internal Standard In Hydrolysates.

An internal standard of Alpha amino adipic acid (Adi) at an approximate 1 uM level was added to the sea-water samples before hydrolysis and a recovery of 89 percent was obtained. It was necessary to add the Adi to each hydrolysis sample, in order to monitor the hydrolysis process, and also to check that the pH adjustment, (section 3.2), was effective in every case.

3.11. Detail Of The Hydrolysis Procedures Involved In The Evacuated Sealed Tube Technique.

1. Pyrex test tubes measuring 150 x 18 mm were cleaned by immersion in a 10% Analar Hydrochloric acid overnight. They were then rinsed many times in MilliQ water and allowed to drain in an inverted position.

2. A constriction approximately 3 mm in diameter was made 2 cm from the rim of the tube using a glass lathe.

3. The batch of constricted tubes was then placed in an inverted position in a beaker. The beaker was covered with aluminium foil to prevent contamination and placed in an oven. The oven temperature was raised to 560°C over a period of 30 mins and maintained at that level for 20 mins, then reduced to 500°C for 6 hours. The oven was then turned off. The tubes remained overnight in the oven

to cool. This pyrolysis was necessary to ensure complete removal of traces of contaminating amino acids.

4. The next stages of the hydrolysis procedure were carried out in a fume cupboard. Clean plastic gloves were worn at all times to prevent contamination of glassware or sample.

5. Samples with appropriate blanks were hydrolysed as follows:

Aristar Hydrochloric acid (1 cm^3) was added to the hydrolysis tube using an acid washed, all glass syringe, followed by the water sample 1 cm^3 (or MilliQ water in the case of blanks). All samples for hydrolysis were filtered using a Millex GV (0.22um) filter attached to the full syringe. 100 ul of a $1x10^{-3}$M solution of alpha amino adipic acid was added using a micropipette with a pre-rinsed plastic tip. The solution was degassed by bubbling helium through it (2 min) using a previously cleaned glass capillary.

After degassing, the samples were frozen by immersion in liquid nitrogen. The sample tubes were then evacuated to a pressure of 0.05 mm Hg and sealed at the constriction with a flame. Hydrolysis was carried out in an oven at 110°C for 16 hours.

6. After hydrolysis the tubes were immersed in liquid nitrogen and opened. The contents were carefully poured into a clean 10 cm^3 round bottom flask and the

hydrolysate evaporated to dryness on a rotary film evaporator. The bath temperature for the evaporation process was 50°C. Final traces of moisture and acid were removed by vacuum desiccation over potassium hydroxide for at least 15 hours. Following the release of the vacuum, 1 cm3 of ultrapure water was added to the round bottomed flask and a 500 mm^3 aliquot removed and analysed for amino acid content.

3.12. Conclusion.

The experiments outlined above show that the most generally used method of sea water hydrolysis i.e. refluxing at atmospheric pressure will only give reliable results at a protein level of approximately $1x10^{-3}$ g/cm^3, which is far in excess of that found in seawater. At the low levels found in seawater the reflux method is unreliable e.g. the recovery of free a.a. at 0.362 uM is 26 percent. This is due to a combination of degradation and contamination. The evacuated sealed tube method gives an average recovery of 87 percent for free amino acids at the 0.362 uM level and a 61 percent recovery of amino acids from a protein at the $3x10^{-7}$ g/cm^3 level.

CHAPTER 4.

THE METHODOLOGY FOR SIZING THE COMBINED AMINO ACID FRACTION.

4.1. Introduction.

A number of different techniques are available for the sizing of organic material in sea water. These are, absorption onto organic resins (used for humic acids), reverse osmosis, gel exclusion chromatography, filtration, ultrafiltration and ultracentrifugation. The most commonly used method is ultrafiltration see Table 4.1.

Table 4.1. The Techniques Used By Previous Workers To Size Organics In Seawater, And The Size Classes Investigated.

Reference	Technique.	Size classes investigated.
Sharp, 1973.	Ultrafiltration.	0.8 um 0.025 um 0.003 um
Maurer, 1976	Ultrafiltration	1,000 10,000 100,000
Ogura, 1977	Ultrafiltration	500-100,000
Lancelot, 1984	Ultrafiltration	500 d
Shoji et al.,1984	Ultrafiltration	1,000 10,000 100,000
Carlson et al.,1985	Ultrafiltration	500 5,000 10,000 20,000 50,000
Suzuki & Sugimura 1985	Gel filtration	<700 700-30,000 30,000-70,000 70,000-150,000 >150,000

A stirred ultrafiltration unit was available for use in this project. The main problem associated with ultrafiltration is concentration polarization. This is due to a layer several molecules thick building up on the surface of the filter, effectively creating a second filter, which can severly affect the size of molecules in the filtrate. This problem is overcome by agitation of the solution. Ultrafiltration cells that employ a stirrer bar as agitator can cause microscopic damage to the filter, which in turn can affect pore size. Therefore the preferred method of ultrafiltration is tangential flow,

which has no destructive effect on the filter.

In tangential flow there are two processes occuring in the filter head. A small positive pressure causes the liquid to be filtered, whilst, at the same time, the solution being filtered sweeps away any build up of large molecules, as it flows across the surface of the filter membrane, and thereby removes any effect of concentration polarization. The flow has no harsh physical effect on the filter and does not cause damage to the delicate membrane. Fig. 4.1 illustrates the principal of tangential flow.

Due to the problems outlined above the stirred ultrafiltration unit was not used. However no money was available for a tangential flow unit and therefore the cheaper, but well established technique of ultracentrifugation was used as an alternative sizing method.

4.2. Methods Of Ultracentrifugation.

Two types of ultrafilters were used. These were Centriflo membrane cones CFLS: Retention cut off 25,000 and Centricon-10 microconcentrators with a cut off at 10,000. Both these products were supplied by Amicon. The two amicon publications dealing with the use of these units are Pub. I-122G for centriflo cones and Pub. I-259C for microconcentrators.

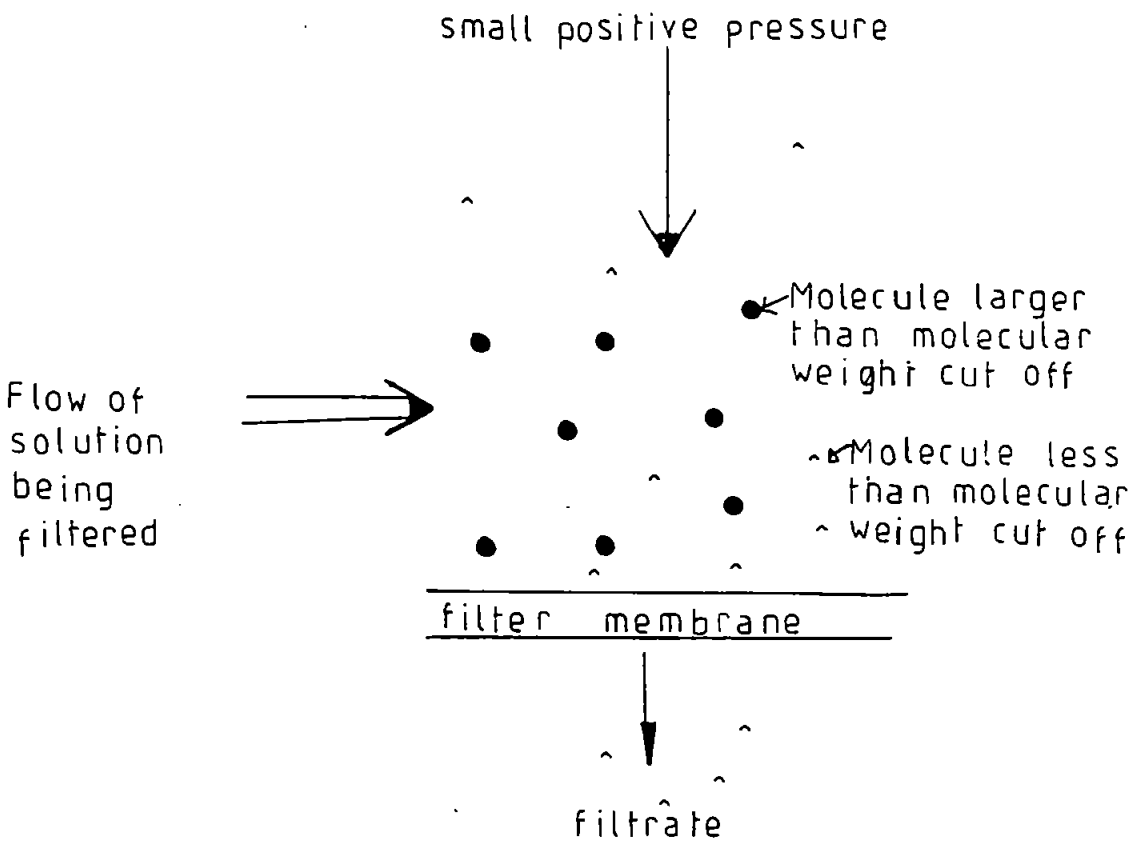


Fig 4.1 Diagram Illustrating The Theory Of Tangential Flow Ultrafiltration.

4.2.1. Use Of Centriflo Cones.

A size determination consisted of a preliminary washing of the cone, a blank determination for combined amino acids, followed by centrifugation of a sea water sample with a similar analysis. Experimental details of this process were as follows.

Two cones were soaked overnight in ultrapure water in an acid washed covered container. The cones were then transferred to their acid washed supports which were fitted into similarly washed centrifuge tubes, see Fig. 4.2. 7 cm^3 of ultrapure water were placed in the cone using an all glass syringe. The cone assembly i.e. cone, support and tube were placed in a centrifuge and spun at 2,000 r.p.m. for 20 mins. The water passed through the cone into the centrifuge tube, carrying with it any molecules less than molecular weight 25,000. (All molecules of greater weight being retained in the cone). The water was discarded and the process repeated another two times using ultrapure water. On the third spinning of the cones 1 cm^3 of the water from the centrifuge tube was taken for hydrolysis and served as a sizing procedural blank. Hydrolysis being by the evacuated sealed tube technique described in chapter 3.

The centrifugation through the cones was then repeated using 7 cm^3 of 0.22 um filtered sea water. 1 cm^3 of the spun sea water was removed from the centrifuge tube and hydrolysed.

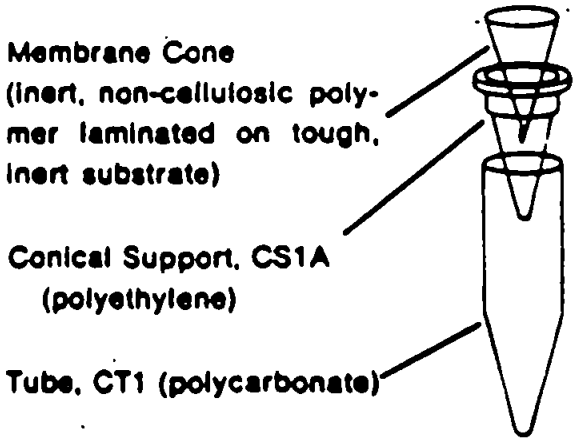


Fig 4.2 A Diagram Illustrating The Amicon Centriflo Cone For Ultrafiltration, (nominal value 25,000 M.W.).

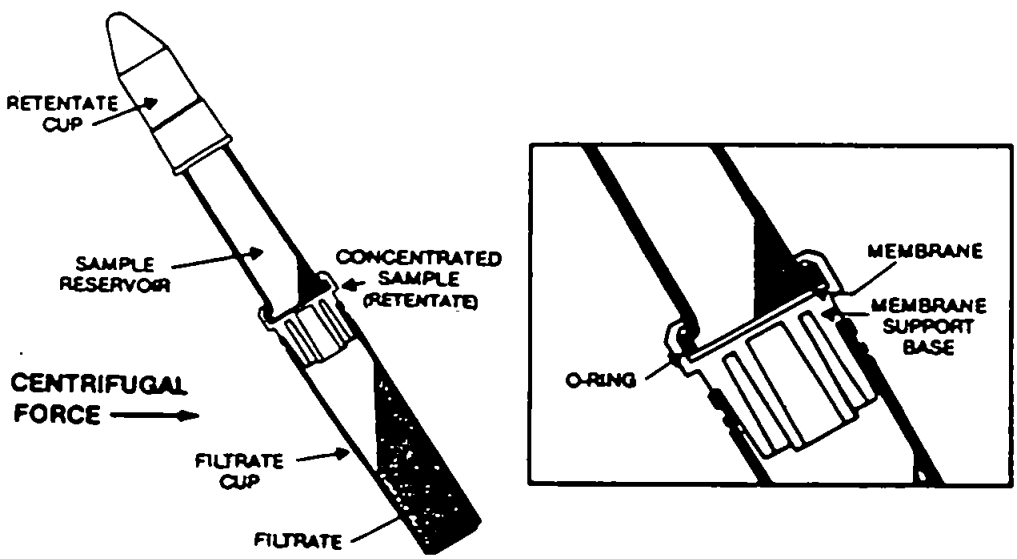


Fig 4.3 A Diagram Of The Amicon Centricon-10 For Ultrafiltration, (nominal value 10,000 M.W.).

4.2.2. Use Of Centricon-10 Microconcentrator.

Two centricon-10 systems were assembled as shown in Fig. 4.3. 1 cm^3 of ultrapure water was placed in the sample reservoir the mouth of which was covered with the retentate cup. The centricon -10's were spun for 20 mins at 3000g. The water passed through the membrane into the filtrate cup. All molecules, molecular weight, less than 10,000 enter the filtrate cup, while molecules larger than 10,000 stay in the sample reservoir. The cup was removed and the water discarded, the filtrate cup was then replaced and the process repeated twice more using pure water. On the third centrifugation the 1 cm^3 of water was taken for hydrolysis i.e. a sizing procedural blank. 1 cm^3 of 0.22 um filtered sea water was then centrifuged as described above and subsequently hydrolysed using the evacuated sealed tube method.

4.3. Sizing Procedural Blanks.

Sizing procedural blanks (SPB) were run to monitor the levels of contamination introduced during the sizing process. For the centriflo tubes average SPB's were 0.8 uM while the centricon-10 gave levels of 0.4 uM.

4.4. Treatment Of Results.

Sizing the dissolved combined amino acid fraction by the two techniques described above will produce data from which the percentage of the combined amino acids

smaller than the nominal m.w. cut off of the filter can be found. To determine this percentage several steps are necessary and these are outlined below using arbitary values as an illustration of the data needed and the calculations used.

The first step is the analysis of the dissolved free amino acid content of the seawater sample (DFAA). Subsequent hydrolysis of the seawater sample followed by amino acid analysis gives the total amino acid levels i.e. free plus combined amino acids in the water (TAA). Subtraction of the procedural blank for the experiment gives an adjusted total amino acid level.(TAA').

TAA - PB = adjusted TAA (TAA')

The hydrolysis of the sized fraction gives the TAA levels less than e.g 25,000 (STAA) again the relevant sized procedural blank (SPB) is subtracted (STAA') Having acquired all the relevant data the percentage of combined amino acids less than 25,000 can be calculated: as an example consider the following, if

DFAA = 0.5 uM

TAA = 2.5 uM

PB = 0.5 uM

Then TAA'= 2.0 uM

Also if,

STAA = 1.5 uM

SPB = 0.5 uM

Then STAA'= 1.0 uM

Free amino acids have a molecular weight of the order of 100 and will therefore appear in both the STAA' and TAA' figures. To find the concentration of combined amino acids (CAA) only the DFAA levels are subtracted from both sets of figures:

TAA'-DFAA = 2.0-0.5 = 1.5 uM CAA'

STAA'- DFAA = 1.0-0.5 = 0.5 um SCAA'

% of CAA < 25,000 = SCAA'/CAA'x 100 =

0.5/1.5 x 100 = 33 % of CAA < 25,000 m.w.

Thus in this example 33 percent of the combined amino acids would have a molecular weight less than 25,000.

4.5. Sizing Bovine Serum Albumin (BSA) To Test The Retention Of The Centriflo Cones.

Bovine Serum Albumin has a molecular weight of approximately 67,000 and therefore should not pass through the cetriflo cones with molecular weight cut off of 25,000. This was tested as follows; duplicate samples of a BSA solution were placed in two centriflo cones as described for procedural blanks and sea water samples. The protein solutions were spun and the filtrates obtained hydrolysed by the evacuated sealed tube technique. Recovery of amino acids from the protein in

the filtrate were 10 and 8 percent. Therefore 90 and 92 percent of the protein was retained within the cones. This compares well with the amicon company values for retention for BSA of >90 percent. The results of the sizing experiments described above can be found in Chapter 5 Coastal Results.

CHAPTER 5.

SEASONAL LEVELS AND COMPOSITION OF DISSOLVED FREE AND DISSOLVED COMBINED AMINO ACIDS IN LOCAL COASTAL WATERS.

5.1. Introduction.

Before presenting the results of the seasonal survey of the dissolved combined and dissolved free amino acids in coastal waters it is necessary to consider some factors relevant to the strategy for collection and interpretation of environmental data.

In analytical chemistry much emphasis is placed on obtaining a representative sample of the material under investigation and there are numerous texts which deal with the methodologies employed for different types of sample to achieve this result.(Allen et al. 1974, Reid 1981, Beyermann 1984). In the case of a dynamic system such as the oceans there is a difficulty in measuring the levels of micronutrients due to factors such as patchiness of distribution, water movement, production or consumption of the analyte, time of the year, time of day, turbidity, the level of biological activity and the location. Changes of the sample once collected due to the labile nature of dissolved free amino acids and the possibility of containment effects etc. is another difficulty encountered when dealing with environmental samples. The problems outlined above mean that the concept of a "representative sample" cannot be strictly applied instead the result of an analysis may be best

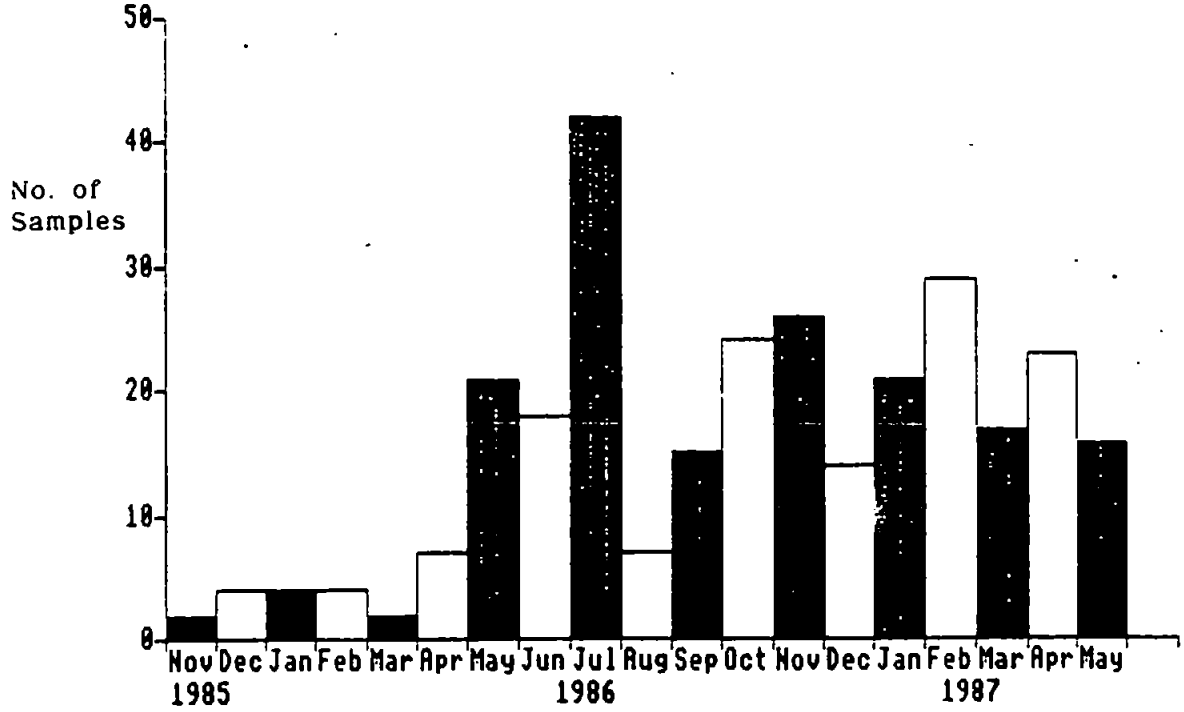


Fig 5.1a Number of Samples Analysed for DFAA per Month.

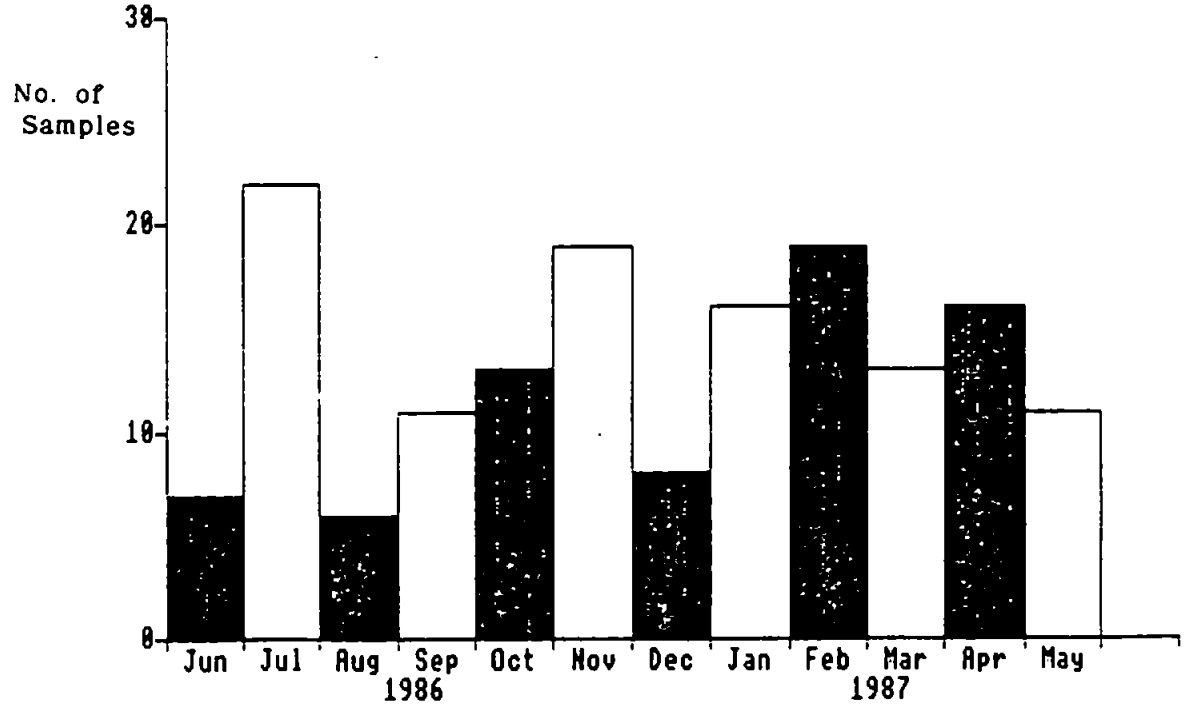


Fig 5.1b Number of Samples Analysed for DCAA per Month.

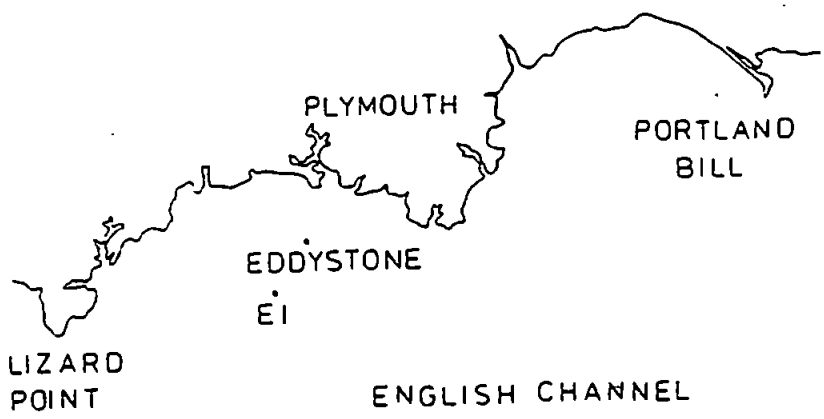


Fig 5.2 Map Of The Sampling Area, (from Braven et al. 1984).

amino acids and the relationship between the total amino acids (free plus combined) and the total dissolved nitrogen and dissolved organic nitrogen.

4. The percentage amino acid composition of the free and combined fractions.

5. The molecular size of the dissolved combined amino acid fraction.

In order to examine the five areas listed above, the raw data has been converted to graphs, histograms, pie charts and tables, depending upon which was the most appropriate form of data presentation for the topic under discussion. Appendix III details the calculations used to convert the data to the relevant graphs and charts.

The chapter finishes with a discussion (section 5.9) of the salient points of the environmental results.

5.3 Results.

5.3.1.

The average concentrations and range of the free and combined acids observed during the sampling period are given Table 5.1.

Table. 5.1 The Range, Average Concentrations, Co-efficient of Variation (CV) and Number of Samples Analysed (n) for the Dissolved Free and Combined Amino Acids Over the Sampling Period.

	Dissolved free amino acids	Dissolved combined amino acids.
Range.	BLD - 9.372 (uM).	0.246 - 6.741 (uM).
Average.	0.403 (uM).	1.700 (uM).
CV	113	27
n	295	158

BLD= below the limits of detection of the method. See chapter 2.

Comparison of the results found in this study (Table 5.1), with those of previous workers, can be found in the discussion section (5.9) at the end of the chapter. The levels of dissolved combined amino acids found in this study are approximately 4 times those of the free amino acids. Thurman (1986) attributes the difference in levels to the dissolved free amino acids being removed by microbial activity.

Graphs of the average concentration for total dissolved free and combined amino acids for each batch of samples collected over the sampling period are given in Fig. 5.3 (a+b).

The graphs of dissolved free and dissolved combined amino acid concentrations show fluctuations in levels over the sampling period, which are particularly noticeable for the free acid fraction (Fig. 5.3a).

The variation of each data point is shown graphically by a vertical line drawn to scale. The

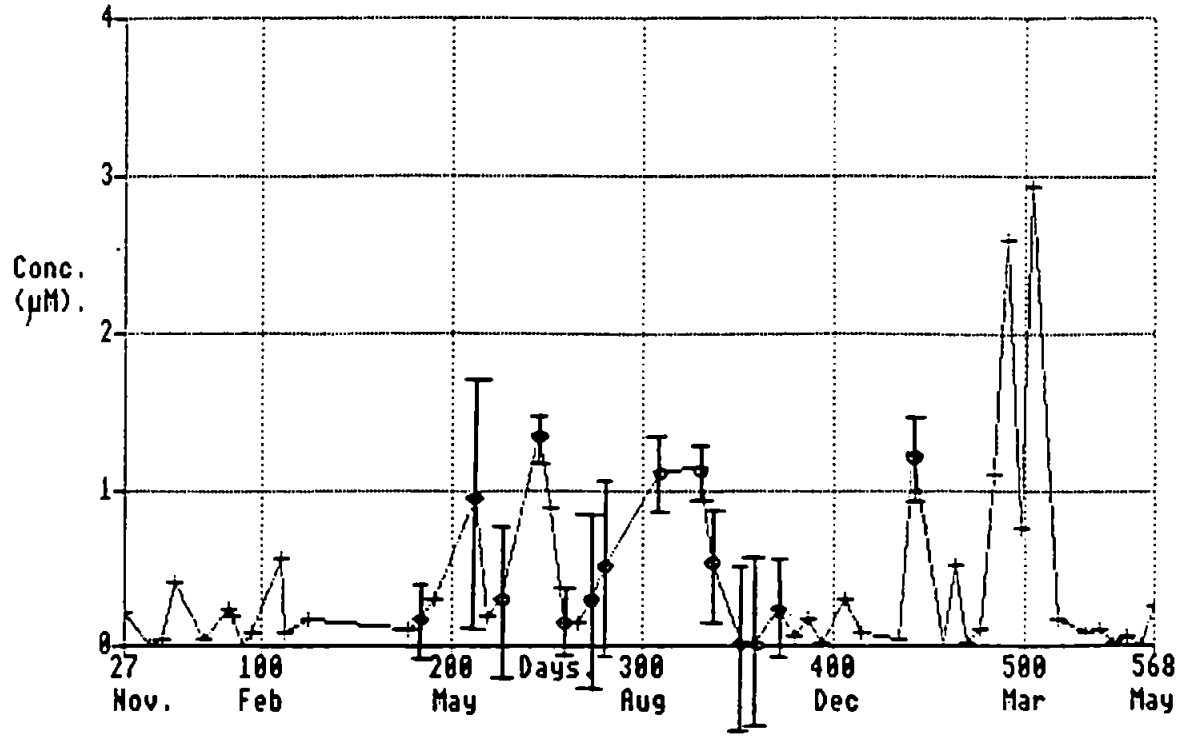


Fig 5.3a Batch Averages for DFAA During the Sampling Period: Nov 1985 - May 1987.

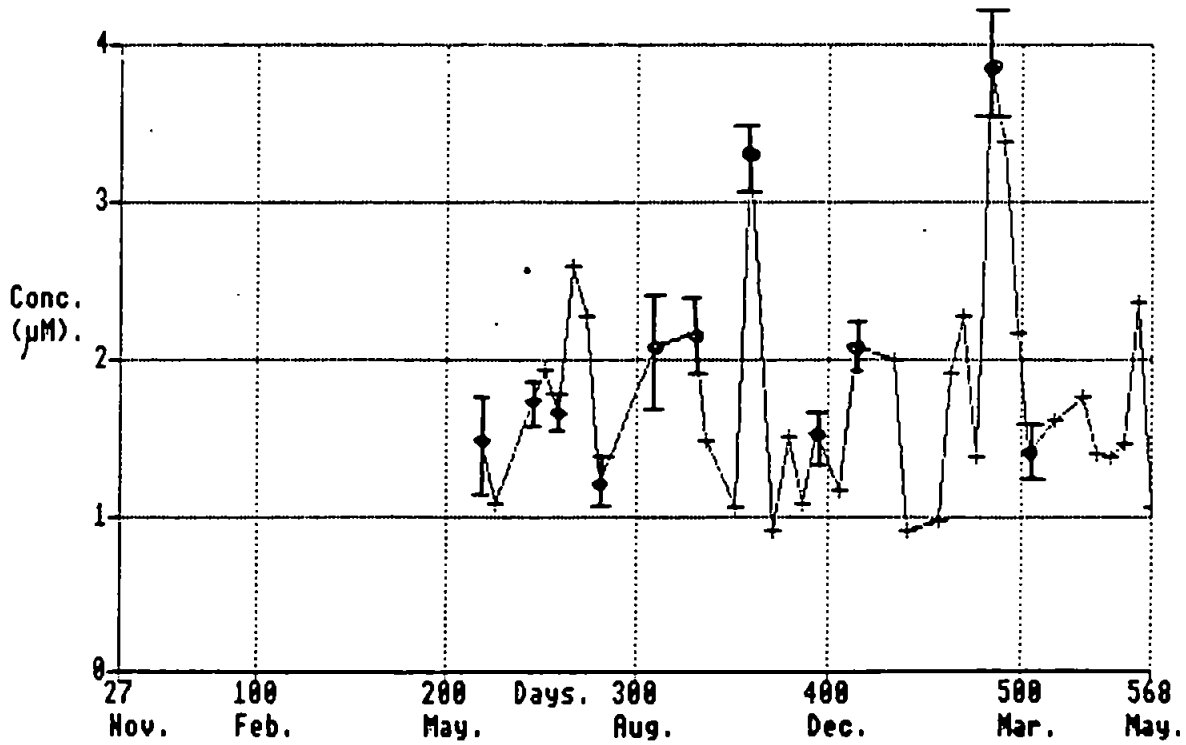


Fig 5.3b Batch Averages for DCAA During the Sampling Period: May 1986 - May 1987.

results for the combined amino acids show much less variation (CV=27), than those for the free acids (CV=113). Futhermore high levels of free acids have less variability than low levels. From chapter two, it can be seen that the presicion of the analytical technique improves with increasing concentrations. The dissolved free amino acids vary in levels from approximately 1 uM to below the limits of detection. As many of the dissolved free amino aids levels found in the environmental samples are very close to the limits of detection for the analytical method there is a large co-efficient of variation for the free amino acid figures. There is therefore a problem in the interpretation of the dissolved free amino acid data in that the variability found for this fraction, is related to both the analytical technique, as well as to the behaviour of the free acids in the environment.

A summary of the free and combined acid levels on a monthly basis can be seen in Fig.5.4 (a+b).

5.3.2 Individual Amino Acid Levels in the Free and Combined Fraction Over the Sampling Period.

Graphs of the average batch concentration for individual amino acids over the sampling period can be seen in Fig. 5.5a-5.19a for the free acids and Fig. 5.5b-5.19b for the combined amino acids.

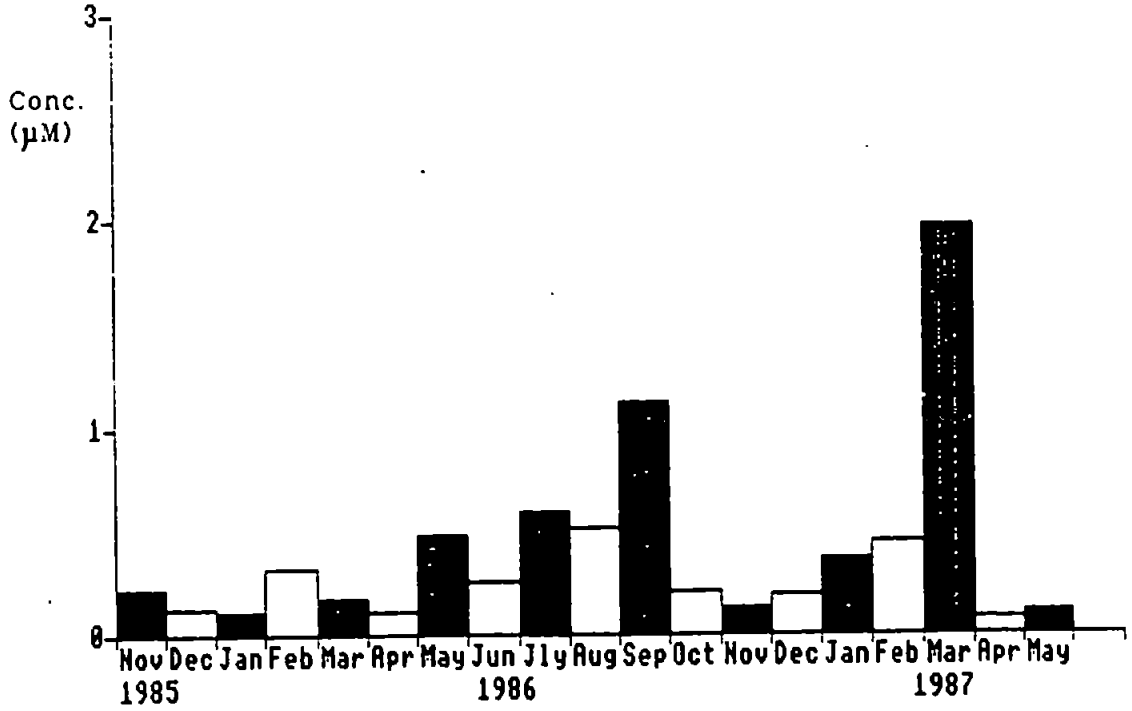


Fig 5.4a Average Monthly Values for the Total DFAA.

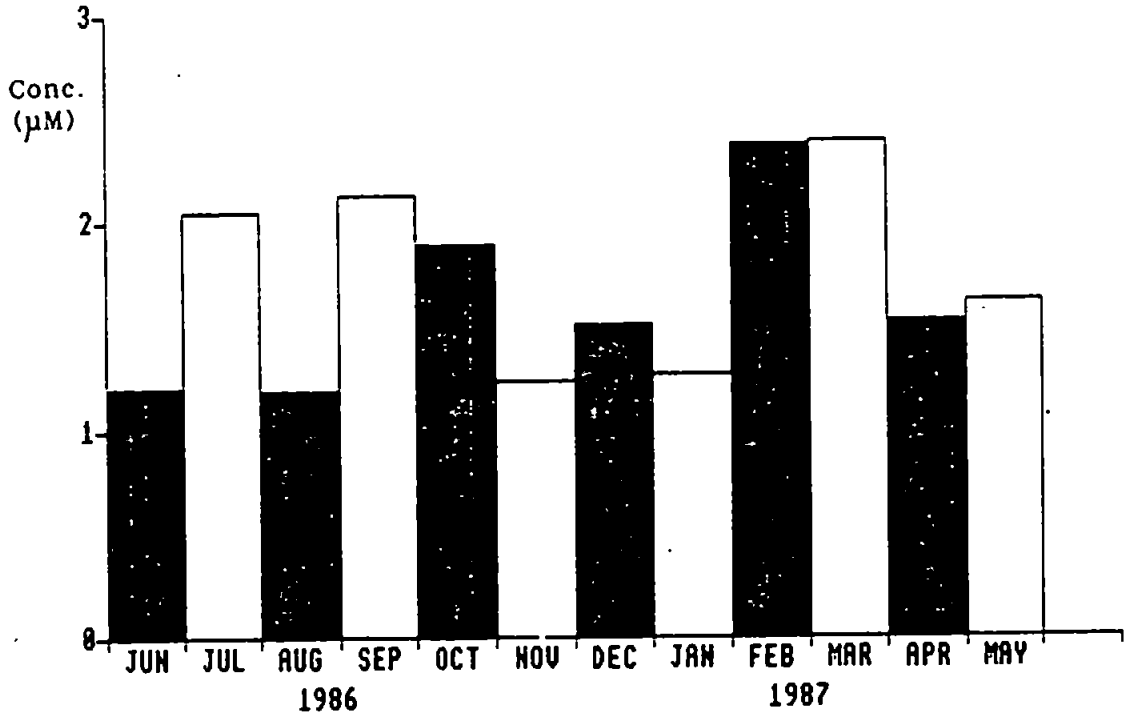


Fig 5.4b Average Monthly Values for the Total DCAA.

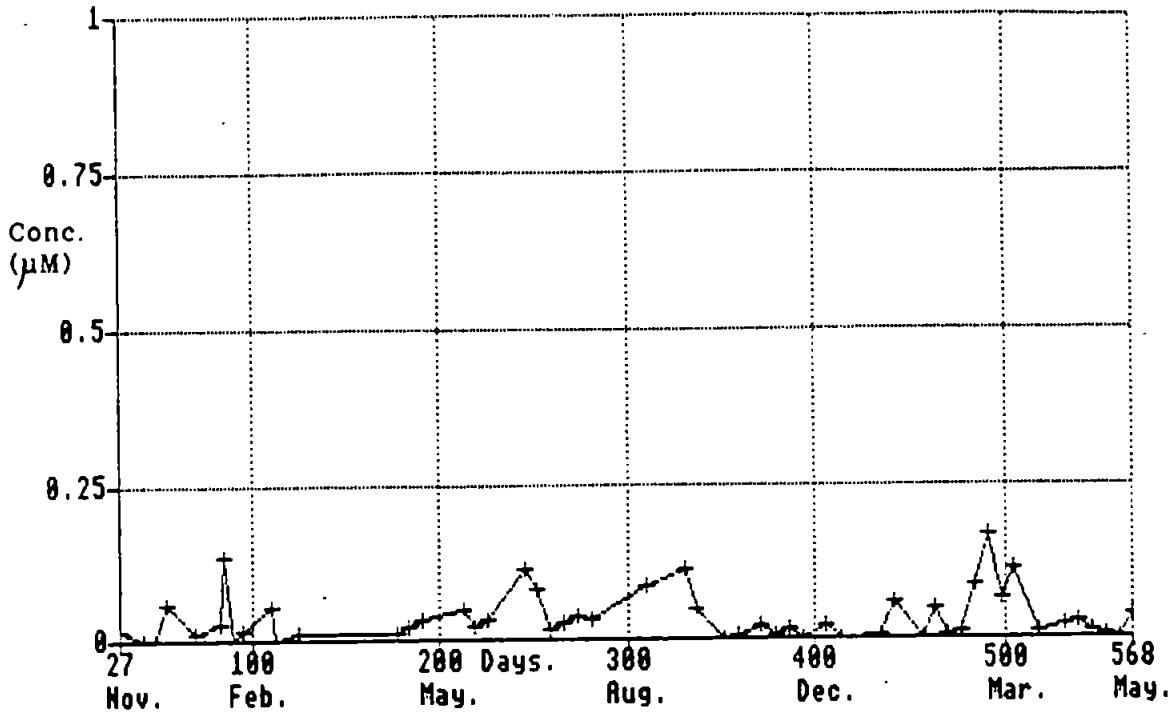


Fig 5.5a Average Concentration of Asp. in DFAA During Sampling Period: Nov 1985 - May 1987.

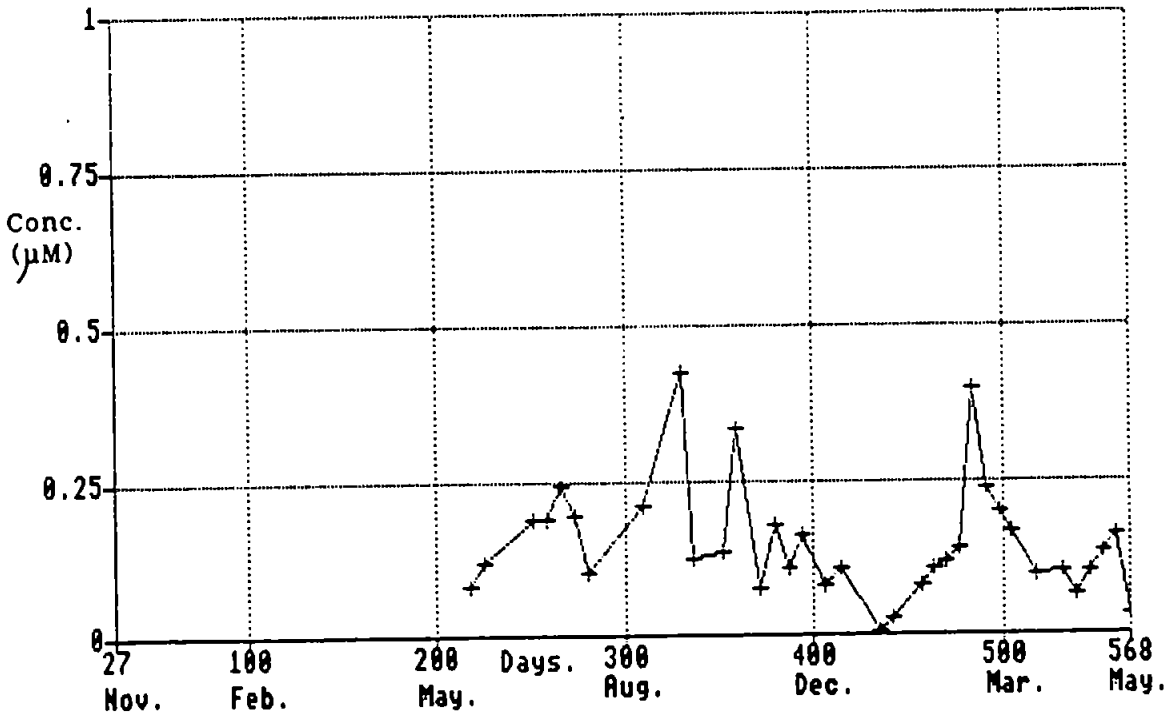


Fig 5.5b Average Concentration of Asp. in DCAA During Sampling Period: May 1986 - May 1987.

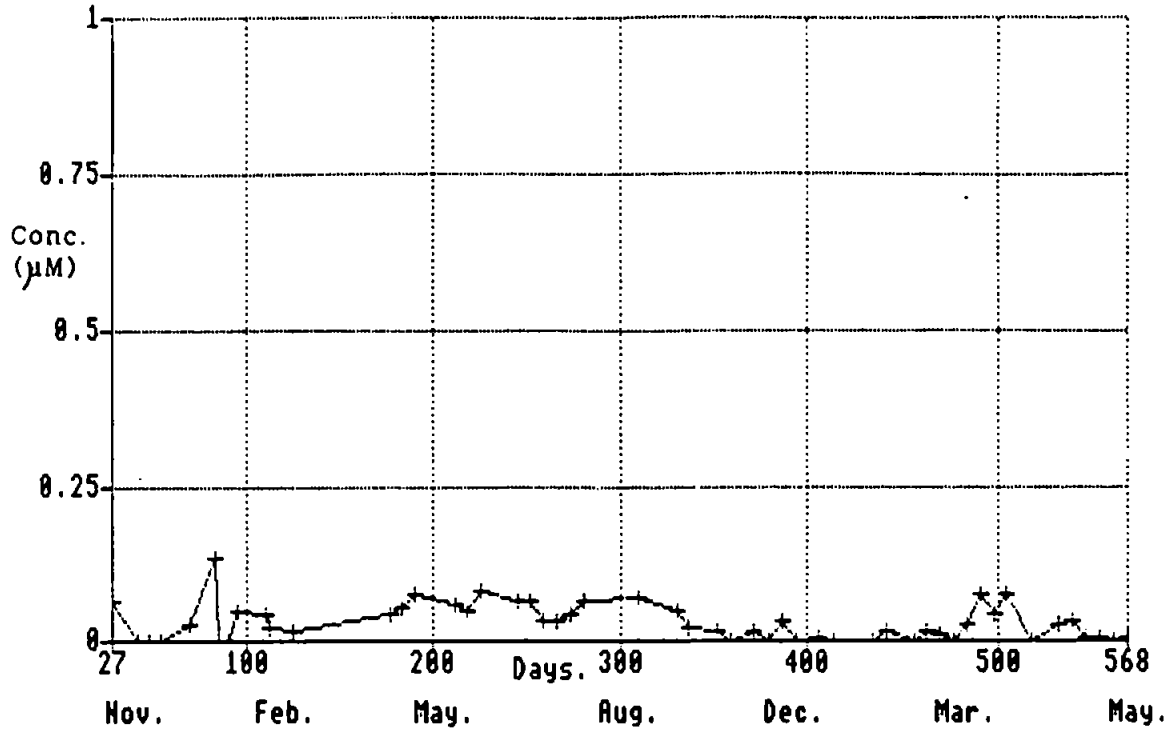


Fig 5.6a Average Concentration of Glu. in DFAA During Sampling Period: Nov 1985 - May 1987.

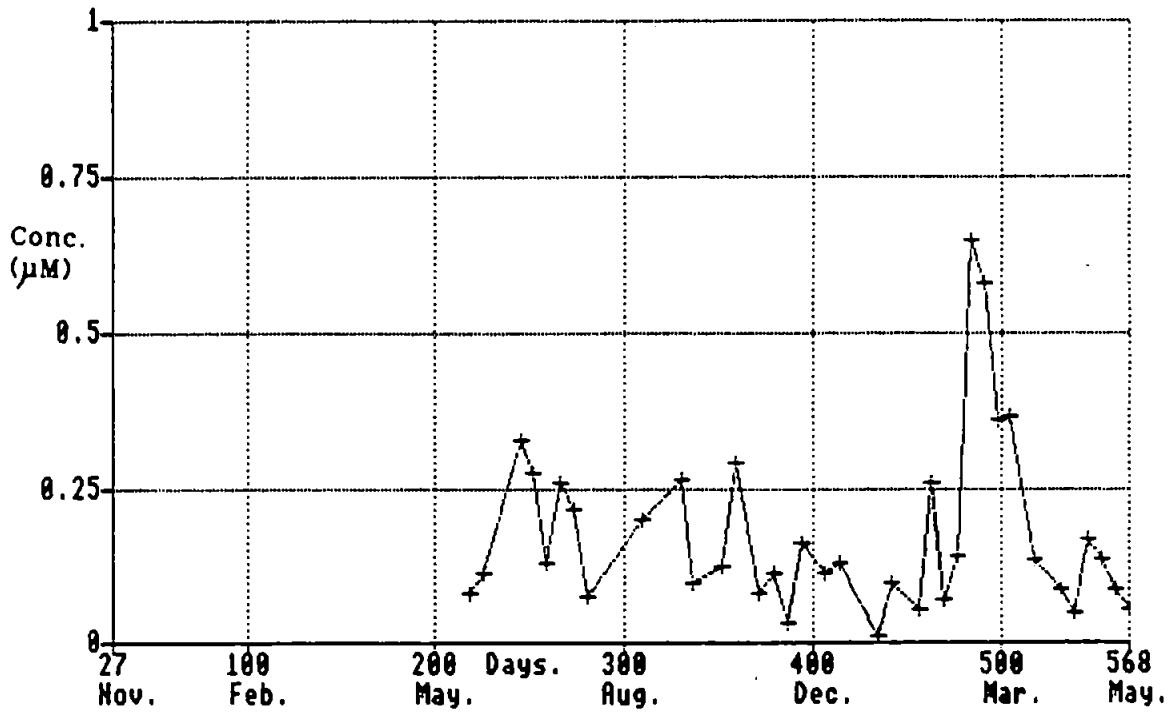


Fig 5.6b Average Concentration of Glu. in DCAA During Sampling Period: May 1986 - May 1987.

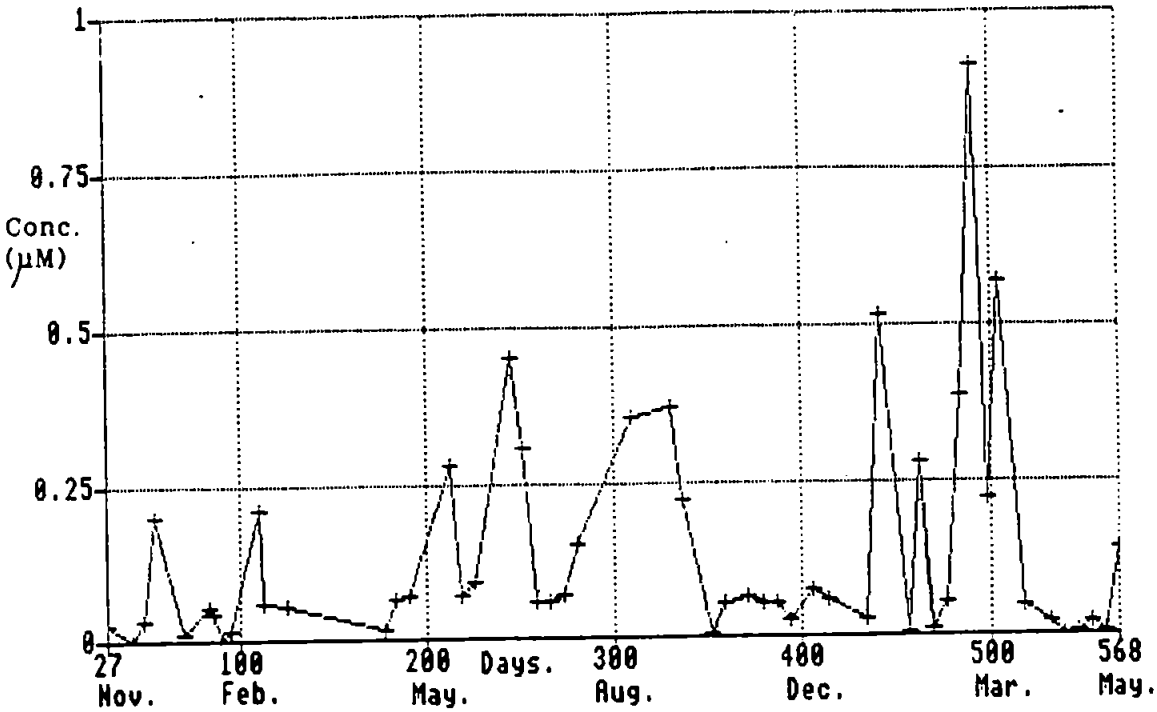


Fig 5.7a Average Concentration of Ser. in DFAA During Sampling Period: Nov 1985 - May 1987.

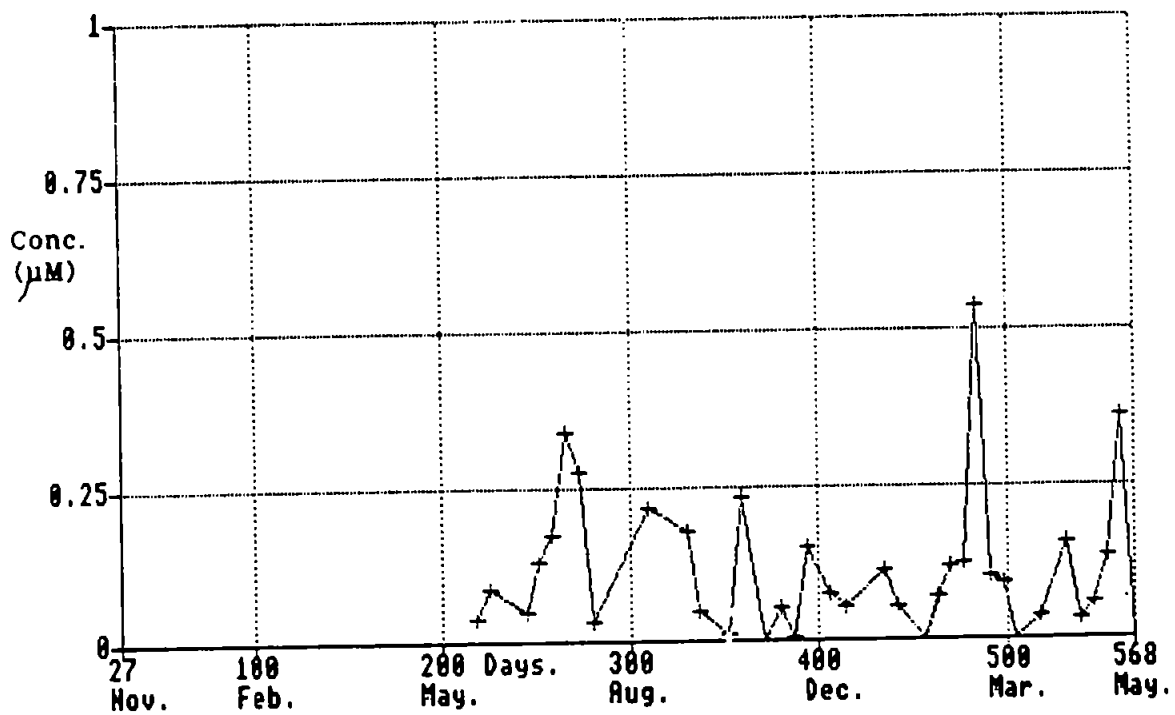


Fig 5.7b Average Concentration of Ser. in DCAA During Sampling Period: May 1986 - May 1987.

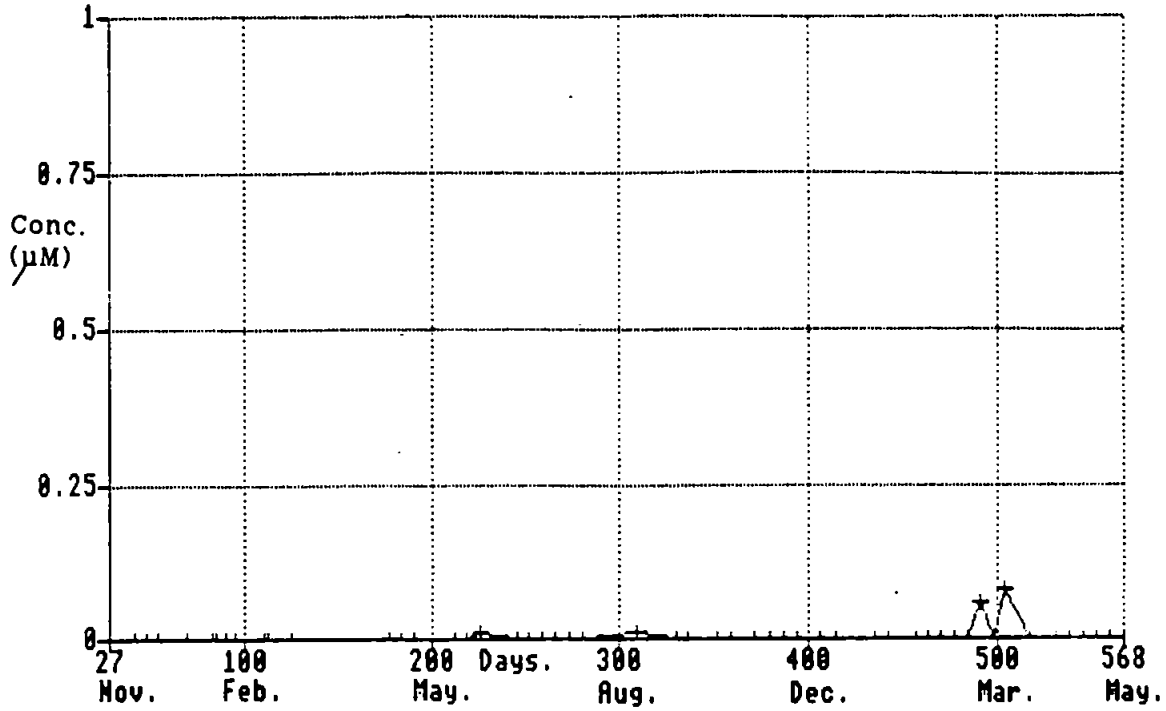


Fig 5.8a Average Concentration of His. in DFAA During Sampling Period: Nov 1985 - May 1987.

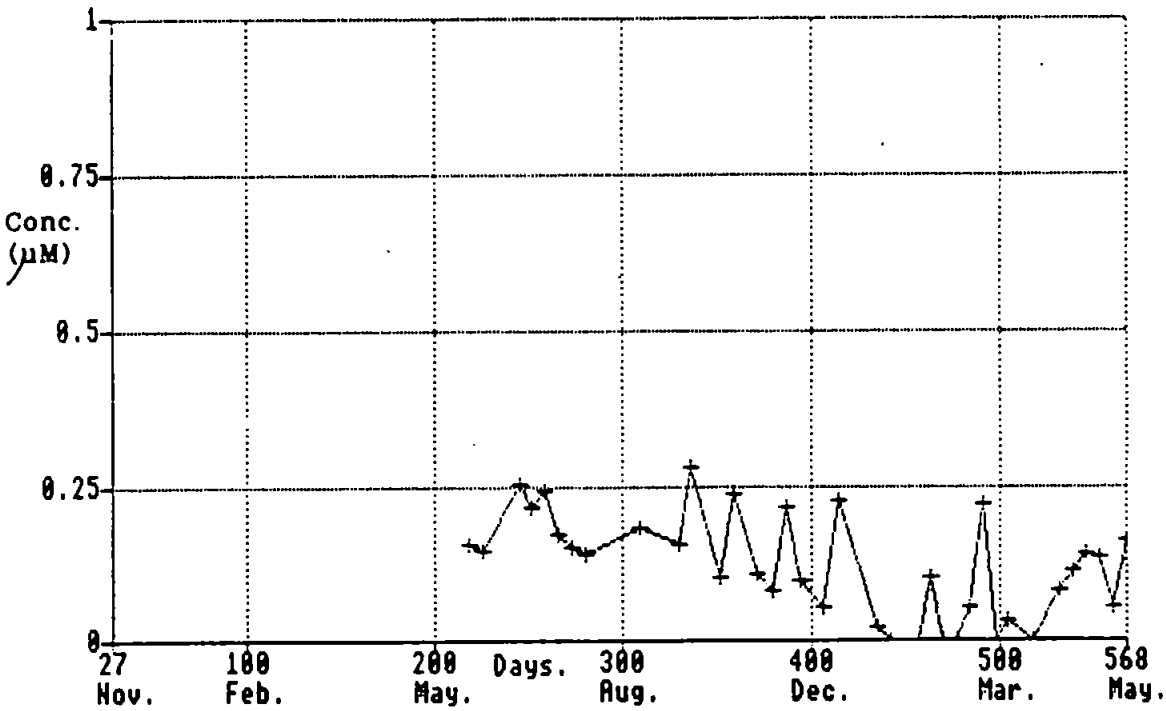


Fig 5.8b Average Concentration of His. in DCAA During Sampling Period: May 1986 - May 1987.

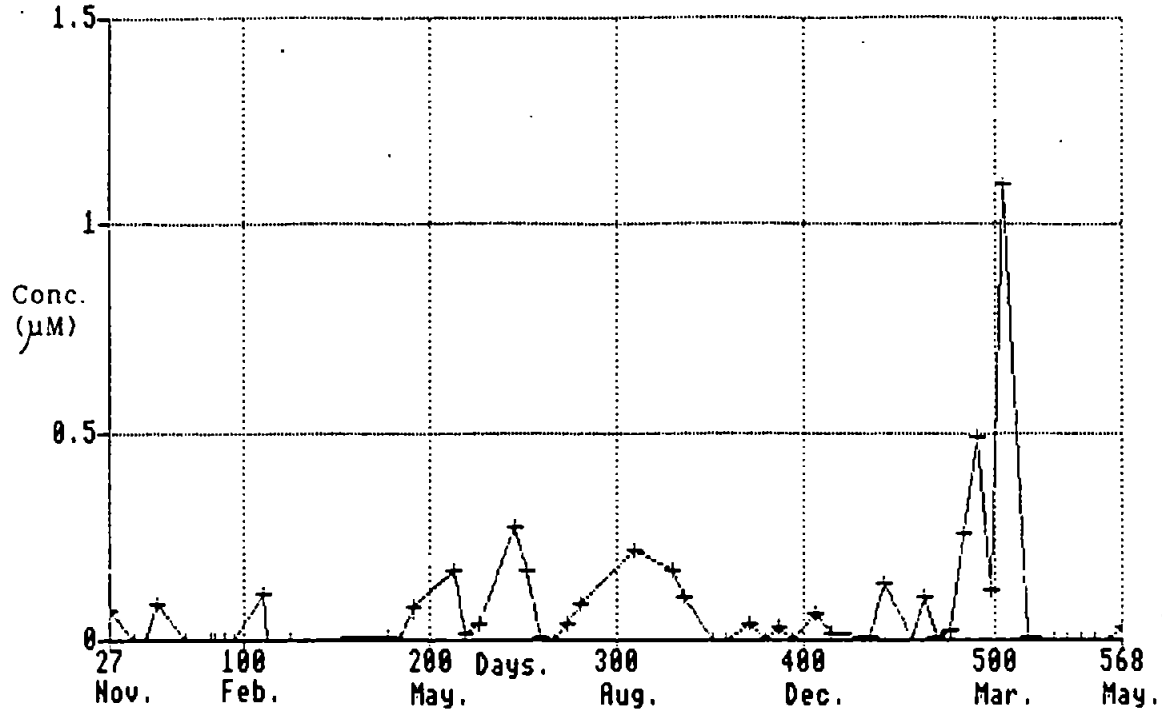


Fig 5.9a Average Concentration of Gly. in DFAA During Sampling Period: Nov 1985 - May 1987.

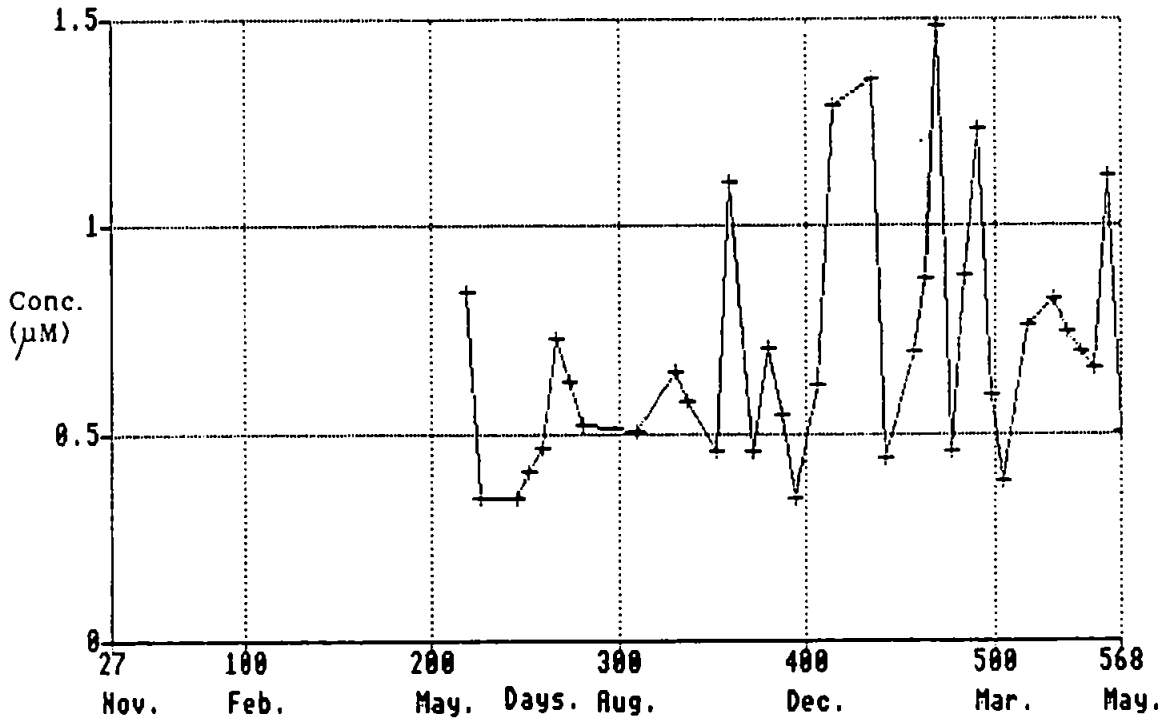


Fig 5.9b Average Concentration of Gly. in DCAA During Sampling Period: May 1986 - May 1987.

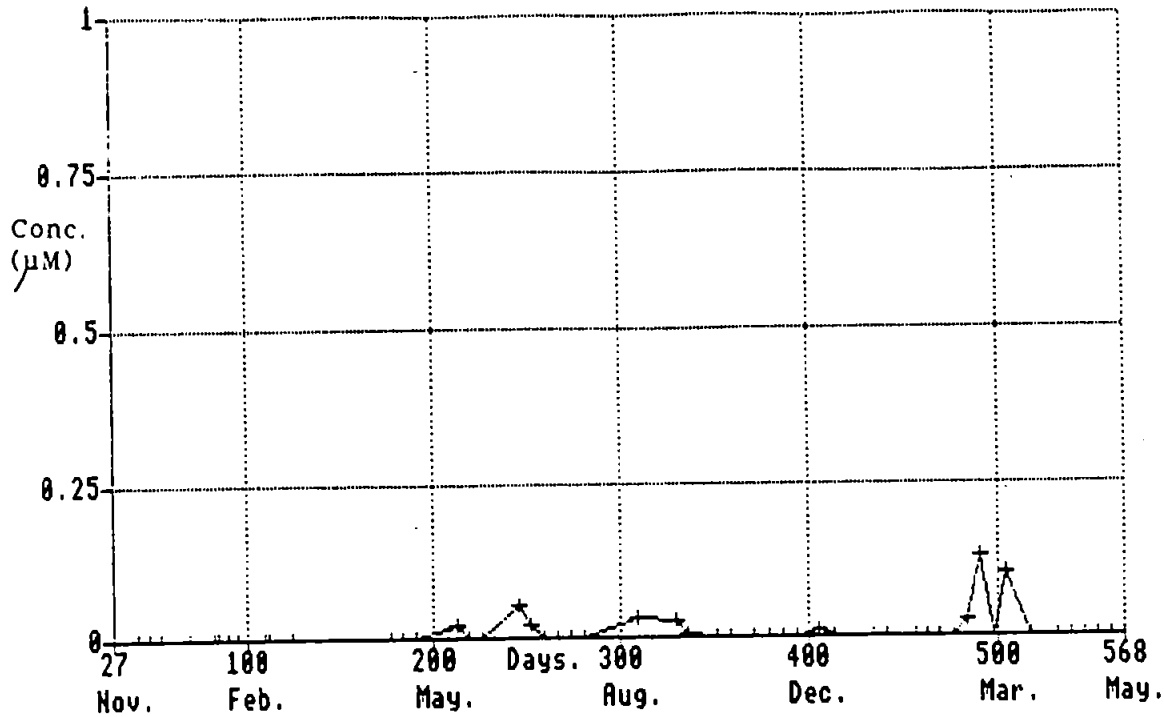


Fig 5.10a Average Concentration of Thr. in DFAA During Sampling Period: Nov 1985 - May 1987.

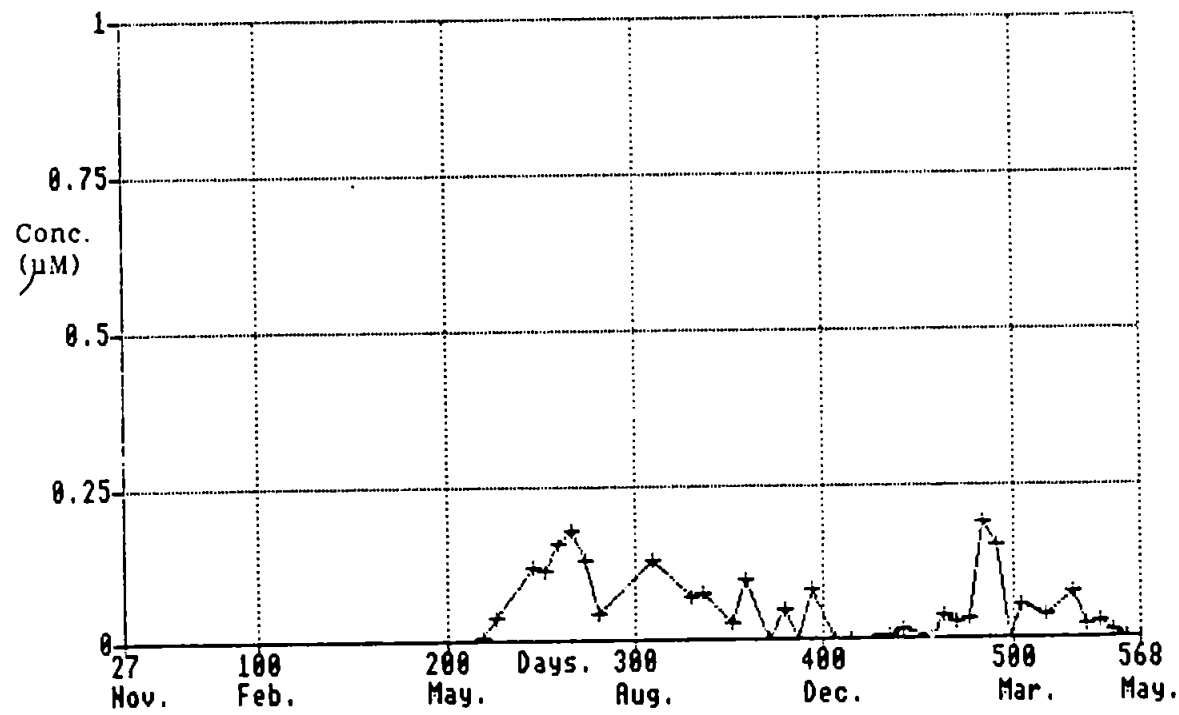


Fig 5.10b Average Concentration of Gly. in DCAA During Sampling Period: May 1986 - May 1987.

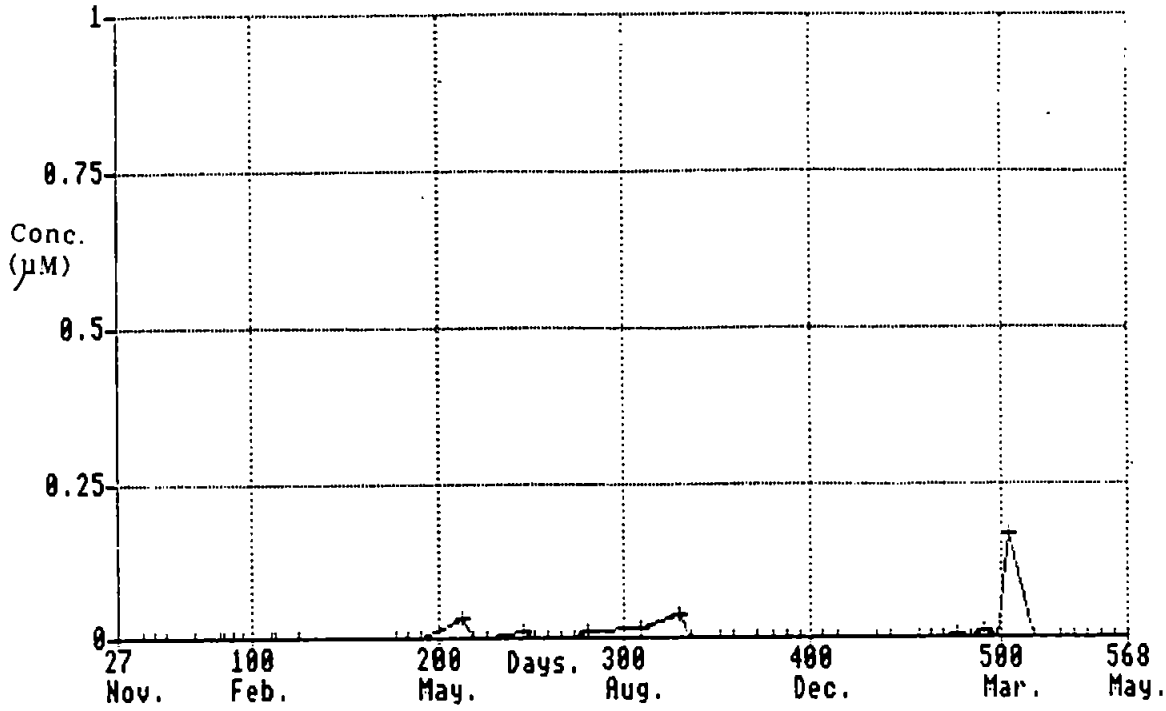


Fig 5.11a Average Concentration of Arg. in DFAA During Sampling Period: Nov 1985 - May 1987.

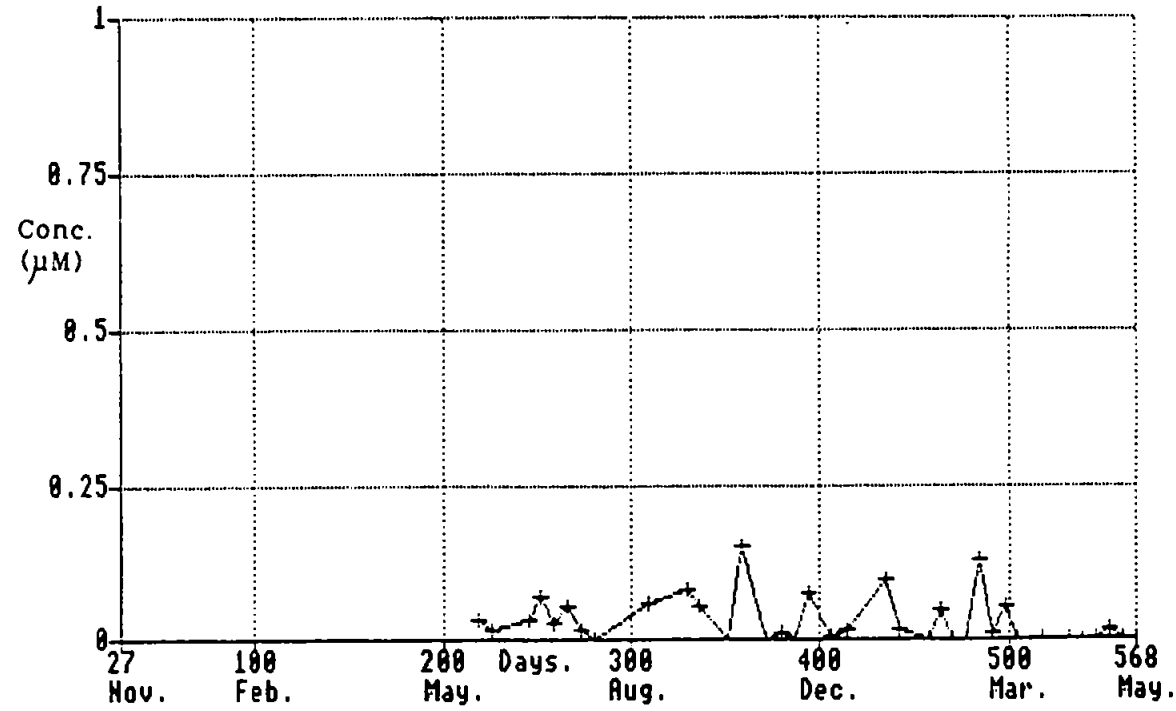


Fig 5.11b Average Concentration of Arg. in DCAA During Sampling Period: May 1986 - May 1987.

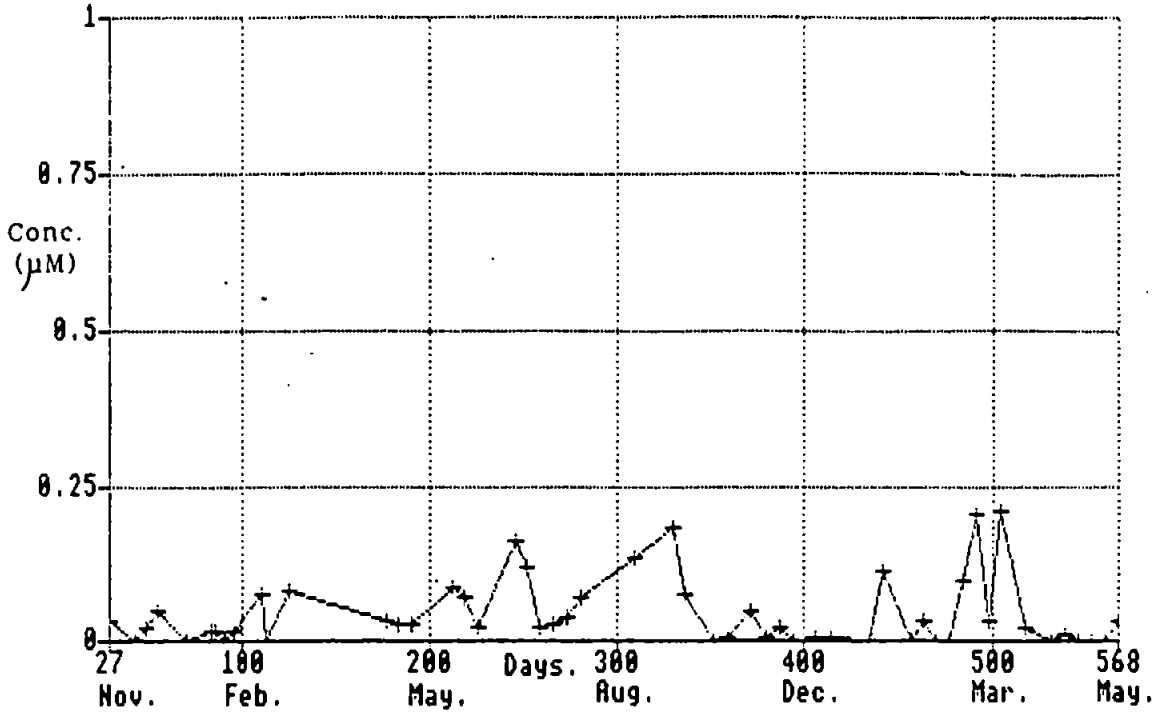


Fig 5.12a Average Concentration of Ala. in DFAA During Sampling Period: Nov 1985 - May 1987.

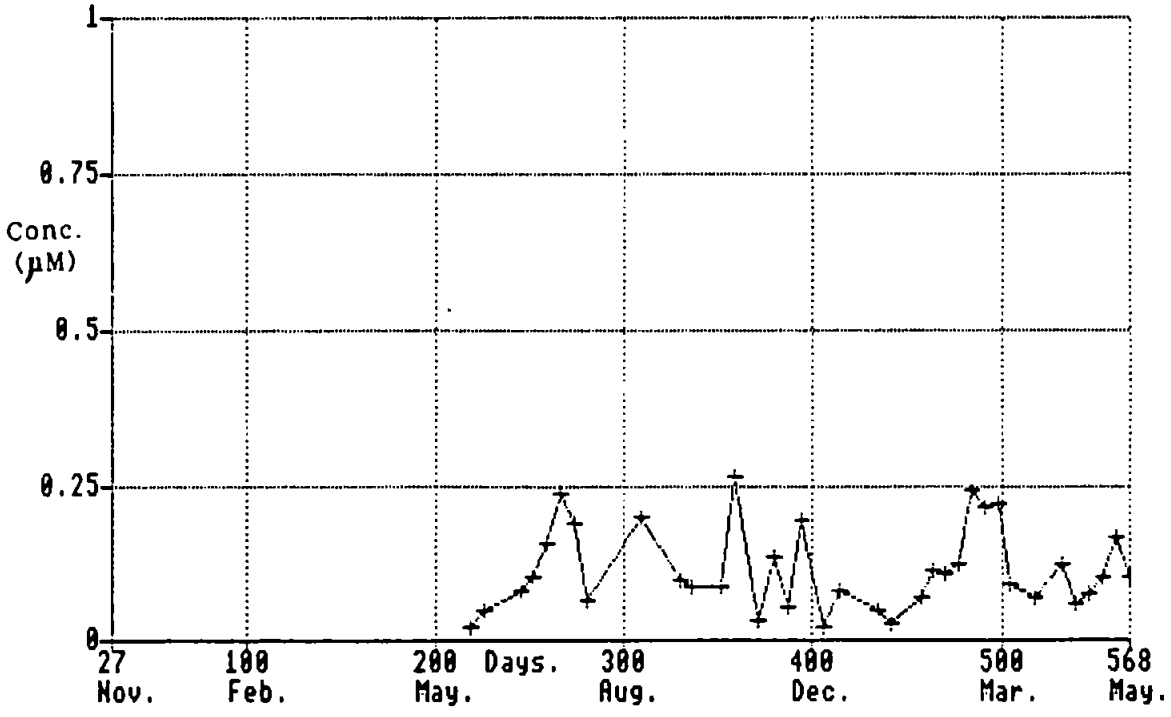


Fig 5.12b Average Concentration of Ala. in DCAA During Sampling Period: May 1986 - May 1987.

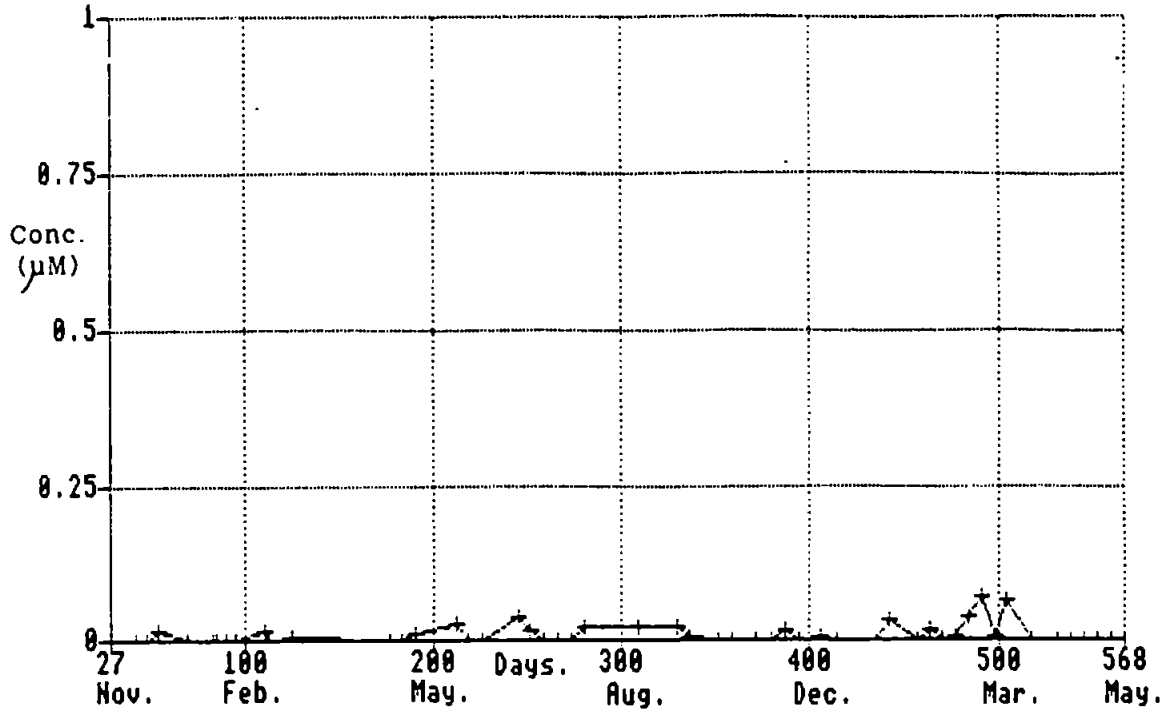


Fig 5.13a Average Concentration of Tyr. in DFAA During Sampling Period: Nov 1985 - May 1987.

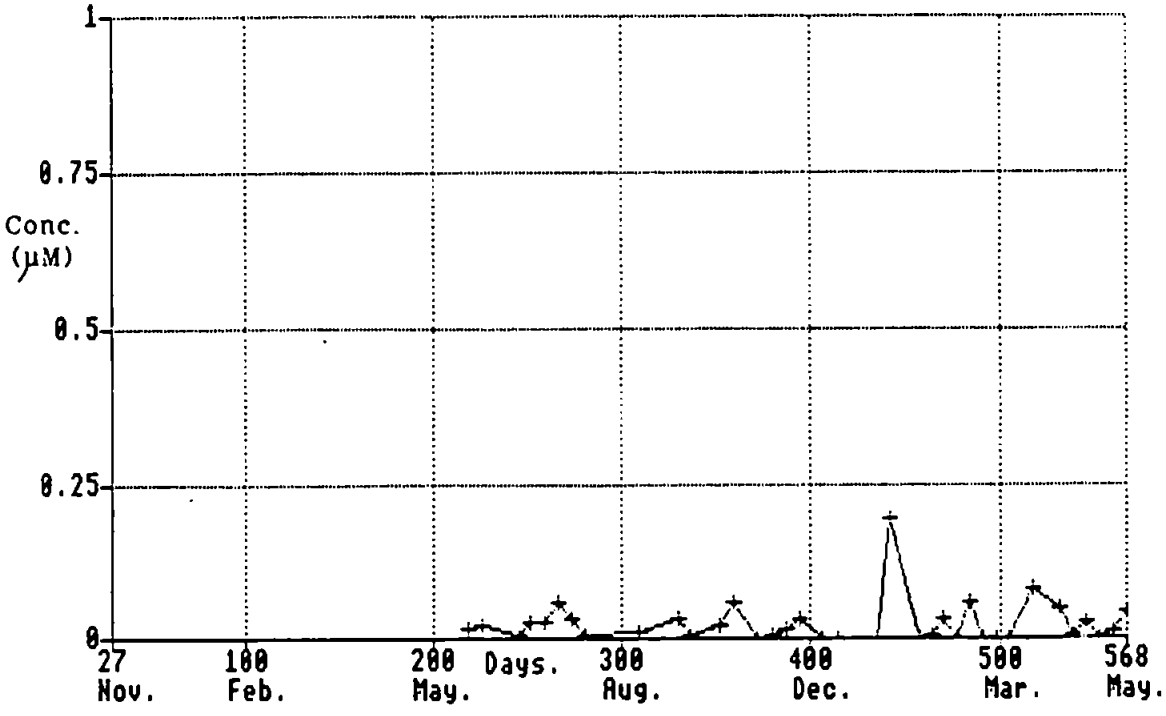


Fig 5.13b Average Concentration of Tyr. in DCAA During Sampling Period: May 1986 - May 1987.

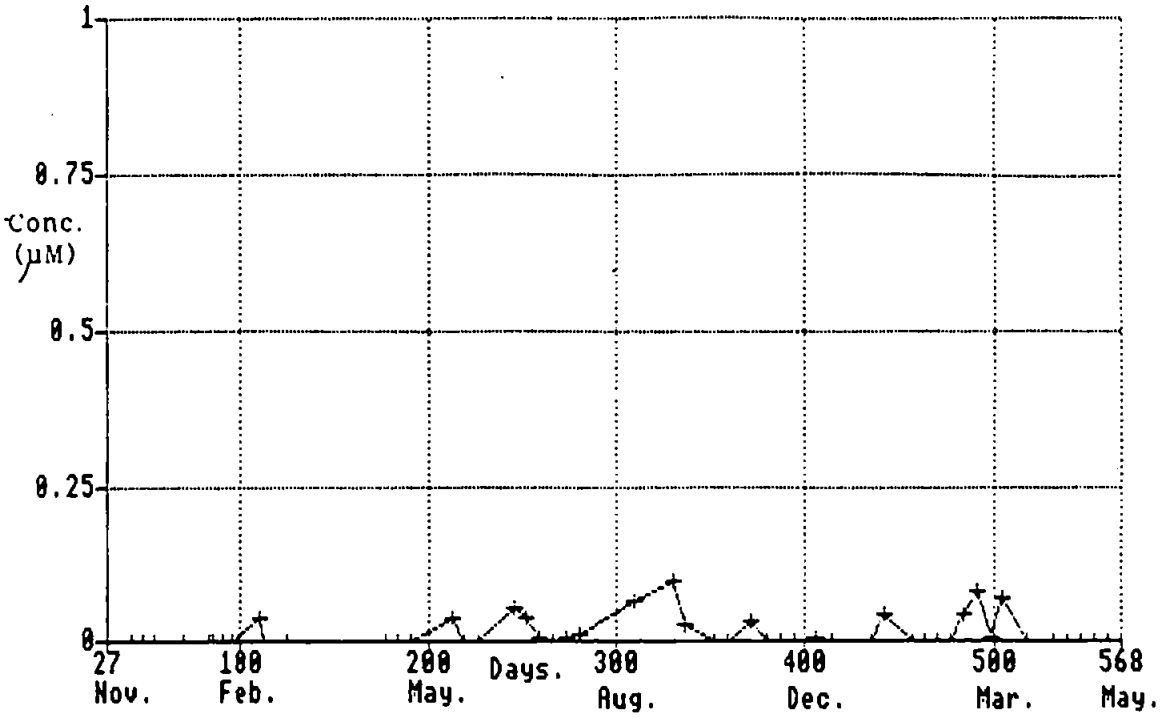


Fig 5.14a Average Concentration of Val. in DFAA During Sampling Period: Nov 1985 - May 1987.

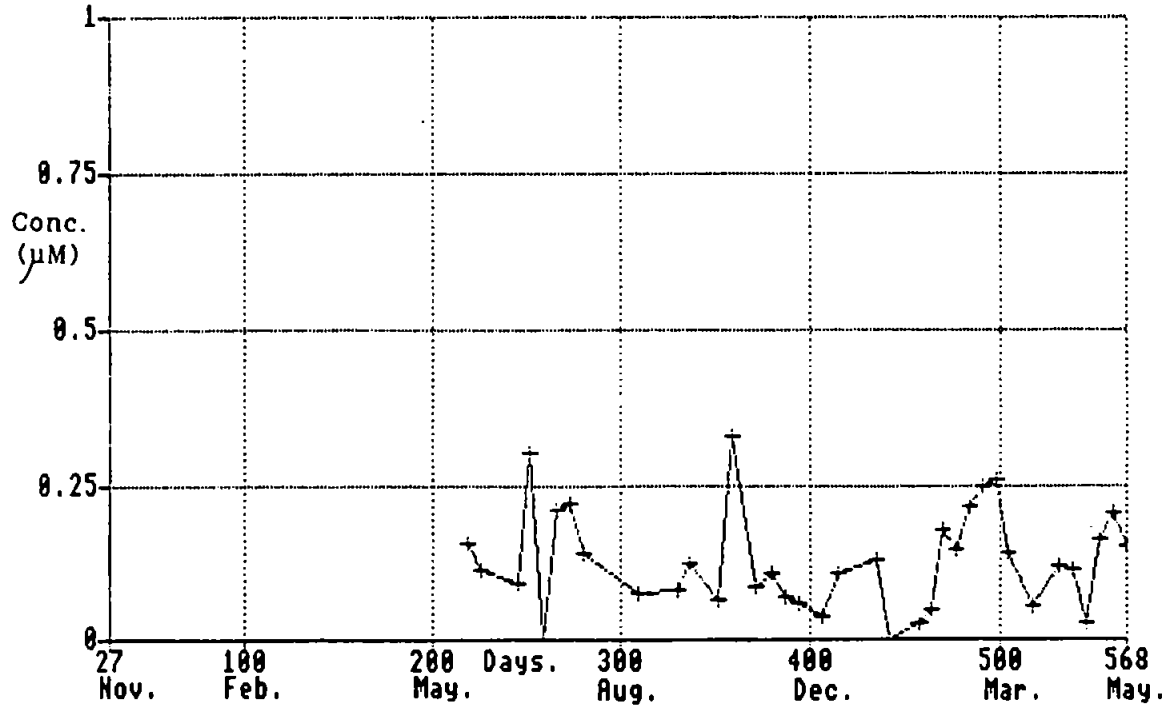


Fig 5.14b Average Concentration of Val. in DCAA During Sampling Period: May 1986 - May 1987.

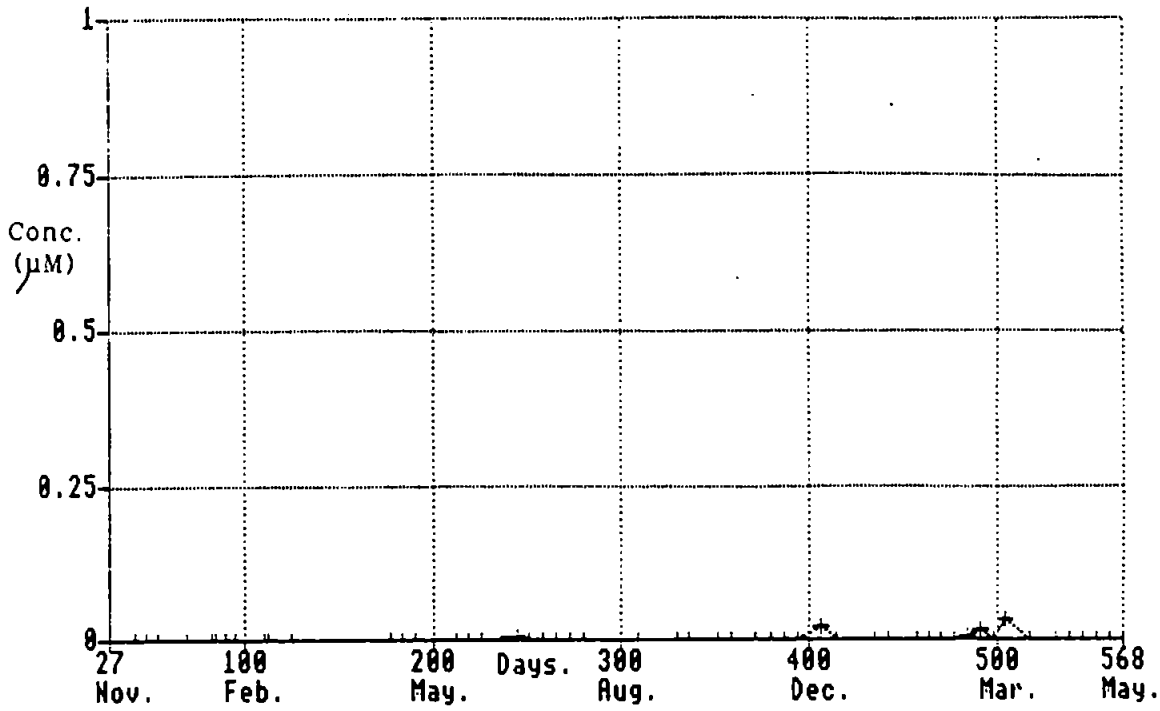


Fig 5.15a Average Concentration of Trp. and Ile. in DFAA During Sampling Period: Nov 1985 - May 1987.

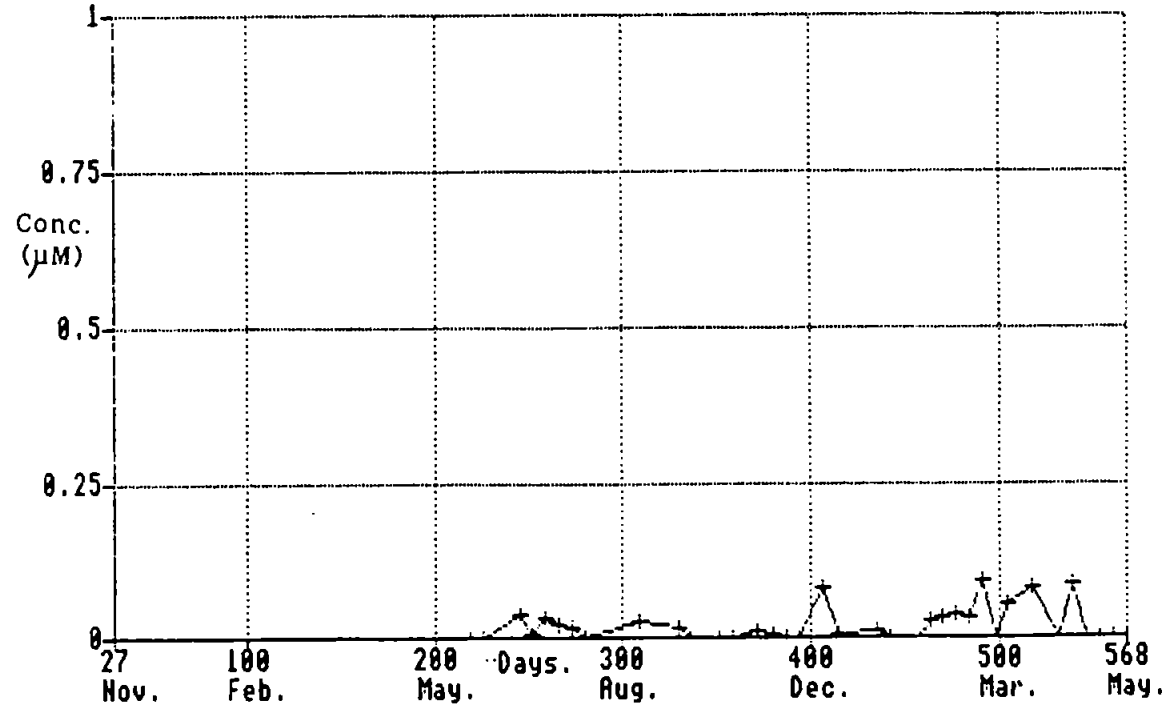


Fig 5.15b Average Concentration of Trp. and Ile. in DCAA During Sampling Period: May 1986 - May 1987.

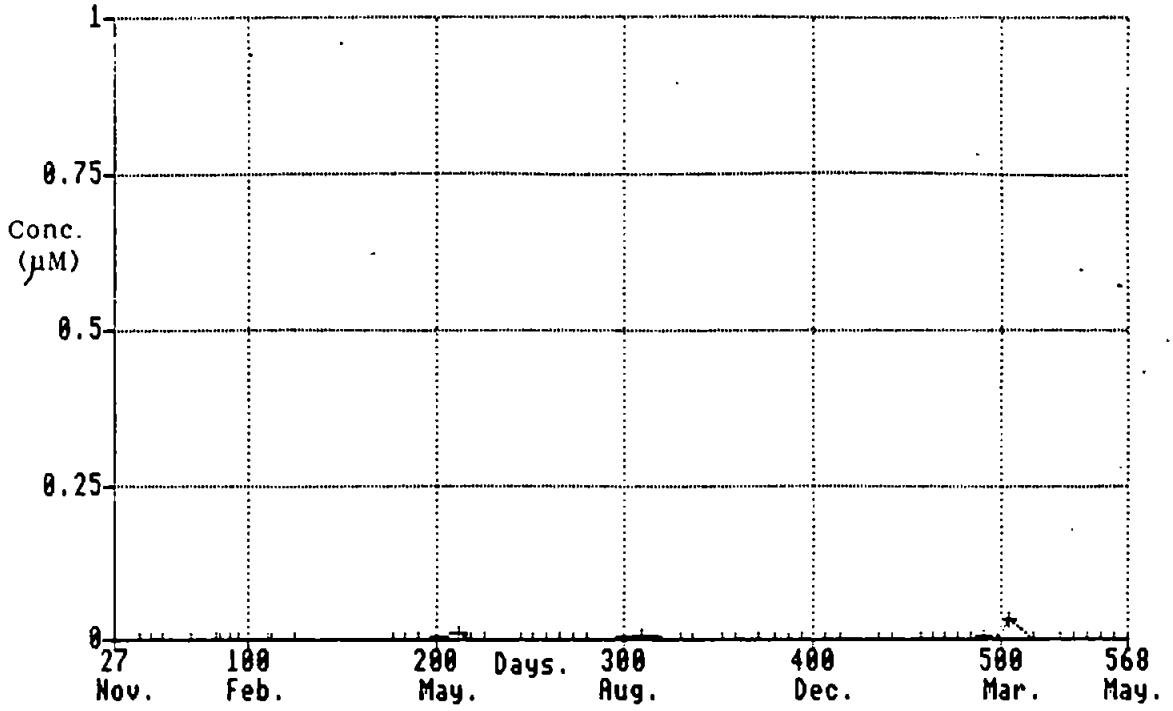


Fig 5.16a Average Concentration of Phe. in DFAA During Sampling Period: Nov 1985 - May 1987.

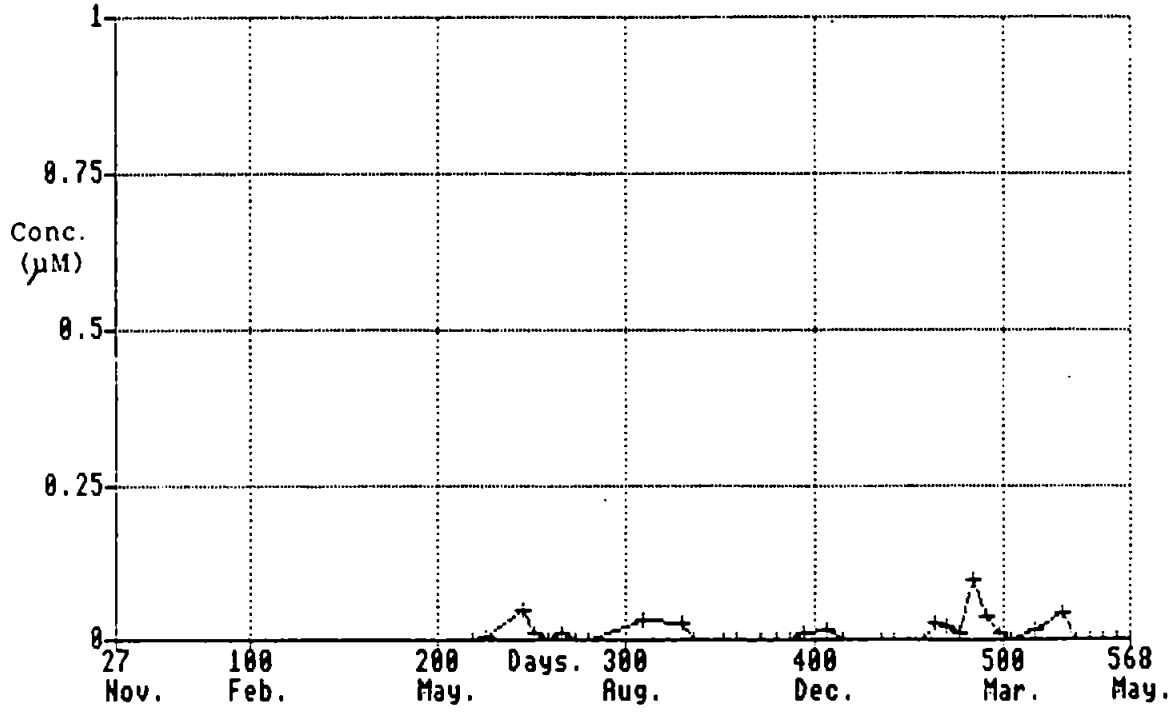


Fig 5.16b Average Concentration of Phe. in DCAA During Sampling Period: May 1986 - May 1987.

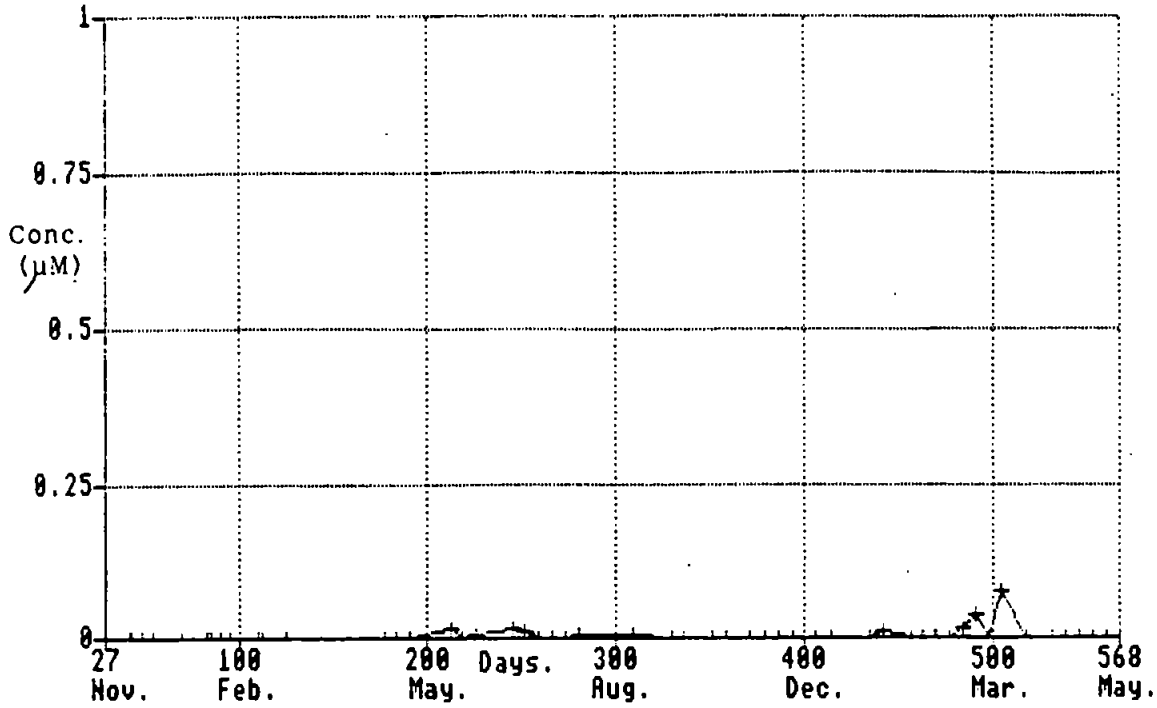


Fig 5.17a Average Concentration of Leu. in DFAA During Sampling Period: Nov 1985 - May 1987.

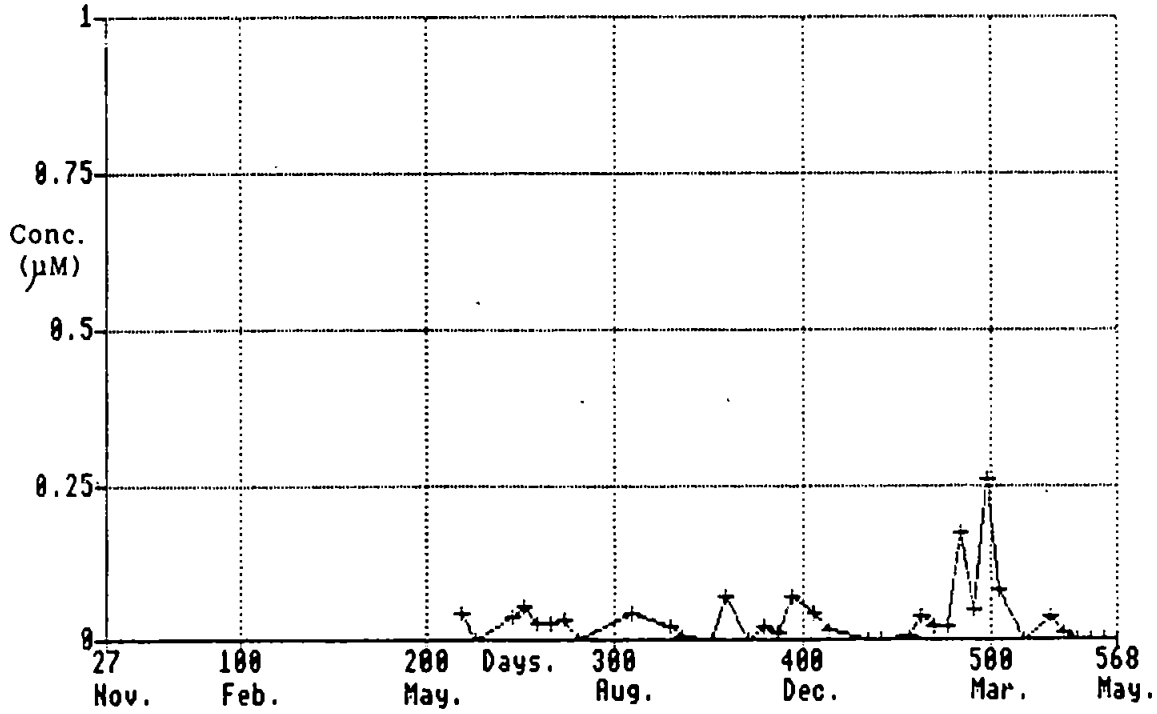


Fig 5.17b Average Concentration of Leu. in DCAA During Sampling Period: May 1986 - May 1987.

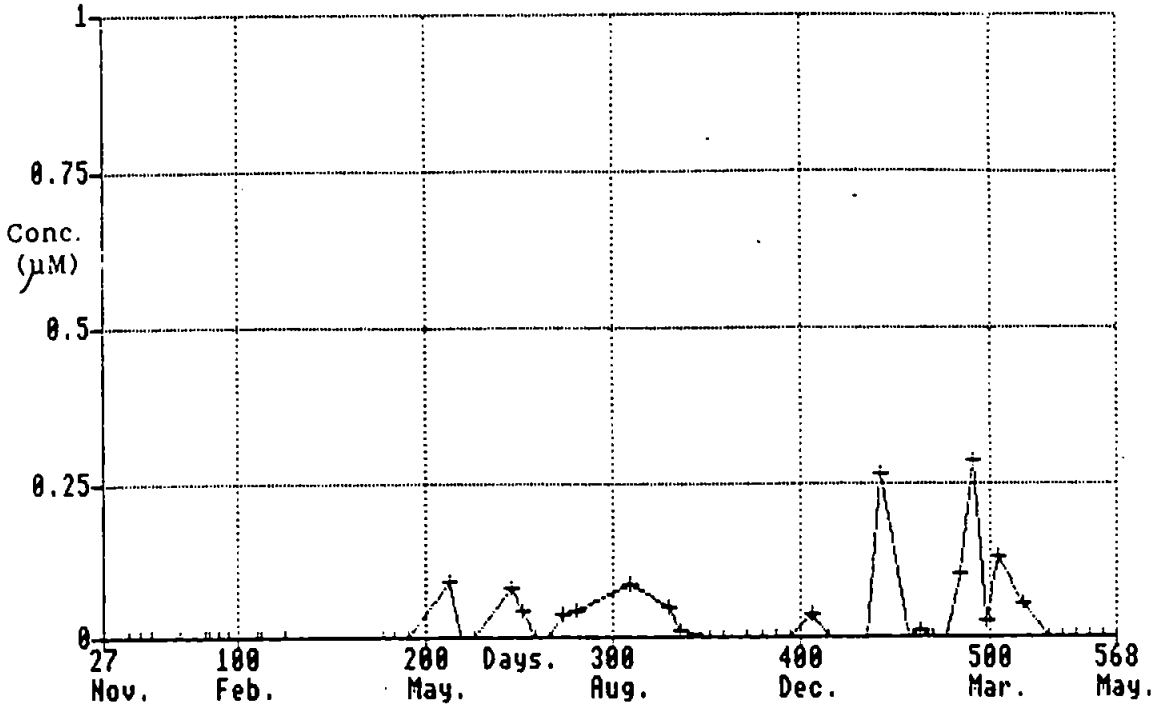


Fig 5.18a Average Concentration of Orn. in DFAA During Sampling Period: Nov 1985 - May 1987.

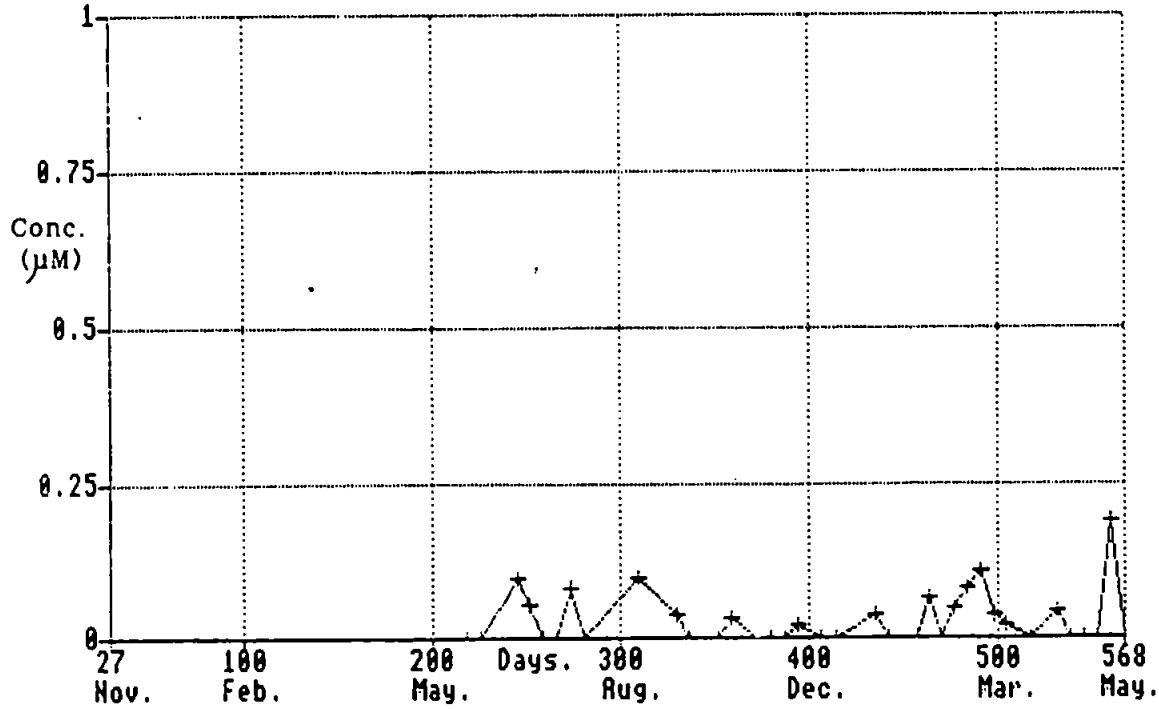


Fig 5.18b Average Concentration of Orn. in DCAA During Sampling Period: May 1986 - May 1987.

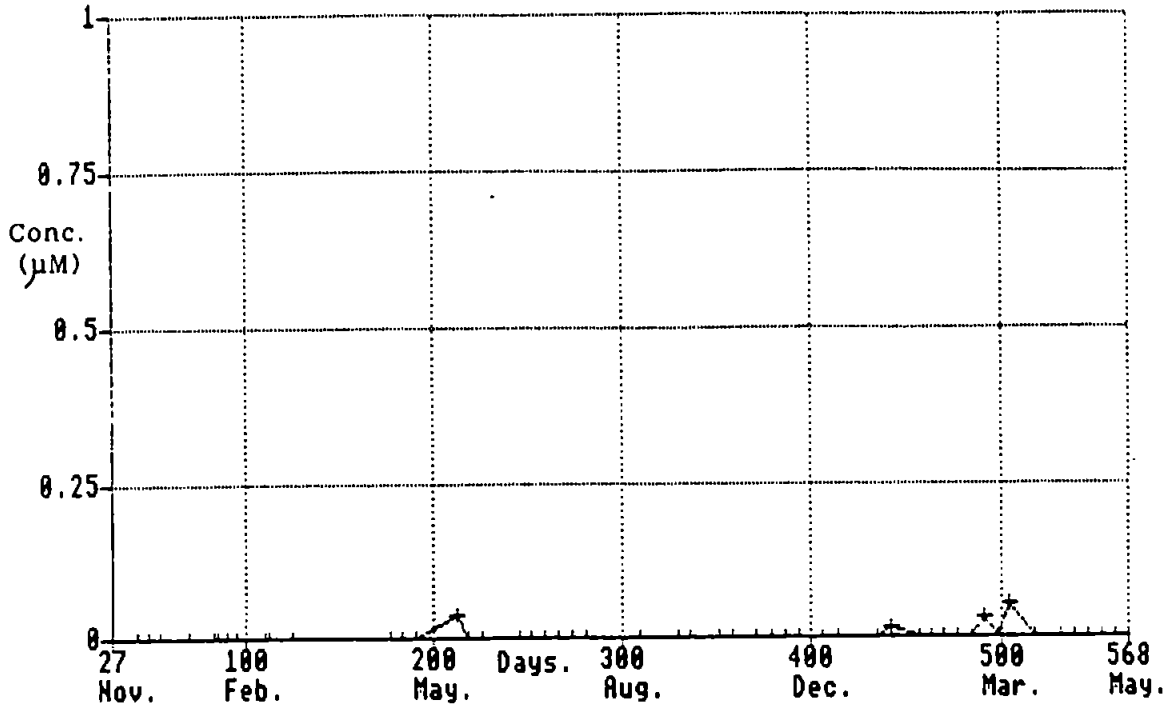


Fig 5.19a Average Concentration of Lys. in DFAA During Sampling Period: Nov 1985 - May 1987.

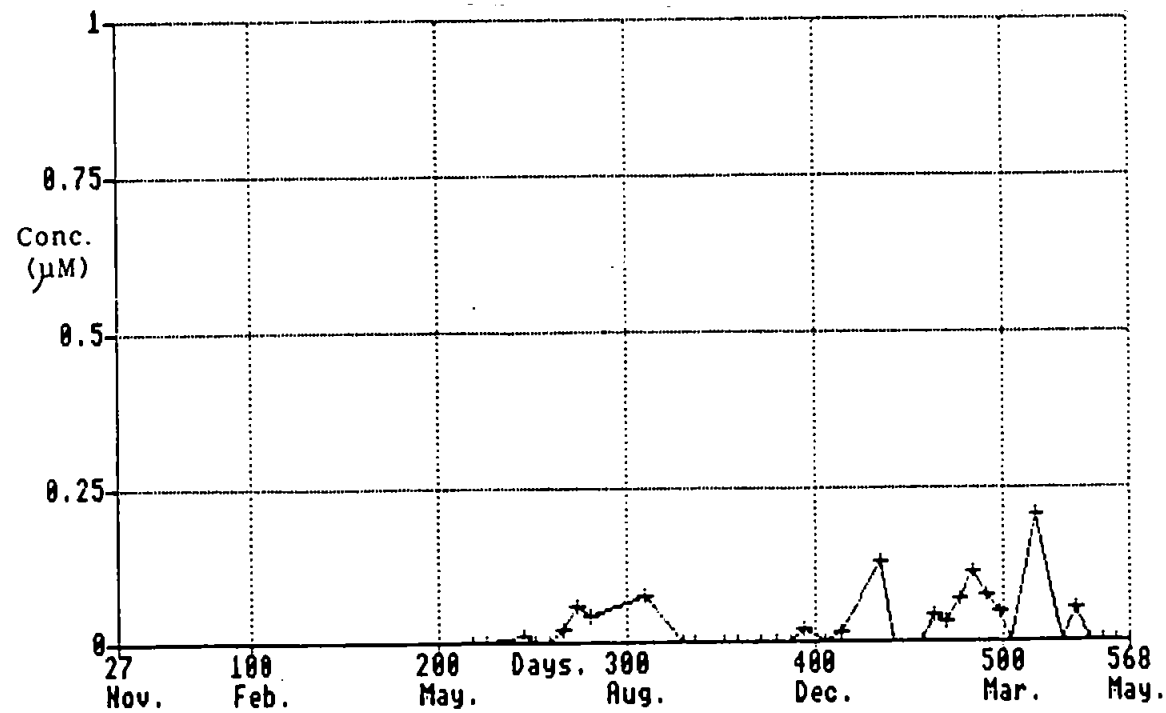


Fig 5.19b Average Concentration of Lys. in DCAA During Sampling Period: May 1986 - May 1987.

Three distinct patterns are observed for individual amino acids in the free fraction. Firstly, those amino acids which are present over most of the sampling period; these acids exhibit a seasonal trend having lower levels between October 1986 and January 1987. Acids showing this pattern are aspartic, glutamic, serine, alanine and glycine (Fig. 5.5a, 5.6a, 5.7a, 5.9a and 5.12a). All these acids with the exception of glutamic are small molecules i.e. they have low molecular weights (Doolittle 1985). Andrews and Williams (1971), state that low molecular weight compounds yield less energy on respiration than those of high molecular weight. Bolter and Dawson (1982) believe that aspartic, glutamic, serine, glycine and alanine, represent the "left overs" of biological processes subsequent to release. (The results for March and April 1986 are based on very few analyses and are therefore not included in this discussion. Fig.5.1.).

The second set of free amino acids is only present at times of high total free amino acid concentrations, in May, August, September 1986 and March 1987. This pattern is shown by histidine, threonine, phenylalanine, arginine, leucine and lysine. (Fig.5.8a, 5.10a, 5.11a, 5.16a, 5.17a and 5.19a). All these acids with the exception of threonine are larger molecules. (Doolittle 1985). The low levels of aromatic compounds in the free fraction were noted by Daumas (1976) and Thurman (1986).

Lastly, some of the amino acids are never found in seawater samples, these are methionine and asparagine. Daumas (1976) comments on the absence of sulphur

containing amino acids from the free spectrum. There are two possible reasons for the absence of methionine and asparagine from the dissolved free amino acid spectrum. Firstly, they are not present in or are not released from the phytoplankton cells. Secondly, the acids are released but are rapidly removed from the seawater. Experiments detailed in chapter 8, show that both methionine and asparagine are present in water containing concentrations of phytoplankton cells.

For the combined amino acid fraction the seasonal patterns of individual acids are not as pronounced as those for the free fraction. However, some acids of the combined fraction have lower levels between October and January. These acids are aspartate, histidine and threonine. (Fig. 5.5b, 5.8b and 5.10b).

5.4. Relationship Between Free and Combined Amino Acid Levels and the Relationship of Total Amino Acid Levels (free plus combined) to Total Dissolved Nitrogen and Dissolved Organic Nitrogen.

5.4.1 Relationship of dissolved free amino acids to dissolved combined amino acids.

Table 5.2 below lists the values for the monthly ratio of DCAA/DFAA levels from June 1986 to May 1987. This data is presented graphically in Fig.5.20.

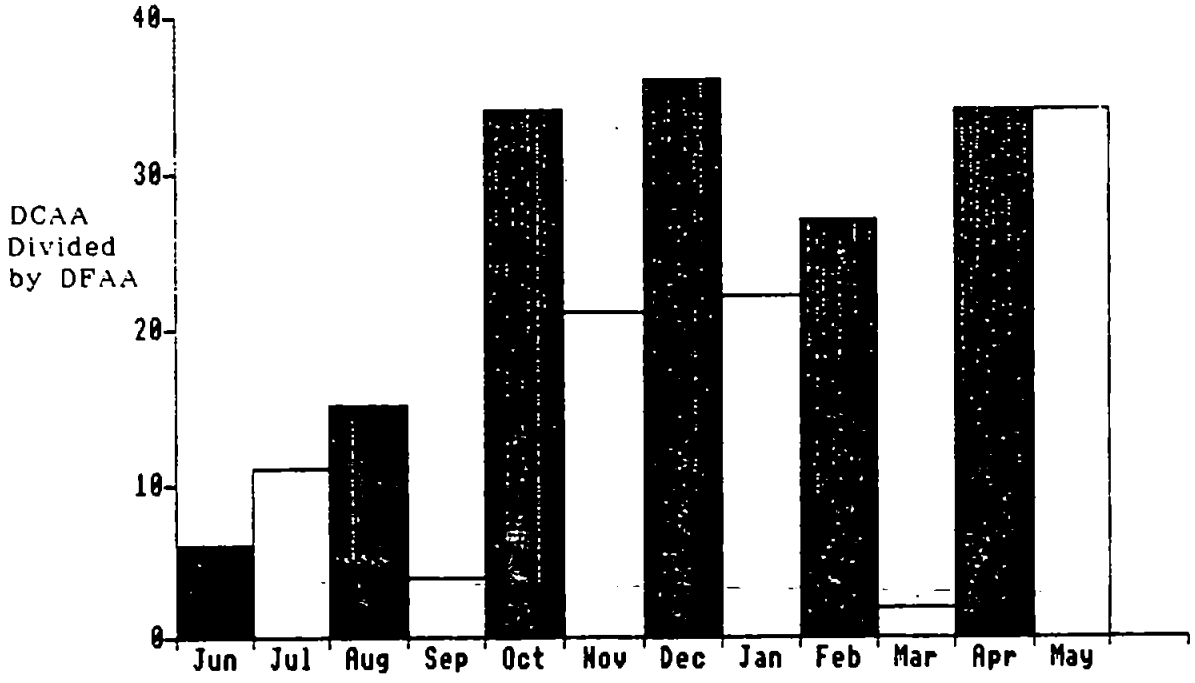


Fig 5.20 The Relationship Between DCAA and DFAA.
June 1986 - May 1987

Table 5.2 The monthly ratio of dissolved combined amino acids to dissolved free amino acids (data from appendix 1).

Month	DCAA/DFAA
Jun	6
Jul	11
Aug	15
Sep	4
Oct	34
Nov	21
Dec	36
Jan	22
Feb	27
Mar	2
Apr	34
May	34

From an inspection of table 5.2 and Fig. 5.20 it can be seen that the ratio of combined to free acids varies widely over the season. The ratio is higher during times of low free amino acids levels (October to January and April to May). The possible reasons for the change in the ratio are discussed in section 5.7.

5.4.2. Relationship of Total Dissolved Amino Acids to Total Dissolved Nitrogen.

Figure 5.21a below shows the monthly total dissolved nitrogen (TDN) levels for a season, redrawn from Butler et al. (1979). Fig. 5.21b presents graphically the percentage of total dissolved nitrogen present as total dissolved amino acids (TAA, free plus combined). It can be seen from Fig. 5.21b that the TDN present as amino acids ranges from 12 to 32 percent. Nagel & Liemann (1987) state that approximately 28% of the TDN is present

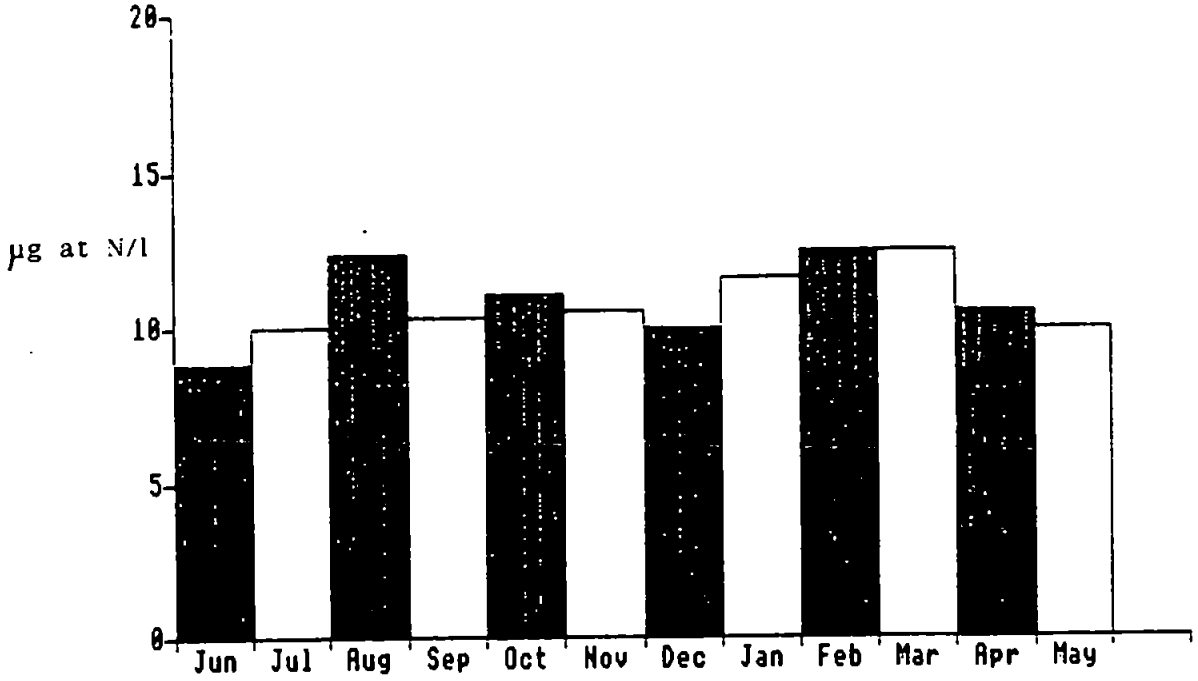


Fig 5.21a Total Dissolved Nitrogen in the Waters of the Western English Channel, (redrawn from Butler et al. 1979).

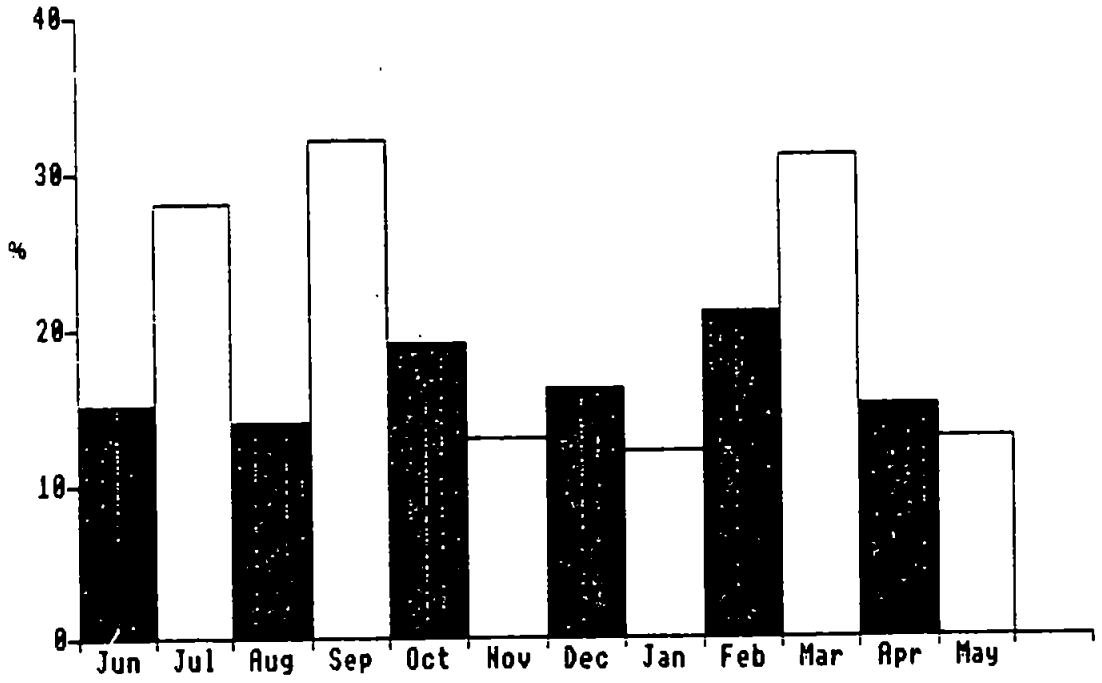


Fig 5.21b The Percentage of Total Dissolved Nitrogen Which Can Be Accounted for by Total Dissolved Amino Acids. June 1986 - May 1987

as total amino acids (free plus combined)

The figures quoted above, for this study, are however, likely to be an over estimation of the percentage of the total dissolved nitrogen accountable for by amino acids. This is due to the fact that the figures in Butler et al. (1979) are the average for station E1, twenty miles out in the English channel. TDN figures for this station are seldom in excess of 12 uM nitrogen. The coastal waters, analysed in this study, can have considerably higher values than this. Unfortunately no measurement of the actual total dissolved nitrogen of the coastal samples were made. .

5.4.3 The Percentage Of Dissolved Organic Nitrogen (DON) Present As Total Dissolved Amino Acids (Free Plus Combined).

The figures for dissolved organic nitrogen (DON) redrawn from Butler et al. (1979) can be seen in Fig. 5.22a. The data for the percentage of the DON present as total amino acids, found in this study, over a season is summarised in Fig. 5.22b. The DON levels were not directly measured. It can be seen from Fig. 5.22b that the DON present as amino acids ranges from 18 to 65 percent.

The data of monthly averages for dissolved free and combined acids (June 1986 to May 1987) were correlated against total dissolved nitrogen, dissolved organic nitrogen and other environmental factors using the microtab statistical package. The results of these

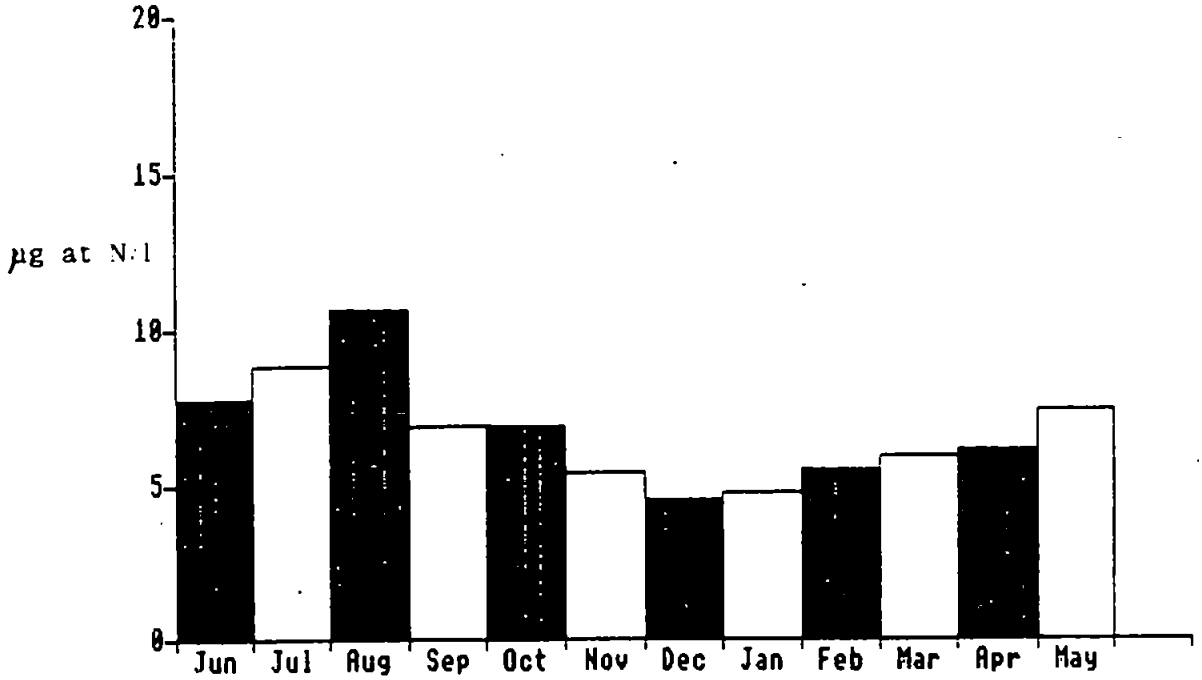


Fig 5.22a Dissolved Organic Nitrogen in the Waters of the Western English Channel, (redrawn from Butler et al. 1979).

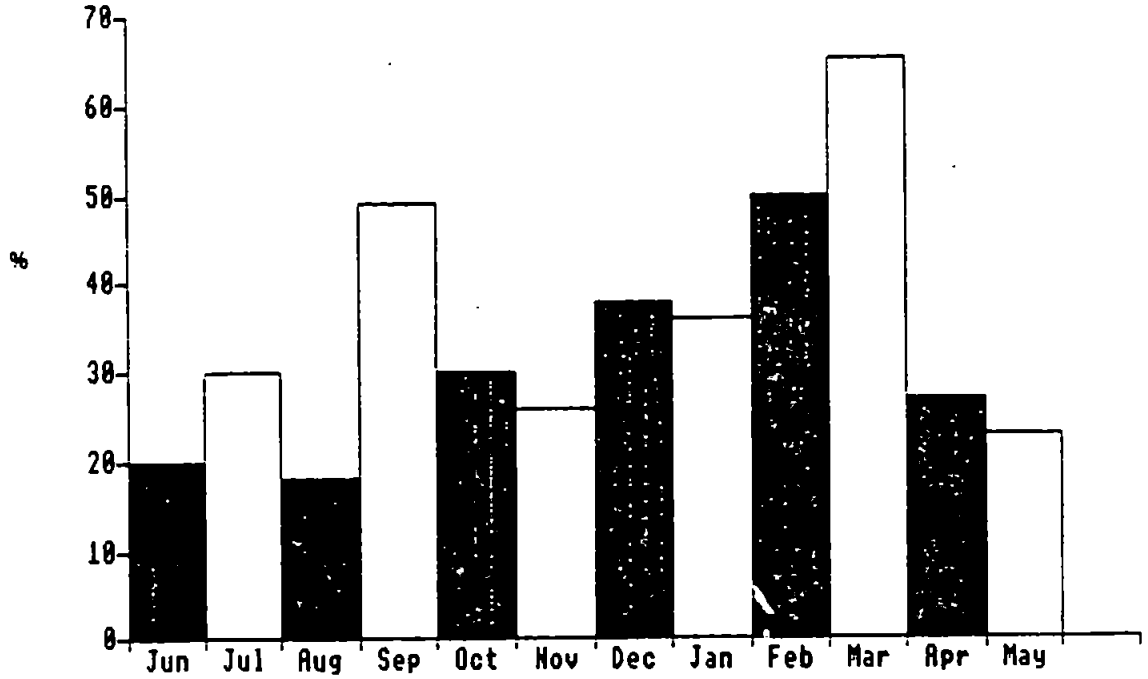


Fig 5.22b The Percentage of Dissolved Organic Nitrogen Which Can Be Accounted for by Total Dissolved Amino Acids. June 1986 - May 1987

correlations can be found in the discussion at the end of this chapter.

5.5. Percentage Composition Data for Individual Dissolved Free and Dissolved Combined Amino Acids.

The percentage compositions for both dissolved free and dissolved combined amino acids, on a monthly basis, have been calculated using data from appendix II and are given in Fig. 5.23- 5.37.

The dominant amino acids for the free fraction were aspartic, glutamic, serine, glycine and alanine. Whereas the dominant acids in the combined fraction were glycine, histidine, aspartic, glutamic, alanine and valine.

From Fig. 5.27b, it can be seen that the percentage of glycine, present in the dissolved combined amino acid fraction, is high throughout the year reaching exceptional levels in December and January (Maximum sixty five percent of totoal dissolved combined amino acids.). The possibility that the chromatographic peak thought to be glycine could be an artefact produced by the hydrolysis of seawater samples was investigated. Control experiments involving co-injection of glycine with hydrolysed seawater samples and studies of the percentage recovery of Glycine from Bovine Serum Albumin, (chapter 3), indicate that an artefact was not present, and that the chromatographic peak genuinely represented high Glycine levels.

Jorgensen (1968), discusses the adaptation of

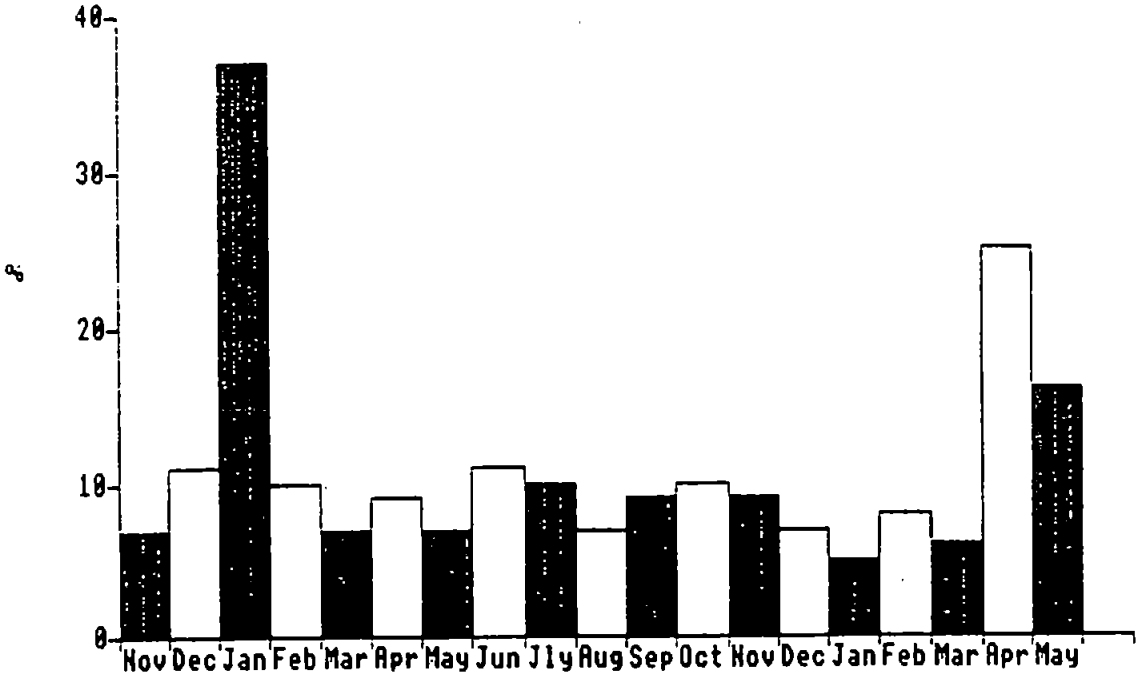


Fig 5.23a Monthly Percentage Composition of Asp. in the DFAA, Nov 1985 - May 1987.

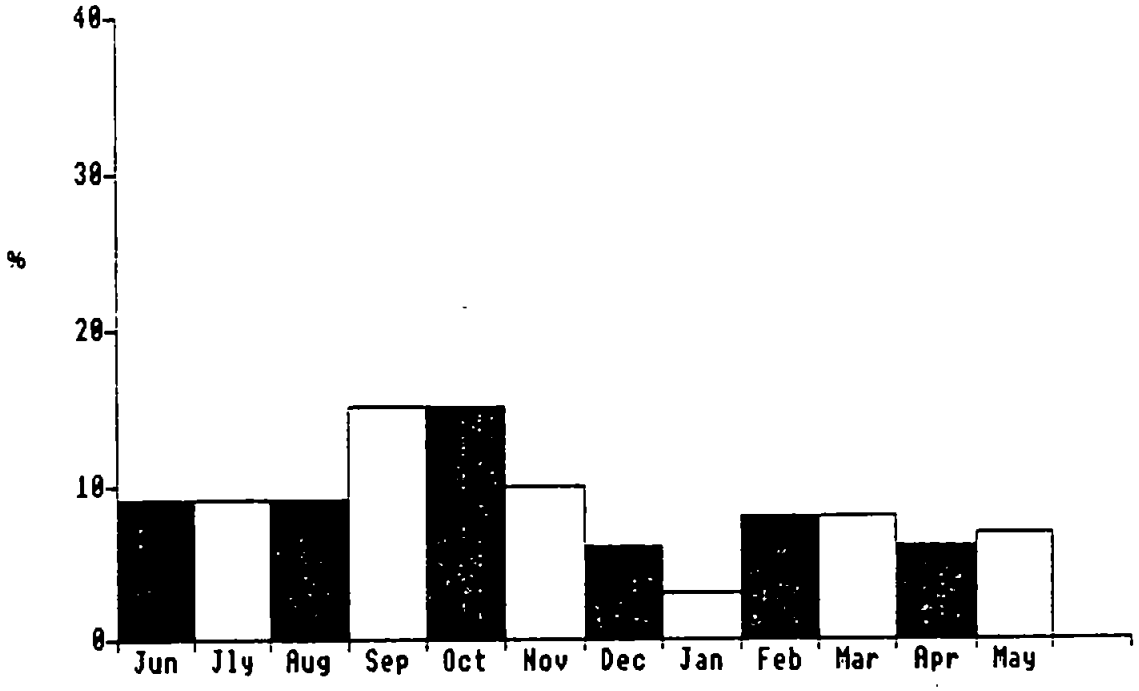


Fig 5.23b Monthly Percentage Composition of Asp. in the DCAA, June 1986 - May 1987.

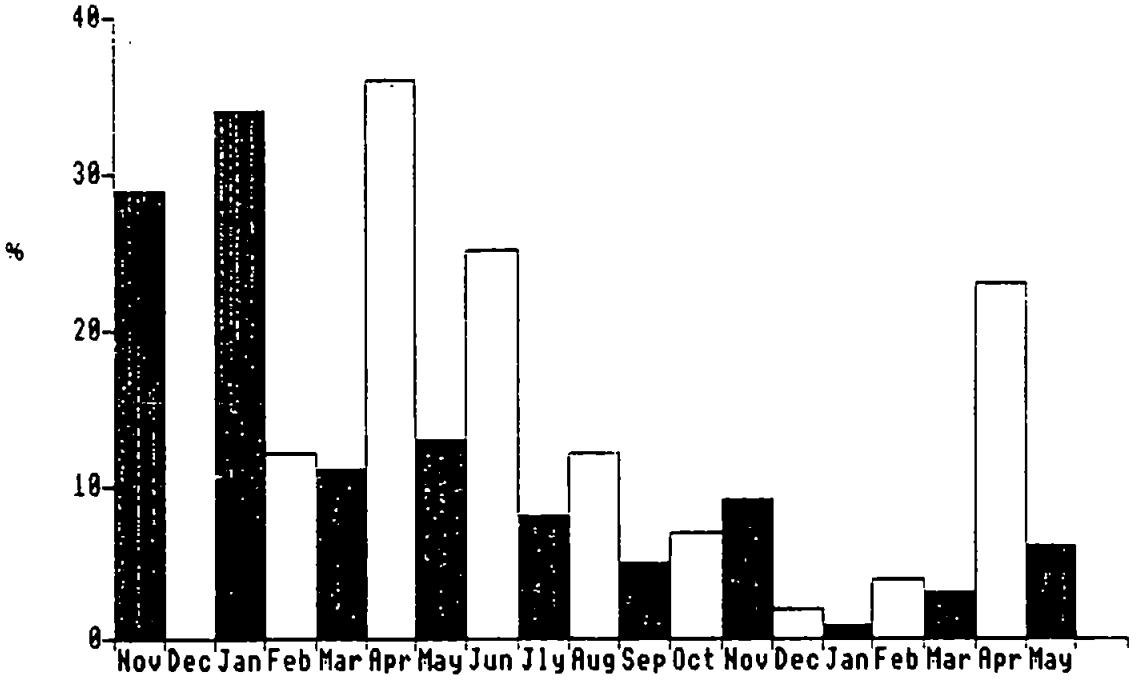


Fig 5.24a Monthly Percentage Composition of Glu. in the DFAA, Nov 1985 - May 1987.

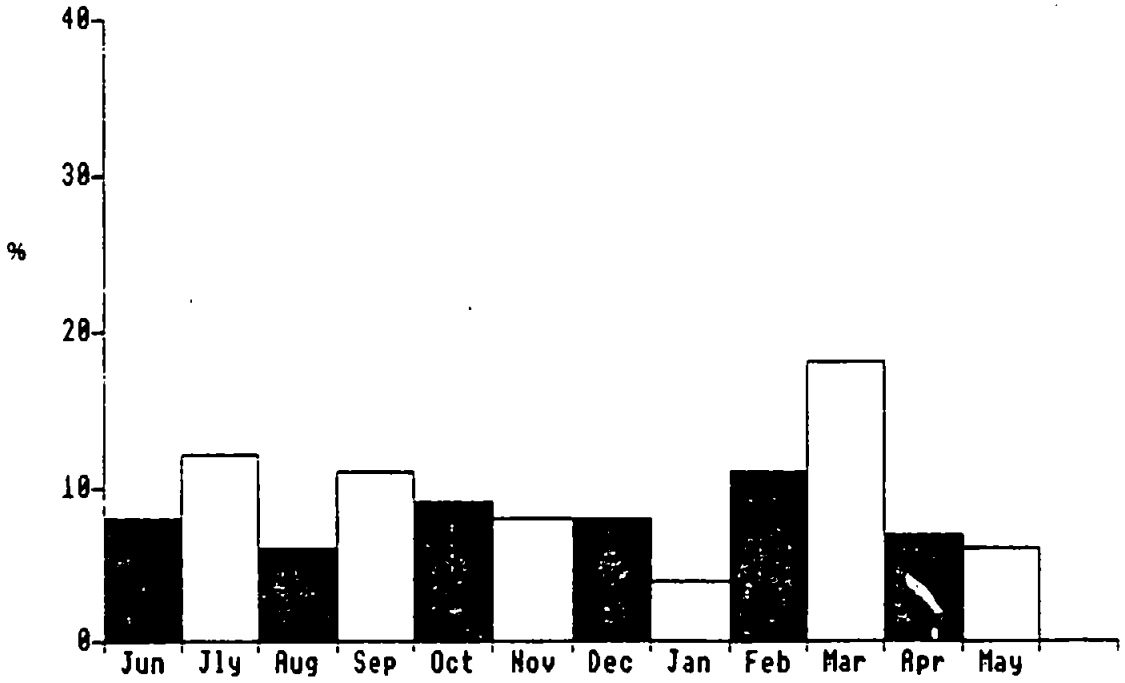


Fig 5.24b Monthly Percentage Composition of Glu. in the DCAA, June 1986 - May 1987.

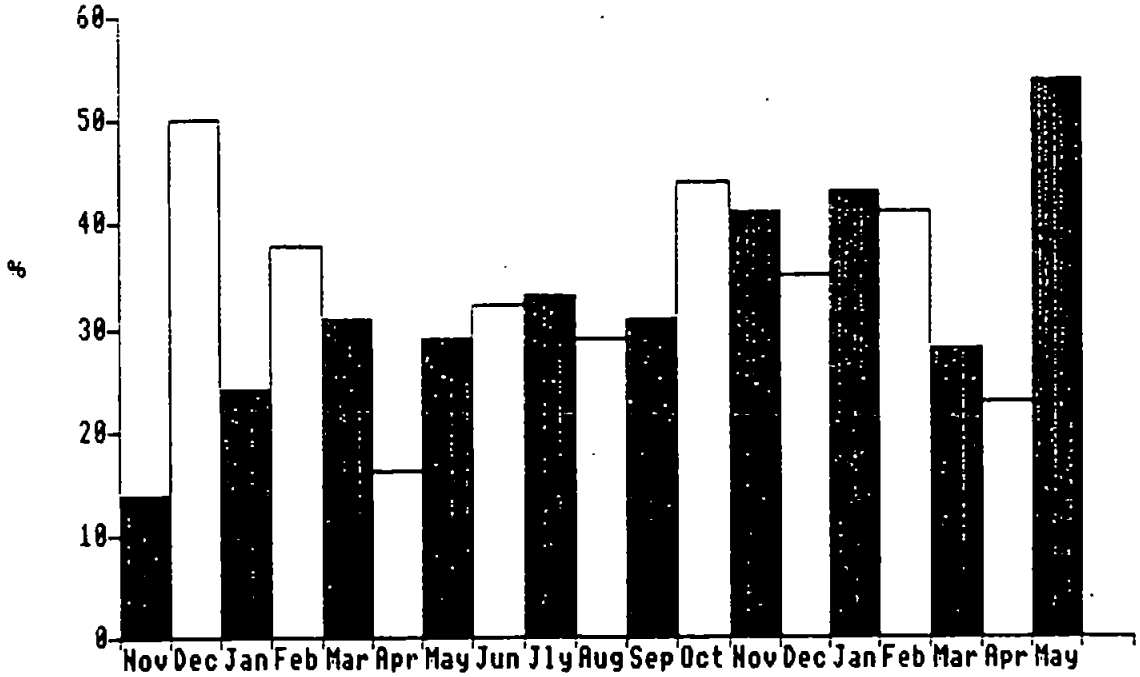


Fig 5.25a Monthly Percentage Composition of Ser. in the DFAA, Nov 1985 - May 1987.

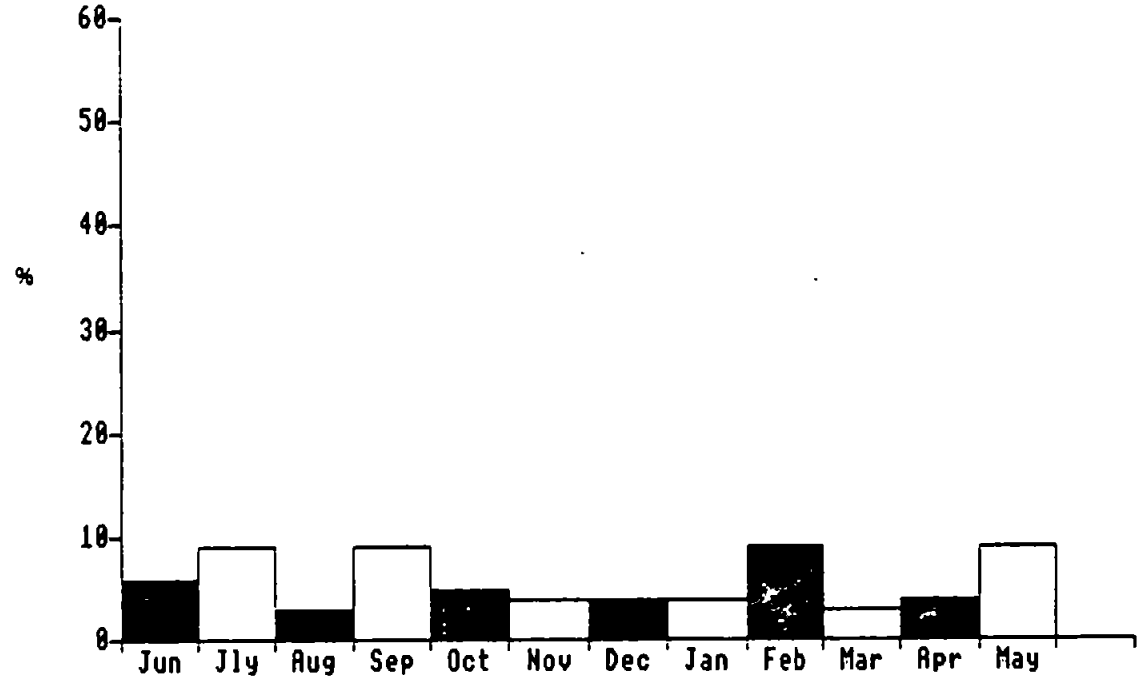


Fig 5.25b Monthly Percentage Composition of Ser. in the DCAA, June 1986 - May 1987.

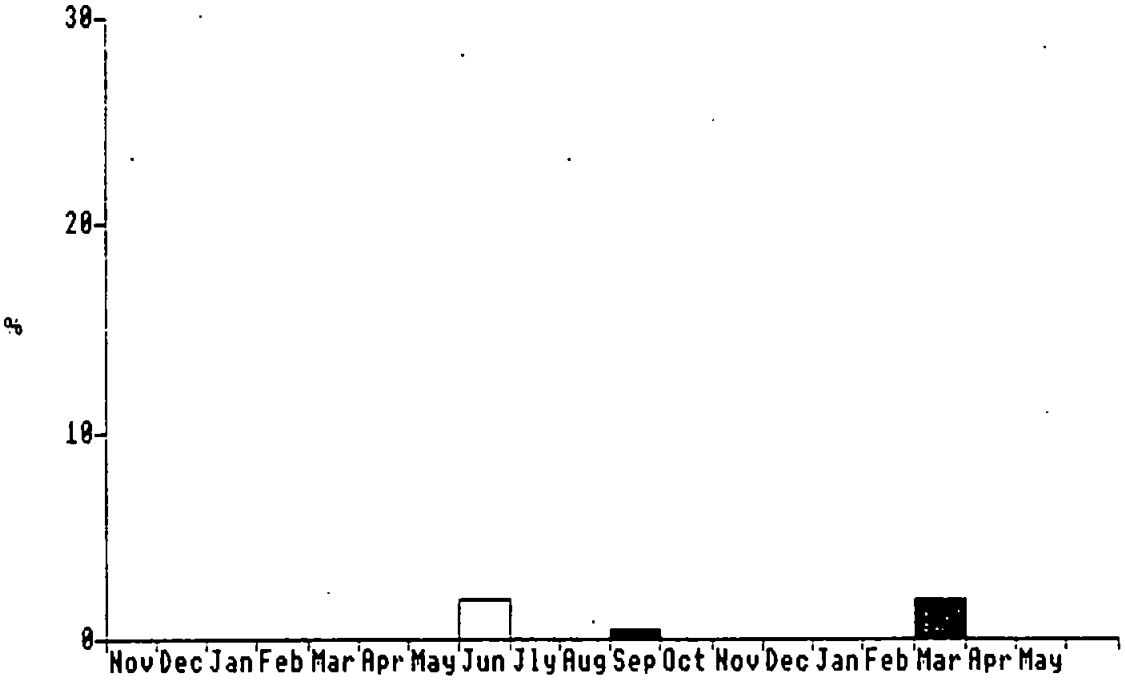


Fig 5.26a Monthly Percentage Composition of His. in the DFAA, Nov 1985 - May 1987.

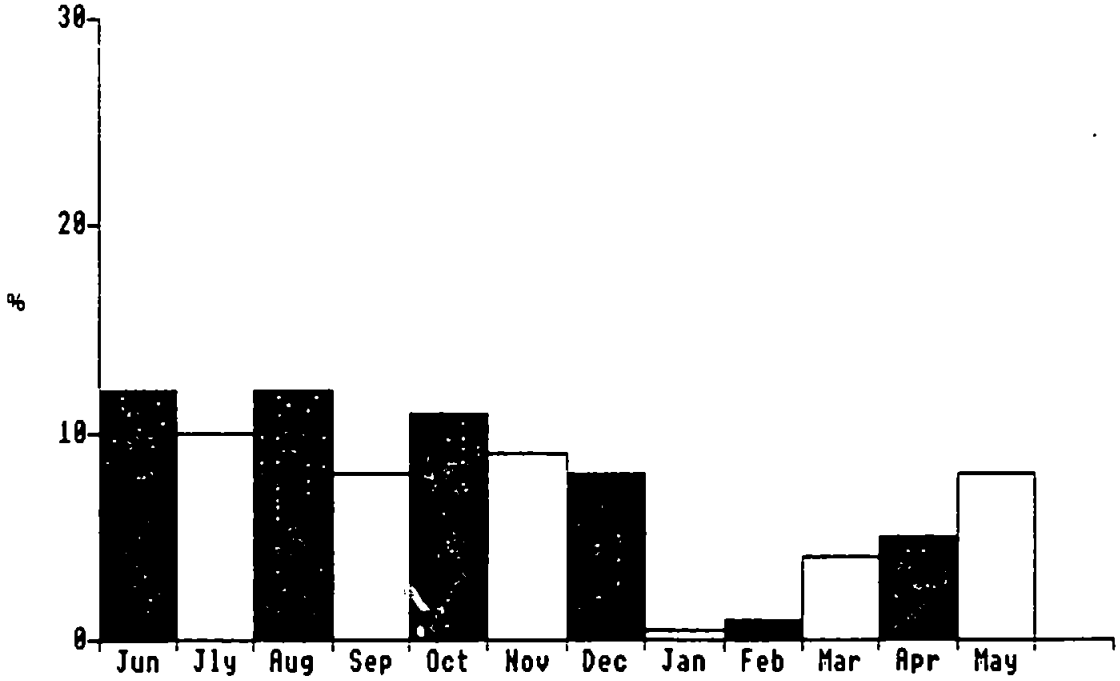


Fig 5.26b Monthly Percentage Composition of His. in the DCAA, June 1986 - May 1987.

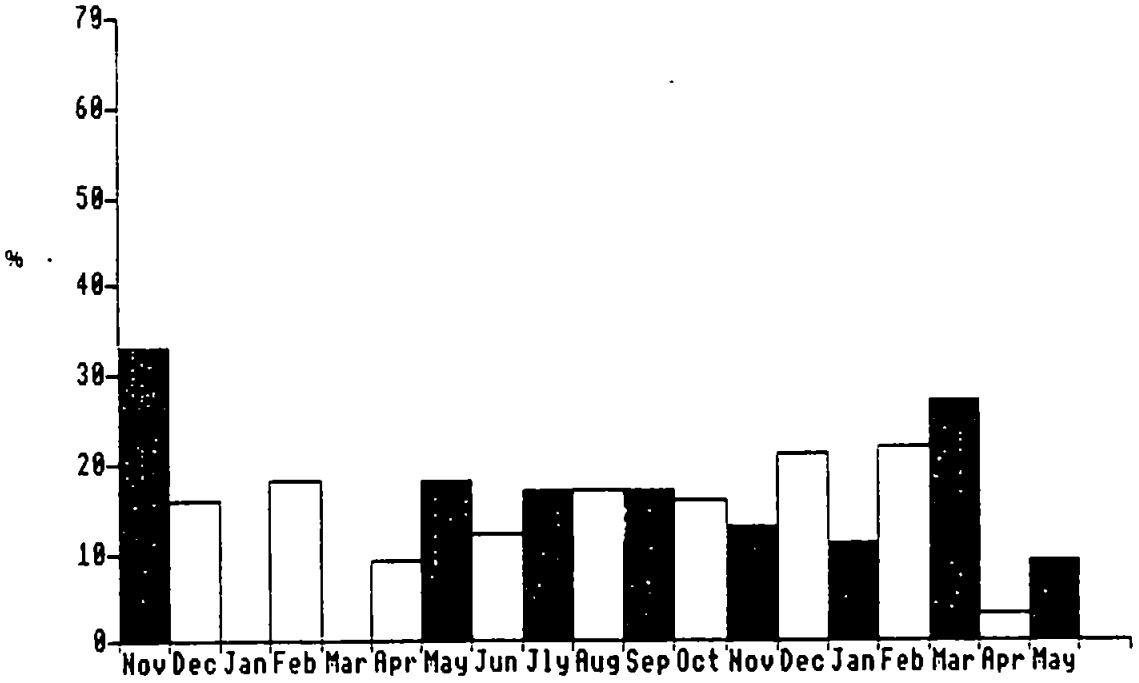


Fig 5.27a Monthly Percentage Composition of Gly. in the DFAA. Nov 1985 - May 1987.

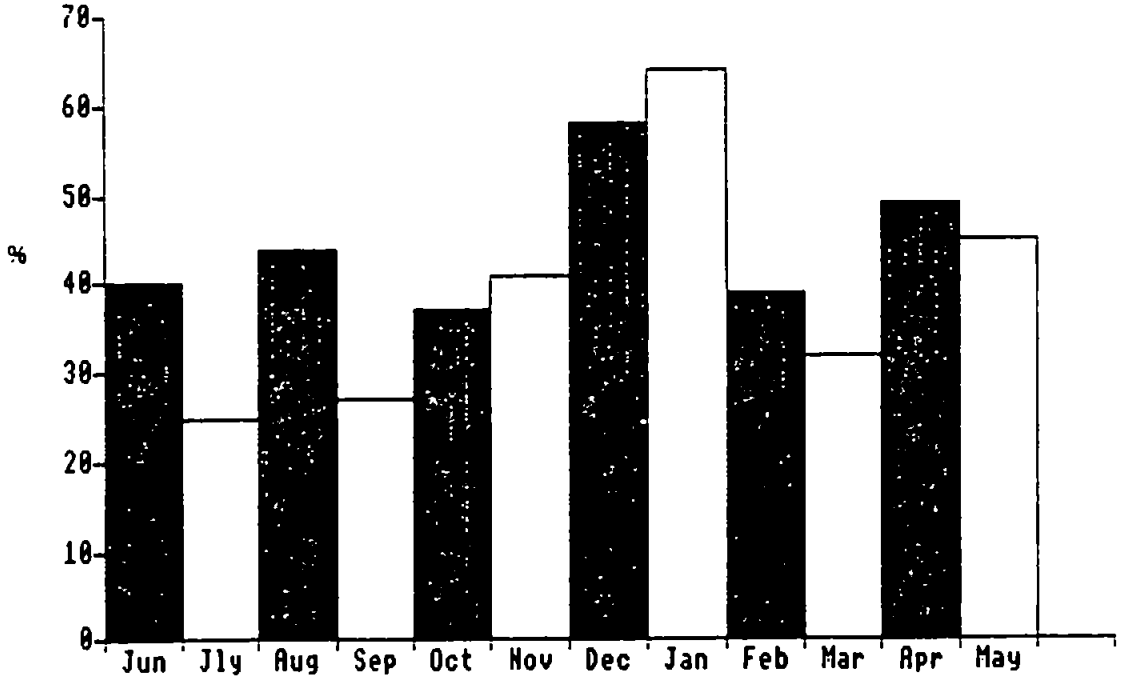


Fig 5.27b Monthly Percentage Composition of Gly. in the DCAA. June 1986 - May 1987.

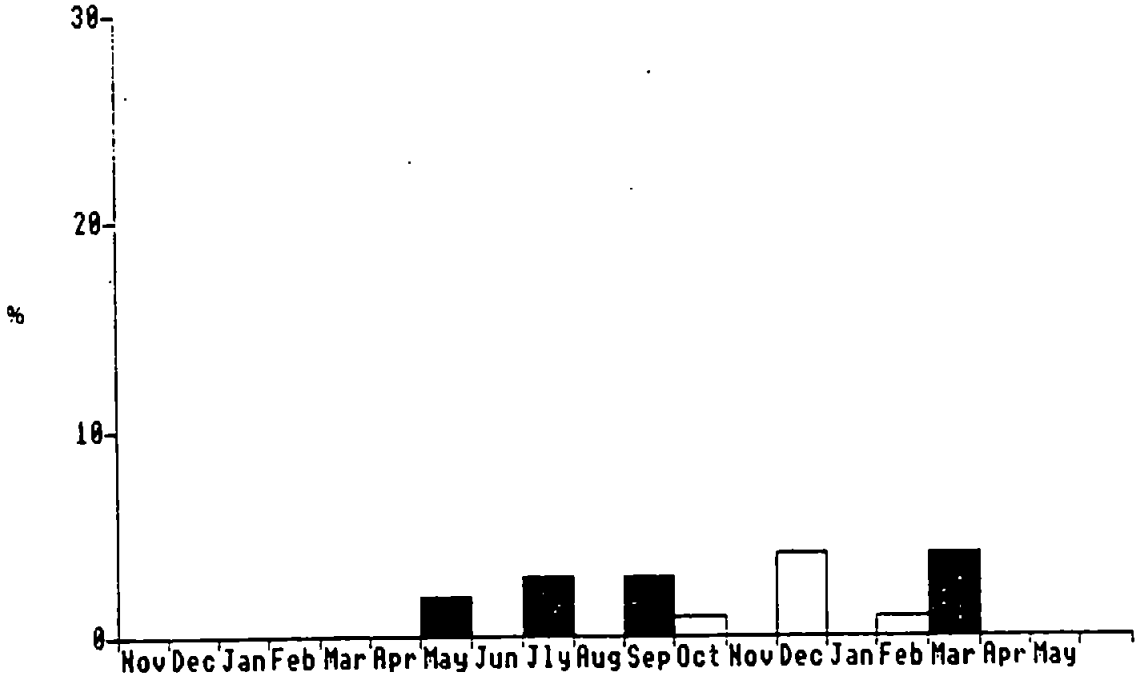


Fig 5.28a Monthly Percentage Composition of Thr. in the DFAA, Nov 1985 - May 1987.

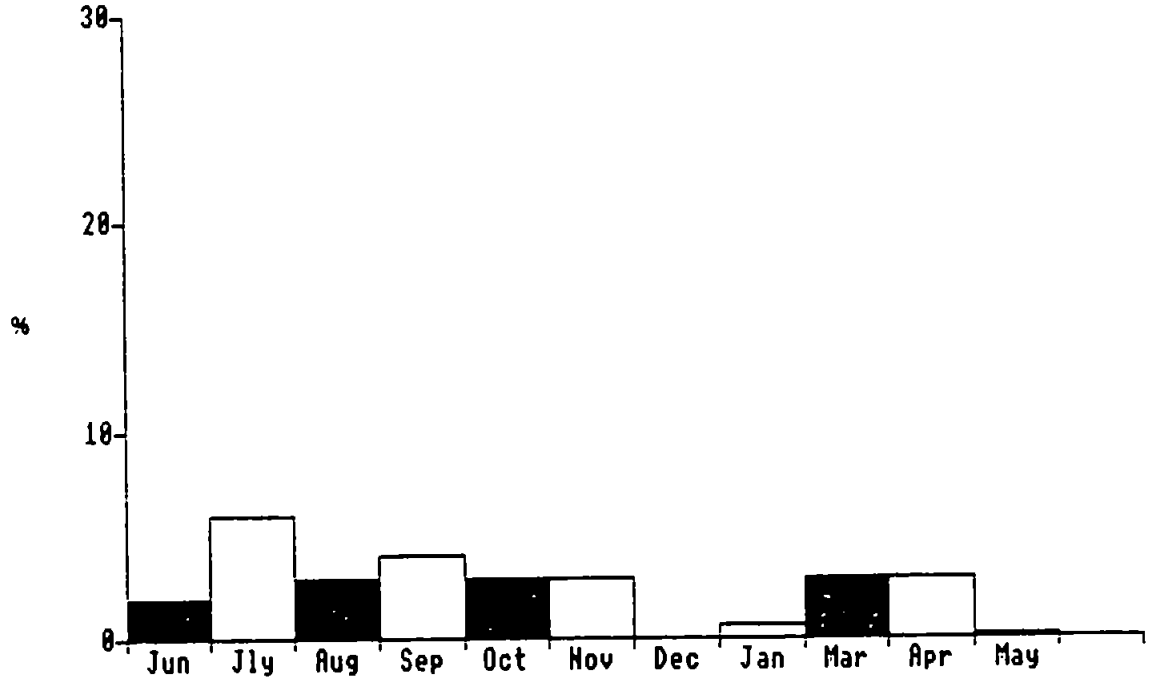


Fig 5.28b Monthly Percentage Composition of Thr. in the DCAA, June 1986 - May 1987.

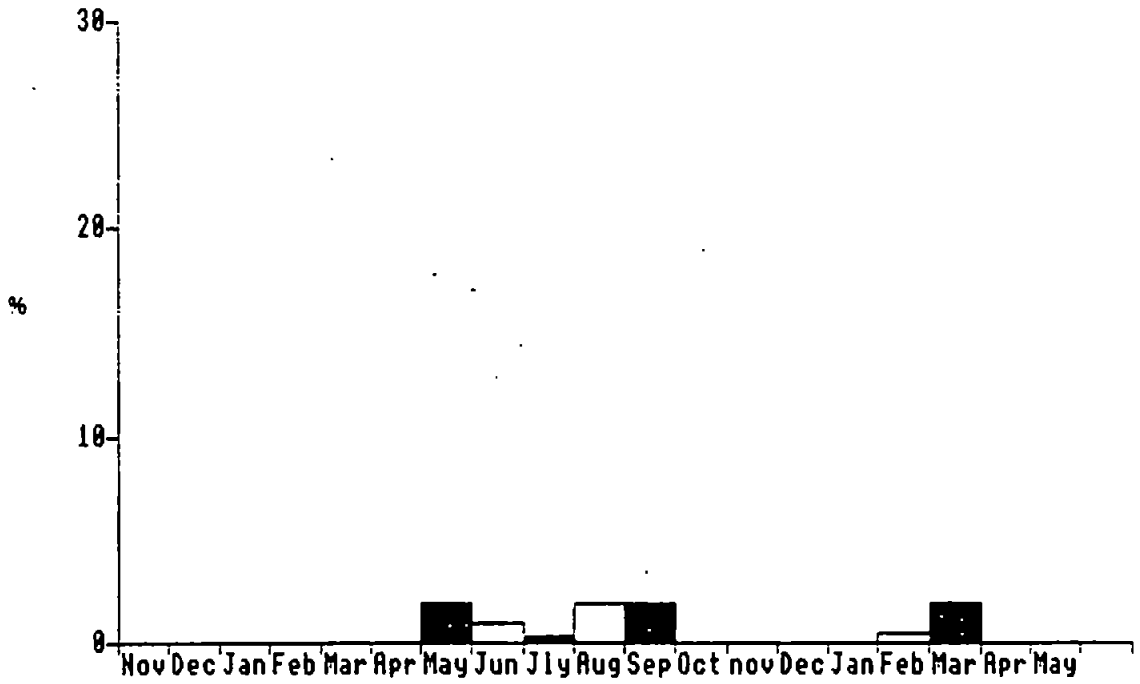


Fig 5.29a Monthly Percentage Composition of Arg. in the DFAA, Nov 1985 - May 1987.

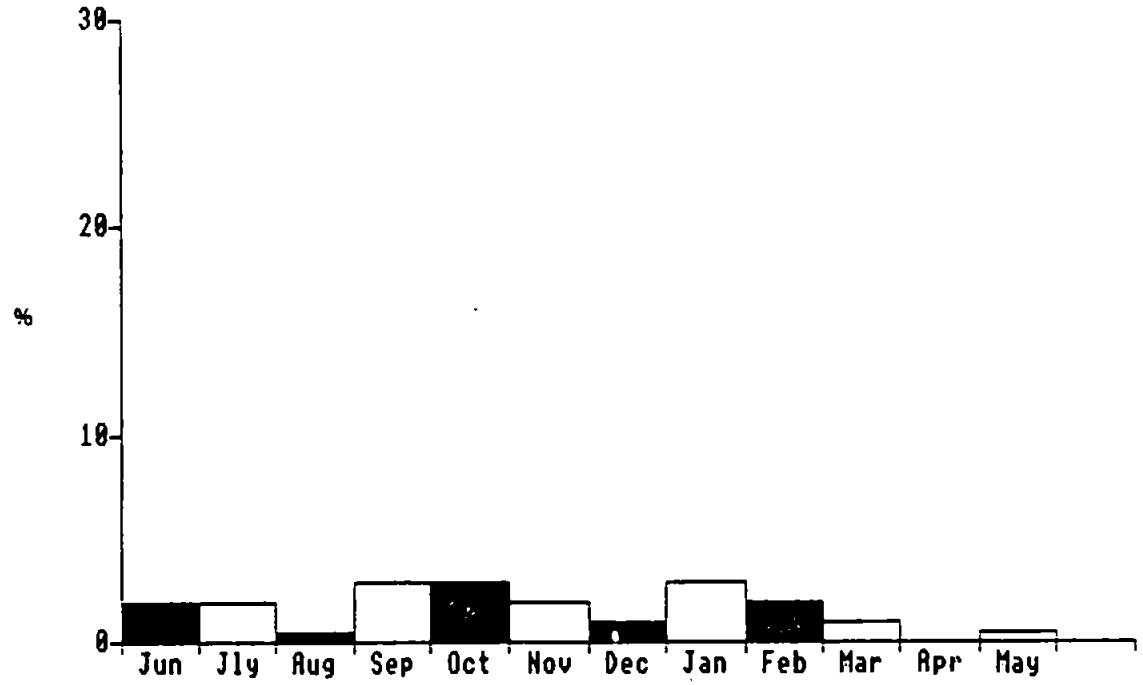


Fig 5.29b Monthly Percentage Composition of Arg. in the DCAA, June 1986 - May 1987.

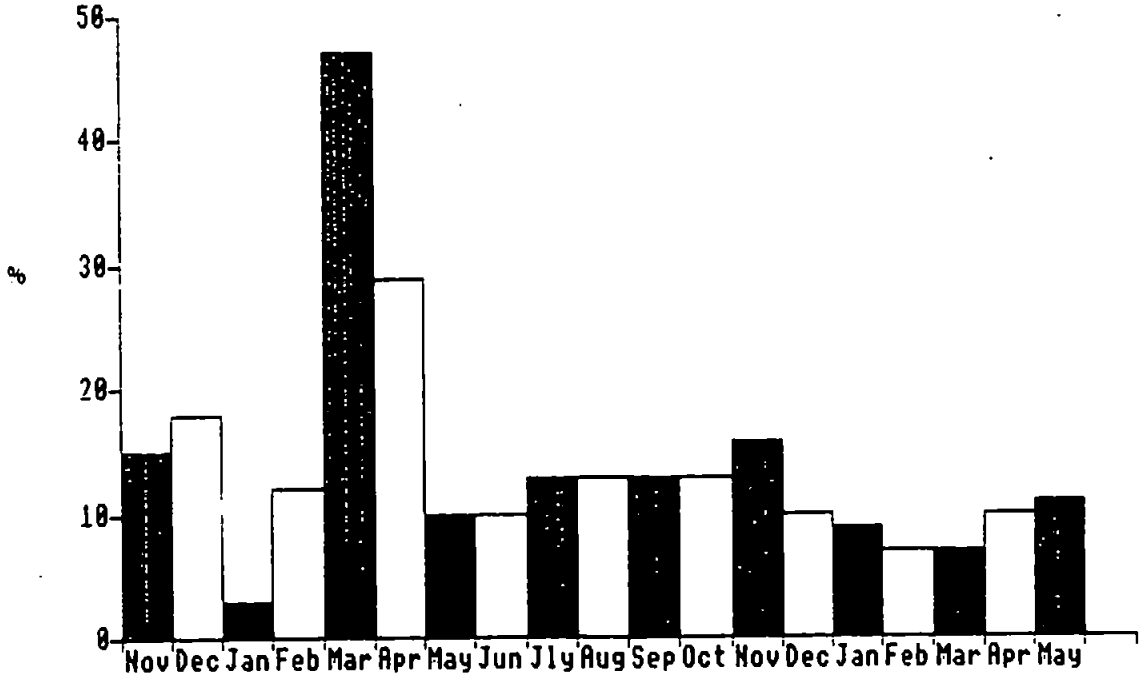


Fig 5.30a Monthly Percentage Composition of Ala. in the DFAA, Nov 1985 - May 1987.

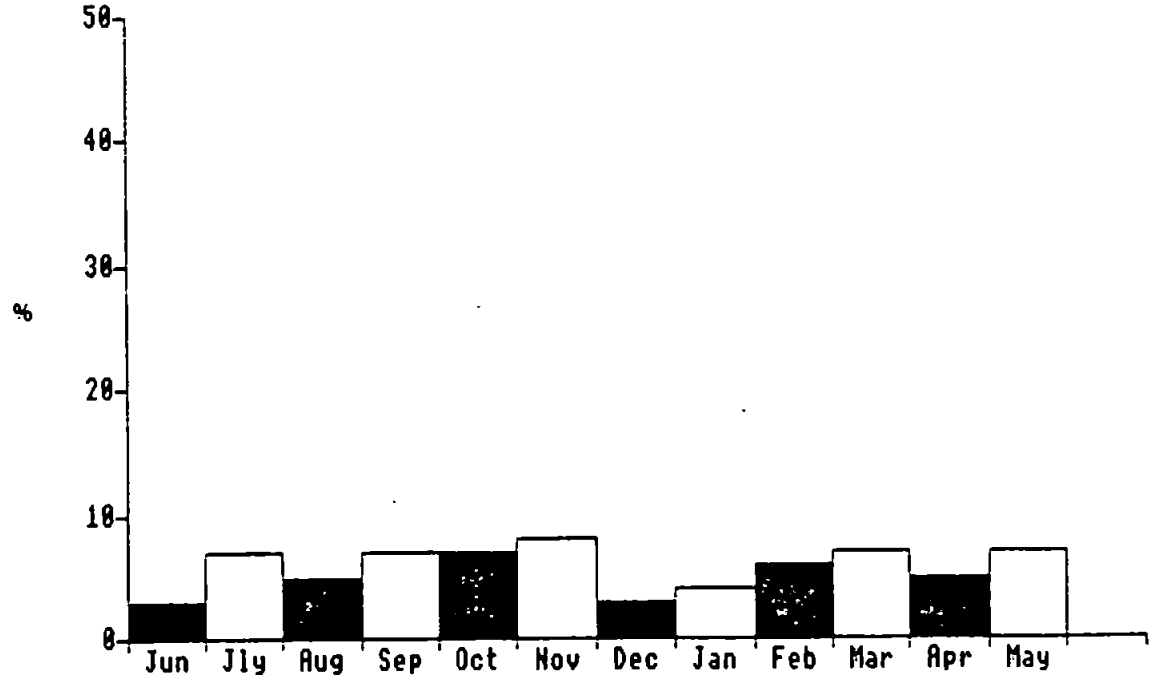


Fig 5.30b Monthly Percentage Composition of Ala. in the DCAA, June 1986 - May 1987.

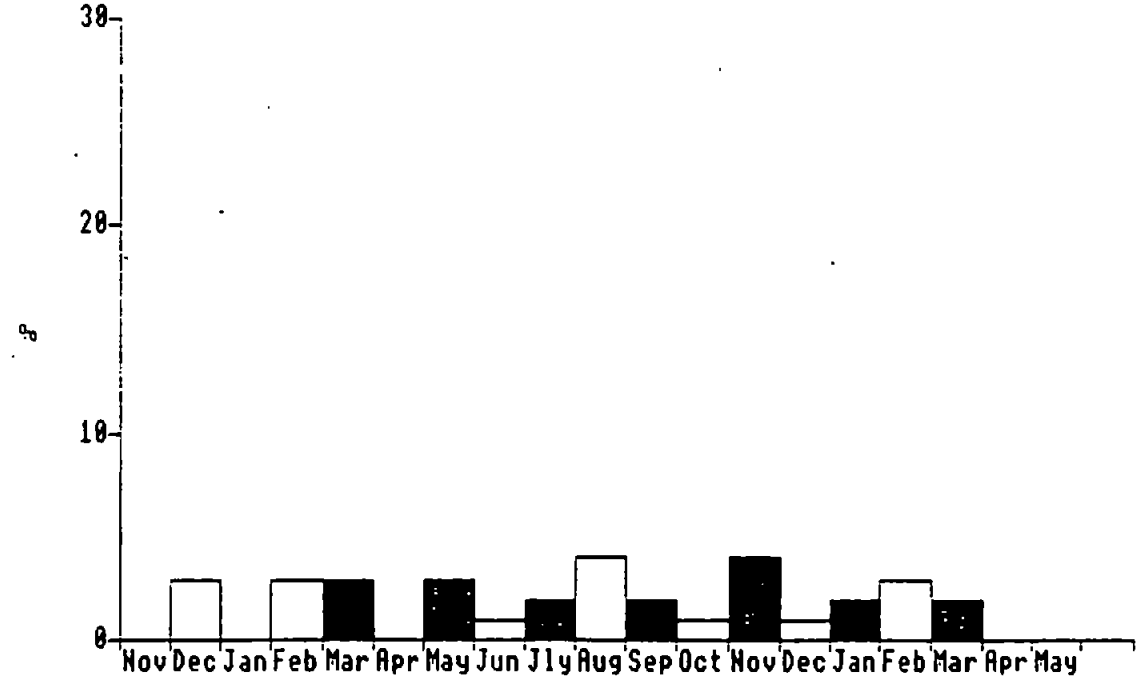


Fig 5.31a Monthly Percentage Composition of Tyr. in the DFAA. Nov 1985 - May 1987.

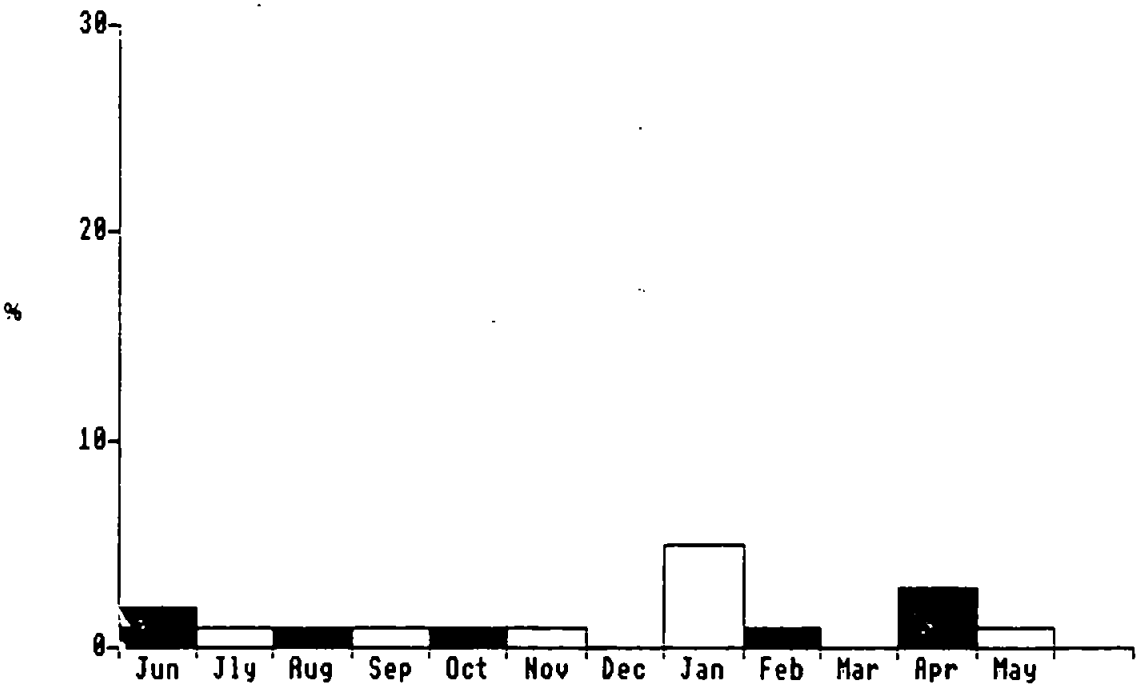


Fig 5.31b Monthly Percentage Composition of Tyr. in the DCAA. June 1986 - May 1987.

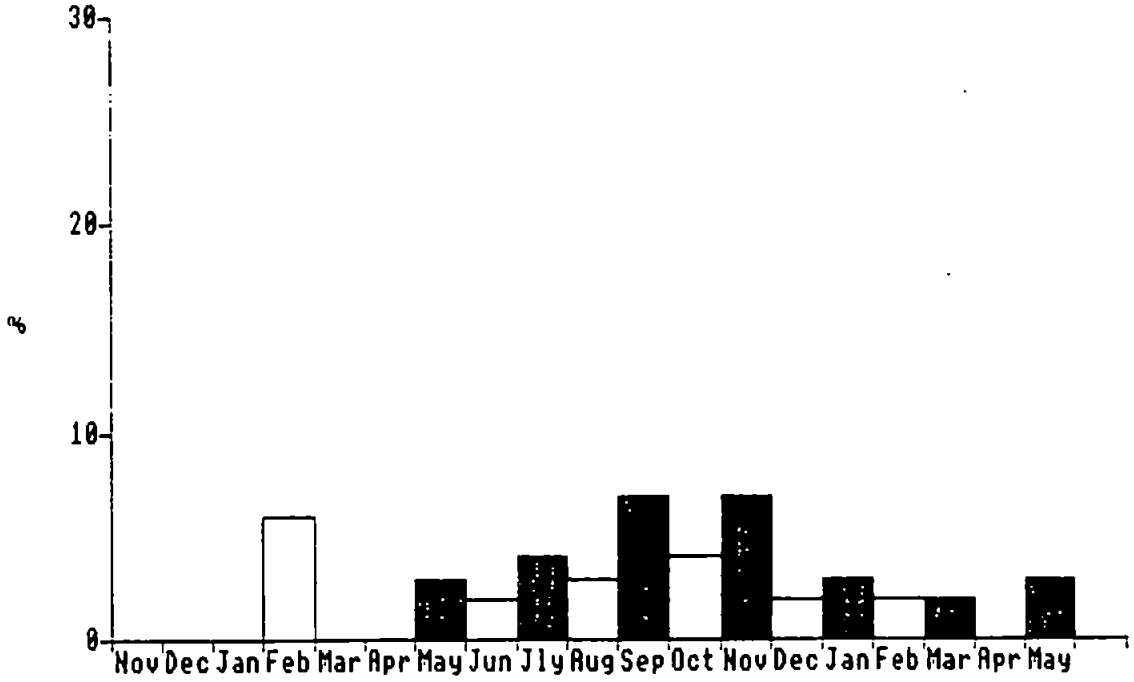


Fig 5.32a Monthly Percentage Composition of Val. in the DFAA, Nov 1985 - May 1987.

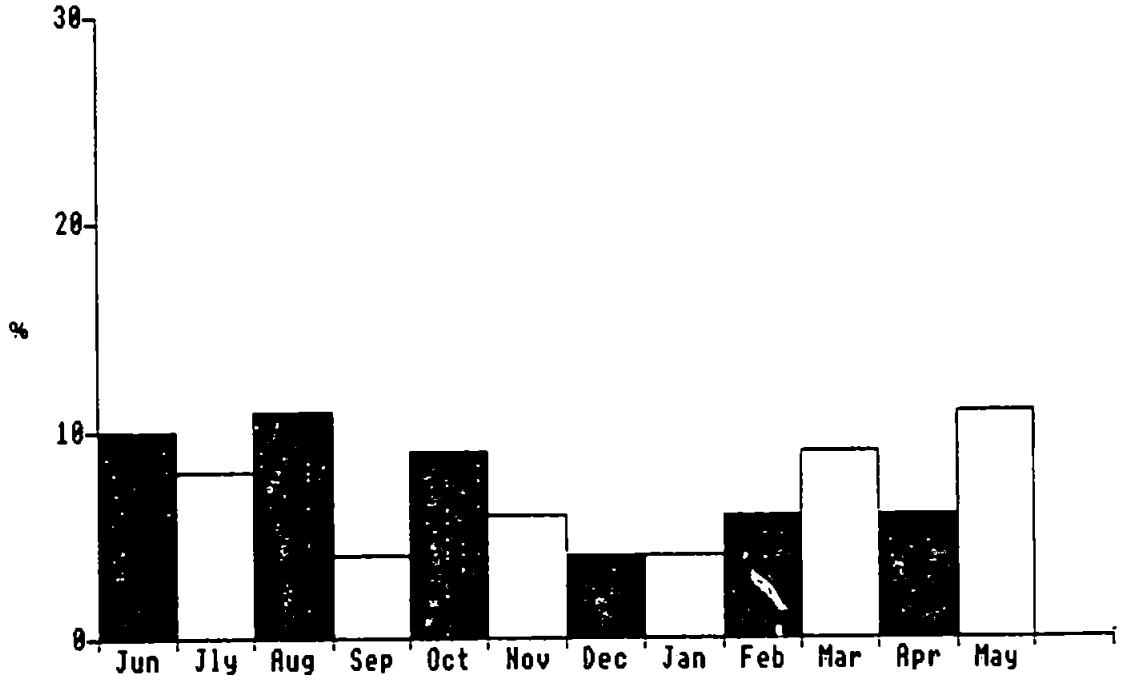


Fig 5.32b Monthly Percentage Composition of Val. in the DCAA, June 1986 - May 1987.

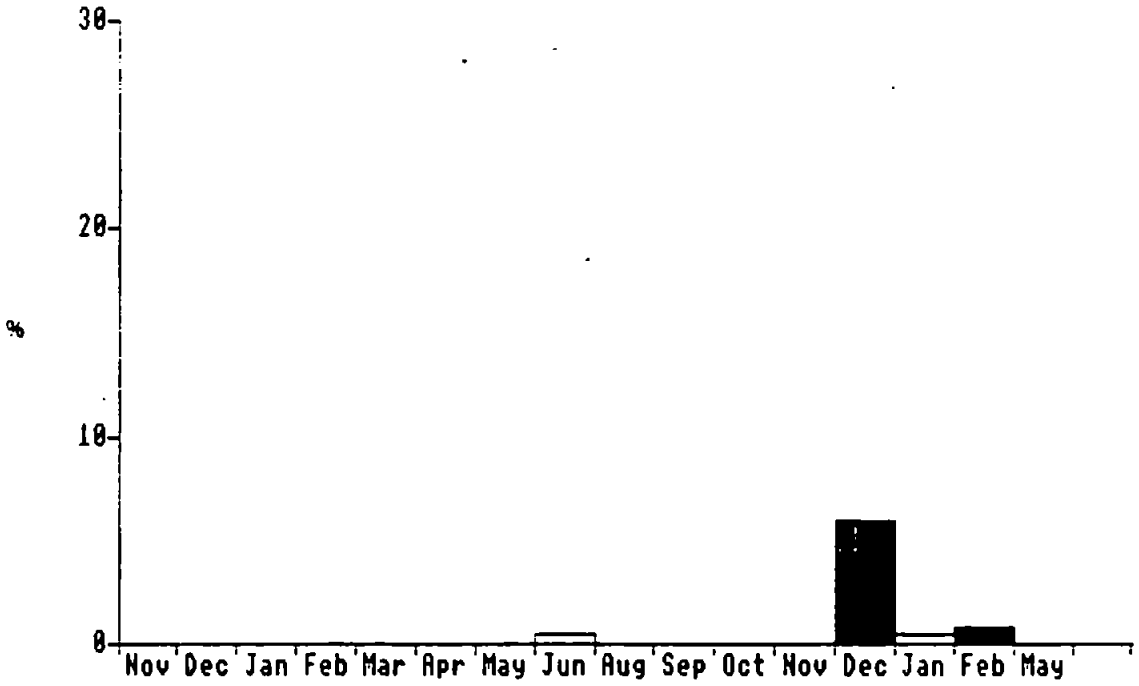


Fig 5.33a Monthly Percentage Composition of Trp. and Ile. in the DFAA, Nov 1985 - May 1987.

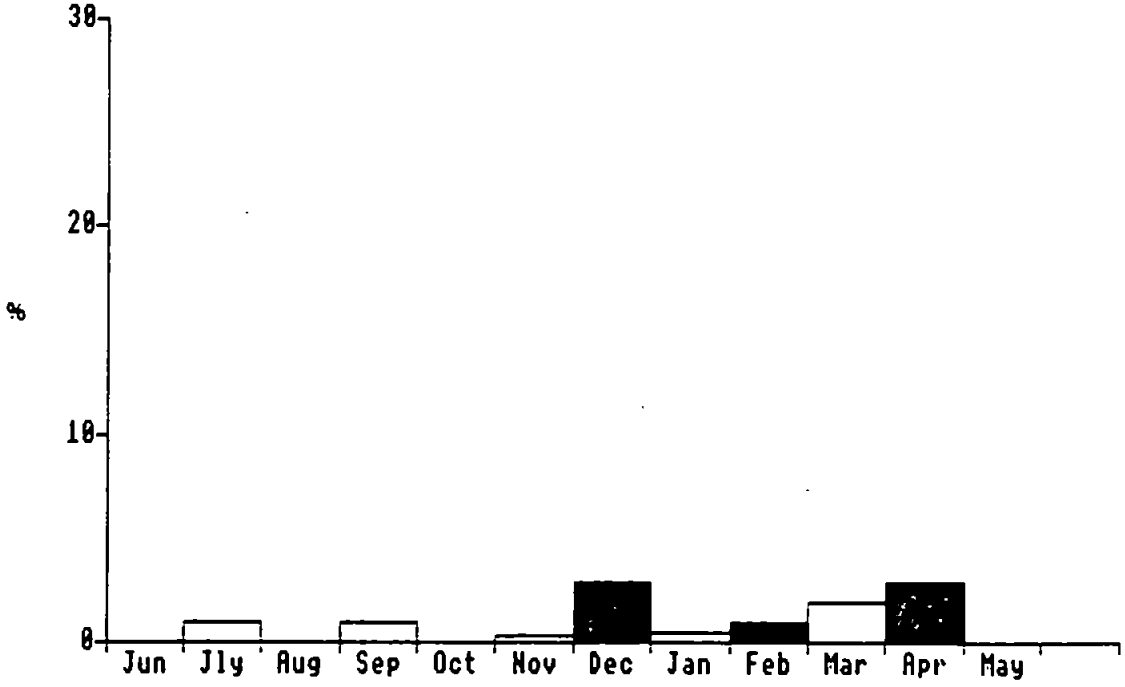


Fig 5.33b Monthly Percentage Composition of Trp. and Ile. in the DCAA, June 1986 - May 1987.

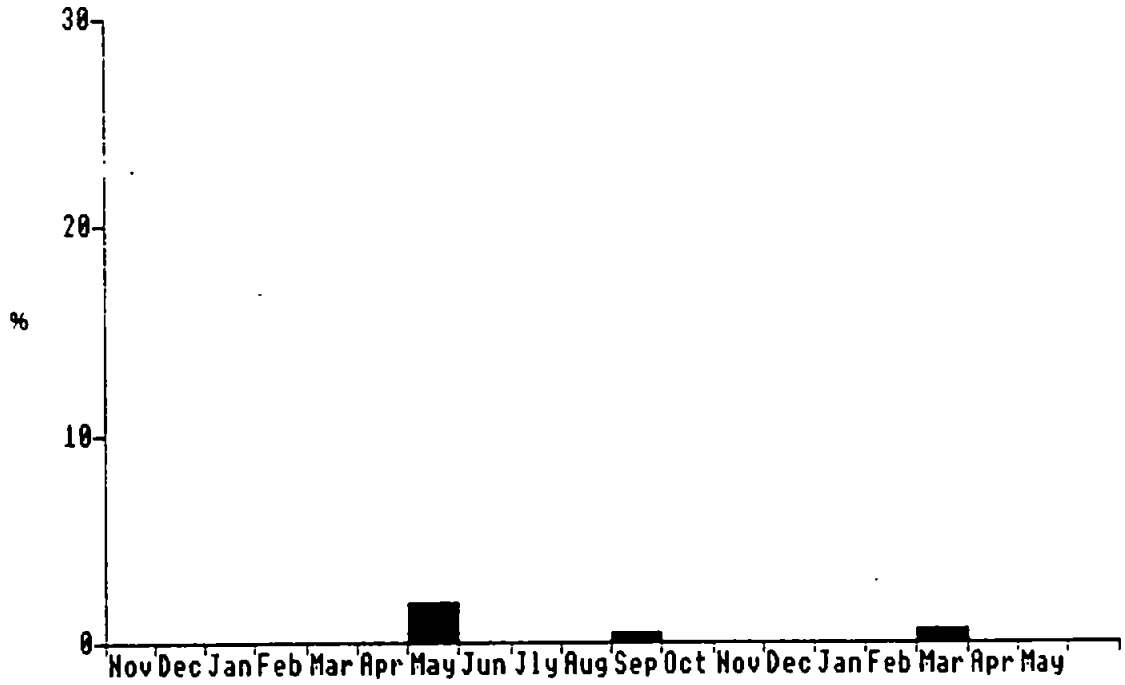


Fig 5.34a Monthly Percentage Composition of Phe. in the DFAA, Nov 1985 - May 1987.

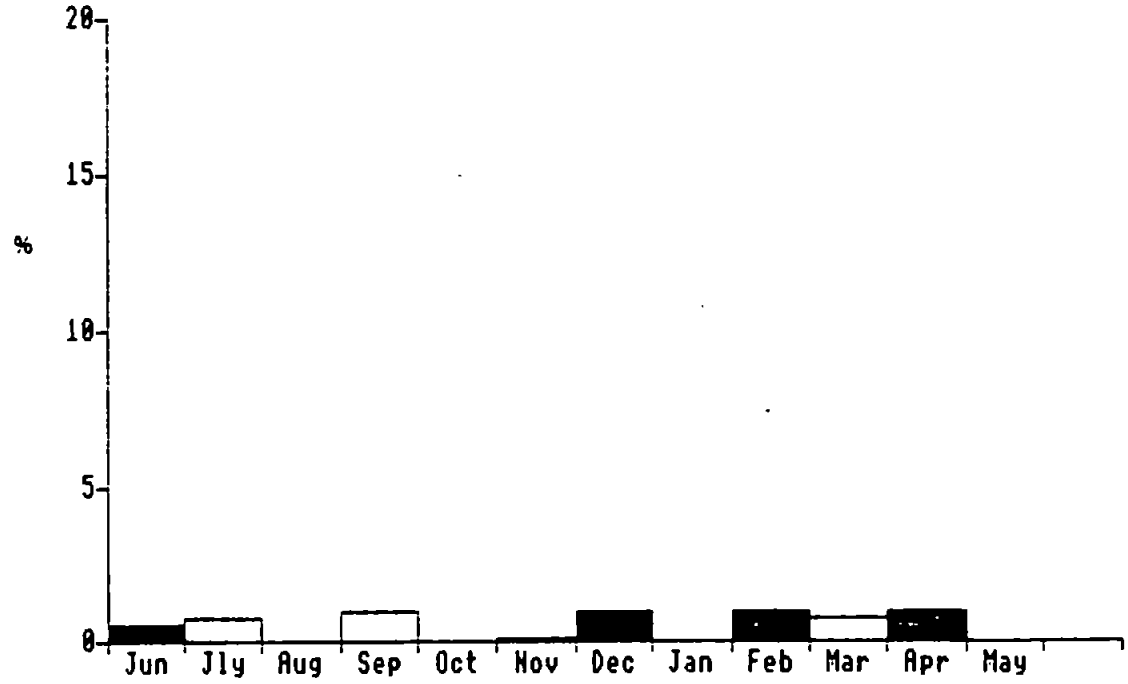


Fig 5.34b Monthly Percentage Composition of Phe. in the DCAA, June 1986 - May 1987.

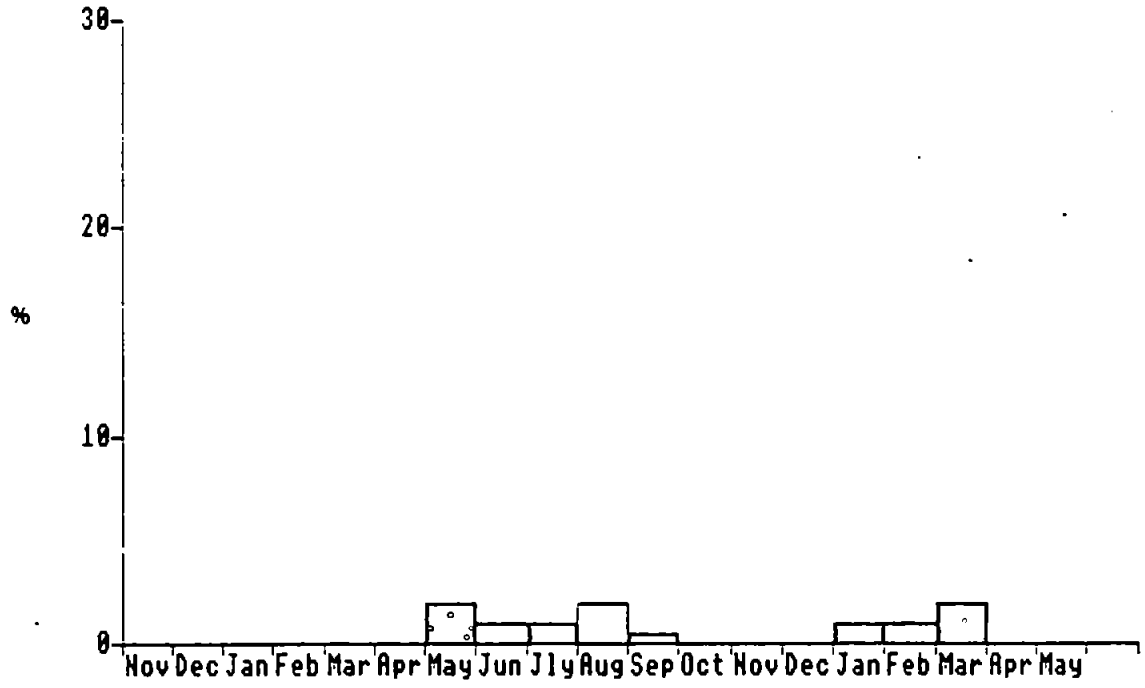


Fig 5.35a Monthly Percentage Composition of Leu. in the DFAA, Nov 1985 - May 1987.

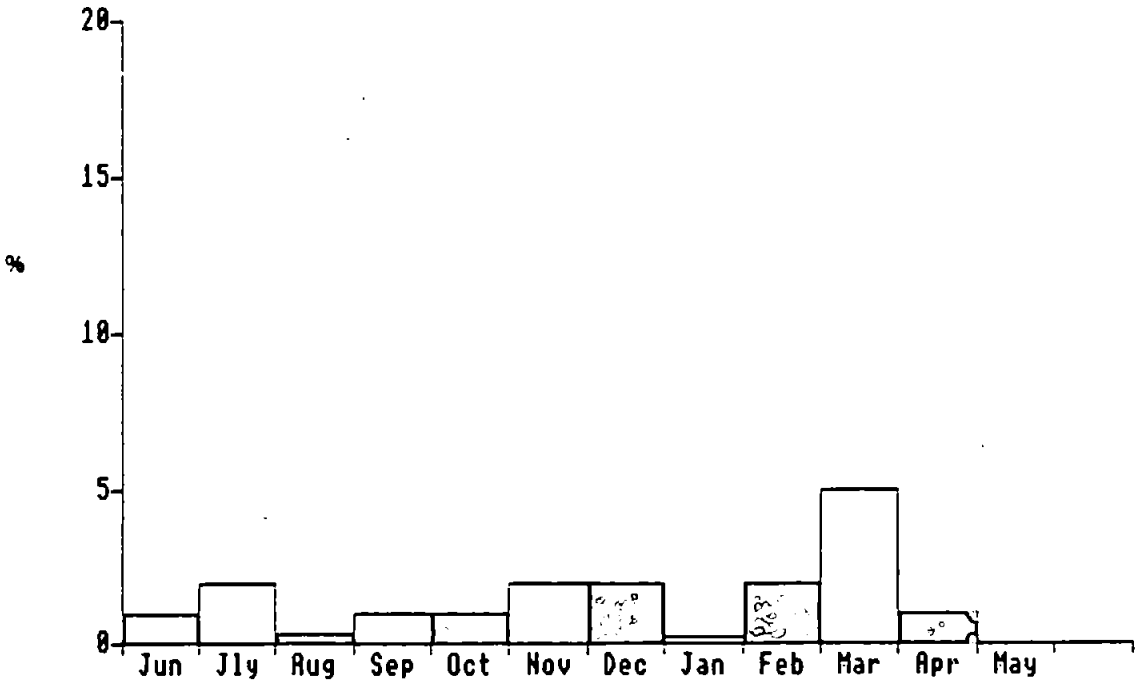


Fig 5.35b Monthly Percentage Composition of Leu. in the DCAA, June 1986 - May 1987.

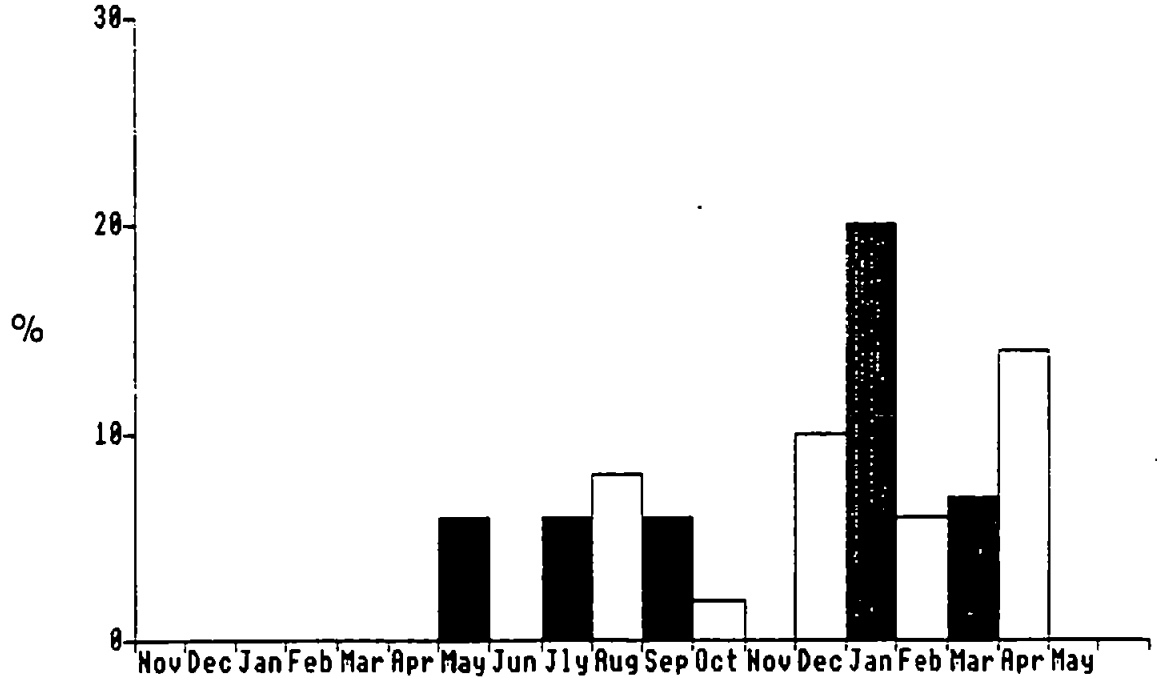


Fig 5.36a Monthly Percentage Composition of Orn. in the DFAA, Nov 1985 - May 1987.

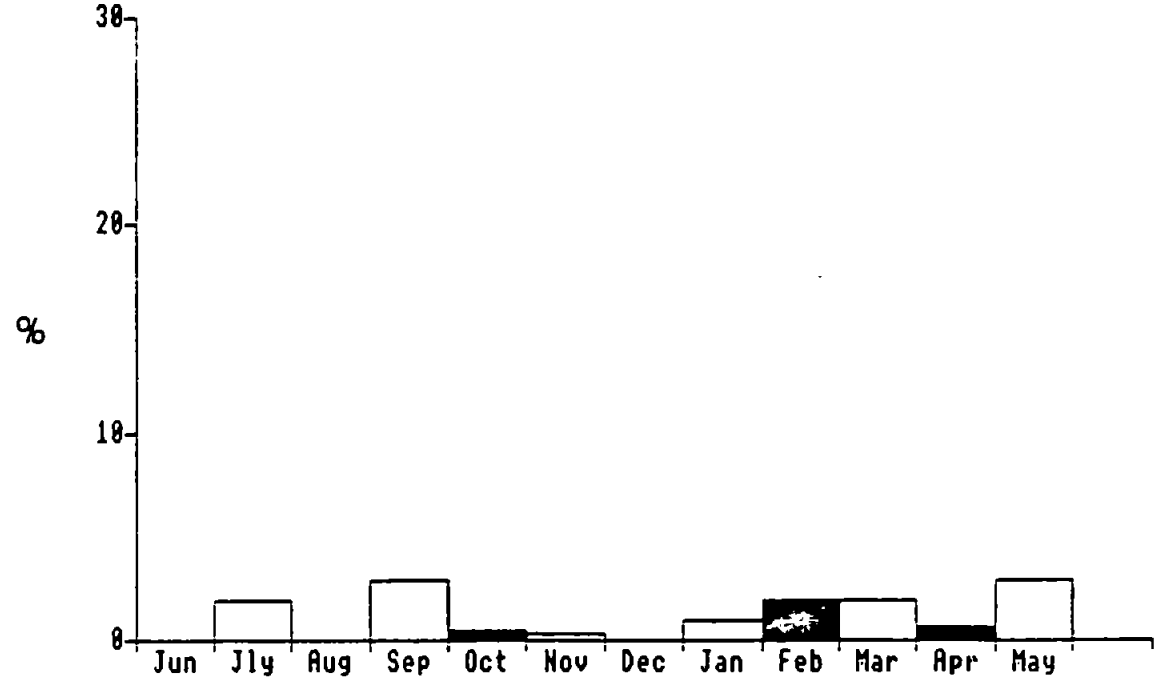


Fig 5.36b Monthly Percentage Composition of Orn. in the DCAA, June 1986 - May 1987.

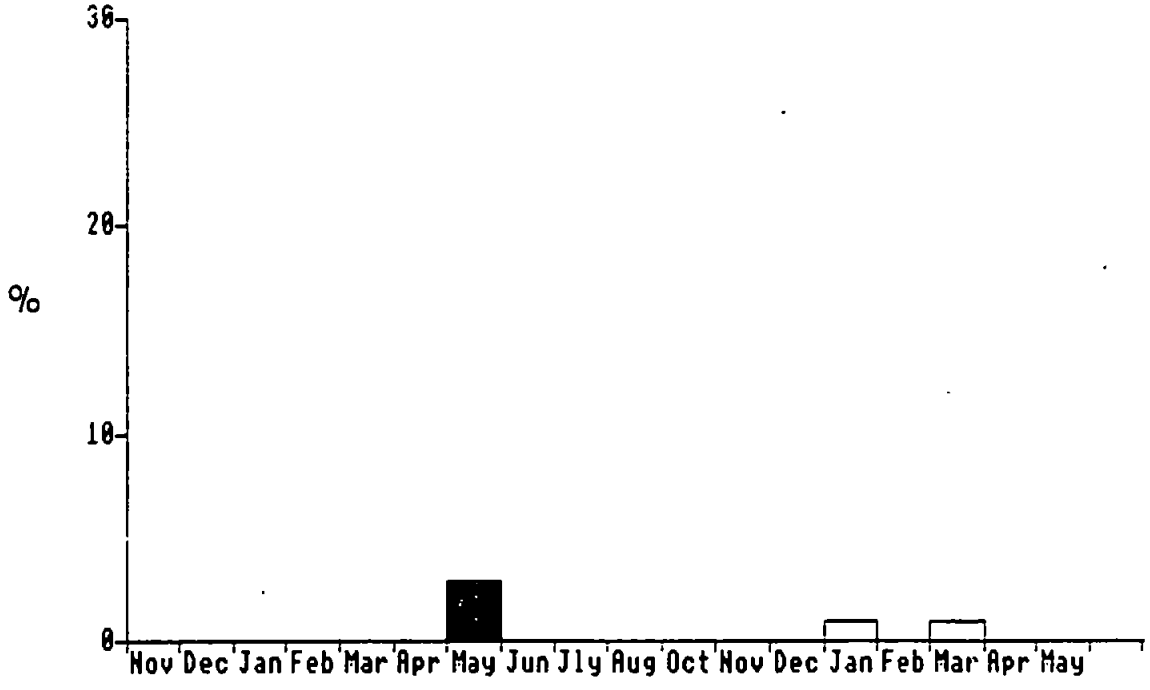


Fig 5.37a Monthly Percentage Composition of Lys. in the DFAA, Nov 1985 - May 1987.

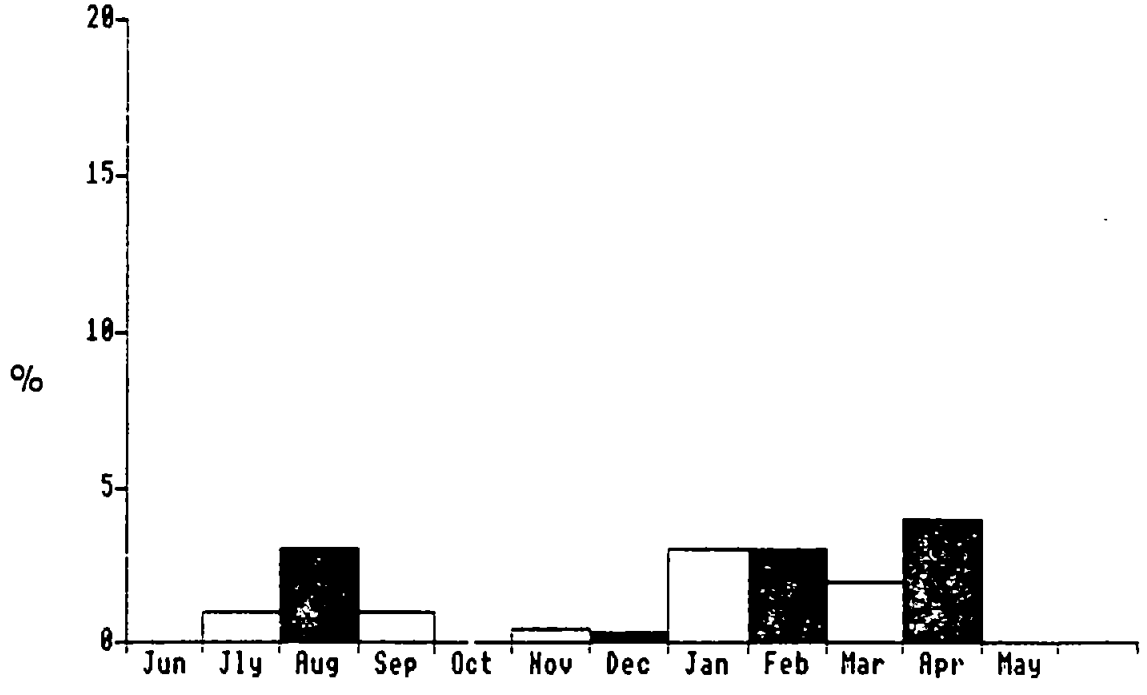


Fig 5.37b Monthly Percentage Composition of Lys. in the DCAA, June 1986 - May 1987.

Skeletonema Costatum to decreases in temperature. At lower temperatures the cells increase their enzyme (protein) levels. The cell protein at 7°C being twice that at 20°C. The increase in glycine, could possibily be due to a temperature adaptation, or to storage of glycine to produce ammonia (see section 5.7.5).

Figures 5.23 - 5.37 highlight the fact that several of the major acids disappeared from the free and/or combined fractions for short periods of time. These acids were glutamic, glycine, threonine, and tyrosine. (Fig. 5.24a, 5.27a, 5.28b, 5.31a & b). These figures also highlight changes in percentage composition of amino acids present in seawater as the season progressed. The average percentage amino acid composition for a given month is shown in a series of pie charts Fig. 5.38-5.56.

There is a pronounced seasonal change in the composition of the dissolved free amino acid fraction. When the dissolved free amino acid levels are low, e.g. October 1986 to January 1987 (Fig. 5.49a to 5.52a) and April to May 1987 (Fig. 5.55a, 5.56a), fewer amino acids are present in the spectrum. This can be seen by comparing the pie charts for October to January and April to May, which contain 6 to 10 acids with that of the free amino acid fraction in March 1987 (Fig. 5.54a) which has 16 amino acids in the spectrum. Possible reasons for the increase in the dissolved free amino acid levels with the concomitant rise in the number of acids present in the spectrum are discussed at the end of this chapter.

The dissolved combined amino acid fraction has a

DISSOLVED FREE AMINO ACIDS NOVEMBER 1985 SAMPLE

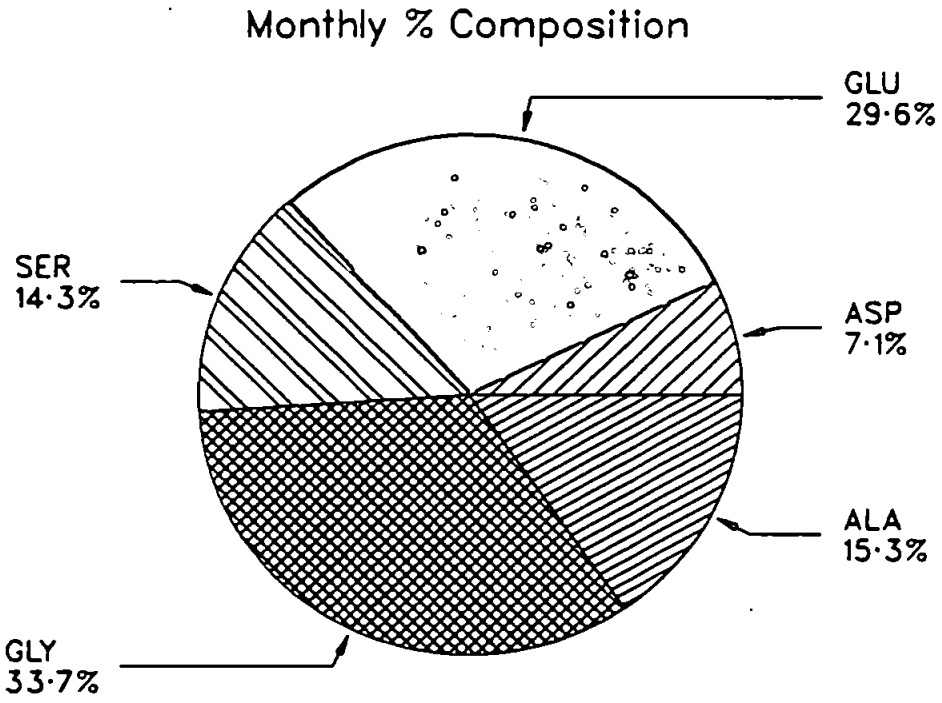


Fig 5.38 The DFAA Composition for Nov 1985.

DISSOLVED FREE AMINO ACIDS DECEMBER 1985 SAMPLE

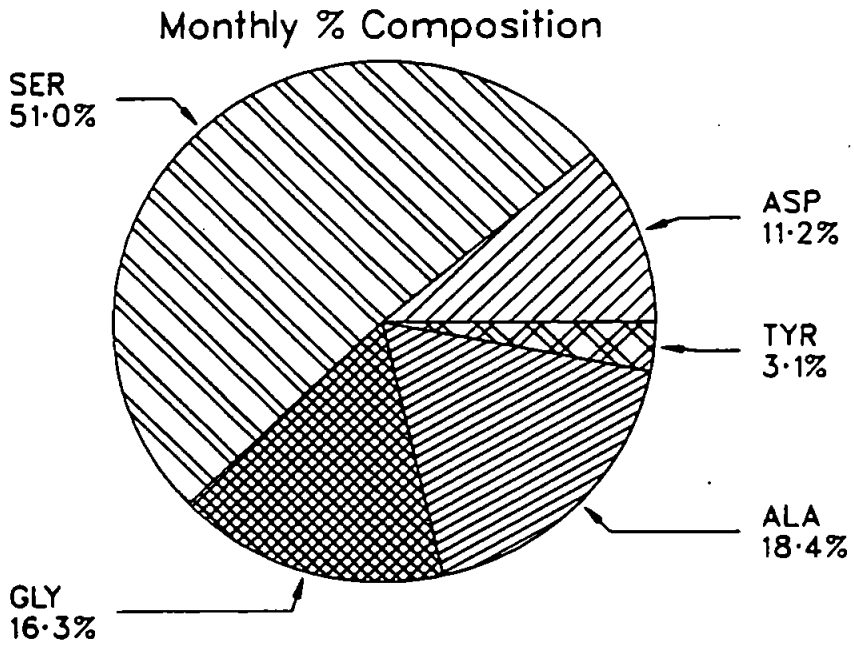


Fig 5.39 The DFAA Composition for Dec 1985.

DISSOLVED FREE AMINO ACIDS JANUARY 1986 SAMPLE

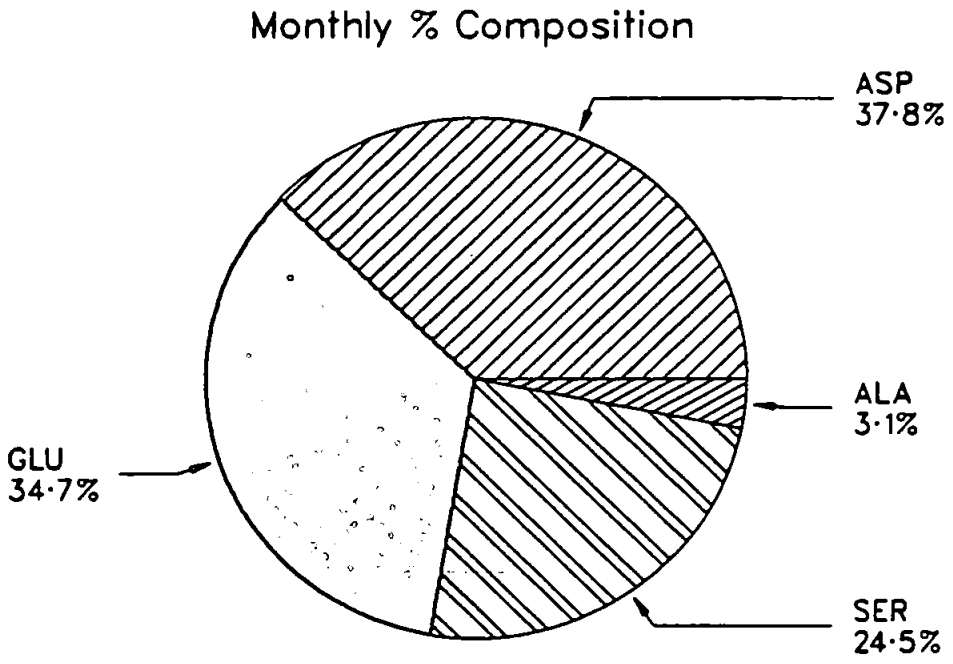


Fig 5.40 The DFAA Composition for Jan 1986.

DISSOLVED FREE AMINO ACIDS FEBRUARY 1986 SAMPLE

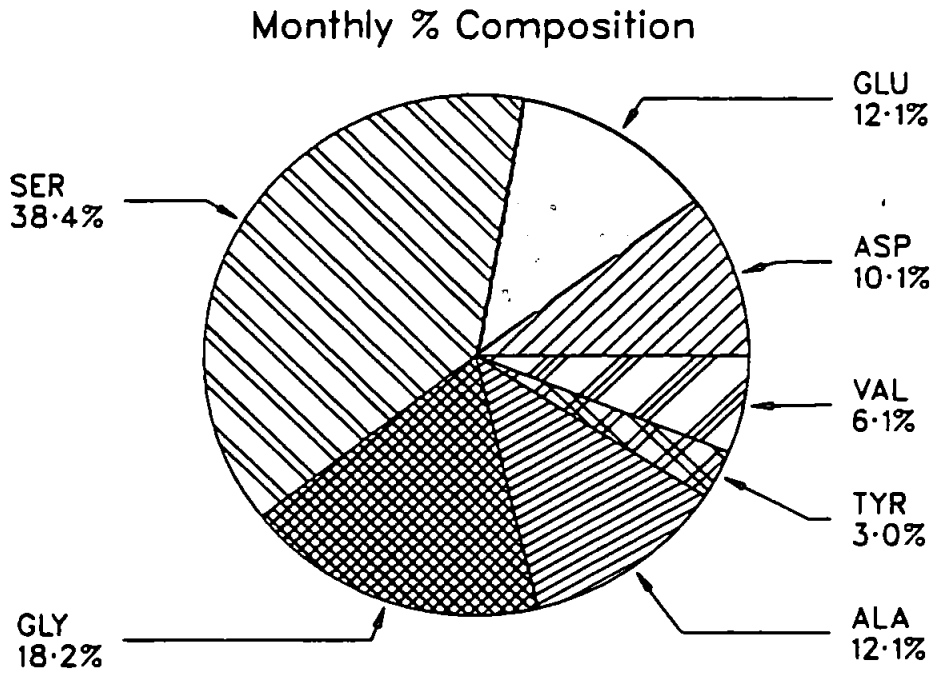


Fig 5.41 The DFAA Composition for Feb 1986.

DISSOLVED FREE AMINO ACIDS MARCH 1986 SAMPLE

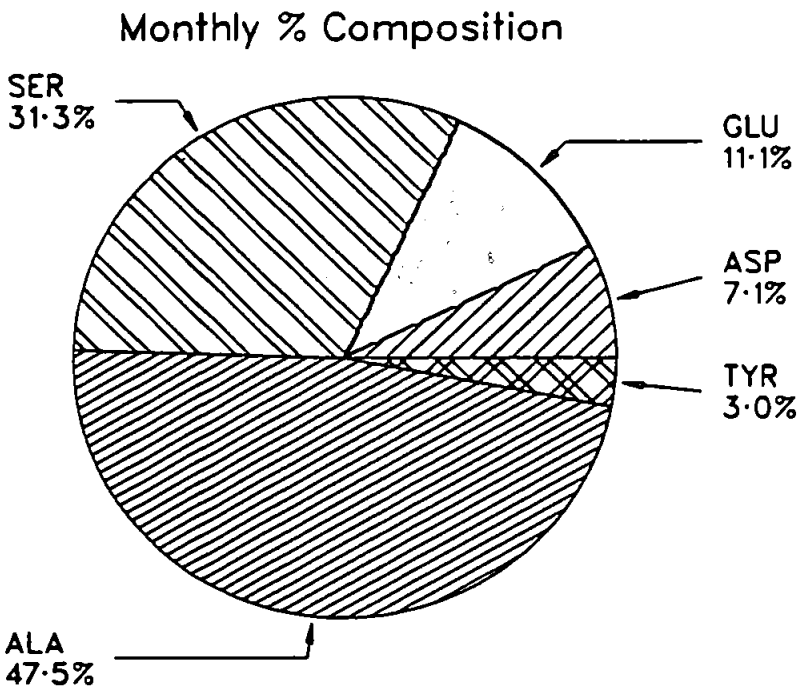


Fig 5.42 The DFAA Composition for Mar 1986.

DISSOLVED FREE AMINO ACIDS
APRIL 1986 SAMPLE

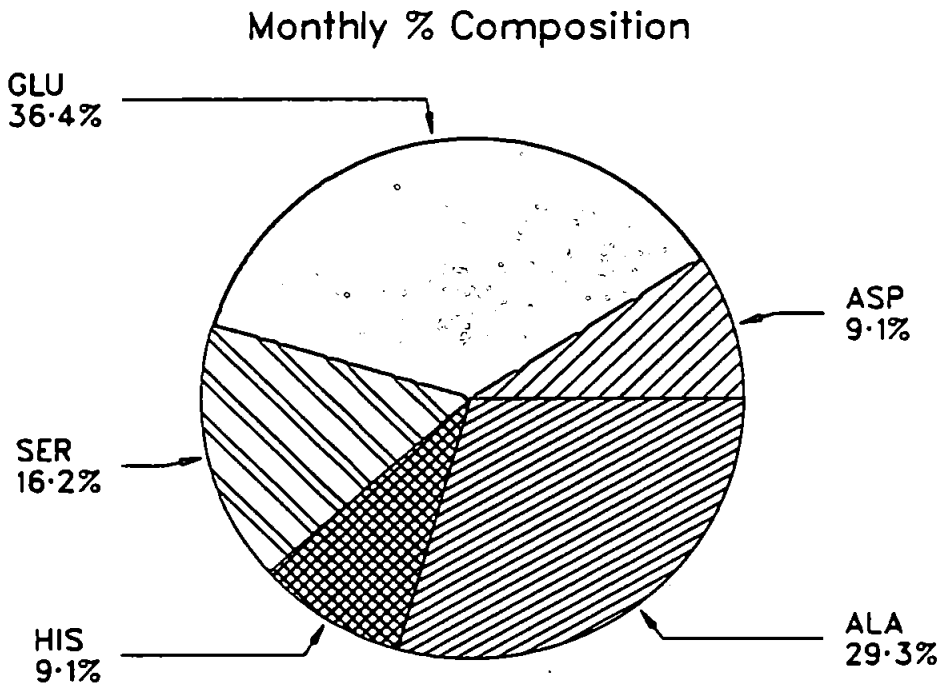


Fig 5.43 The DFAA Composition for Apr 1986.

DISSOLVED FREE AMINO ACIDS MAY 1986 SAMPLE

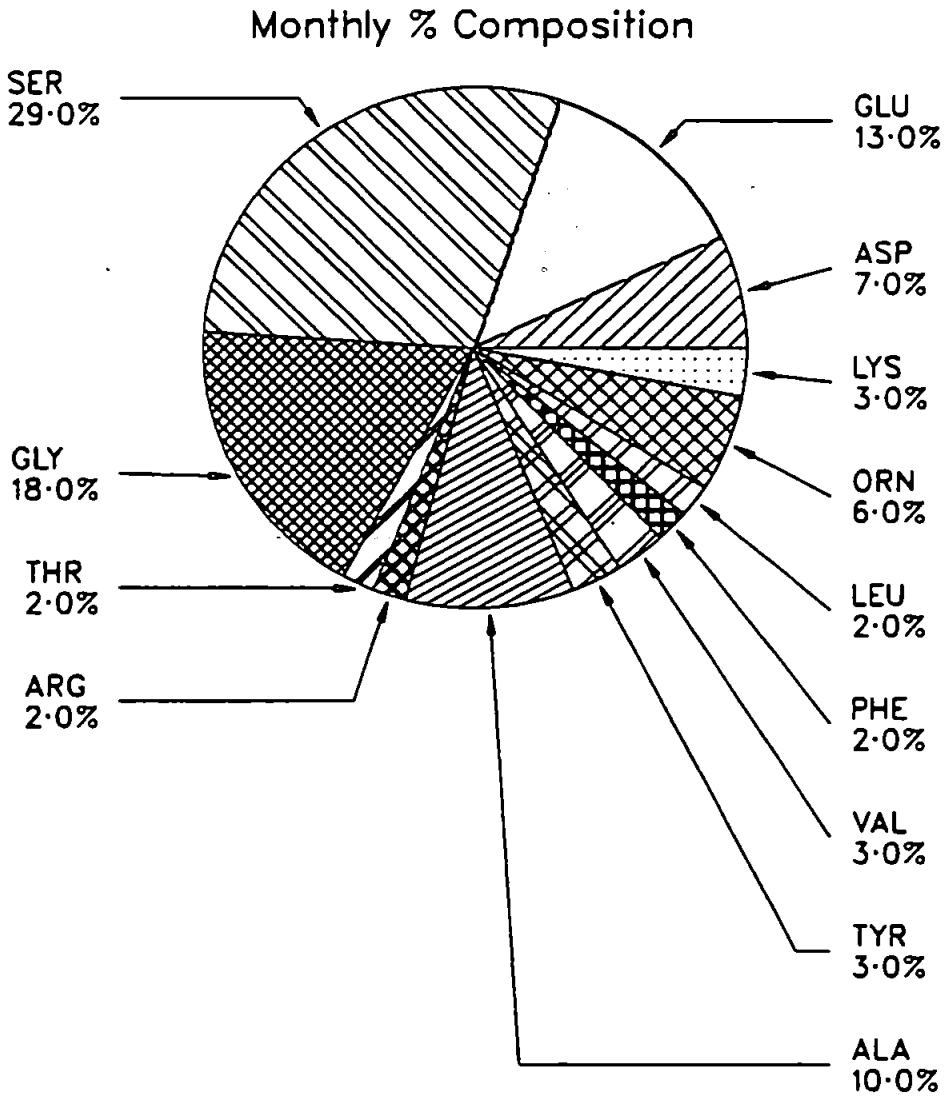


Fig 5.44 The DFAA Composition for May 1986.

DISSOLVED FREE AMINO ACIDS
JUNE 1986 SAMPLE

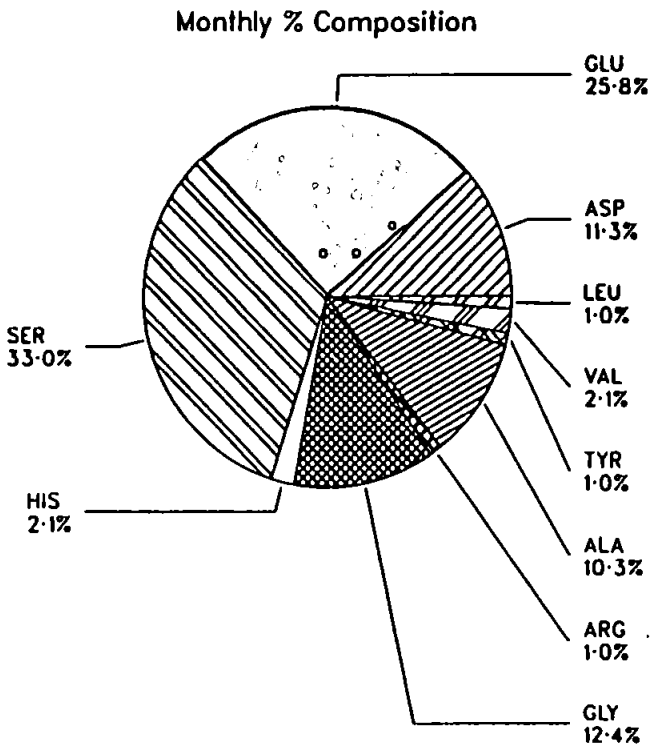


Fig 5.45a The DFAA Composition for Jun 1986.

DISSOLVED COMBINED AMINO ACIDS
JUNE 1986 SAMPLE

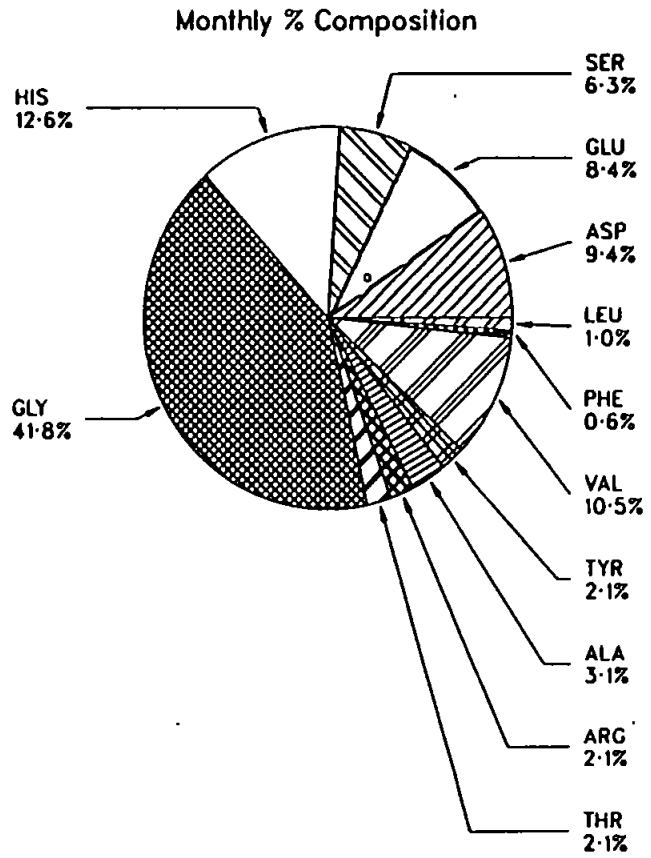


Fig 5.45b The DCAA Composition for Jun 1986.

DISSOLVED FREE AMINO ACIDS
JULY 1986 SAMPLE

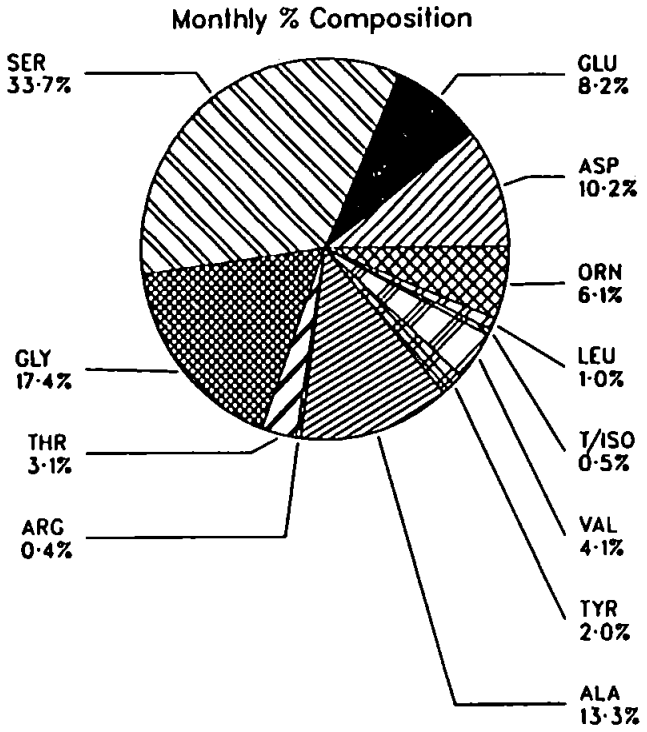


Fig 5.46a The DFAA Composition for Jly 1986.

DISSOLVED COMBINED AMINO ACIDS
JULY 1986 SAMPLE

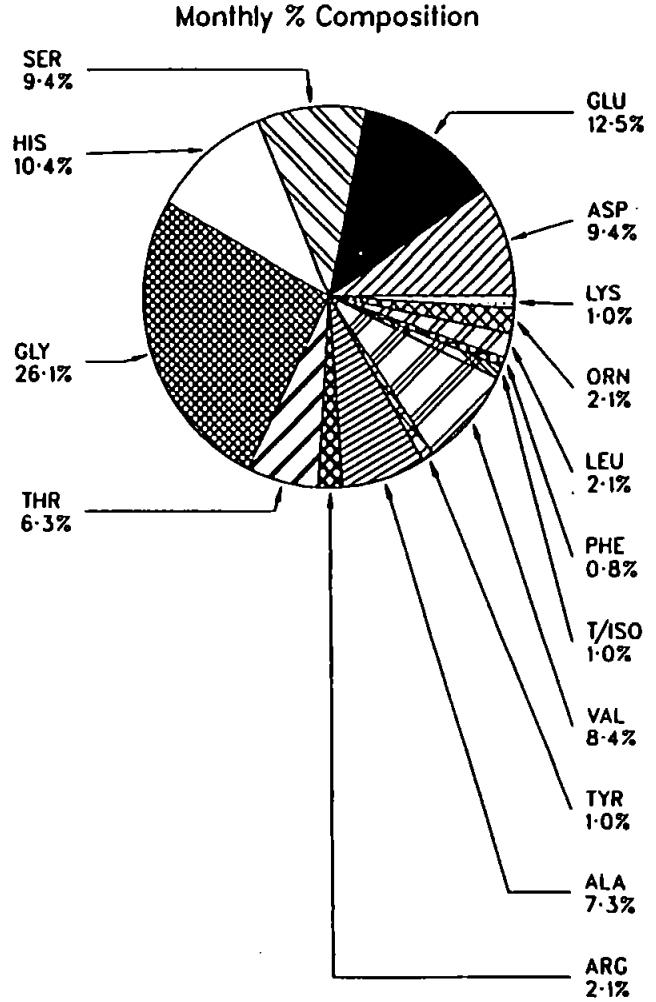


Fig 5.46b The DCAA Composition for Jly 1986.

DISSOLVED FREE AMINO ACIDS
AUGUST 1986 SAMPLE

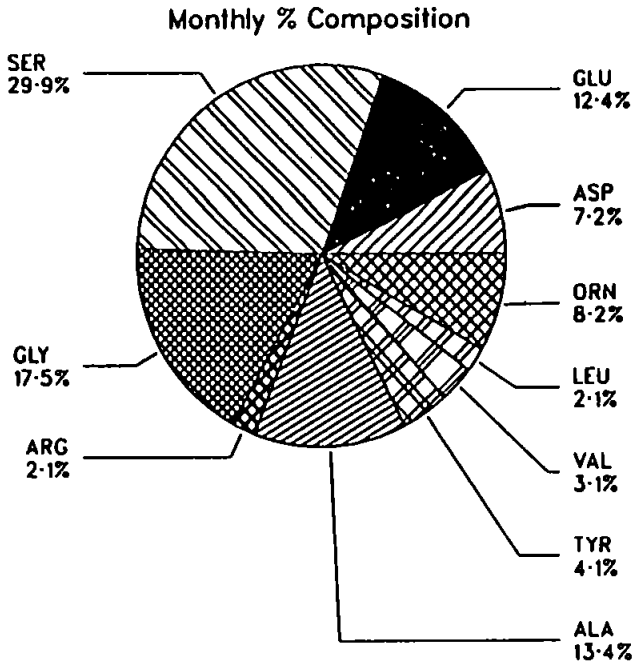


Fig 5.47a The DFAA Composition for Aug 1986.

DISSOLVED COMBINED AMINO ACIDS
AUGUST 1986 SAMPLE

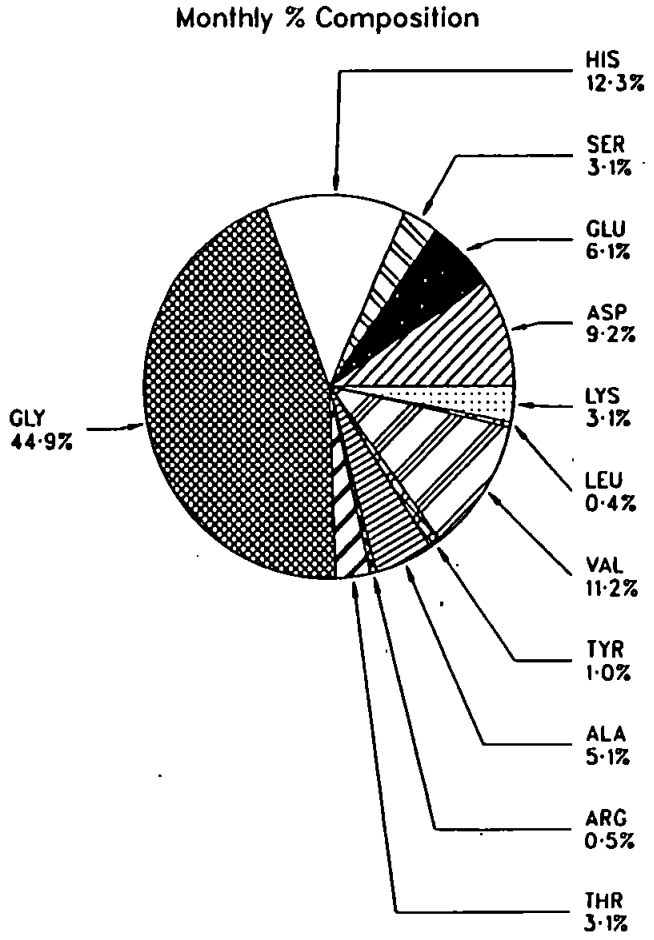


Fig 5.47b The DCAA Composition for Aug 1986.

DISSOLVED FREE AMINO ACIDS SEPTEMBER 1986 SAMPLE

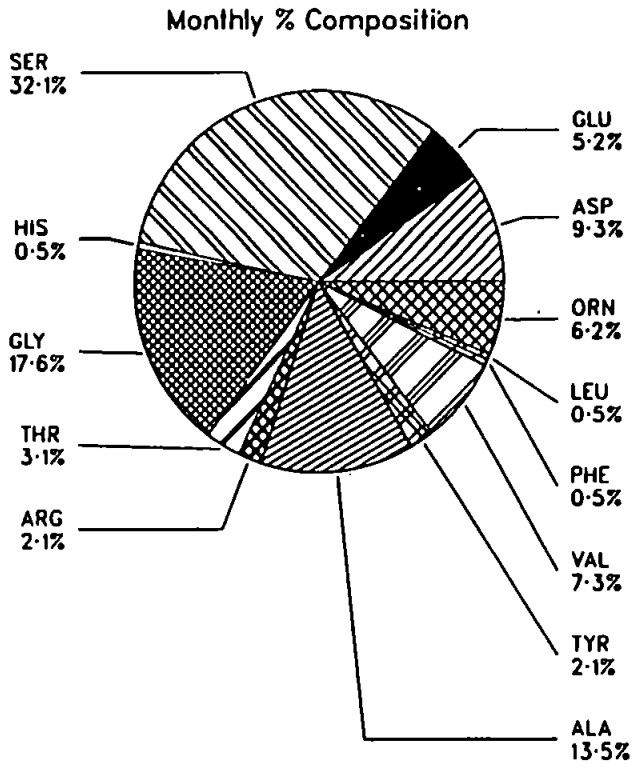


Fig 5.48a The DFAA Composition for Sep 1986.

DISSOLVED COMBINED AMINO ACIDS SEPTEMBER 1986 SAMPLE

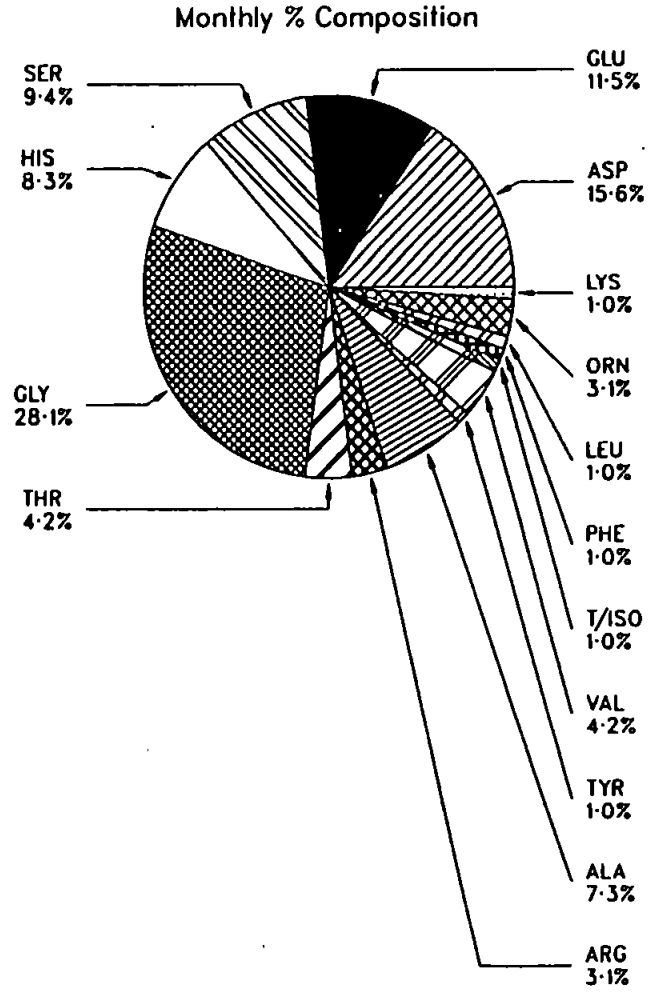


Fig 5.48b The DCAA Composition for Sep 1986.

DISSOLVED FREE AMINO ACIDS
OCTOBER 1986 SAMPLE

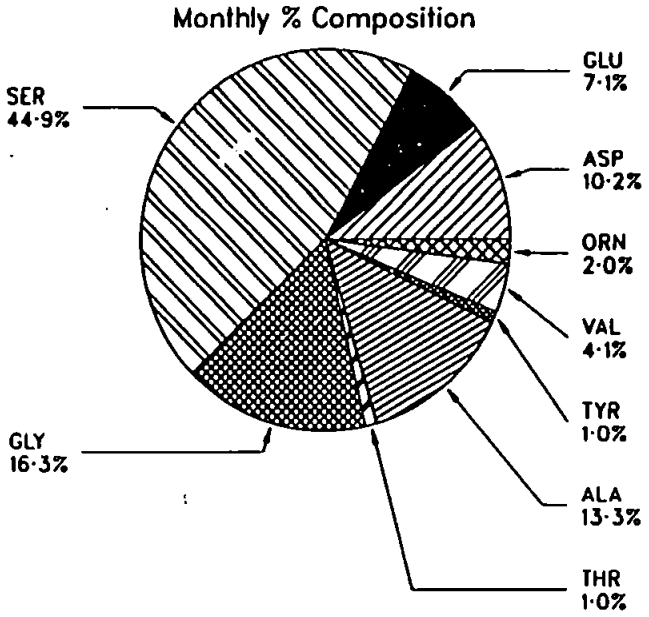


Fig 5.49a The DFAA Composition for Oct 1986.

DISSOLVED COMBINED AMINO ACIDS
OCTOBER 1986 SAMPLE

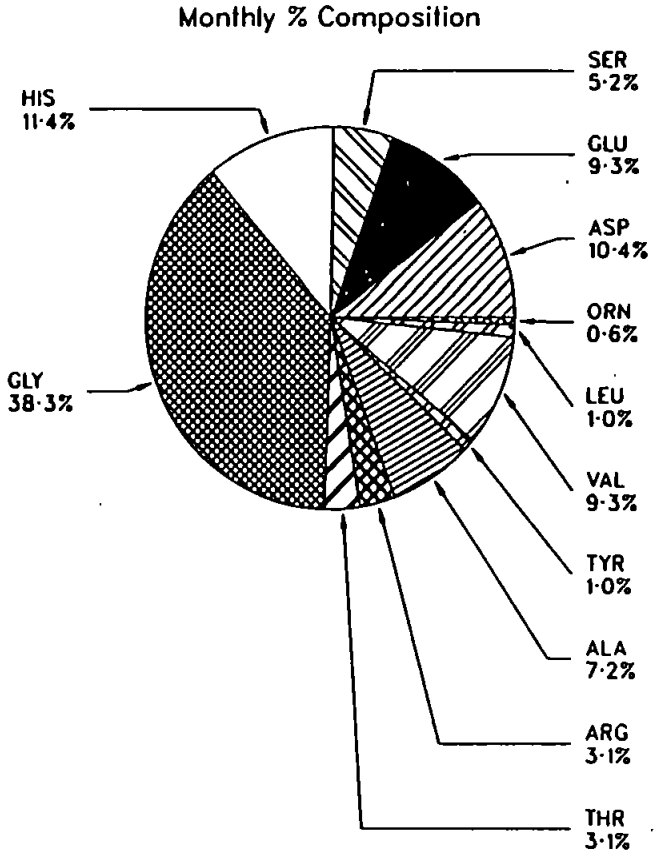


Fig 5.49b The DCAA Composition for Oct 1986.

DISSOLVED FREE AMINO ACIDS
NOVEMBER 1986 SAMPLE

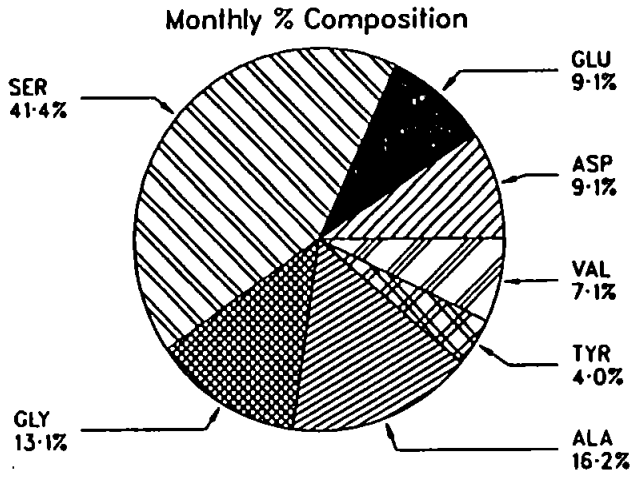


Fig 5.50a The DFAA Composition for Nov 1986.

DISSOLVED COMBINED AMINO ACIDS
NOVEMBER 1986 SAMPLE

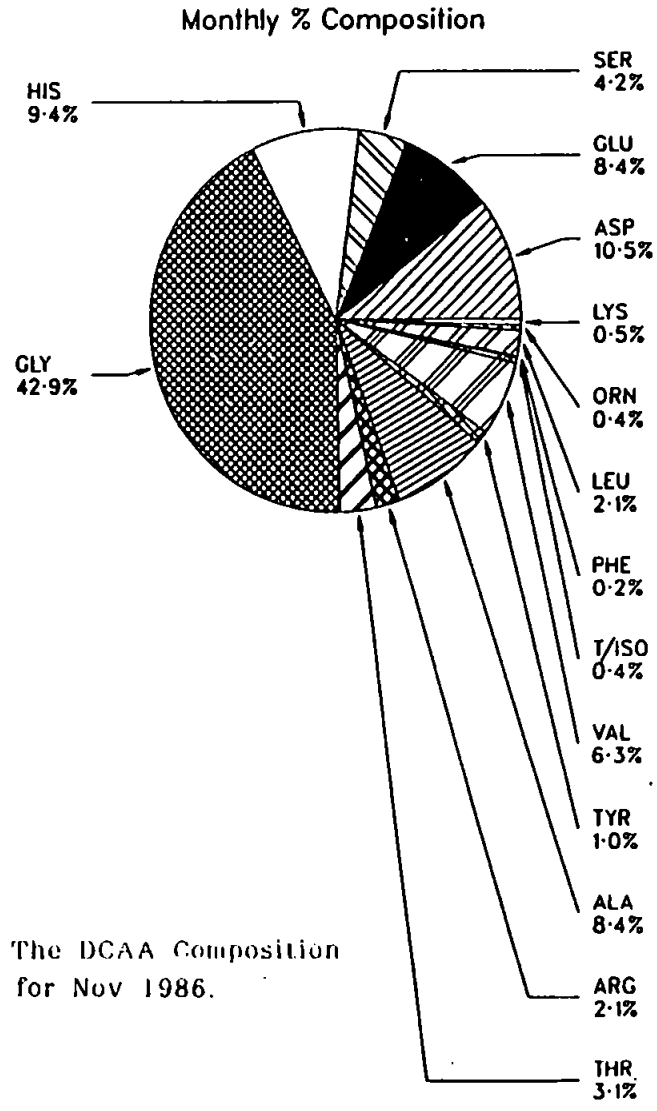


Fig 5.50b The DCAA Composition for Nov 1986.

DISSOLVED FREE AMINO ACIDS
DECEMBER 1986 SAMPLE

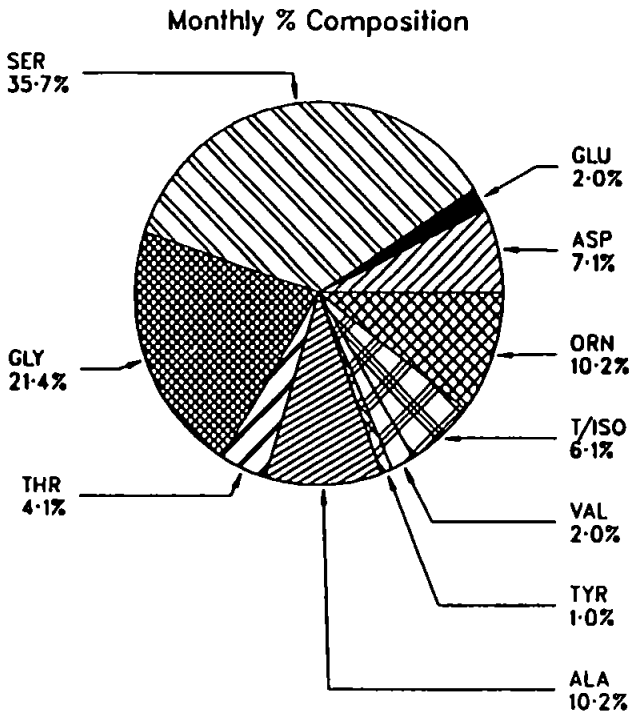


Fig 5.51a The DFAA Composition for Dec 1986.

DISSOLVED COMBINED AMINO ACIDS
DECEMBER 1986 SAMPLE

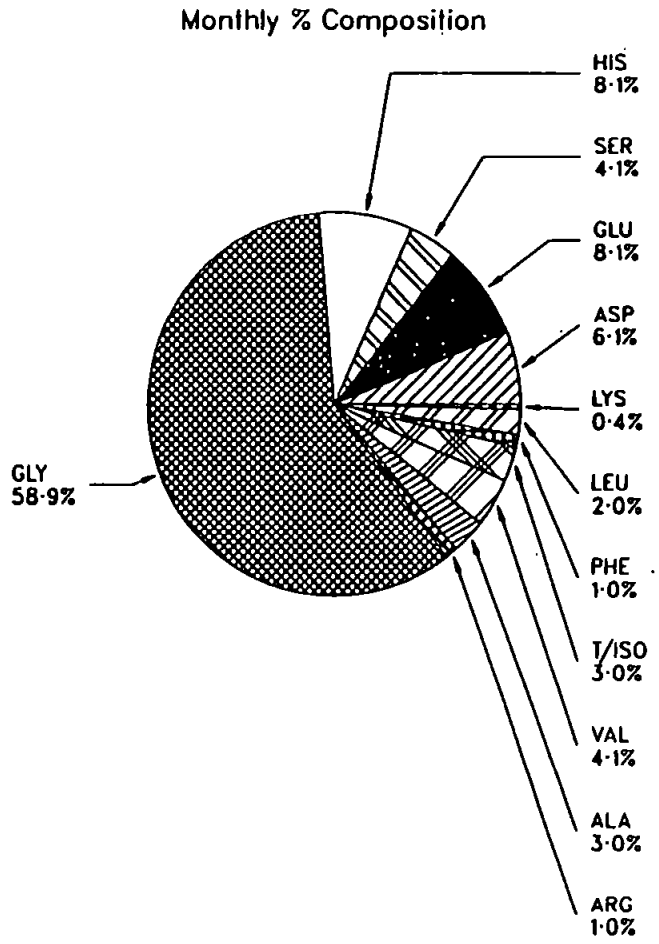


Fig 5.51b The DCAA Composition for Dec 1986.

DISSOLVED FREE AMINO ACIDS JANUARY 1987 SAMPLE

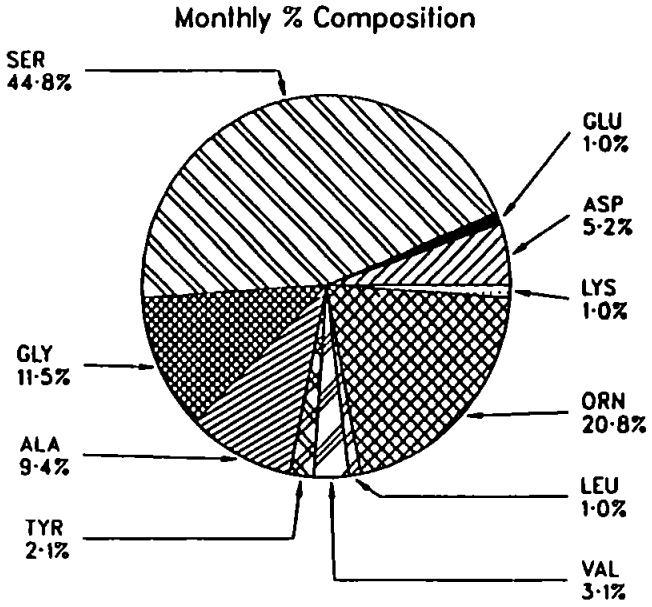


Fig 5.52a The DFAA Composition for Jan 1987.

DISSOLVED COMBINED AMINO ACIDS JANUARY 1987 SAMPLE

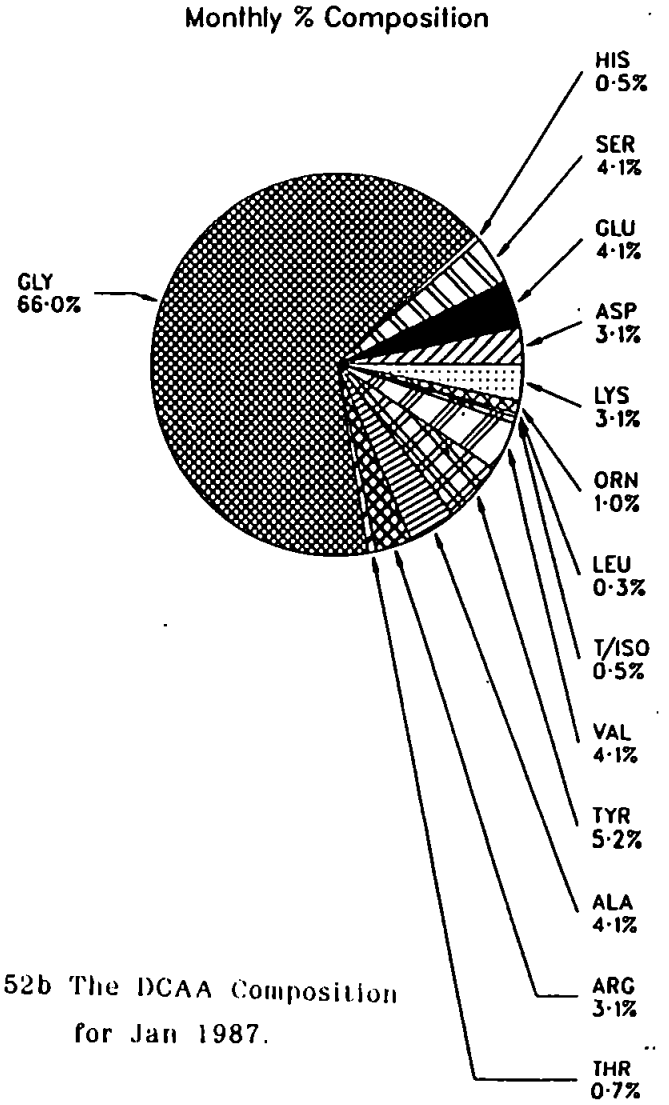


Fig 5.52b The DCAA Composition for Jan 1987.

DISSOLVED FREE AMINO ACIDS
FEBRUARY 1987 SAMPLE

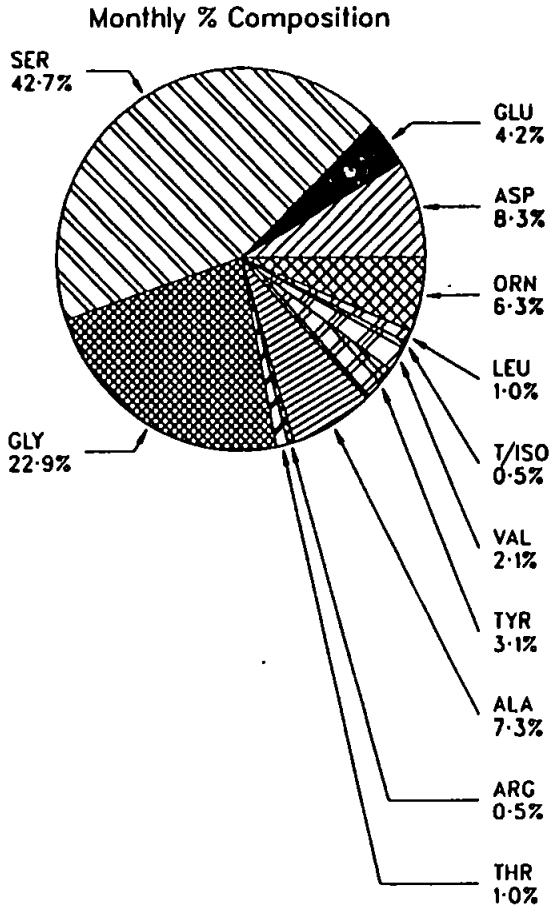


Fig 5.53a The DFAA Composition for Feb 1987.

DISSOLVED COMBINED AMINO ACIDS
FEBRUARY 1987 SAMPLE

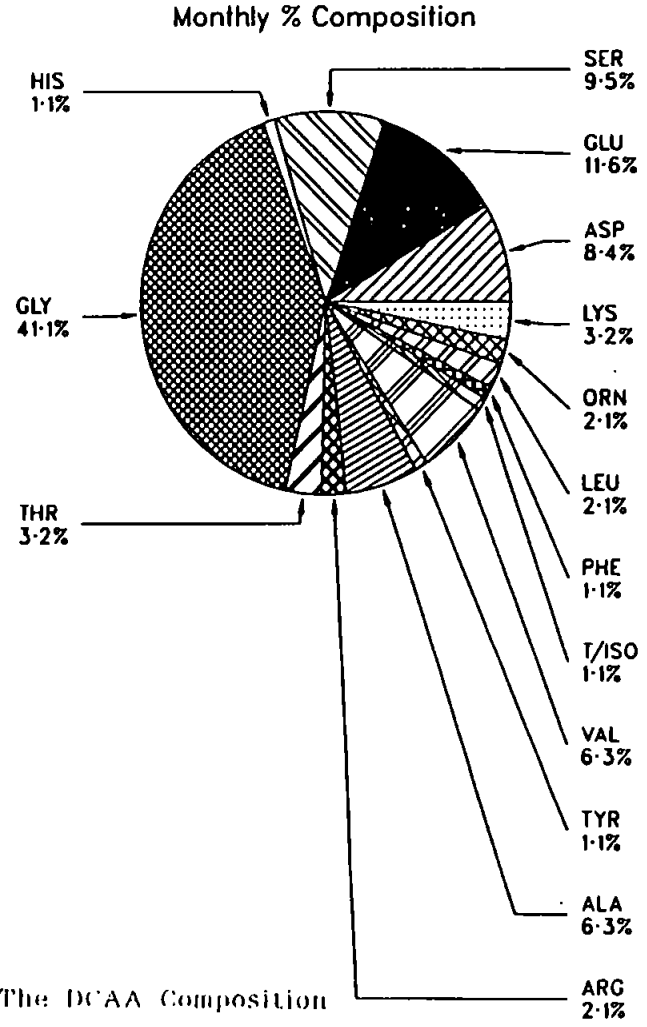


Fig 5.53b The DCAA Composition for Feb 1987.

DISSOLVED FREE AMINO ACIDS MARCH 1987 SAMPLE

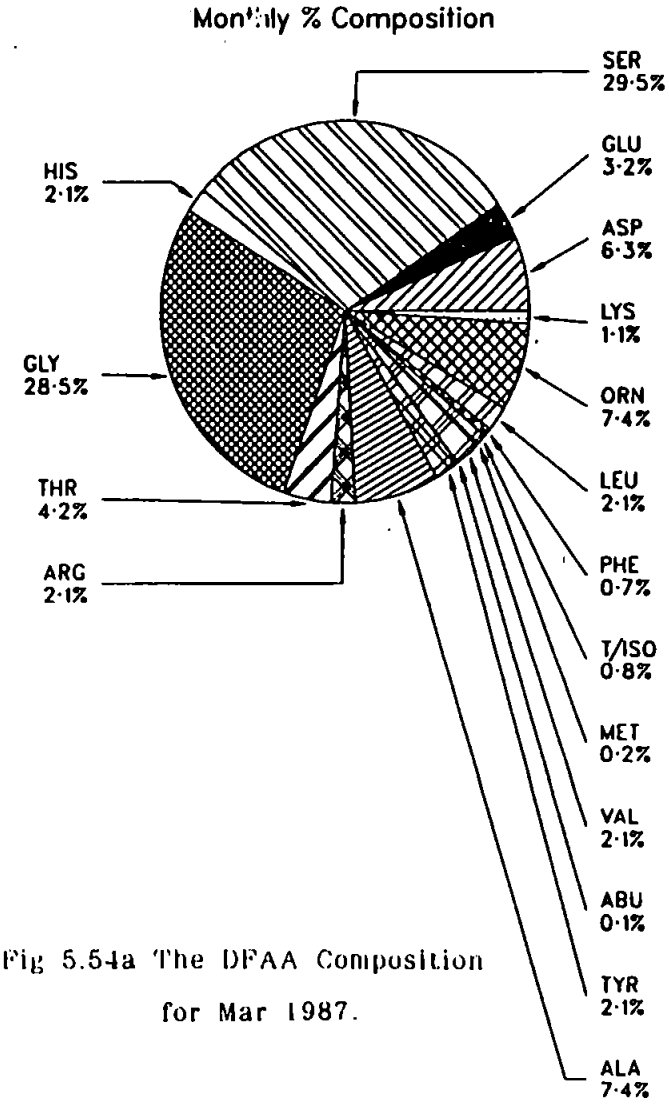


Fig 5.54a The DFAA Composition for Mar 1987.

DISSOLVED COMBINED AMINO ACIDS MARCH 1987 SAMPLE

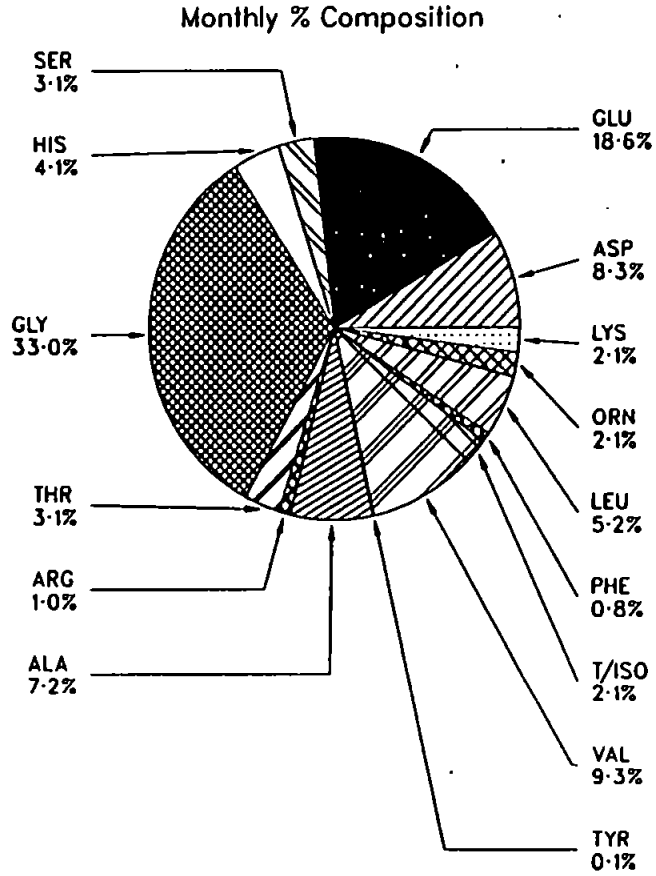


Fig 5.54b The DCAA Composition for Mar 1987.

DISSOLVED FREE AMINO ACIDS
APRIL 1987 SAMPLE

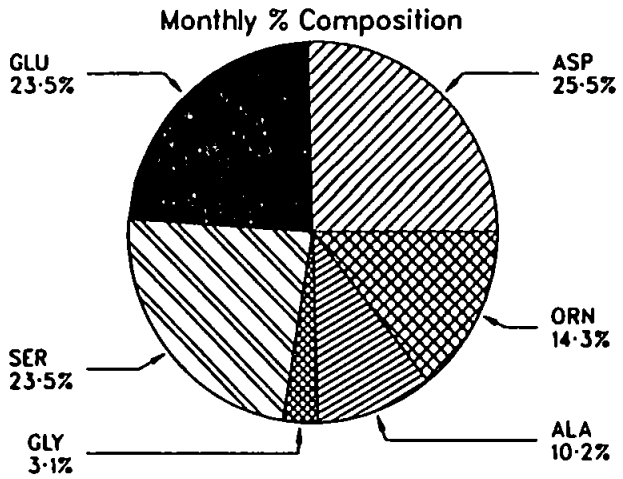


Fig 5.55a The DFAA Composition for Apr 1987.

DISSOLVED COMBINED AMINO ACIDS
APRIL 1987 SAMPLE

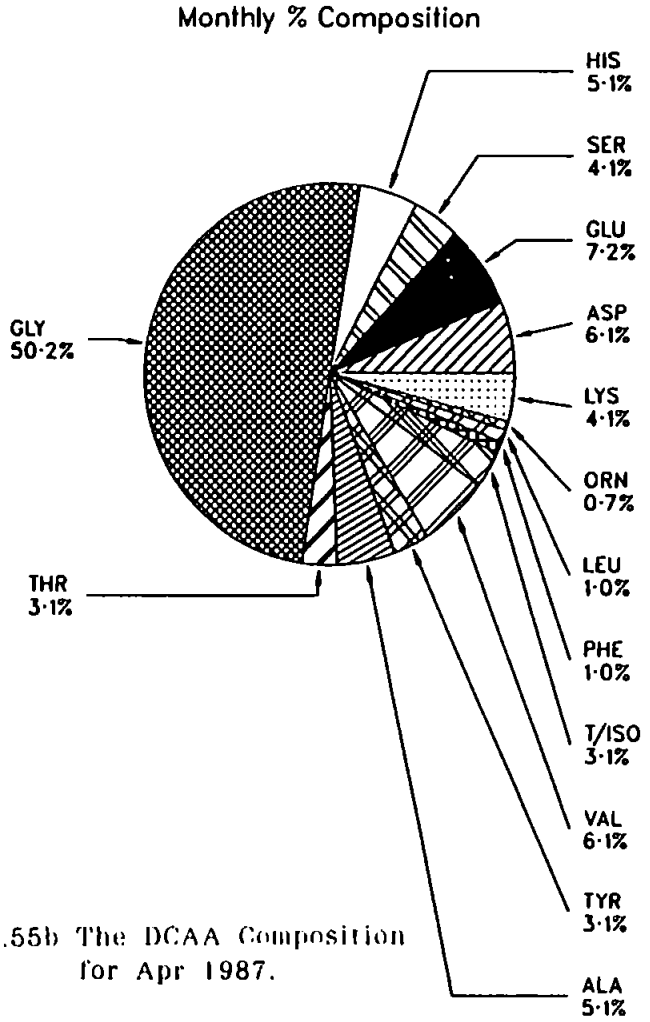


Fig 5.55b The DCAA Composition for Apr 1987.

DISSOLVED FREE AMINO ACIDS
MAY 1987 SAMPLE

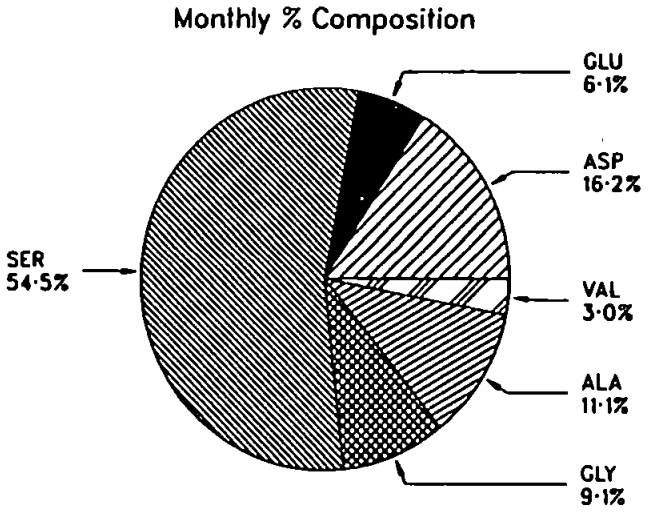


Fig 5.56a The DFAA Composition for May 1987.

DISSOLVED COMBINED AMINO ACIDS
MAY 1987 SAMPLE

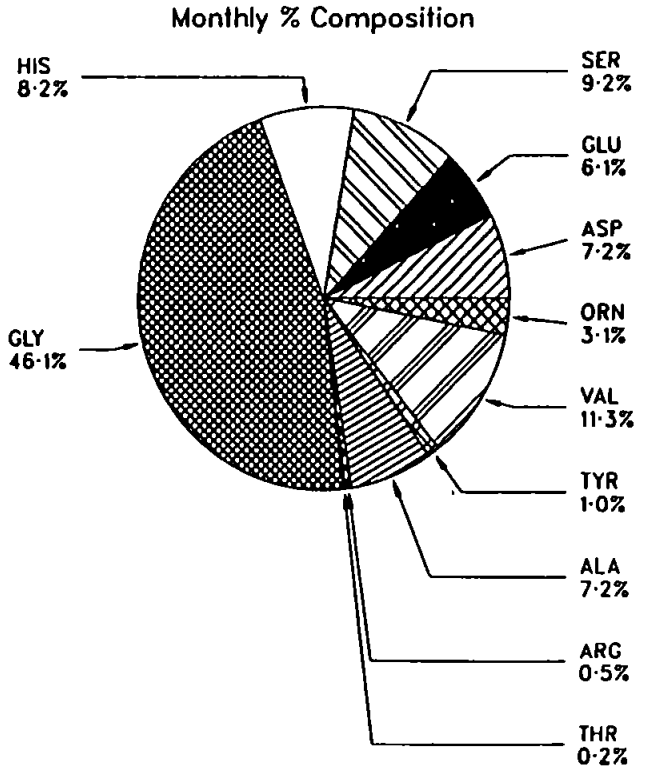


Fig 5.56b The DCAA Composition for May 1987.

more constant composition with less distinct seasonal trends. However aspartate, histidine and threonine show a reduction in levels, in the combined fraction, from October 1986 to January 1987.

The fundamental idea of this project, which was stated in chapter 1, was to determine the levels and composition of both the dissolved combined and dissolved free amino acid fractions.The composition of the combined fraction was expected to remain fairly constant, and from this stable base, comparisons with the composition of the free fraction could be made. Therefore acids present in the combined but not in the free fraction could be identified and "disappearence" values calculated. This process would identify the acids most likely to be being used by the biota in the natural environment. The small seasonal variation of the dissolved combined amino acid fraction produces a difficulty in the interpretation of the "missing" acids. For example, histidine is present for most of the time in the combined but not in the free fraction. Therefore a "disappearence" value can be calculated. If however the histidine then disappears from the combined fraction this will affect the calculation of the "disappearence" values. Due to the seasonal variation of some acids in the combined fraction the "disappearence" values stated in section 5.7.4. can only be tentative.

5.6. Sizing The Dissolved Amino Acid Containing Macromolecule.

The results of the sizing experiments, carried out using the centriflo cones and centricon-10 microconcentrators detailed in Chapter 4, are shown overleaf in table 5.3.

TABLE 5.3. Sizing of the dissolved amino acid containing macromolecule.

Date.	Sample No.	% < 25,000	% < 10,000 m.w.
1.10.86	1	110	
Day:336	4	120	
8.11.86	4	100	
Day:371			
13.11.86	9	32	
Day:379	10	23	
15.1.87	3	70	
Day:442	6	50	
21.1.87	1	93	
	7	119	
30.1.87	4	89	51
Day:457	6	56	33
12.2.87	1	36	16
Day:470	4	24	
26.2.87	1	25	
Day:484	4	60	
5.3.87	1	109	
Day:491	4	40	
12.3.87	6	108	
Day:498	7	108	
19.3.87	1		35
Day:505	4		84
1.4.87	1		84
Day:518	3		35
7.4.87	1		84
Day:532			
23.4.87	1		95
Day:540	2		119

The average value of material with a molecular weight less than 25,000 was 72 percent. While the average value of material less than 10,000 molecular weight was 63 percent.

In nearly all the molecular weight cut off experiments, two samples of a batch were sized. Only in one sizing experiment, on the 5.3.87, when a small bloom was present, was there a large difference between the percentage of the material less than 25,000 i.e. 40 and 109 percent. It is interesting to note that a similar phenomenon was found, in bloom conditions, for phosphatase cut off experiments. (Personal communication E.I. Butler). In twelve of the nineteen sizing experiments at molecular weight cut off 25,000, over fifty percent of the macromolecular material was less than the cut off value. In six of the ten samples sized at 10,000, again, over fifty percent of the macromolecular species were below the nominal value.

The results of the sizing experiments indicate that the macromolecular species sized in this study are relatively small molecules generally having less than 250 amino acids in their composition. The possible reason for the small size of the macromolecular species is discussed in section 5.7.

There appears to be no correlation between molecular size and total dissolved free or combined amino acid concentrations.

5.7. Discussion

5.7.1 Introduction.

The discussion of the results of the coastal analyses has been subdivided into six sections.

First, the levels of dissolved combined and dissolved free amino acids found in this study are compared to the values determined by previous workers. The seasonal levels of the dissolved combined and dissolved free amino acids and the ratio of dissolved free to dissolved combined acids are examined. The idea that the levels and ratios found may reflect two different processes occurring during the year is introduced.

The second section contains a seasonal correlation of the dissolved free and dissolved combined fractions with factors such as hours of sunshine, rainfall and productivity etc.

Section three considers the compositional difference between the dissolved combined and dissolved free amino acid fraction.

Section four looks at the possible role of serine and glycine as nutrient sources.

Section five attempts to explain how species succession may be related to the "disappearence" of major acids from the compositional spectrum.

The final section attempts to explain the small size of the dissolved combined amino acid fraction found in

this study and relates this to composition.

As has been seen the results presented in this chapter have produced a large number of graphs and for ease of presentation several of these will be repeated in the discussion to illustrate salient features of the analyses.

5.7.2 Examination Of The Total Dissolved Combined And Dissolved Free Amino Acid Levels Over A Season.

Comparison of the range of values for dissolved free amino acids (BLD to 9.372 uM) and dissolved combined amino acids (0.246 to 6.741 uM) found in this study, with those of previous workers (Table 5.4), shows that the dissolved free amino acid range is wider than that generally quoted in the literature. The results in this study have a distribution which is skewed towards low dissolved free amino acid levels. The problems of contamination found in trace organic analysis of this type already have been discussed in chapter 2 and 3. It is possible that the occasionally high values reported e.g. 9.372 may be due to contamination of the sample. However similar high values have been found in other studies (Evens 1986) and there is no evidence to support their removal from the analyses. The average values for both the dissolved free (0.403 uM) and dissolved combined amino acids (1.7 uM) fall well within those quoted in the literature.

TABLE 5.4: Amino acid levels from previously published work. Abbreviations are as follows: $Fe(OH)_3$ = precipitation of organics with ferric hydroxide, P.C.=paper chromotography, L.E.=Ligand exchange chromatography, I.E. Ion exchange chromatography TLC= Thin layer chromatography. GC=Esterification of a.a. and separation and identification on a gas chromatograph with FID. OPA/MCE/RPLC=The reversed phase liquid chromatographic method based on the work of Lindroth and Mopper 1979.

Author	Pre-concentration method.	Detection	Levels. uM
Tatsumoto et al 1961	$Fe(OH)_3$	P.C.	DAA 0.65-0.78
Park et al 1963	$Fe(OH)_3$	I.E.	DAA 0.33
Degens et al 1964	I.E.	P.C.	DFAA 0.16-1.25 DCAA 0.06-0.20
Chau & Riley 1966	$Fe(OH)_3$	TLC	DAA 0.02-0.16
Siegel & Degens 1966	L.E.	I.E.	DFAA 0.38-0.77 DCAA 1.85-2.90
Webb & Wood 1967	L.E.	I.E.	DAA 0.21-0.77
Hobbie et al.1968	L.E.	I.E.	DAA 0.38
Riley & Segar 1970	I.E.	TLC	DFAA 0.045-0.31 DCAA 0.02-1.19
Bohling 1970	I.E.	I.E.	DFAA 0.8
Andrew & Williams 1971	L.E.	I.E.	DFAA 0.2-0.8
Kawahara & Maita 1971	I.E.	GC	DFAA 0.33-0.92
North 1975	None	Fluorescence	DAA 0.48-1.31

TABLE 5.4 Continued.

Reference	pre-concentration	Detection	Levels (uM).
Daumas 1976	L.E.	I.E.	DAA 0.39-2.826 x 0.9-1.2
Josefsson et al. 1977	None	OPA/MCE No separation	DAA 0.09-1.74
Lee & Bada 1977	L.E.	I.E.	DFAA 0.04-0.12 DCAA 0.12-0.48
Dawson & Pritchard 1978	L.E.	I.E. with OPA/MCE post column fluorescence	DFAA 0.045-0.84 x 0.25 DCAA 4.38-8.05
Garrasi et al 1979	None	I.E. with OPA/MCE post column fluorescence	DFAA 0.187-0.497 DCAA 0.327-2.289
Bolter & Dawson 1982	None	OPA/MCE/RPLC	DFAA 1.067 DCAA 4.534
Ittekkot 1982	None	I.E. with OPA/MCE post column fluorescence	DFAA 0.1-0.85
Mopper & Lindroth 1982	None	OPA/MCE/RPLC	DFAA 0.03-0.4
Laane 1983	None	Fluorescence (fluorescamine)	DFAA 0.066-1.53
Poulet et al 1984	None	OPA/MCE/RPLC	DFAA 0.169-0.347
Jorgenson & Sondergaard 1984	None	OPA/MCE/RPLC	DFAA 0.155-0.72
Braven et al 1984	None	OPA/MCE/RPLC	DFAA 1.4-3.7

Table 5.4 Continued.

Reference	pre-concentration	Detection	Levels (uM).
Henrichs 1985	None	OPA/MCE/RPLC	DFAA 0.011-0.136
Eberlein et al 1985	None	OPA/MCE/RPLC	DFAA 0.1-1.2
Liebezeit 1985	None	OPA/MCE/RPLC	DFAA 0.846-2.014 x 1.3 (n=5) DFAA 0.822-1.067 x 0.927 (n=3)
Hammer & Kattner 1986	None	OPA/MCE/RPLC	DFAA BLD-0.3
Furham & Ferguson 1986	None	OPA/MCE/RPLC	DFAA BLD-$5x10^{-4}$
Williams & Poulet 1986	None	OPA/MCE/RPLC	DFAA 0.024-1.689

The sampling period covered November 1985 to May 1987. During the bloom period for phytoplankton of 1986 (March to May) not many samples were collected because the hydrolysis method development was at a critical stage. Furthermore, 1987 was an unusual year in that there was a small phytoplankton bloom in March, but no large blooms in April or May. Therefore, this study does not include many analyses of samples from bloom conditions.

The monthly averages for total dissolved free amino acids (Fig. 5.57) show a gradually increasing level from May 1986 to September 1986 and a very high level in March 1987. Low levels of free acids were observed from October 1986 to January 1987, i.e. dissolved free amino acid levels were low in the winter and high in the summer. This pattern of dissolved free amino acid levels is in contrast to other nutrients, which are low in the summer and high during the winter, e.g. nitrate, ammonia and urea. (Flynn & Butler 1986). The raised free amino acid levels in the summer/autumn, with lower levels during the winter months, shows a similar trend to that of dissolved organic nitrogen Fig. 5.58. The dissolved free amino acid spectra for the months with high free amino acid levels, differ from those with low free amino acid levels. (Fig.5.59). This difference in both levels and spectra for the free acids may indicate that there are different processess occuring during the winter and summer/autumn months. This idea is expanded in section 5.7.3.

The ratio of the combined to the free acids for the monthly figures can be seen in Fig.5.60. Again, two

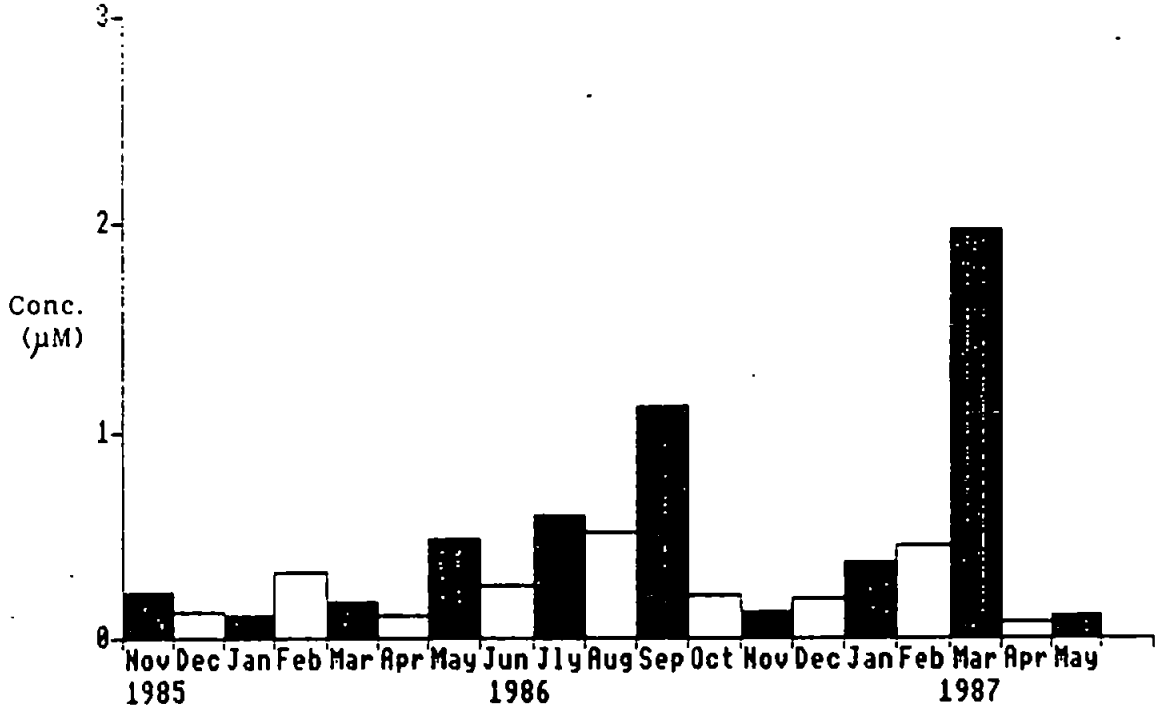


Fig 5.57a Average Monthly Figures for the Total DFAA.

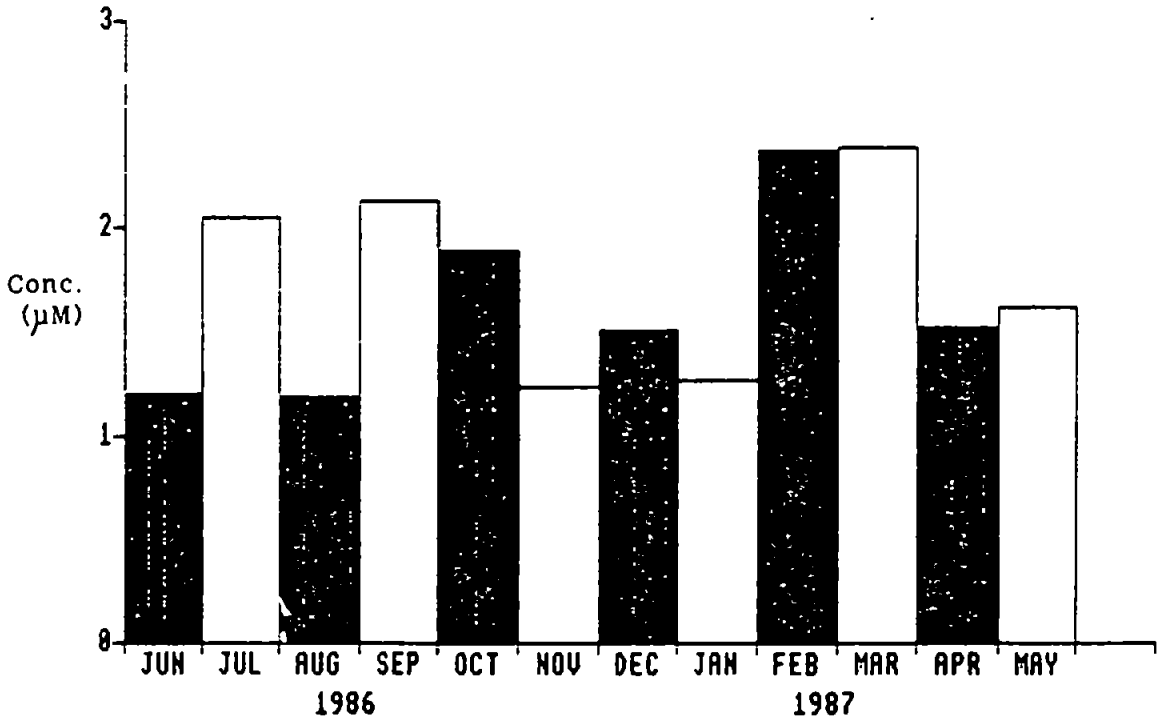


Fig 5.57b Average Monthly Figures for the Total DCAA.

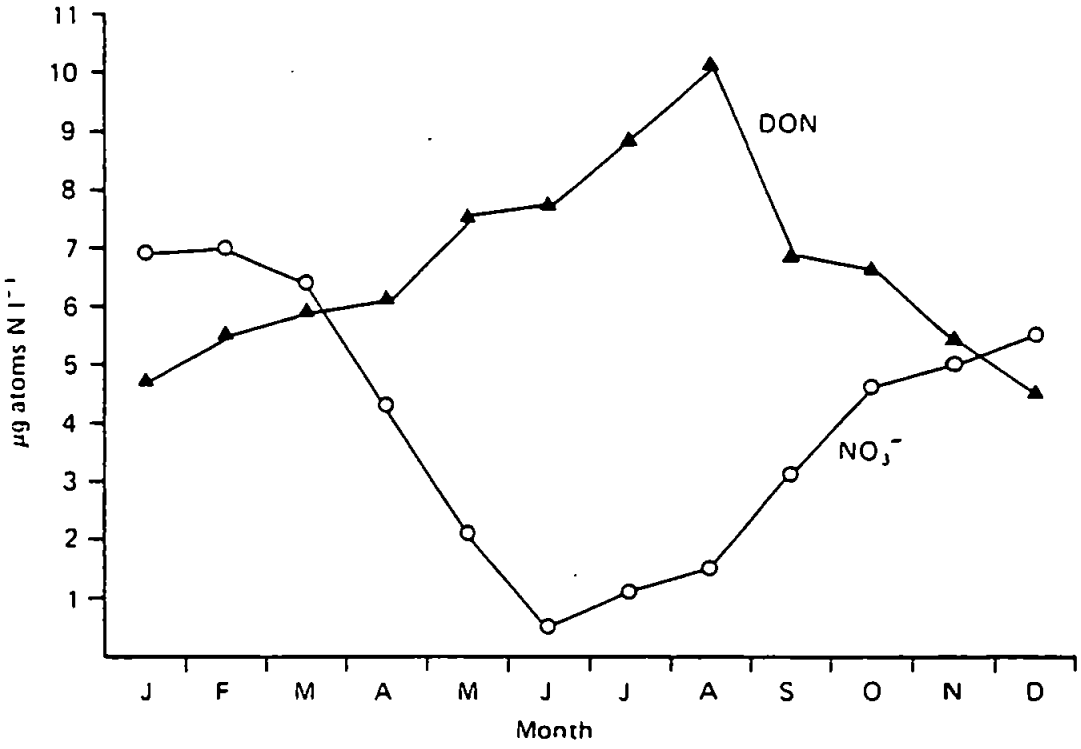


Fig 5.58 Levels of Nitrate and DON at Station E1, (taken from Butler et al. 1979).

DISSOLVED FREE AMINO ACIDS
MARCH 1987 SAMPLE

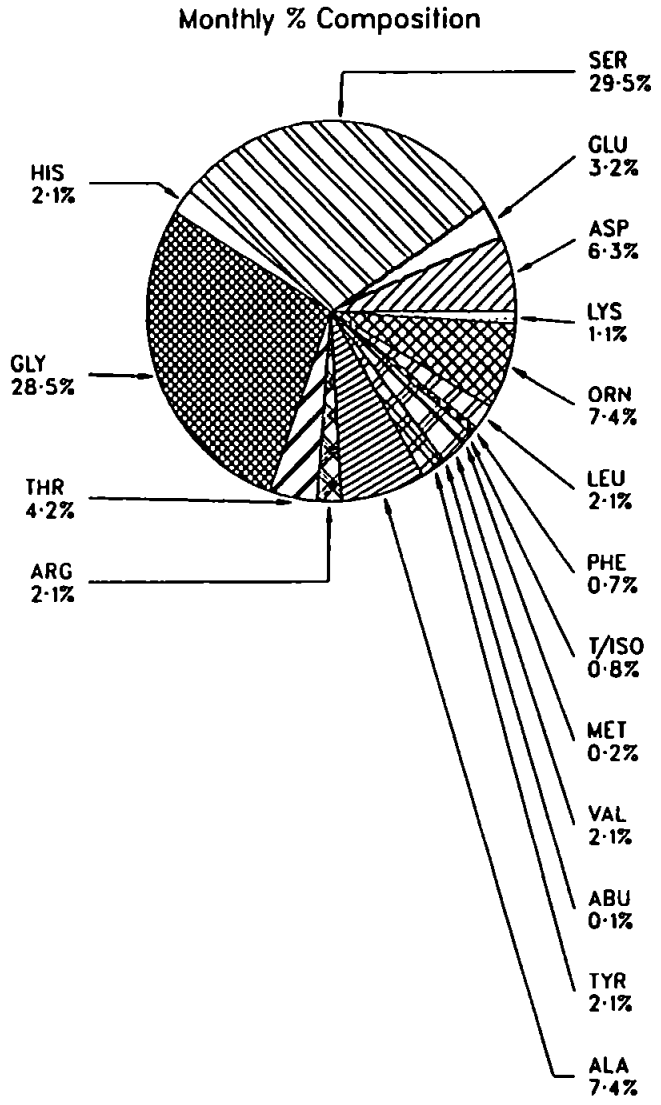


Fig 5.59a The DFAA Composition for Mar 1987.

DISSOLVED FREE AMINO ACIDS
APRIL 1987 SAMPLE

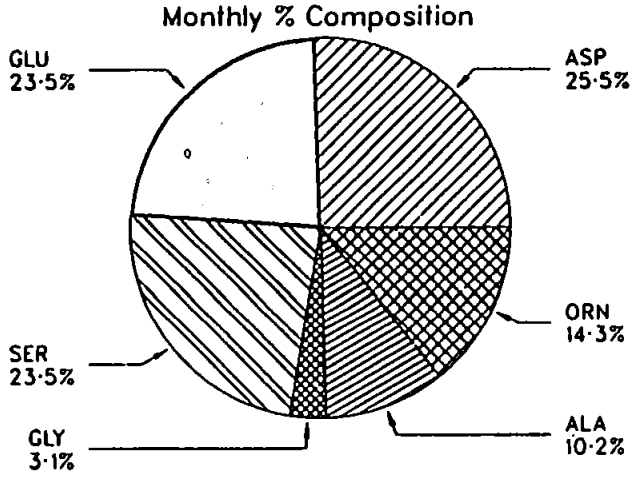


Fig 5.59b The DFAA Composition for Apr 1987.

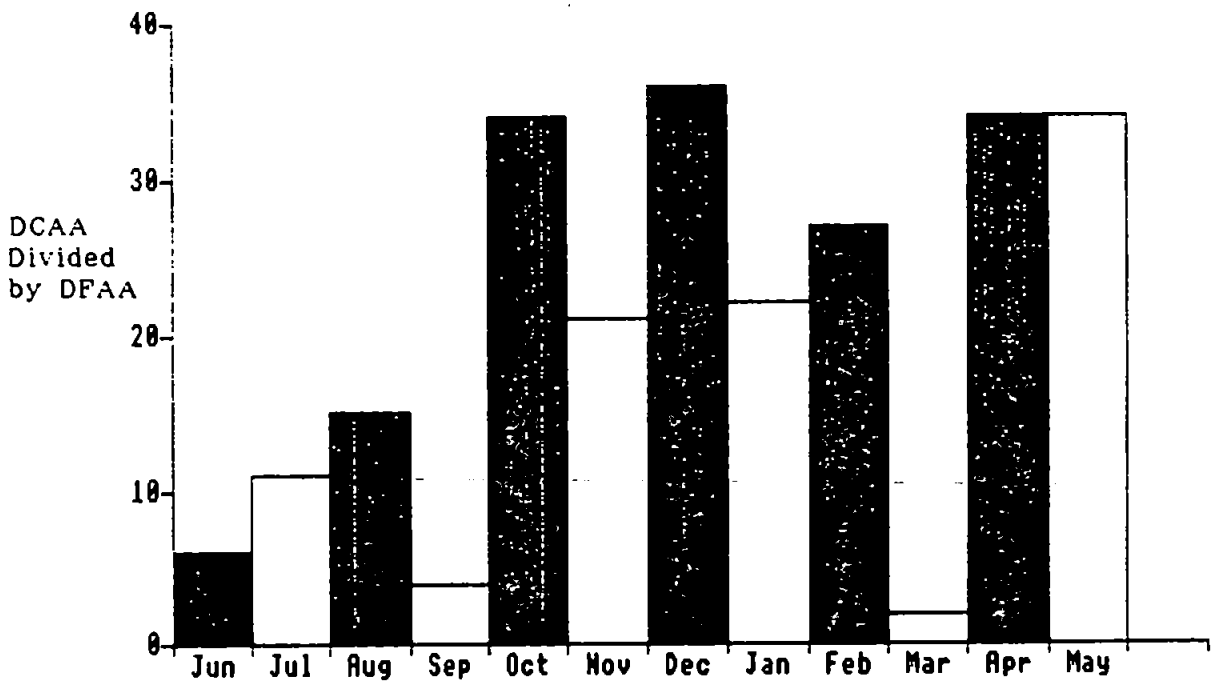


Fig 5.60 The Relationship Between DCAA and DFAA.
June 1986 - May 1987.

different patterns are observed. In October 1986 to Febuary 1987 and April 1987 to May 1987, the ratio of the combined to the free acids is high (approx.30:1-see Table 5.2), while in June 1986 to September 1986 and March 1987 the ratio is low (approx. 10:1). The possible reasons for these differences are that the dissolved combined amino acids are not broken down to free acids in October to February, April to May; or conversely the breakdown occurs but with greater uptake during the winter months. This disappearence of the dissolved free amino acids during the winter months may be due to bacterial uptake. However, Keller et al. (1982) report reduced levels of extracellular release of dissolved organic carbon during the winter period, while Flynn & Butler (1986) suggest that the reduction in light experienced during the winter months, may induce the development of microalgal amino acid uptake systems. The low ratio of combined to free acids,(June to September 1986 and March 1987), may be due to lack of uptake of the free acids, or, to the production of free acids at a rate greater than their removal. The production of acids may be due to excretion/decay of phytoplankton or the sloppy feeding of zooplankton.

Examination of the data, for each batch of analyses, shows large fluctuations in the dissolved free amino acid concentrations. (Fig. 5.61). To determine if these fluctuations were due to random variation of the data, or were statistically significant peaks, the means and standard deviations of the maxima and minima of each "peak" was tested using the student-t test.(Caswell

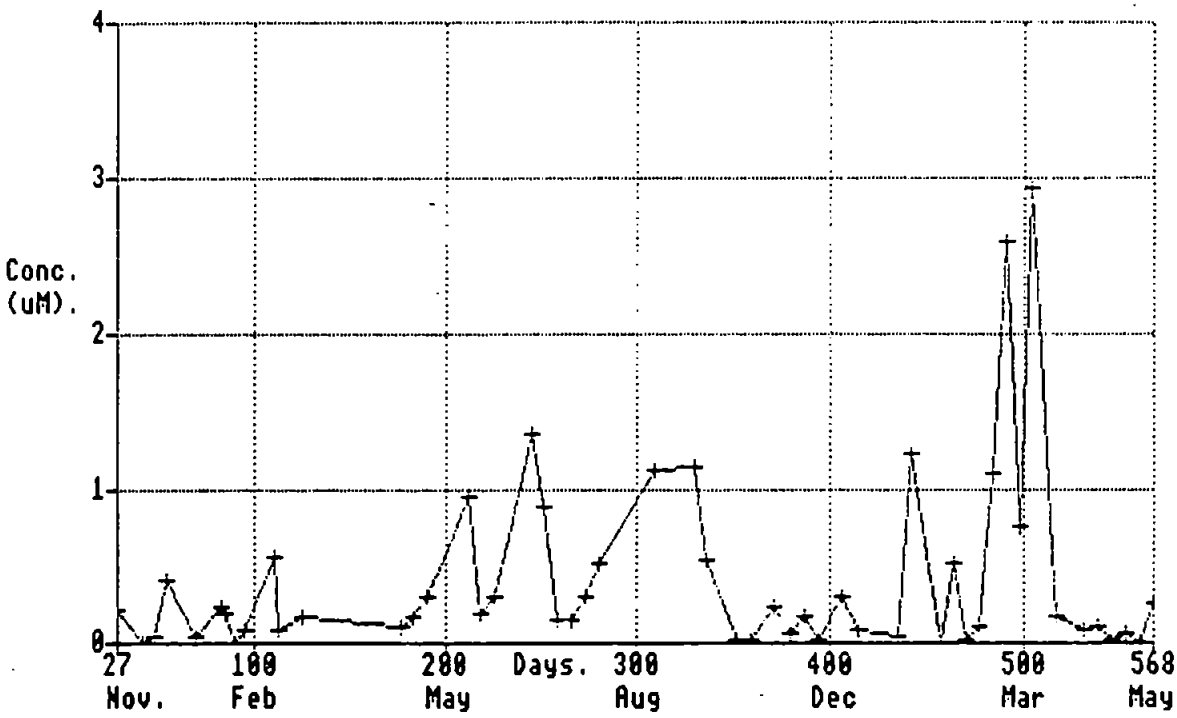


Fig 5.61 Average Total DFAA Values Over Sampling Period: Nov 1985 - May 1987.

1982). It was found that the large fluctuations in dissolved free amino acid levels were statistically significant (5% level).

Regular cyclic oscillations have been depicted in theoretical studies of population dynamics by Lotka and Volterra (Solomon 1969). Using the method of moving averages (Caswell 1982), an attempt was made to fit the fluctuations of dissolved free amino acid levels to a specific time cycle. No regular cycle could be determined, possibly due to the small and incomplete nature of the data set.

The increased concentrations of the dissolved free amino acids may be explained by extracellular release, change in the usage or breakdown of the dissolved acids, terrestrial run off and grazing by zooplankton (sloppy feeding). Phytoplankton have a greater arginine content than the zooplankton that graze them. The excess arginine is removed by the formation and excretion of urea and ornithine (Flynn & Butler 1986). Therefore the presence of ornithine has been used as an indicator of zooplankton activity. Comparison of the total dissolved free amino acid levels over the sampling period, with those of ornithine over the same period, indicates that in general, where the total dissolved free amino acid levels are high ornithine is present. Ornithine does not occur in the free amino acid spectrum when the total free acid levels are low. Therefore the ornithine levels may indicate that grazing is linked with high dissolved free amino acid levels.

Similar peaks in dissolved free amino acid levels

over a season were found by Keller et al. (1982) who stated that "seasonal peaks in dissolved free amino acid levels were not wholly dependent on phytoplankton release although a clear relationship was evident". They suggest that other sources for the increase in dissolved free amino acids may be zooplankton and macroalgae. While Flynn & Butler (1986) suggest an input of dissolved free amino acids from invertebrates, as part of their excretion and osmo-regulation.

5.7.3. Correlation Of Dissolved Free And Dissolved Combined Amino Acids Levels With Environmental Factors.

The monthly figures for dissolved combined and dissolved free amino acids from June 1986 to May 1987 were correlated against monthly figures for primary productivity (Boalch 1978), dissolved organic nitrogen (Butler et al 1979), phytoplankton numbers (Maddock et al. 1981), hours of sunshine, air temperature, rainfall and wind speed using a statistical program for microcomputers called Microtab. The data for primary productivity, dissolved organic nitrogen and phytoplankton numbers are from past papers and therefore are not directly related to data collected in this study. Meteorological data were taken from the "Metenq" program on Plymouth Polytechnic prime computer system. The meteorological data are for the same month and year as the dissolved combined and dissolved free amino acid data.

The results of the correlation of data for June 1986 to May 1987 can be seen in Table 5.5.

Table 5.5 Correlation on a monthly basis (June 1986 to May 1987) of dissolved combined and dissolved free amino acid levels with environmental factors.

	DFAA r	DCAA r	Statistically significant at the stated percentage level.
DFAA		0.633	(2%)
DCAA	0.633		
DON	0.036	-0.117	
PROD	-0.151	0.021	
SUN	-0.009	-0.147	
TEMP	-0.109	0.336	
RAIN	-0.176	-0.737	(0.1%)
WIND	-0.233	0.266	
PHYTO	-0.121	0.003	

Degree of freedom = 10 To be statisically significant $r > 0.5670$ (5%), $r > 0.4973$ (10%).

Using the full twelve months data, there are only two parameters that correlate with the environmental data, i.e. a positive correlation of the dissolved combined with dissolved free acids, and a negative correlation of dissolved combined acids with rainfall. The negative correlation of dissolved combined amino acid levels with rain is unexpected, since the work of Mopper & Zika (1987) shows that marine rains tend to be high in both dissolved free and dissolved combined acids. One possible reason for this negative correlation is that rain washes particulate matter, such as soils and debris, from the rivers into the coastal waters. The dissolved combined amino acids present in the water may possibly become complexed with this material and are lost from the water column when the particulates settle out. It has been found in model studies by Evens et al. (1988), that whereas as free dissolved amino acids are not

co-precipitated, or removed by ferric hydroxide or rich mud particles in seawater, dissolved proteins are completely removed by both these systems.

Dissolved organic nitrogen levels (Fig 5.62) and primary productivity (Fig 5.63) both show a pattern of rising levels in the summer/autumn, followed by a fall during the winter months. The dissolved free amino acid data also show this pattern and therefore the correlation was repeated using dissolved free amino acid data from June 1986 to Febuary 1987, omitting spring data. The results of this second correlation exercise can be seen in Table 5.6. There are positive correlations between dissolved free amino acid levels, with both primary productivity and phytoplankton numbers in the summer, autumn and winter months. During the spring this relationship appears to break down. There is negative correlation between free amino acids and wind. It is known that phytoplankton blooms occur in calm weather conditions and so the negative correlation of wind speed and phytoplankton numbers would seem to be a realistic result.

over much of the area. The salinity range is from 34.2 to 35.4 parts per thousand. (Braven et al 1984).

5.2. Data Presentation.

Due to the large number of samples analysed and the fact that each analysis reports information on up to 19 amino acids, it was felt necessary to report the individual chemical analysis figures in a series of appendices which are found at the end of this thesis. Appendix I contains the total dissolved free and combined amino acid levels per sample and shows how these relate to total dissolved nitrogen and dissolved organic nitrogen. It should be noted that the figures for total dissolved nitrogen and dissolved organic nitrogen values were not determined experimentally on the sea water samples analysed for dissolved free and dissolved combined acids, but are average figures taken from Butler et al.(1979). Appendix II presents data for the levels of the individual amino acids in each sample.

The following five categories of results have been derived from the data in appendices I and II:

1. Seasonal levels for total dissolved combined and total dissolved free amino acids.

2. Individual amino acid levels in the free and combined fractions.

3. The ratio of dissolved free to dissolved combined

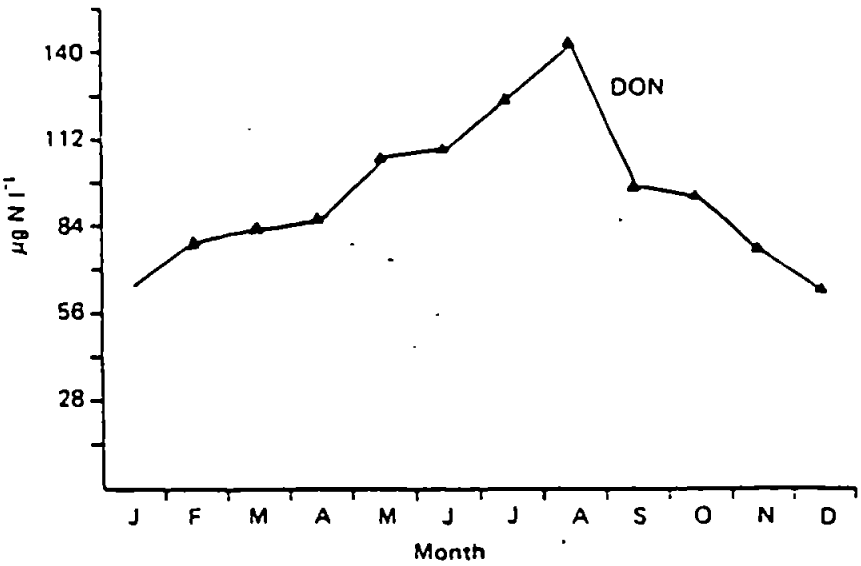


Fig 5.62 Dissolved Organic Nitrogen Levels Over A Season. (from Butler et al. 1979).

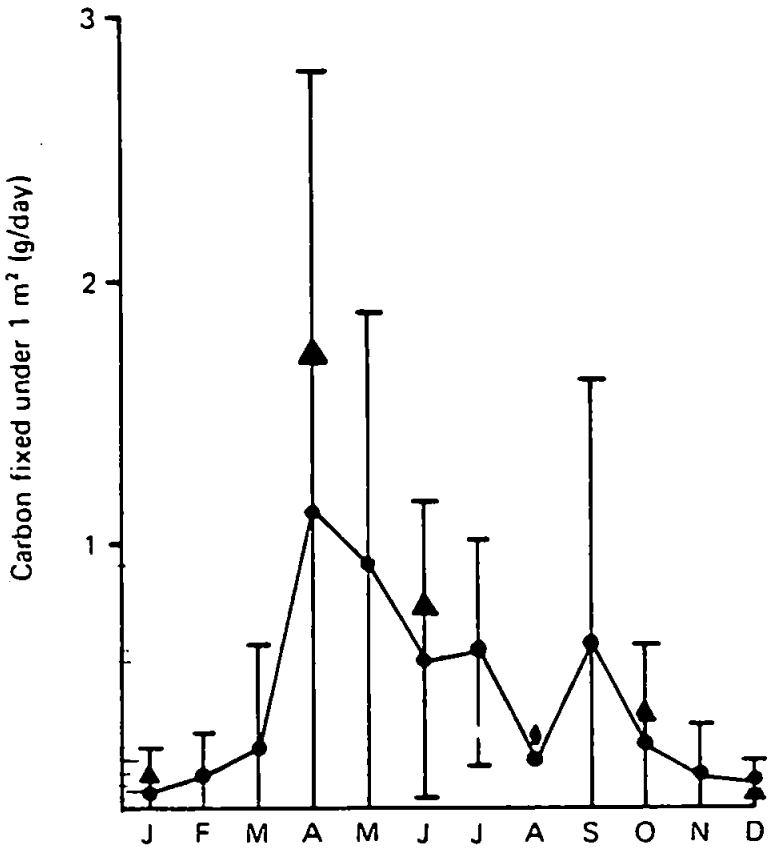


Fig 5.63 Mean Carbon Fixation Rates For Each Month. Expressed As Grams Of Carbon Fixed Per Day.
● Monthly Means For The Period, 1964-74, Excluding 1966. With 95% Confidence Limits. ▲ 1966 Values. (from Boalch et al. 1978)

Table. 5.6. Correlation on a monthly basis (June 1986 to Febuary 1987) with environmental factors.

	DFAA r	Statistically significant at the stated percentage level.
DFAA		
DCAA	0.046	
DON	0.322	
PROD	0.603	(10%)
SUN	0.433	
TEMP	0.163	
RAIN	-0.582	(10%)
WIND	-0.806	(1 %)
PHYTO	0.852	(0.1%)

Degrees of freedom 7.To be statistically significant r>0.6640 (5%), r>0.5822 (10%).

The different correlations obtained from the two data sets (9 months vs 12 months) may indicate that different processes are occuring during a season. For example, during the summer and autumn the dissolved free amino acid levels may be related to the presence of phytoplankton, while in the spring, amino acid levels may relate to specific environmental or biological factors not tested in this study. Removal of the 3 months data for spring, drastically reduces the correlation between dissolved combined and dissolved free acids i.e. 0.633 to 0.046. This indicates that only in spring is there a strong relationship between the two amino acid fractions which may be due to the intense activity of the biota.

One of the central ideas of Lovelocks Gaia hypothesis (Lovelock 1979) is that systems containing life are characterised by being maintained in a state of disequilibrium or "confusion", when compared to inorganic

(lifeless) systems. The springtime is a very dynamic biologically active phase of the year and it is therefore not surprising that the inclusion of the spring data, in the correlation exercise above, leads to a breakdown in the relationships of the tested parameters i.e. increased confusion.

There is a problem in undertaking correlations of the dissolved combined and dissolved free amino acid data collected in this study, with data from past papers. For example, the primary productivity figures quoted in the paper by Boalch et al. (1978) are high in March to May, but, as mentioned earlier, the March to May period for 1987 showed no major plankton bloom, implying an unusual spring season (personal communication E.I.Butler). Therefore, the correlation discussed above can only be very tentative and further work using data from the same season will be necessary to elucidate the relationship of dissolved combined and dissolved free amino acids to environmental and biological factors.

The dissolved combined amino acid fraction only shows a significant positive correlation with dissolved free amino acids and a negative correlation with rainfall for 1986 to 1987. Using the nine months data as opposed to the twelve months does not dramatically improve the correlation figures as was the case for the free acids. This may be due to the fact that dissolved combined amino acids are complex molecules and the process of production and breakdown may make these compounds less related to direct physical influence.

5.7.4 The Comparison Of The Amino Acid Composition Of Phytoplankton With That Found For The Free And Combined Acids.

The amino acid composition of different species of phytoplankton determined by Minghou et al. (1986) can be seen in table 5.7. The phytoplankton have been hydrolysed and therefore the amino acids reported are both free and combined. It should be noted that the studies of phytoplankton composition have employed cell cultures. The particular feeding regime of the culture may affect the amino acid composition of the phytoplankton. Therefore the amino acid composition of laboratory cultures may not resemble those of natural populations. Comparison of total amino acid composition of phytoplankton, with the composition of the dissolved free and dissolved combined amino acids, indicates that the dissolved amino acids found in sea water do not resemble those of phytoplankton. If however, the amino acid composition of phytoplankton in both cultures and the natural environment are similar this suggests that processes, other than, simple enzymatic hydrolysis of particulate and dissolved combined amino acids, with the subsequent release of free amino acids are occurring in the environment. The reason for these compositional differences may be preferential uptake of amino acids following proteolytic bacterial activity. (Amano et al 1982, Hollibough & Azam 1983). The findings of this study, are supported by Maurer (1976) who states that the

Amino Acid	*Platymonas subsordiformis* (Wille) Hazen	*Chaetoceros minutissimus* Maker et Pr-Laver	*Phaeodactylum tricornutum* Bohl	*Dunaliella* sp.	*Nitzschia* sp.
Asp	1.60	2.27	3.07	3.74	4.16
Thr	0.69	0.95	1.66	1.97	2.02
Ser	0.68	1.29	1.73	1.79	2.02
Glu	1.90	3.60	3.18	4.28	4.78
Pro	0.85	0.99	1.36	2.00	1.75
Gly	1.01	1.49	1.86	2.10	2.29
Ala	1.20	1.49	1.98	2.55	2.60
Cys	0.09	0.15	0.14	0.13	0.20
Val	0.99	0.09	1.84	1.84	2.11
Met	0.23	0.39	0.46	0.41	0.57
Ile	0.62	1.21	1.51	1.43	1.94
Leu	1.26	1.76	2.31	2.86	2.70
Tyr	0.85	1.30	1.65	1.67	2.00
Phe	0.86	1.37	2.00	2.09	2.28
Lys	0.89	1.49	1.39	1.82	1.89
His	0.31	0.48	0.53	0.60	0.62
Arg	1.05	1.47	1.63	2.35	2.09
Total amino acids	15.07	21.79	28.30	33.62	36.01
Total amino acids-N (A)	2.08	2.98	3.82	4.49	4.82
Total amino-N (B)	1.80	2.66	3.46	4.05	4.33
Total N content (C)	3.37	4.56	6.38	7.10	7.61
Crude protein (N × 6.25)	21.06	28.50	39.87	44.38	47.56
A.C(%)	61.7	65.4	59.9	63.2	58.4
B.C(%)	53.5	58.3	54.3	57.0	57.0

Table 5.7 Total Content of Amino Acids in Phytoplankton (% on dry wt.), from Minghou et al. 1986.

higher molecular weight organic compounds do not resemble their assumed source i.e. excretory products, soluble cell debris etc. Degens et al. (1964), Siegel & Degens (1966), Garrasi et al. (1979), Bolter & Dawson (1982) all comment on the difference between both the compositions and the levels of the dissolved combined and the dissolved free amino acid fraction.

Flynn & Butler (1986) stress that the concentration of a particular compound in seawater need not be indicative of its use. A nitrogen source with low levels in the water column supports two contrasting arguments: either there is too little of the compound to support growth, or it it is being rapidly used to support growth and therefore does not appear in the water column. It is the flux of a compound through the ecosystem which is of importance. In the present study, it is possible to identify amino acids which are present in the combined form but not as dissolved free amino acids i.e. acids that have "disappeared". Table 5.8 gives details of the percentage loss of individual acids between the combined form (taken as 100%) to the free form, over the season. The calculations used to construct this table can be seen in appendix III.

Table 5.8 percentage disappearence of individual acids between the dissolved combined and dissolved free amino acids. (NB. a value of 100 percent means removed to below the limits of detection for that acid).

amino acid	Range of disappearence	Average disappearence	number of batches.
Asp	33-100	78	36
Glu	44-100	85	36
Ser	0-100	51	36
His	32-100	97	30
Gly	54-100	90	36
Thr	40-100	91	29
Arg	26-100	90	23
Ala	28-100	74	36
Tyr	0-100	67	32
Abu			
Val	1-100	89	36
Met			
Trp/Ile	54-100	96	21
Phe	71-100	97	16
Leu	25-100	87	28
Orn	0-100	63	19
Lys	0-100	87	18

Amino acids with "disappearence" values of ninety percent or greater are histidine, glycine, threonine, arginine, tryptophan/isoleucine and phenylalanine. These disappearences could be due to uptake by algae, invertebrates or bacteria. Therefore the values obtained in Table 5.8 were compared to those of Lu & Stephens (1984) on algal preference (see Table 5.9).

Table 5.9 Percentage removal of free amino acids by phytoplankton from Lu & Stephens (1984).

amino acid	percentage removal
Asp	73
Glu	55
Ser	84
Arg	100
Ala	95
Tyr	85
Val	86
Ile	88
Leu	88
Orn	100
Lys	100

The values in Tables 5.8 and 5.9 show some similarities, with low figures for the "disappearence" of the acids aspartate and glutamate, and high values for arginine. No literature on bacterial or invertebrate preference for free amino acids could be found. Preliminary work on the uptake of dissolved free amino acids by phytoplankton can be found in chapter 8.

5.7.5 The Possible Role Of Serine And Glycine In Plant Nutrition.

In the marine environment flux is a two way process of uptake and production. Examination of the graphs for serine in the dissolved combined and dissolved free amino acid fractions (Fig.5.64) coupled wih the low value for its disappearence (51%) suggests that serine may be produced as the free acid. This together with the high levels of glycine in the combined fraction (Fig. 5.65)

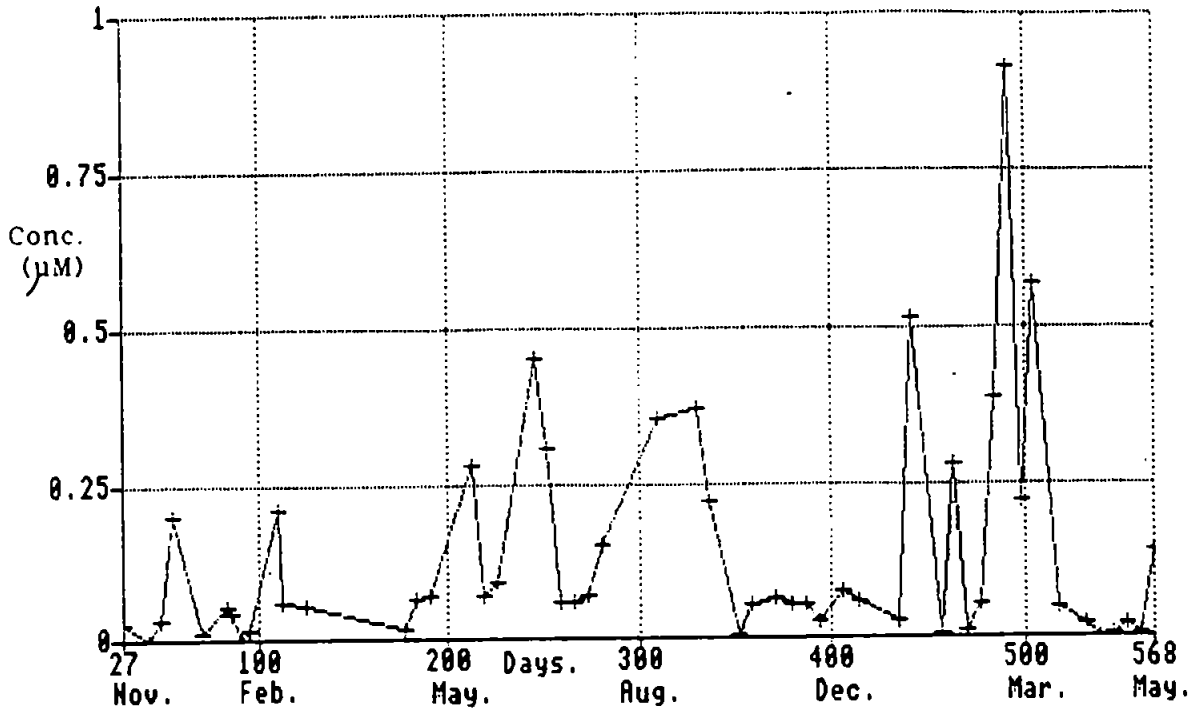


Fig 5.64a Average Concentration of Ser in DFAA During Sampling Period: Nov 1985 - May 1987.

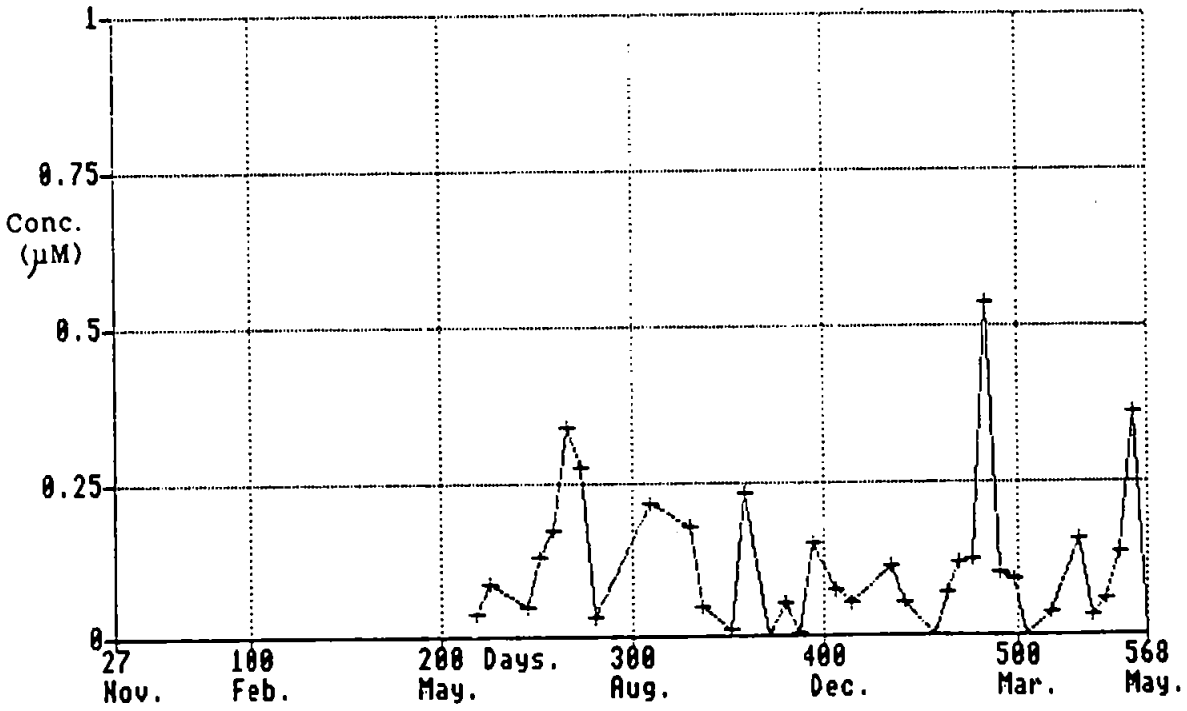


Fig 5.64b Average Concentration of Ser in DCAA During Sampling Period: May 1986 - May 1987.

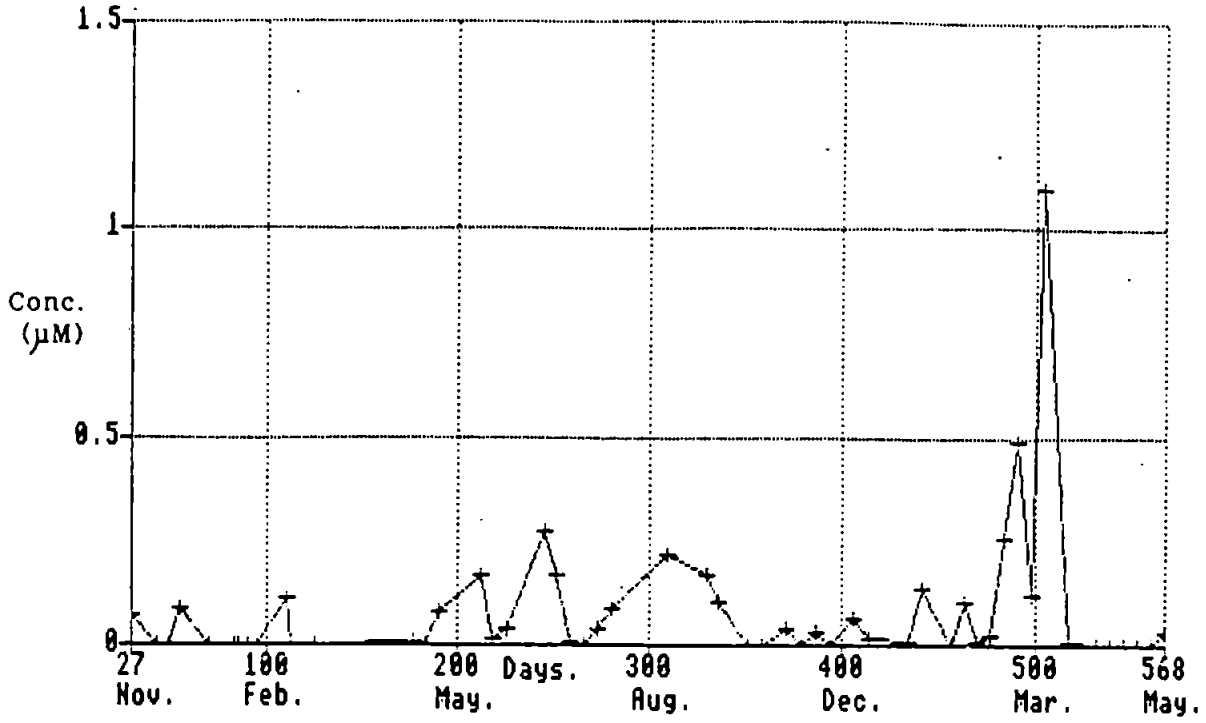


Fig 5.65a Average Concentration of Gly in DFAA During Sampling Period: Nov 1985 - May 1987.

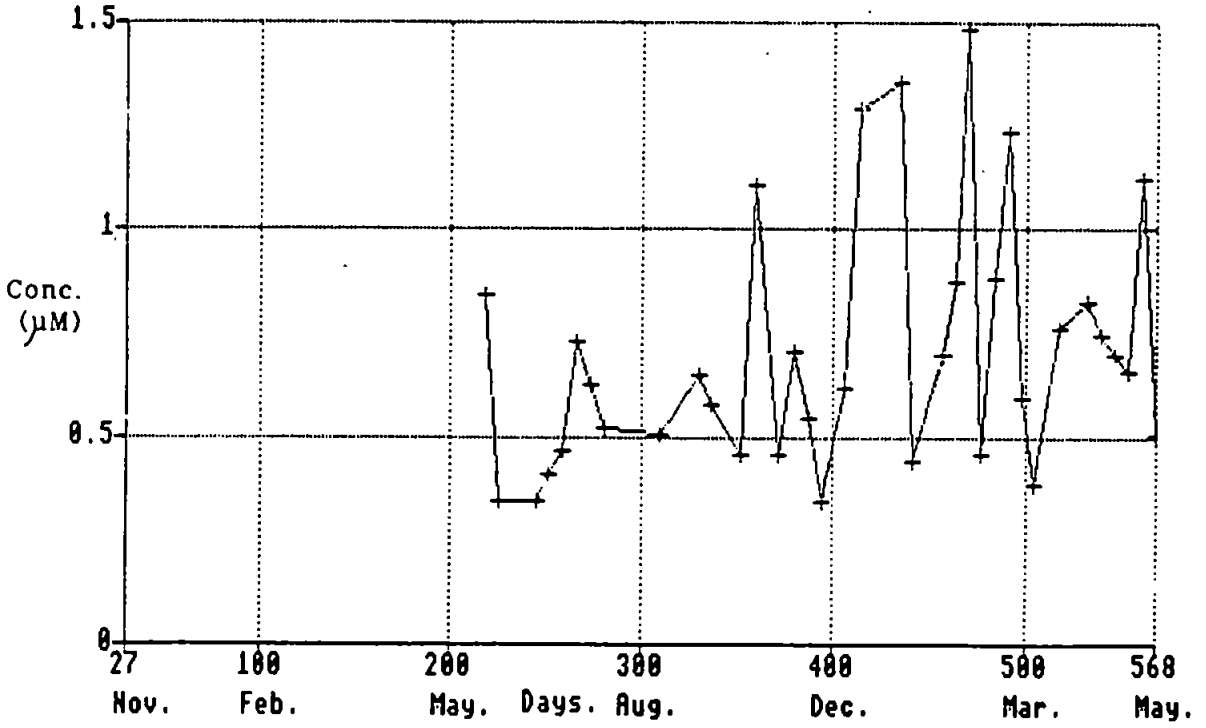


Fig 5.65b Average Concentration of Gly in DFAA During Sampling Period: May 1986 - May 1987.

and the high value for its disappearance (90%) suggests that the following biochemical pathway known to occur in plants (Lea et al. 1985) may produce ammonia for use as a nitrogen source.

2 x glycine ——————> serine + ammonia + carbon dioxide.

This would be an interesting area, using cultures, for further research.

5.7.6. Species Succession.

It has been shown that (section 5.3) the dissolved free amino acids can be divided into three groups, depending upon their persistence in the spectrum. Those present over most of the year were aspartate, glutamate, glycine and tyrosine. An examination of the graphs of monthly percentage composition show that even these major acids may disappear for short periods of time, e.g. glutamate from the dissolved free fraction for the month of December 1985 (Fig. 5.66), glycine from the dissolved free for January and March 1986 (Fig. 5.67), and tyrosine from the free fraction for November 1985, January 1986 April 1986 and 1987 and May 1987 (Fig. 5.68). Any phytoplankton species that could utilize these sources of energy and/or nitrogen would have a competitive advantage, and the disappearance of these major acids may be linked to species succession. Again, this is an area for further work.

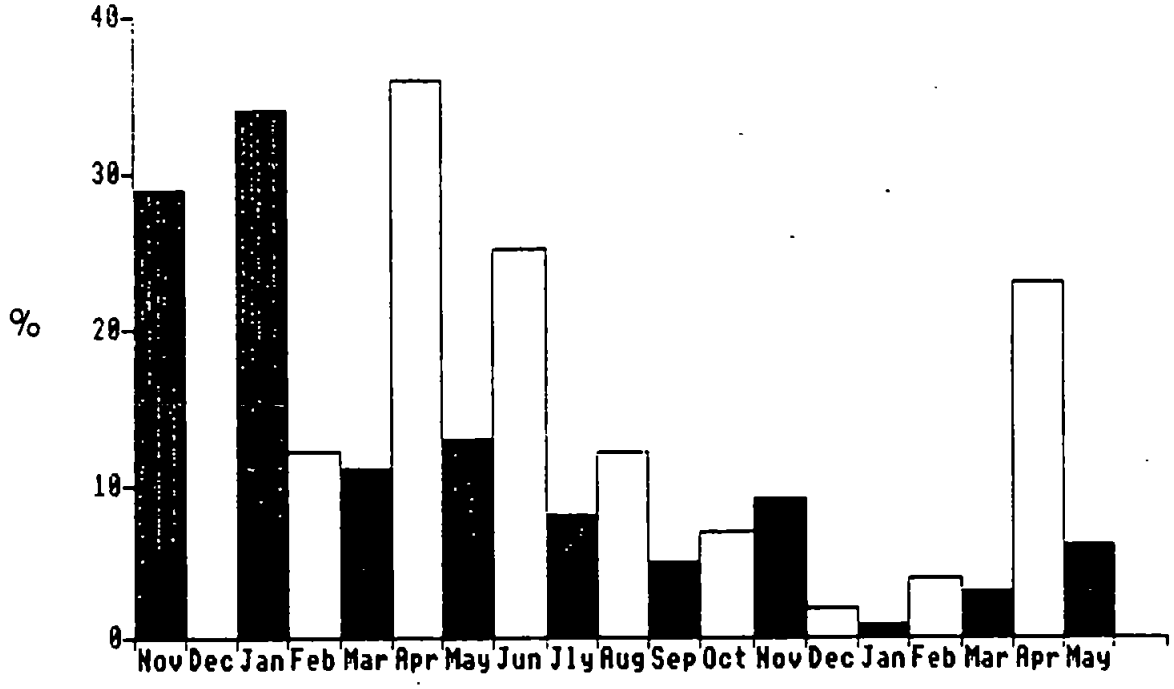


Fig 5.66 Monthly Percentage Composition of Glu in the DFAA, Nov 1985 - May 1987.

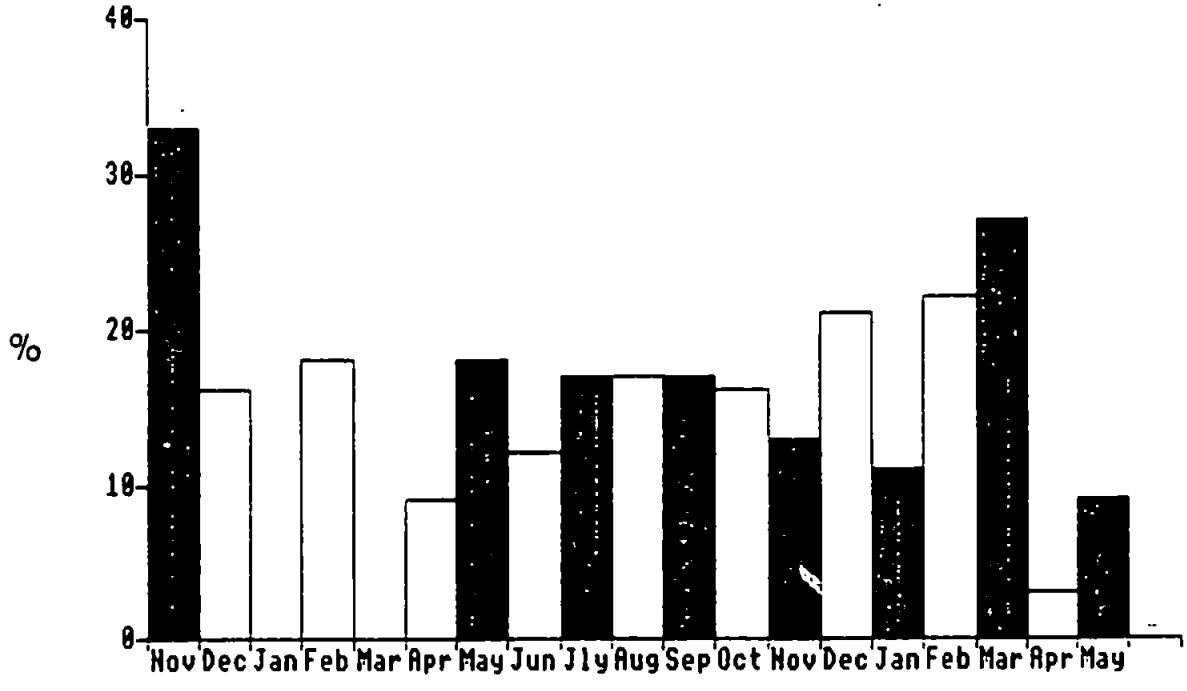


Fig 5.67 Monthly Percentage Composition of Gly in the DFAA, Nov 1985 - May 1987.

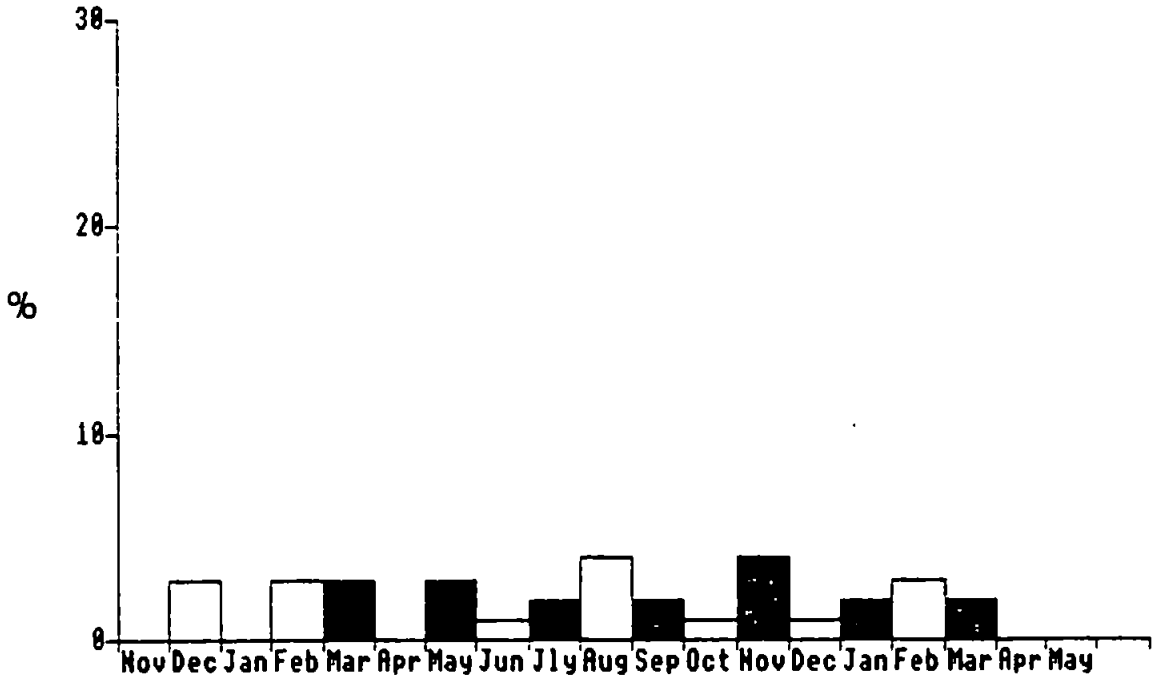


Fig 5.68 Monthly Percentage Composition of Tyr in the DFAA, Nov 1985 - May 1987.

5.7.7. Size Of The Dissolved Combined Amino Acid Containing Macromolecule.

The results of the sizing experiments carried out during the course of this study indicate that the macromolecular species are of a small size, 72% having a molecular weight less than 25,000 (~250 amino acids) and 63% having a molecular weight less than 10,000 (~100 amino acids in length). Table 5.10 gives the results obtained by previous workers who have sized the dissolved organic matter, rather than simply the dissolved combined fraction. Table 5.10 indicates that the majority of the dissolved organic matter, of which the dissolved combined amino acids are a part, is of a small size.

Table 5.10. The results, obtained by previous workers, for the sizing of the dissolved organic material (DOM).

Author	Size of DOM.
Wiebe & Smith (1977).	95% < 3,500 d
Lancelot. (1984).	14 to 48% < 500 d 25 to 40% 500 to 5,000 d 26 to 57% > 5,000 d
Carlson et al. (1985)	59% < 10,000 mw 34% > 10,000 mw 6% > 30,000 mw 1% > 700,000 mw
Suzuki & Sugimara (1985).	45% 1,500 to 30,000 mw.

The total amino acid composition of phytoplankton shows high levels of the basic acids arginine and lysine (Minghou et al 1986). Both the dissolved combined and

dissolved free amino acid fractions are low in those acids. Also, the macromolecular species, sized as part of this study, has been found to be small. These observations may be due to the fact that the large macromolecules derived from the phytoplankton undergo a "snipping" process, e.g. by an arginase enzyme, (Crawford et al 1974) to preferentially remove that amino acid resulting in smaller molecules, containing fewer basic amino acids.

In general the glycine content of the sized fraction was observed to be less than the glycine content of the original unsized DCAA fraction. This may indicate the presence of a larger macromolecular species, consisting of predominantly glycine. The reasons for this are not understood. However, Liebezeit et al. (1985) states that high glycine levels indicate a material of a refractory nature.

CHAPTER 6.

LEVELS AND COMPOSITION OF THE DISSOLVED FREE AND DISSOLVED COMBINED AMINO ACID FOUND IN DEPTH PROFILE SAMPLES.

6.1 Introduction.

The oceans may be divided into vertical zones depending upon such factors as light, temperature, density and nutrient concentrations.

The three vertical zones, relating to the availability of sunlight as an energy source for primary productivity, are the photic (euphotic) zone, in which there is sufficient light for photosynthesis, the disphotic zone, which is too dim for photosynthesis, and the aphotic zone, where there is no light. (Parsons et al. 1984, Monkhouse and Small 1978 and Darley 1982). The intensity and wavelength of light is dependent upon the sun's position, the season and the cloud cover. In the northern temperate regions, daily illumination reaches a maximum intensity in May-June, and a minimum intensity in December-January. (Boney 1975). The depths of the light zones change with latitude, season, amount of suspended material and the local terrain. At the hydrographic station E1, twenty two miles off Plymouth in the English Channel, the euphotic zone extends to a depth of approximately 24 to 36 m, with an overall depth from surface to sea bed of 70m.

The seasonal light intensity is closely associated

with temperature changes. During the periods of increased sunlight, the surface waters become warmer and less dense than the cooler deeper waters. Eventually, with continued warming and calm conditions, the two layers of water will no longer mix, giving rise to two bodies of water with different temperatures and densities. The area of sharp temperature change between the two bodies of water is known as a thermocline. During the summer months a thermocline develops at station E1 at a depth of approximately 20 m. Due to this lack of mixing, the two water masses can diverge in their nutrient content, especially if different processes are occurring in the water body e.g. in the warmer upper waters, the nitrate levels can be reduced to below the limits of detection, while cooler deeper waters do not lose nitrate. (O'Neill 1985).

A study of the dissolved free and dissolved combined amino acid levels and composition with depth, may show differing results for the photic zone, where both photosynthesis and respiration occur during daylight hours, and the disphotic and aphotic zones, where only respiration occurs. Any difference between the results of the light zones must be attributable to the activity of the phyto- and photosynthetic bacterioplankton. Furthermore, an investigation of the dissolved combined and dissolved free amino acid levels may lead to a clearer understanding of the possible role of free and combined amino acids as nutrients in water bodies with low nitrate concentrations.

The depth profile data presented in this chapter

considered as a "snapshot" of the concentration of the analyte in that aliquot of water at the moment of analysis.

Despite the problem of representative sampling, outlined above, it has been possible, using numerous results obtained over an approximately twelve month sampling period, to construct an overall picture of the levels and spectrum of free and combined amino acids in local coastal waters.

During this period the sampling frequency depended upon weather conditions and the availability of boats but for much of the year one batch of samples was collected per week. Each batch comprised between 5 to 10 samples collected on the same day from different localities in the sampling area.(section 5.1.2).

5.1.1. Period of Sampling.

Dissolved free amino acids were sampled from November 1985 to May 1987 with a total of 295 samples. Of these samples, 158 were also analysed for dissolved combined amino acids during the period June 1986 to May 1987. The number of samples analysed each month can be seen below in Fig.5.1 (a+b).

5.1.2. The Sampling Area.

A map of the sampling area is given in Fig. 5.2. (taken from Evens 1986). The average depth of the water is 50m. During the summer months a thermocline is present

falls into two groups. Firstly, those from station E1 taken at intervals during the sampling programme; (Fig. 6.1 to 6.6) and secondly analyses performed at various stations (Fig. 6.7) during the July 1987 cruise on the RRS Frederick Russell, (Fig. 6.8 to 6.14). Samples taken during the cruise were only analysed for free amino acids, due to the lack of access to a clean working area and the technical difficulty of sealing tubes in unstable ship conditions.

The results for the depth profile analyses are presented in the form of tables showing individual amino acids levels and total amino acid concentrations. Graphs of total levels versus depth are included for each set of analyses.

6.2. Results.

Date: 13.3.86 Dissolved free amino acids (DFAA).(uM).

Station E1. Depth (m)

a.a.	0	5	10	20	35	50	70
Asp	----------		0.124	0.023	-----	0.064	0.017
Glu	----------		0.033	----------		0.024	0.020
Ser	0.074	0.051	0.616	0.089	0.051	0.208	0.082
His	----------		----------	----------	----------	----------	----------
Gly	0.049	-----	0.259	-----	0.062	0.144	0.033
Thr	----------		----------	----------	----------	0.039	-----
Arg	----------		0.049	----------		----------	
Ala	0.014	0.022	0.258	0.041	0.022	0.089	0.044
Tyr	----------		0.057	----------		0.036	-----
Abu	----------		----------	----------	----------	----------	----------
Val	----------		0.096	----------		0.026	-----
Met	----------		----------	----------	----------	----------	----------
Trp/Ile	----------		0.045	----------		----------	
Phe	----------		----------	----------	----------	----------	----------
Leu	----------		0.046	----------		----------	
Orn	----------		----------	----------		0.229	-----
Lys	----------		----------	----------	----------	----------	----------
TOTAL(uM)	0.137	0.073	1.583	0.153	0.135	0.859	0.196

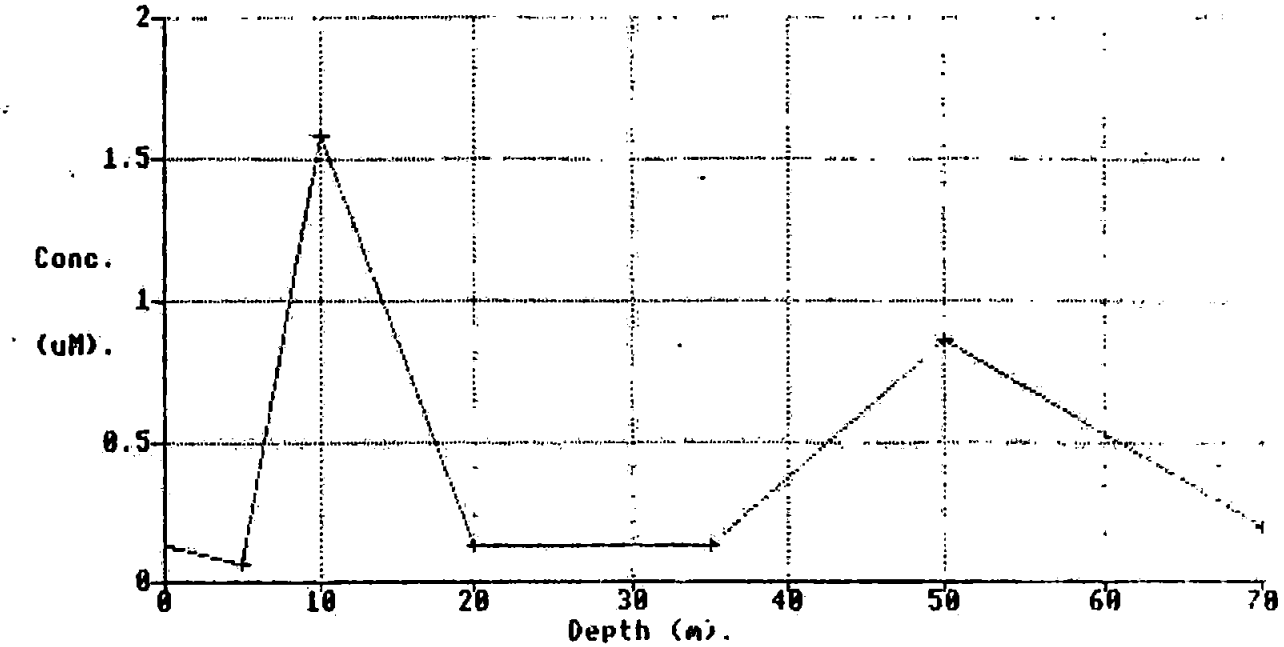


Fig 6.1 Total Dissolved Free Amino Acid Levels For Station E1, 13.3.86.

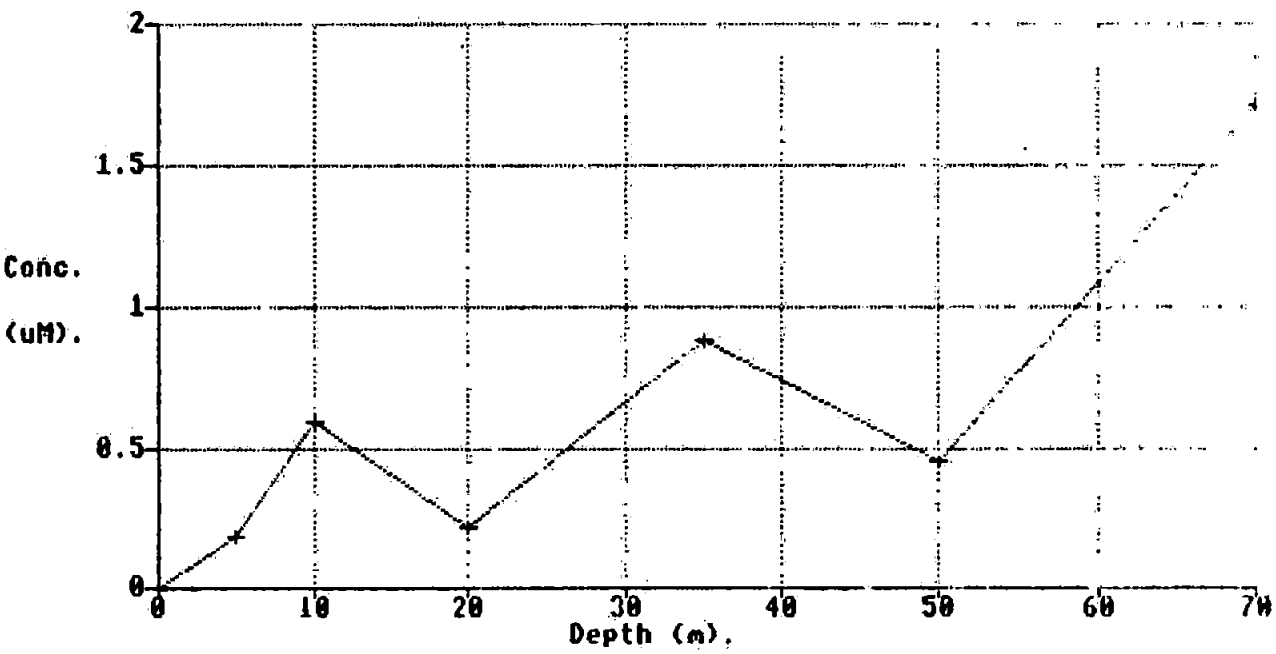


Fig 6.2 Total Dissolved Free Amino Acid Levels For Station E1, 23.5.86.

Date: 23.5.86 Dissolved free amino acids.(uM).

Station E1. Depth (m)

a.a.	0	5	10	20	35	50	70
Asp	-----	0.032	0.059	0.036	0.069	0.042	0.119
Glu	-----	0.027	0.032	0.026	0.056	0.042	0.059
Ser	-----	0.080	0.207	0.105	0.231	0.117	0.440
His	----------						0.164
Gly	----------		0.109	-----	0.221	0.124	0.275
Thr	----------		0.061	-----	0.088	-----	0.096
Arg	----------				0.043	----------	
Ala	-----	0.049	0.087	0.035	0.104	0.059	0.139
Tyr	----------			0.015	0.033	0.044	0.052
Abu	----------						
Val	----------		0.032	-----	0.039	0.029	0.066
Met	----------						
Trp/Ile	----------						
Phe	----------						
Leu	----------						
Orn	----------						0.304
Lys	----------						
TOTAL(uM)	BLD	0.188	0.587	0.217	0.884	0.457	1.714

Date: 16.7.86 Dissolved free amino acids.(uM).

Station E1. Depth (m)

a.a.	5	10	20	35	50	70
Asp	------	0.030	0.027	0.033	-----	0.016
Glu	------	0.025	-----	0.045	0.027	-----
Ser	------	0.181	0.085	0.078	-----	0.044
His	----------	----------	----------	----------	----------	----------
Gly	------	0.043	----------	----------	----------	----------
Thr	----------	----------	----------	----------	----------	----------
Arg	----------	----------	----------	----------	----------	----------
Ala	----------	----------	0.042	0.078	----------	----------
Tyr	----------	----------	----------	----------	----------	----------
Abu	----------	----------	----------	----------	----------	----------
Val	----------	----------	----------	0.034	----------	----------
Met	----------	----------	----------	----------	----------	----------
Trp/Ile	----------	----------	----------	----------	----------	----------
Phe	----------	----------	----------	----------	----------	----------
Leu	----------	----------	----------	----------	----------	----------
Orn	----------	----------	----------	----------	----------	----------
Lys	----------	----------	----------	----------	----------	----------
TOTAL(uM)	BLD	0.279	0.154	0.268	0.027	0.060

Date: 16.7.86 Dissolved combined amino acids. (uM).

Statin E1. Depth (m)

a.a.	5	10	20	35	50
Asp	0.128	0.138	0.119	0.194	0.116
Glu	0.145	0.137	0.161	0.208	0.086
Ser	0.133	----------		0.068	0.119
His	0.160	0.240	0.066	0.286	-----
Gly	0.388	0.356	0.543	0.538	0.387
Thr	-----	0.170	0.123	0.143	0.107
Arg	-----	0.049	0.083	0.083	0.059
Ala	0.109	0.172	0.124	0.149	0.102
Tyr	0.018	0.026	0.014	0.036	0.021
Abu	----------	----------	----------	----------	----------
Val	0.020	----------		0.014	-----
Met	----------	----------	----------	----------	----------
Trp/Ile	----------	----------		0.089	-----
Phe	0.079	----------	----------	----------	----------
Leu	0.070	0.073	-----	0.074	-----
Orn	----------	----------	----------	----------	----------
Lys	----------	----------	----------	----------	----------
TOTAL(uM)	1.250	1.361	1.223	1.882	0.997

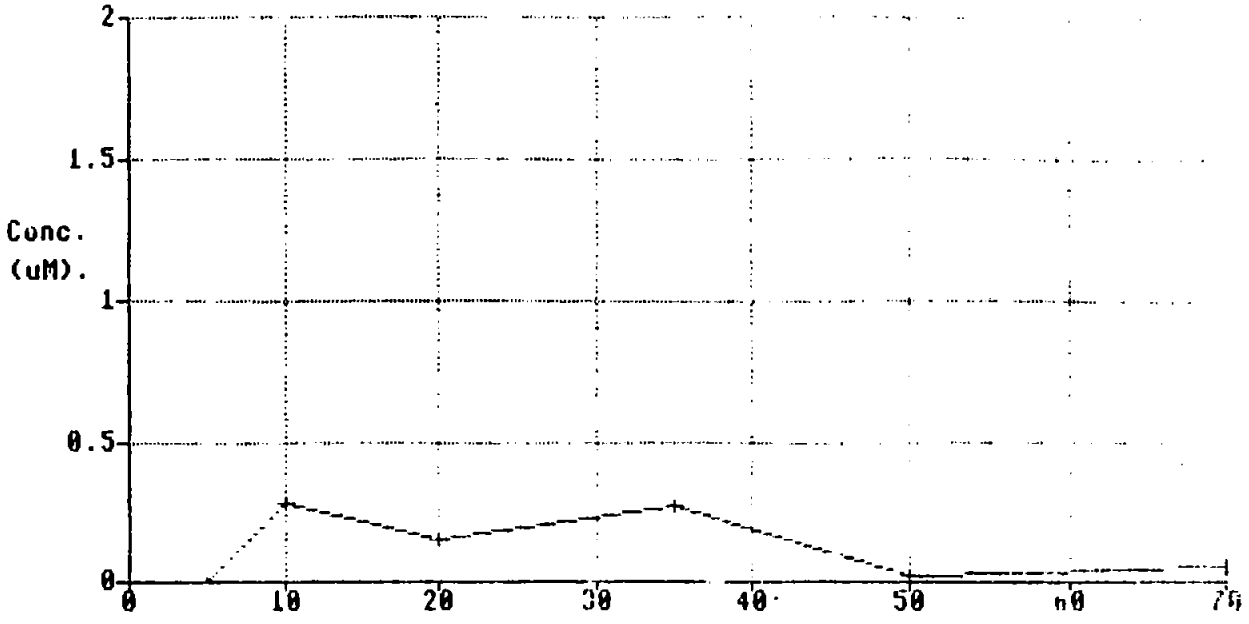


Fig 6.3a Total Dissolved Free Amino Acid Levels
For Station E1, 16.7.86.

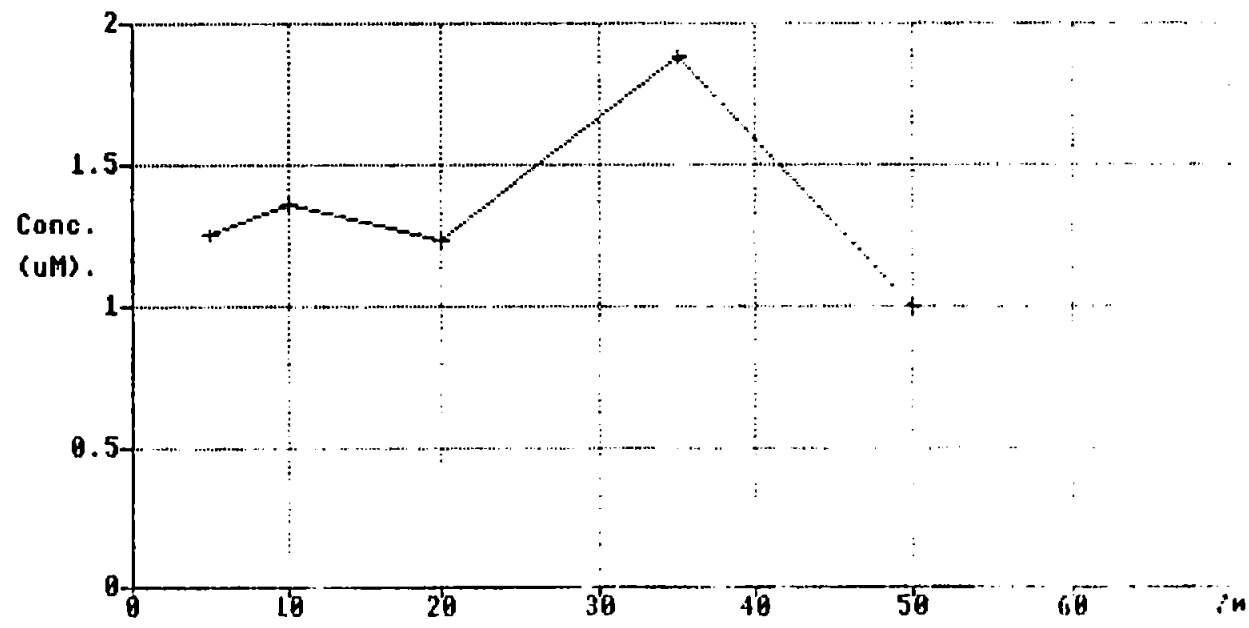


Fig 6.3b Total Dissolved Combined Amino Acid Levels
For Station E1, 16.7.86.

Date: 27.8.86 Dissolved free amino acids.(uM)

Station E1. Depth (m).

a.a.	0	10	15
Asp	0.037	0.023	0.053
Glu	-----	0.046	-----
Ser	0.056	0.078	0.155
His	----------	----------	----------
Gly	------	0.085	-----
Thr	----------	----------	----------
Arg	----------	----------	----------
Ala	------	0.045	0.076
Tyr	----------	----------	----------
Abu	----------	----------	----------
Val	----------	----------	0.024
Met	----------	----------	----------
Trp/Ile	----------	----------	----------
Phe	----------	----------	----------
Leu	----------	----------	----------
Orn	----------	----------	0.107
Lys	----------	----------	----------
TOTAL(uM)	0.093	0.277	0.415

Date: 27.8.86 Dissolved combined amino acids.(uM).

Station E1. Depth (m).

a.a.	0	10	15
Asp	0.133	0.093	0.198
Glu	0.202	0.163	0.567
Ser	----------	----------	0.329
His	0.181	----------	----------
Gly	0.615	0.194	0.859
Thr	0.172	0.122	0.242
Arg	-----	0.088	0.205
Ala	0.057	0.036	0.179
Tyr	0.028	0.028	0.062
Abu	----------	----------	----------
Val	----------	----------	0.142
Met	----------	----------	----------
Trp/Ile	------	0.065	-----
Phe	----------	----------	0.076
Leu	------	0.048	0.099
Orn	------	0.128	0.265
Lys	----------	----------	0.106
TOTAL(uM)	1.388	0.965	3.329

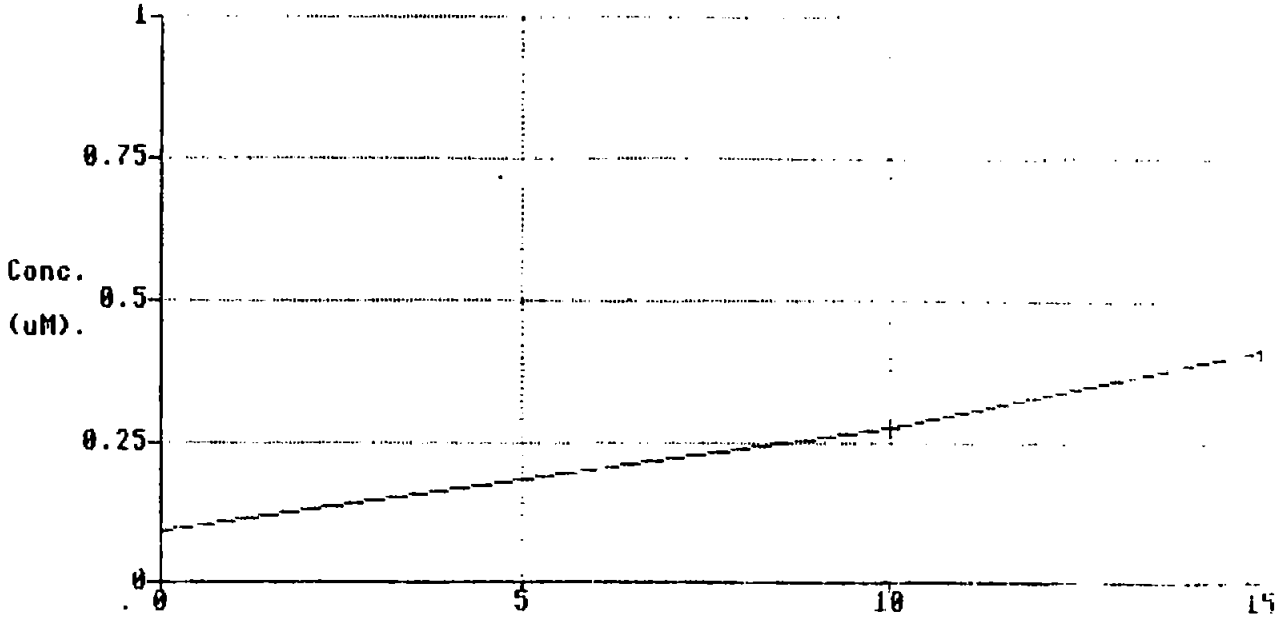


Fig 6.4a Total Dissolved Free Amino Acid Levels
For Station E1, 27.8.86. (Stormy Conditions).

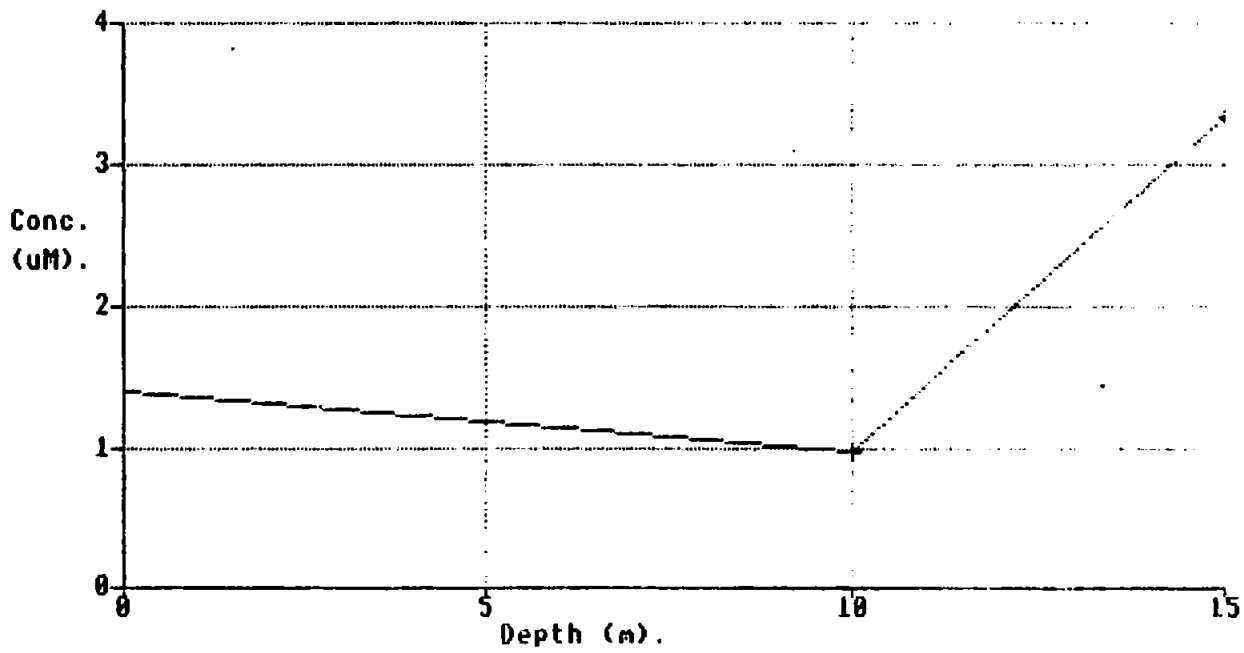


Fig 6.4b Total Dissolved Combined Amino Acid Levels
For Station E1, 27.8.86.

Date: 7.10.86 Dissolved free amino acids.(uM).

Station E1. Depth (m).

a.a.	5	10	20	35	50	70
Asp	-----	0.071	0.032	0.038	0.037	0.088
Glu	0.064	0.089	0.026	----------		0.053
Ser	0.101	0.146	0.142	0.193	0.184	0.499
His	----------					
Gly	----------		0.078	0.140	0.054	0.295
Thr	----------					
Arg	----------					
Ala	-----	0.013	0.041	0.103	0.084	0.166
Tyr	0.032	----------				0.055
Abu	----------					
Val	----------					0.042
Met	----------					
Trp/Ile	----------					
Phe	----------					
Leu	----------					
Orn	----------					0.176
Lys	----------					
TOTAL(uM)	0.197	0.319	0.319	0.474	0.359	1.374

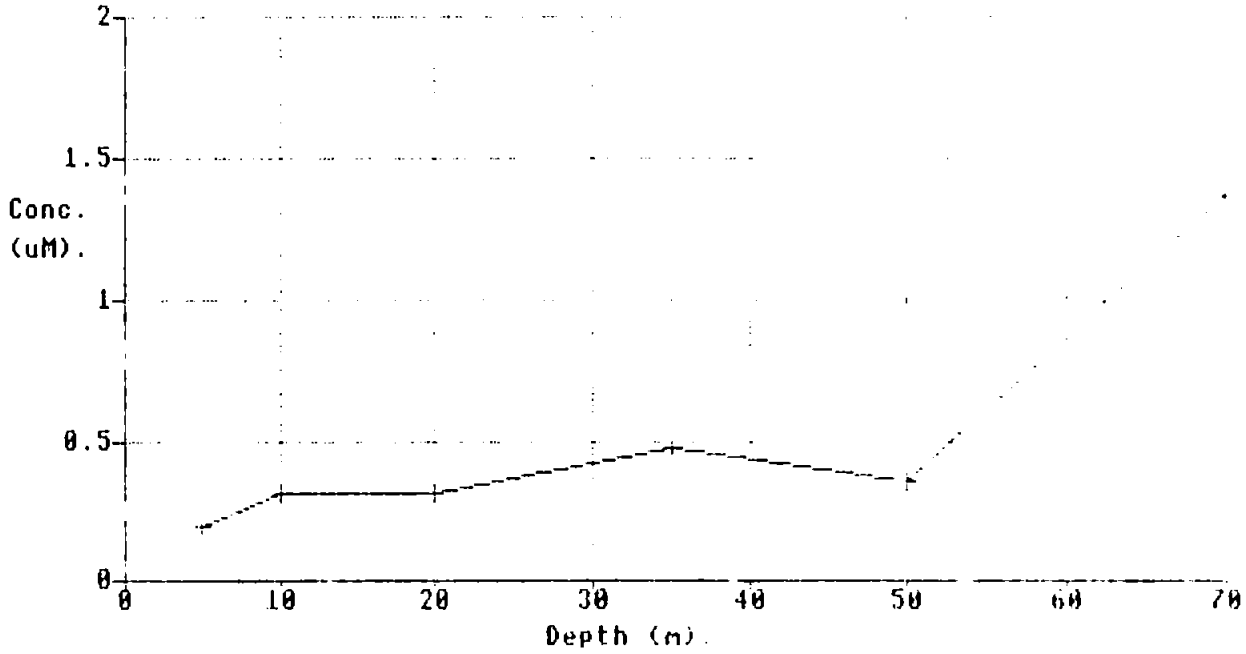


Fig 6.5 Total Dissolved Free Amino Acid Levels
For Station E1, 7.10.86.

Date: 21.1.87 Dissolved free amino acids (uM).

Station E1. Depth (m).

a.a.	0	5	10	20	35	50	70
Asp	0.029	-----	0.068	0.028	-----	0.076	0.103
Glu	----------						0.016
Ser	0.158	-----	0.394	0.165	0.089	0.180	0.477
His	----------						
Gly	0.177	0.191	0.222	----------			0.201
Thr	----------						
Arg	----------						
Ala	----------		0.055	0.048	-----	0.068	0.106
Tyr	----------		0.034	----------			
Abu	----------						
Val	----------		0.037	----------		0.038	0.052
Met	----------						
Trp/Ile	----------						
Phe	----------						
Leu	----------						
Orn	----------		0.100	----------			0.155
Lys	----------						
TOTAL(uM)	0.364	0.191	0.910	0.241	0.089	0.362	1.110

Date: 21.1.87 Dissolved combined amino acids.(uM).

Station E1. Depth (m).

a.a.	0	5	10	20	70
Asp	-----	0.021	0.025	0.026	0.050
Glu	0.025	------	0.125	0.037	0.100
Ser	----------				
His	0.075	-----	0.300	0.287	0.250
Gly	0.040	0.250	0.710	0.552	0.618
Thr	----------				
Arg	----------				
Ala	0.027	0.087	0.100	0.050	0.125
Tyr	----------				
Abu	----------				
Val	----------				
Met	----------				
Trp/Ile	----------				
Phe	----------				
Leu	----------				
Orn	----------				
Lys	----------				
TOTAL(uM)	0.167	0.358	1.260	0.952	1.143

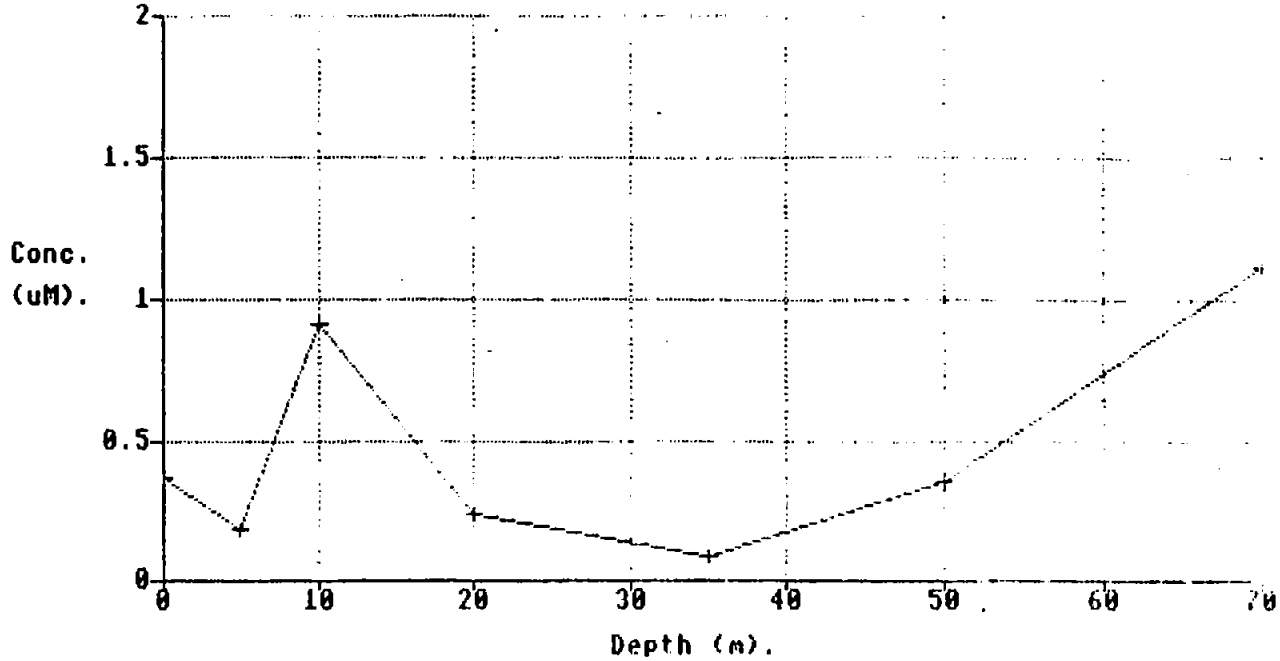


Fig 6.6a Total Dissolved Free Amino Acid Levels
For Station E1, 21.1.87.

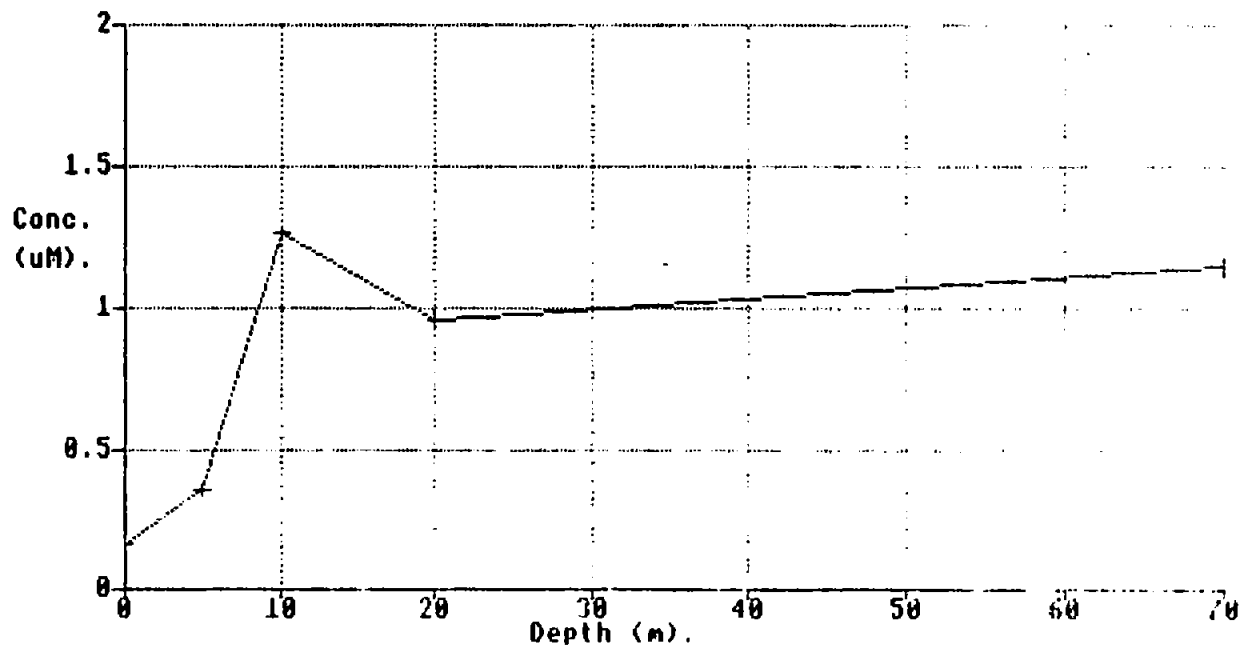


Fig 6.6b Total Dissolved Combined Amino Acid Levels
For Station E1, 21.1.87.

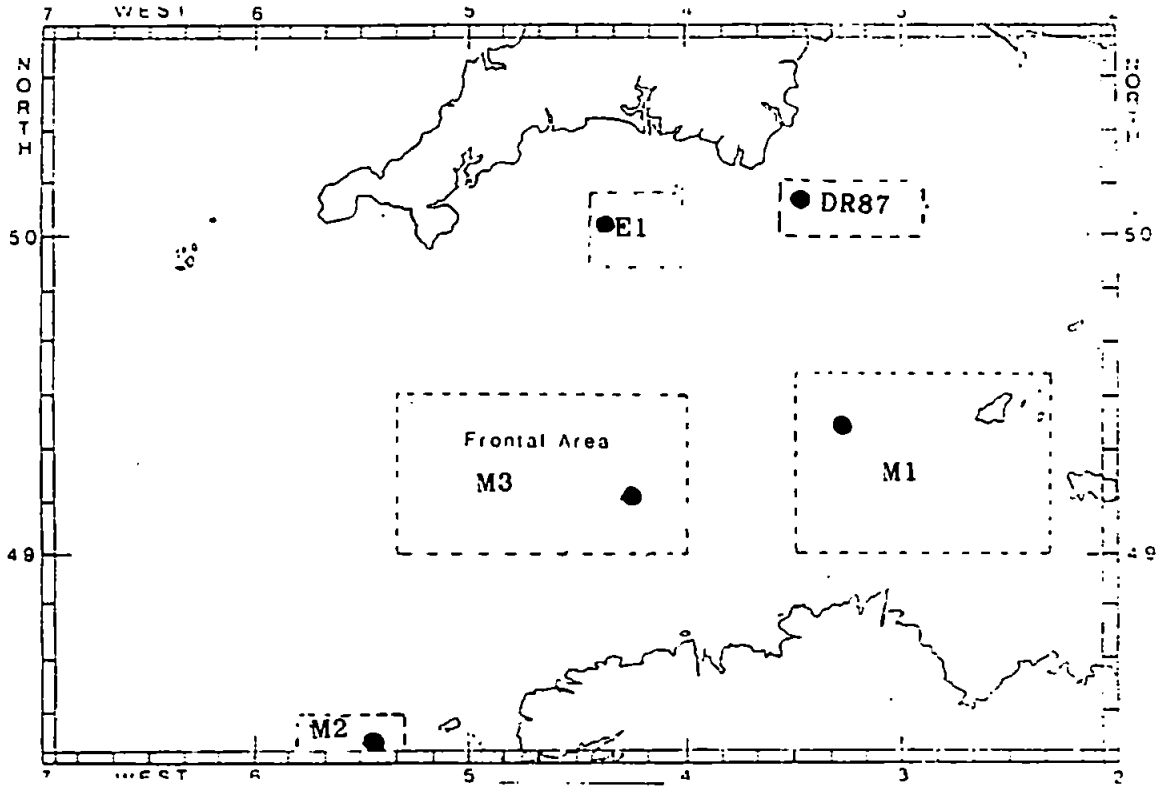


Fig 6.7 Sampling Area for the July 1987 Cruise.

Date: 14.7.87 20.00 hrs. Dissolved free amino acids.(uM)

Station M1. Cruise July 1987.

	Depth (m).					
a.a.	5	10	20	35	50	70
Asp	------	0.088	0.031	0.367	0.037	-----
Glu	----------	----------	----------	0.186	----------	----------
Ser	0.043	0.369	0.091	2.879	0.173	-----
His	----------	----------	----------	----------	----------	----------
Gly	0.128	0.309	0.221	2.135	0.076	-----
Thr	----------	----------	----------	0.241	----------	----------
Arg	0.164	0.138	0.116	0.326	0.095	-----
Ala	0.050	0.144	-----	1.047	0.078	-----
Tyr	-----	0.032	-----	0.097	----------	----------
Abu	----------	----------	----------	----------	----------	----------
Val	-----	0.059	-----	0.316	----------	----------
Met	----------	----------	----------	----------	----------	----------
Trp/Ile	----------	----------	----------	0.103	----------	----------
Phe	----------	----------	----------	0.054	----------	----------
Leu	----------	----------	----------	0.100	0.095	-----
Orn	0.453	0.626	0.552	0.599	0.308	-----
Lys	----------	----------	----------	0.460	----------	----------
TOTAL(uM)	0.838	1.765	1.011	8.910	0.862	BLD

Date: 15.7.87 10.00 Hrs. Dissolved free amino acids. (uM).
Station M1. Cruise July 1987.

	Depth (m).					
a.a.	5	10	20	35	50	70
Asp	-----	0.099	0.058	0.046	0.166	0.044
Glu	----------	----------	----------	----------	0.048	-----
Ser	-----	0.356	0.239	0.160	0.808	0.107
His	----------	----------	----------	----------	----------	----------
Gly	0.147	0.317	-----	0.102	----------	----------
Thr	----------	----------	----------	----------	----------	----------
Arg	----------	----------	----------	----------	----------	----------
Ala	0.059	0.143	0.091	0.052	0.278	-----
Tyr	-----	0.046	----------	----------	0.035	-----
Abu	----------	----------	----------	----------	----------	----------
Val	----------	----------	----------	----------	0.106	-----
Met	----------	----------	----------	----------	----------	----------
Trp/Ile	----------	----------	----------	----------	----------	----------
Phe	----------	----------	----------	----------	----------	----------
Leu	----------	----------	----------	----------	----------	----------
Orn	----------	----------	----------	----------	0.229	------
Lys	----------	----------	----------	----------	----------	----------
TOTAL(uM)	0.206	0.961	0.388	0.360	1.670	0.151

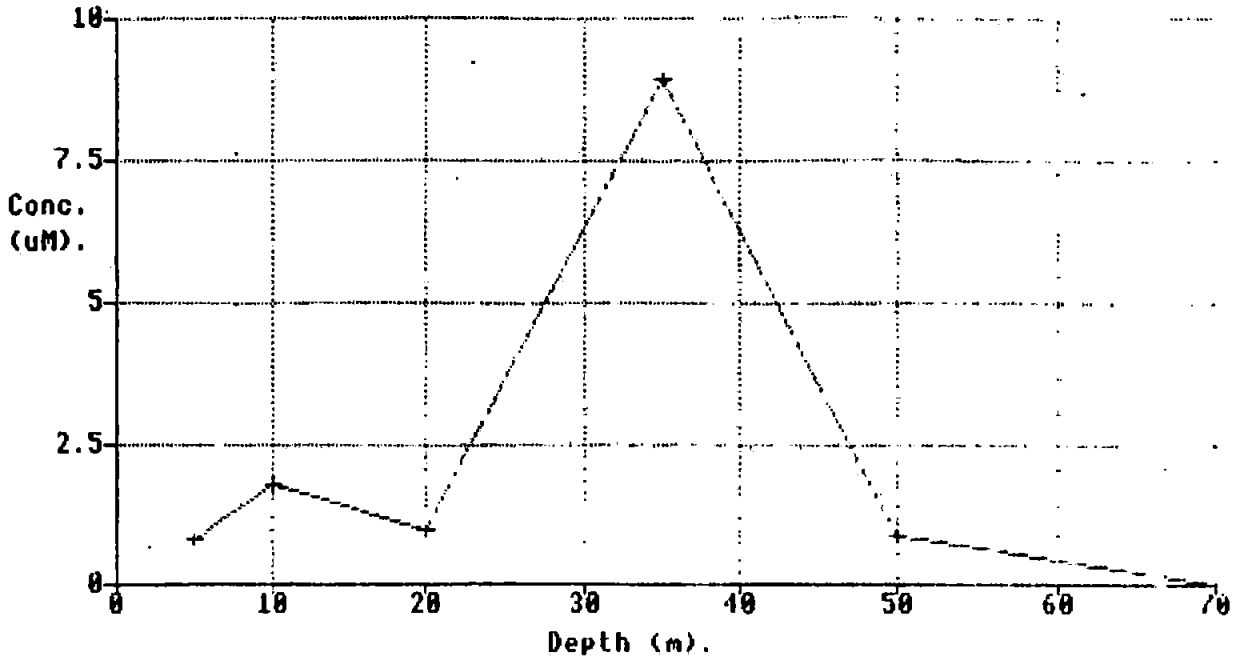


Fig 6.8 Cruise July 1987, Dissolved Free Amino Acid Levels For Station M1, 20.00 Hrs. 14.7.87.

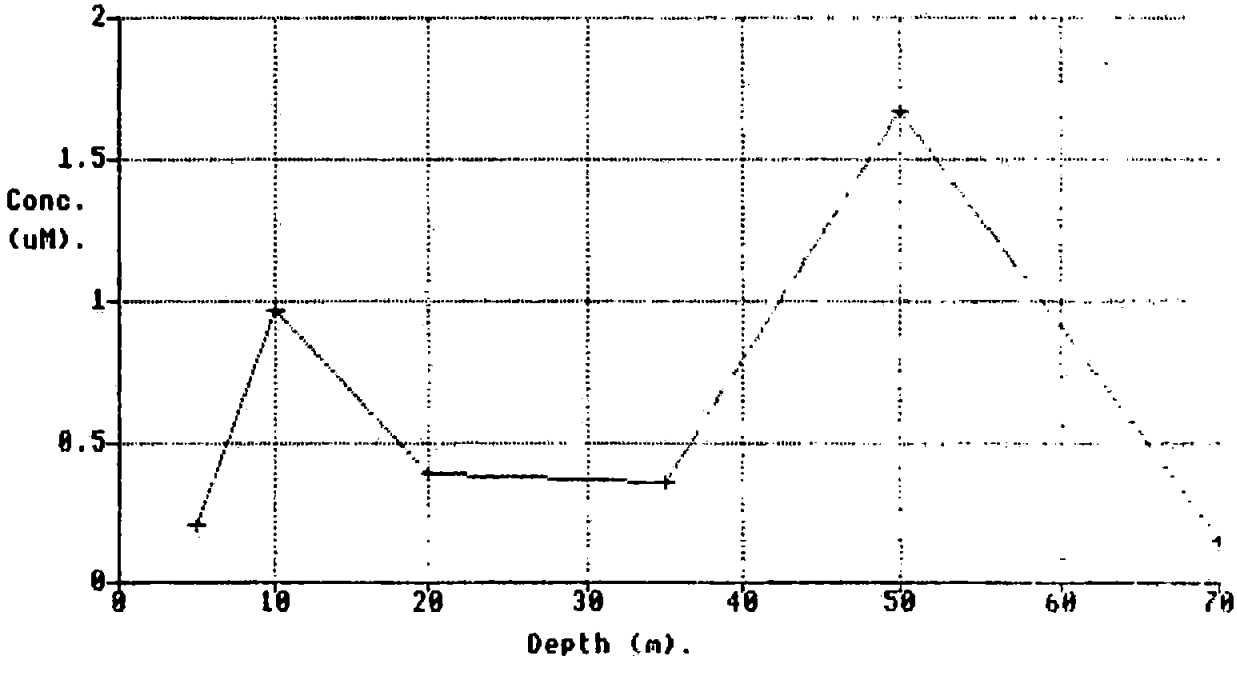


Fig 6.9 Cruise July 1987, Dissolved Free Amino Acid Levels For Station M1, 10.00 Hrs. 15.7.87.

Date: 15.7.87 19.00 Hrs. DFAA Station M1. Cruise. (uM).

	Depth (m)					
a.a.	5	10	20	35	50	70
Asp	0.048	0.102	0.083	0.045	0.092	0.262
Glu	-----	-----	-----	-----	-----	0.073
Ser	0.108	0.859	0.239	0.231	0.328	1.192
His	-----	-----	-----	-----	-----	-----
Gly	-----	0.557	-----	-----	-----	0.164
Thr	-----	0.063	-----	-----	-----	-----
Arg	-----	0.099	-----	-----	-----	-----
Ala	-----	0.297	-----	-----	0.062	0.372
Tyr	-----	0.037	-----	-----	-----	0.031
Abu	-----	-----	-----	-----	-----	-----
Val	-----	0.095	-----	-----	-----	0.162
Met	-----	-----	-----	-----	-----	-----
Trp/Ile	-----	-----	-----	-----	-----	-----
Phe	-----	-----	-----	-----	-----	-----
Leu	-----	-----	-----	-----	-----	-----
Orn	-----	0.188	-----	-----	-----	0.321
Lys	-----	-----	-----	-----	-----	-----
TOTAL(uM)	0.156	2.297	0.322	0.276	0.482	2.577

Date: 16.7.87 07.00 Hrs. DFAA Station M2. Cruise. (uM).

	Depth (m).					
a.a.	5	10	20	35	50	70
Asp	-----	-----	-----	0.029	-----	0.067
Glu	-----	-----	-----	-----	-----	-----
Ser	-----	0.086	0.088	0.026	-----	0.172
His	-----	-----	-----	-----	-----	-----
Gly	0.131	-----	-----	-----	-----	-----
Thr	-----	-----	-----	-----	-----	-----
Arg	-----	-----	-----	-----	-----	-----
Ala	-----	-----	0.052	-----	-----	-----
Tyr	-----	-----	-----	-----	-----	-----
Abu	-----	-----	-----	-----	-----	-----
Val	-----	-----	-----	-----	-----	-----
Met	-----	-----	-----	-----	-----	-----
Trp/Ile	-----	-----	-----	-----	-----	-----
Phe	-----	-----	-----	-----	-----	-----
Leu	-----	-----	-----	-----	-----	-----
Orn	-----	-----	-----	-----	-----	-----
Lys	-----	-----	-----	-----	-----	-----
TOTAL(uM)	0.131	0.086	0.140	0.055	BLD	0.239

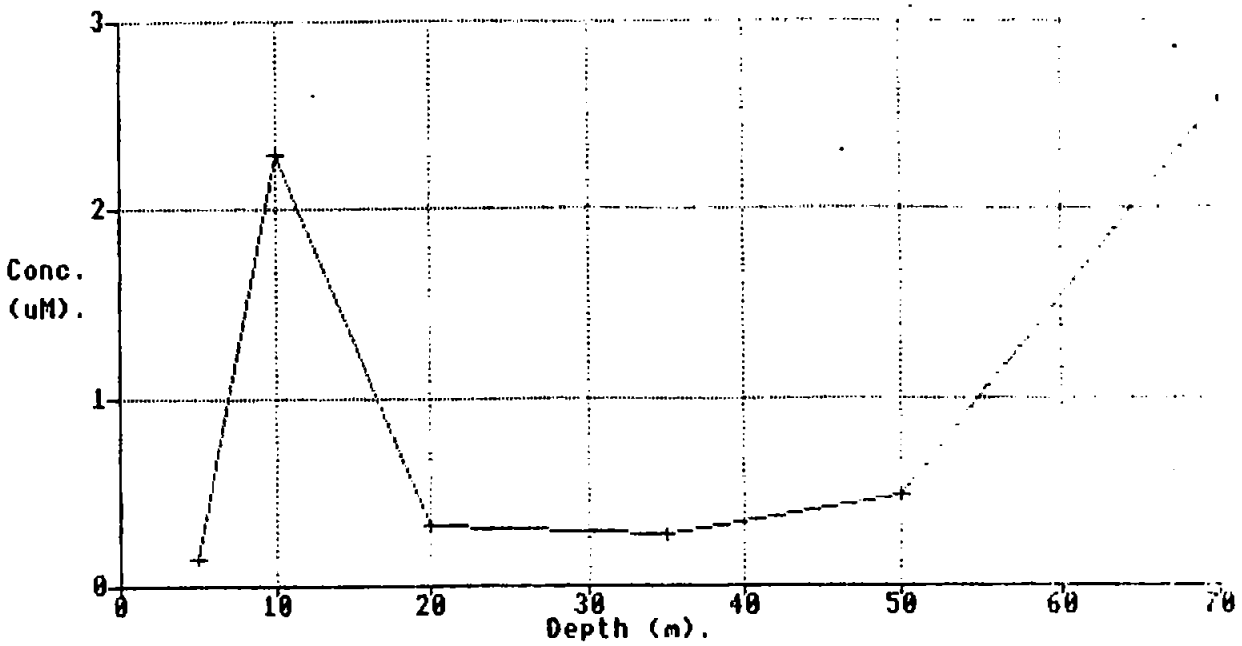


Fig 6.10 Cruise July 1987, Dissolved Free Amino Acid Levels For Station M1, 19.00 Hrs. 15.7.87.

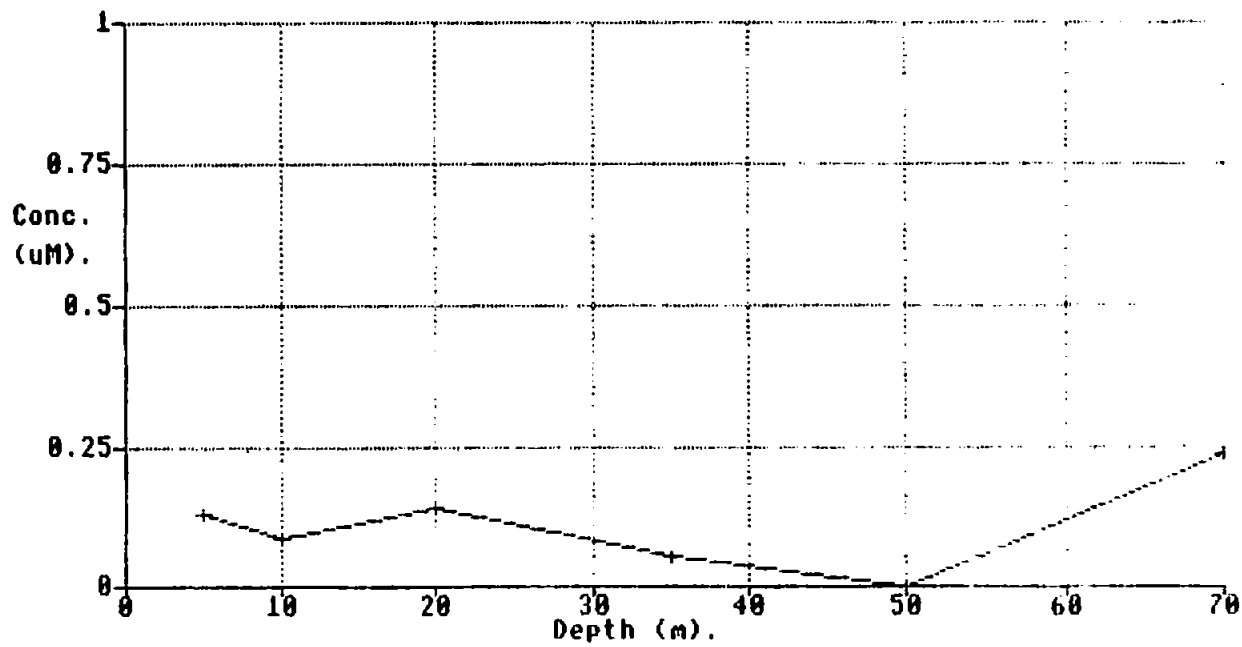


Fig 6.11 Cruise July 1987, Dissolved Free Amino Acid Levels For Station M2, 07.00 Hrs. 16.7.87.

Date: 16.7.87 20.00 Hrs. DFAA Station DR/87. Cruise (uM).

Depth (m).

a.a.	5	10	20	35	50	70
Asp	-----	0.023	-----	0.066	-----	0.045
Glu	-----	-----	-----	-----	-----	-----
Ser	-----	0.091	0.075	-----	-----	0.192
His	-----	-----	-----	-----	-----	-----
Gly	-----	-----	-----	-----	-----	0.108
Thr	-----	-----	-----	-----	-----	-----
Arg	-----	-----	-----	-----	-----	-----
Ala	-----	-----	-----	-----	0.068	-----
Tyr	-----	-----	-----	-----	-----	-----
Abu	-----	-----	-----	-----	-----	-----
Val	-----	-----	-----	-----	-----	-----
Met	-----	-----	-----	-----	-----	-----
Trp/Ile	-----	-----	-----	-----	-----	-----
Phe	-----	-----	-----	-----	-----	-----
Leu	-----	-----	-----	-----	-----	-----
Orn	-----	-----	-----	-----	-----	-----
Lys	-----	-----	-----	-----	-----	-----
TOTAL(uM)	BLD	0.113	0.075	0.066	0.068	0.345

Date: 17.7.87 20.00 Hrs. DFAA Station DR/87. Cruise (uM).

Depth (m).

a.a.	5	10	20	35	50	70
Asp	-----	0.035	0.047	0.034	----	0.093
Glu	0.062	0.059	-----	-----	-----	0.056
Ser	0.074	0.121	0.165	-----	-----	0.332
His	-----	-----	-----	-----	-----	-----
Gly	-----	-----	-----	-----	-----	-----
Thr	-----	-----	-----	-----	-----	-----
Arg	-----	-----	-----	-----	-----	-----
Ala	------	0.074	0.048	-----	-----	0.086
Tyr	-----	-----	-----	-----	-----	-----
Abu	-----	-----	-----	-----	-----	-----
Val	-----	-----	-----	-----	-----	-----
Met	-----	-----	-----	-----	-----	-----
Trp/Ile	-----	-----	-----	-----	-----	-----
Phe	-----	-----	-----	-----	-----	-----
Leu	-----	-----	0.054	-----	-----	-----
Orn	-----	-----	-----	-----	-----	-----
Lys	-----	-----	-----	-----	-----	-----
TOTAL(uM)	0.136	0.289	0.314	0.034	BLD	0.567

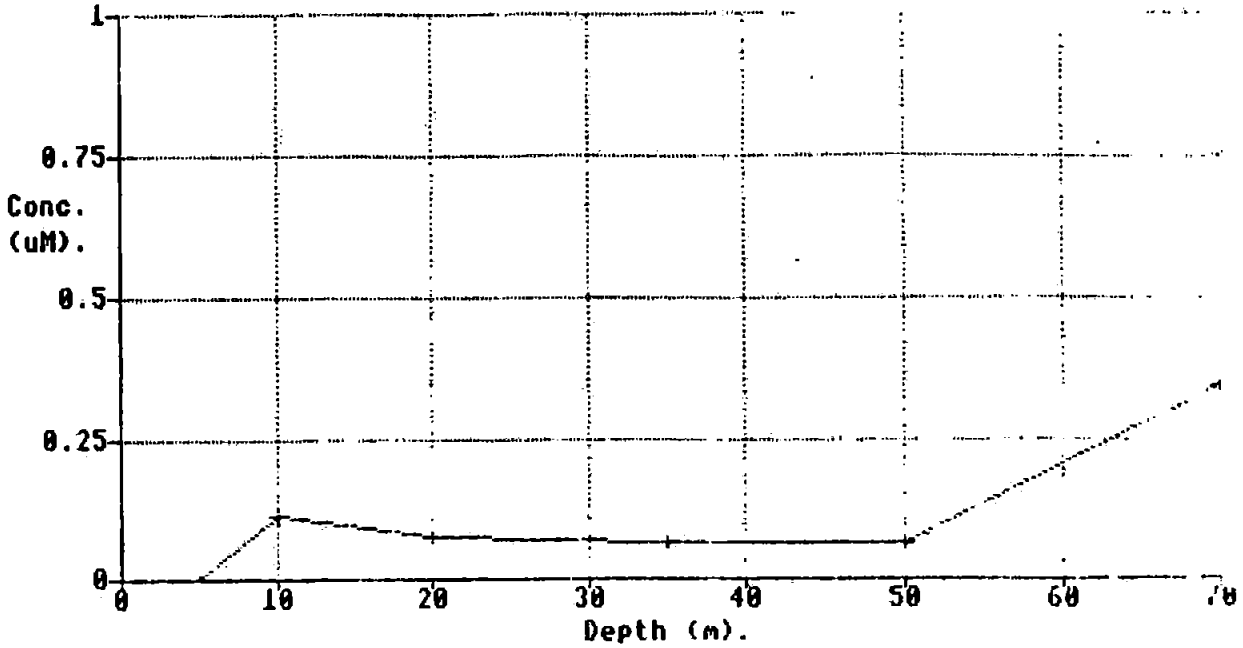


Fig 6.12 Cruise July 1987, Dissolved Free Amino Acid Levels For Station DR/87, 20.00 Hrs. 16.7.87.

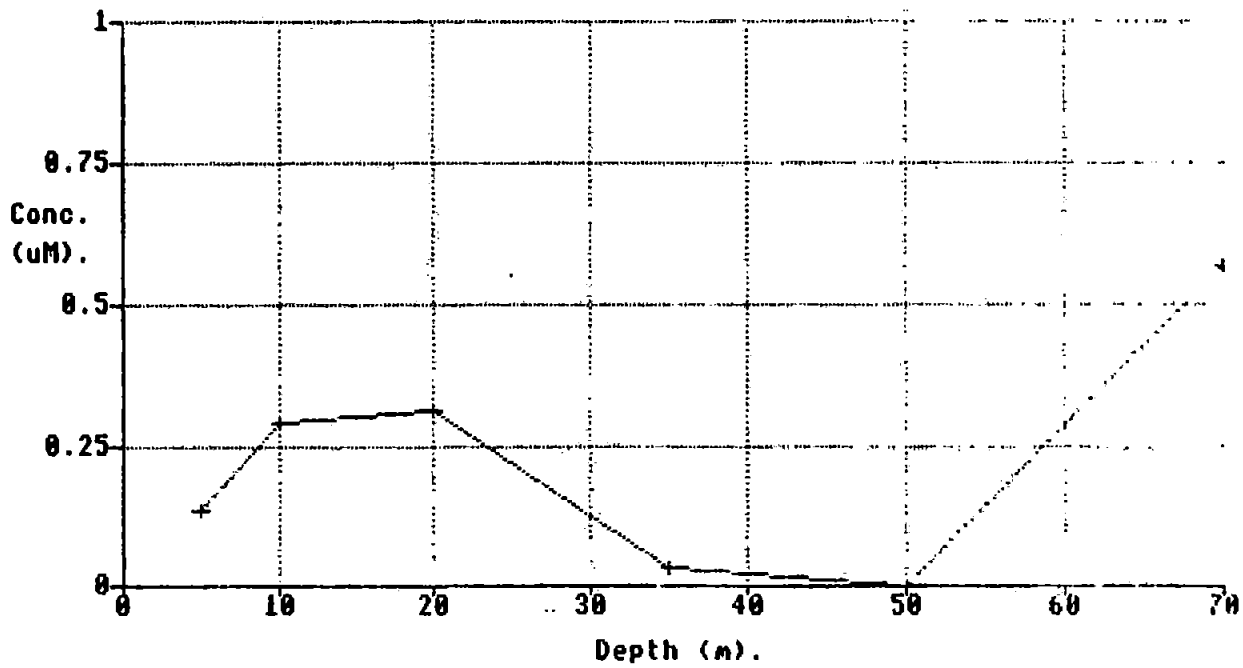


Fig 6.13 Cruise July 1987, Dissolved Free Amino Acid Levels For Station DR/87, 20.00 Hrs. 17.7.87.

Date: 18.7.87 21.00 Hrs. DFAA Station E1. Cruise (uM).

Depth (m).

a.a.	5	10	20	35	50	70
Asp	----------		0.037	------	0.038	-----
Glu	-----	0.039	0.053	----------		
Ser	-----	0.357	0.110	------	0.162	0.069
His	----------					
Gly	-----	0.132	----------			
Thr	----------					
Arg	----------					
Ala	----------				0.064	------
Tyr	----------					
Abu	----------					
Val	----------					
Met	----------					
Trp/Ile	----------					
Phe	----------					
Leu	----------					
Orn	----------					
Lys	----------					
TOTAL(uM)	BLD	0.528	0.200	BLD	0.264	0.069

Date: 19.7.87 08.00 Hrs. DFAA Station E1. Cruise (uM).

Depth (m).

a.a.	5	10	20	35	50	70
Asp	----------		0.053	0.068	-----	0.040
Glu	0.045	------	0.054	0.064	0.052	-----
Ser	-----	0.216	-----	0.171	0.099	0.182
His	----------					
Gly	----------					0.059
Thr	----------					
Arg	----------					
Ala	------	0.118	0.094	0.093	-----	0.064
Tyr	----------					
Abu	----------					
Val	----------					
Met	----------					
Trp/Ile	----------					
Phe	----------					
Leu	----------					
Orn	----------					
Lys	----------					
TOTAL(uM)	0.045	0.334	0.201	0.396	0.151	0.345

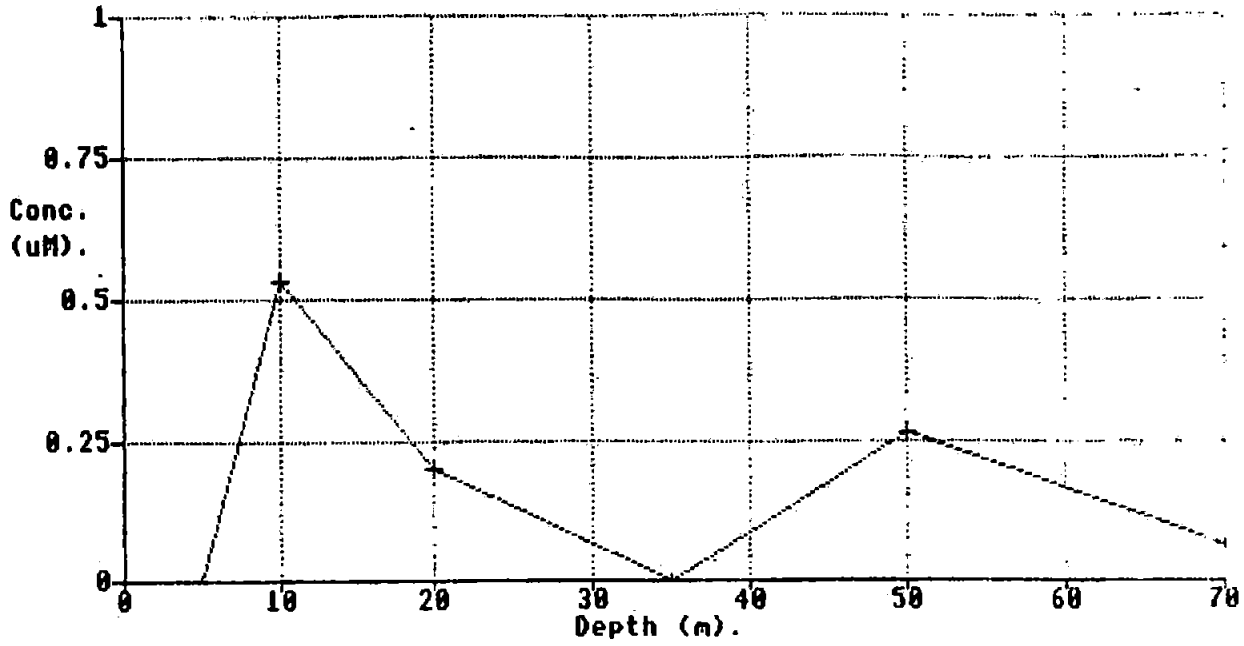


Fig 6.14 Cruise July 1987, Dissolved Free Amino Acid Levels For Station E1, 21.00 Hrs. 18.7.87.

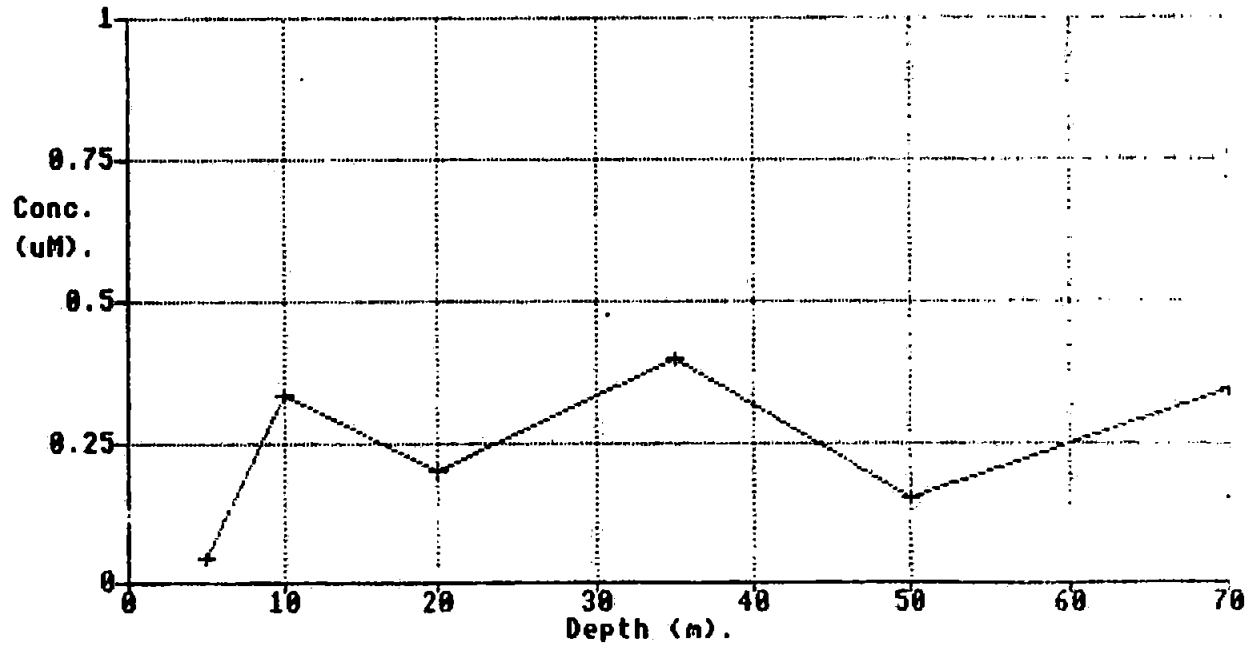


Fig 6.15 Cruise July 1987, Dissolved Free Amino Acid Levels For Station E1, 1800 Hrs. 19.7.87.

6.3. Discussion.

The depth profile results show several patterns which are detailed below. All profiles, with the exception of one taken during bad weather conditions, had dissolved free amino acid maxima at 2 two depths, and also showed low dissolved free amino acid levels at the 5m depth. Again, the exception was the profile taken in adverse weather conditions. Previous workers, Ittekkot (1982), Mopper & Lindroth (1982), Poulet et al. (1984) and Liebezeit & Velimirov (1984), report amino acid maxima in depth profile samples. The possible reasons for the increases and decreases in the dissolved free amino acid content of seawater have been discussed in Chapter 5.

The profiles which have two maxima, show 69 percent with one of the peaks for dissolved free amino acid content, at a depth of 10 m. These raised dissolved free amino acid levels, may be associated with the chlorophyll maximum which often occurs at this depth. Unfortunately there was no chlorophyll data with which to check this hypothesis. 30 percent of the profiles had a maximum at 35 m which is at the lowest depths to which the photic zone may extend. 20 percent of profiles had a peak at 50 m and 54 percent had raised dissolved free amino acid levels at 70 m, which may be due to the close proximity of the sediments. The increased levels for the dissolved free acid fraction shows a concomitant rise in the number of acids present in the spectra, as was the case for

coastal surface samples, (Chapter 5). The overall picture, derived from examination of the depth profiles, is of a maxima within the photic zone (10 to 35m), generally followed by a maxima in the aphotic zone, (50 to 70 m).

It is interesting to note that, ornithine occurs in 54 percent of the samples with raised levels occuring in the 35 to 70m area, compared with only 23 percent of samples at the 10 m depth. This increased ornithine content of the dissolved free fraction with depth, may be due to greater heterotrophic activity. An increase in all the basic amino acids with depth, was not found in this study. This is in contrast to the findings of Mopper & Lindroth (1982).

In the three profiles analysed for dissolved combined amino acids, this fraction is on average 5 times higher than the free acids. The pattern is similar to that found in coastal surface water samples. The difference in levels, between the two fractions, may be due to use of the free acids, by the biota, as a nutrient source. A similar pattern, for the composition of the dissolved combined amino acid fraction in coastal samples, was found in depth profile studies, with the combined fraction having many more acids present in the spectra than the free fraction. Again, the combined fraction is dominated by the presence of glycine. (chapter 5).

CHAPTER 7.

LEVELS AND COMPOSITION OF THE DISSOLVED FREE AMINO ACIDS IN THE TAMAR ESTUARY.

7.1 Introduction.

An estuary is defined as the tidal mouth of a river, where the channel broadens out into a V-shape, within which the tide ebbs and flows, (Monkhouse & Small 1978). An estuary is usually divided into three sections; an upper fluvial (river) area, characterised by fresh water but subject to tidal action. The middle estuary, where large scale mixing of fresh water and sea water occurs, and the lower estuary, in direct connection with the sea.

The estuary and the sea are very different systems. This is illustrated by the changes in salinity, pH, turbidity, ionic and temperature gradients during the passage of the river to the sea. Fig 7.1 reproduced from Morris et al. (1982), shows the changes in pH, salinity and turbidity for the Tamar estuary, which emphasises the complex nature of estuarine processes.

The turbidity of estuarine waters is due to suspended particulate material. Morris et al.(1985) classify the suspended material into three categories. Firstly, inorganic particles derived from erosion and weathering. Secondly, particles generated by processes occurring within the water column. Thirdly, biogenic particles e.g. living biota, dead and decaying remains, faecal pellets, terrestrial plant debris, industrial and

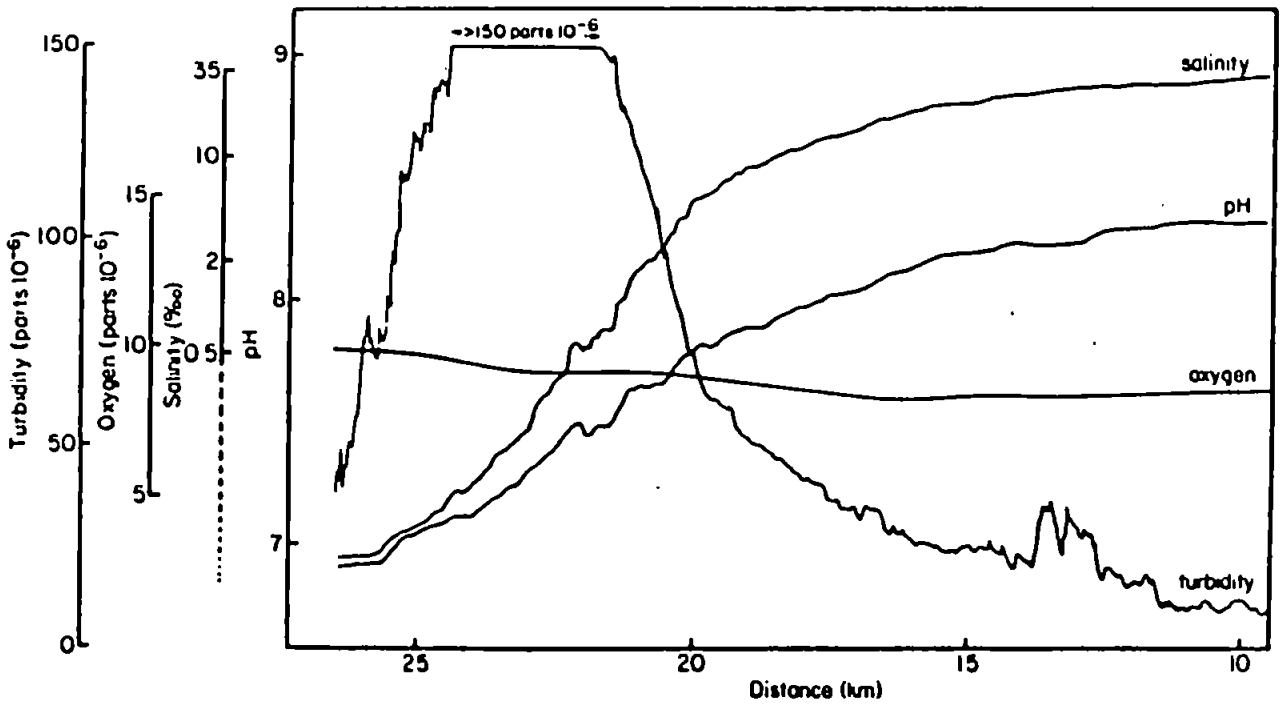


Fig 7.1 Changes In pH, Salinity And Turbidity For The Tamar Estuary, (from Morris 1982).

domestic waste. The suspended and sedimented particulate material will comprise a mixture of these three classes. The levels of suspended material are variable with large increases occurring after heavy rain. (Butler & Tibbits 1972). Suspended matter can greatly affect light penetration and therefore the depth of the photic zone in estuarine waters. In the Tamar estuary the turbidity maximum occurs in the regions with a salinity range of approximately 1 to 5 (Evens 1986).

In the Tamar estuary, where the suspended matter can reach levels of aproximately 1,000 mg per litre, the surface chemistry between particles and dissolved constituents is of great ecological importance. In fact, the concentrations of some dissolved constituents are controlled by these mechanisms, e.g. phosphorus levels.

The fresh water-salt water interface is the location of many chemical and physical changes. Morris (1978) considers this interface to be the area where the biota experience the most severe ionic and osmotic changes and suggests that these effects would be reflected in the amino acid composition of these waters compared to other areas of the estuary. In the Tamar estuary, the fresh water/salt water interface is found in the salinity region 3 to 13, overlapping the turbidity maximum.

The levels and seasonal variation of total dissolved nitrogen, nitrate, nitrite, ammonia and dissolved organic nitrogen, in the Tamar have been reported by Butler & Tibbits (1972); most of the nitrogen is present as nitrate and nitrite. Heavy rainfall causes sharp rises in nitrate and nitrite levels, while

dissolved organic nitrogen shows little variation. Butler & Tibbits (loc cit) suggest that much of the dissolved organic matter in the river never reaches the sea due to precipitation processes.

A dissolved chemical constituent subjected to complex estuarine conditions can behave in a number of ways. The constituent is said to behave conservatively if the concentration of the chemical is directly related to the degree of mixing between the fresh and salt water. (Head 1985). Therefore a constituent displaying conservative behaviour, is suggestive of being unaffected by any process other than dilution. This produces a theoretical dilution line for the dissolved chemical constituent. A graphical representation of this conservative behaviour can be seen in Fig. 7.2. Where a chemical constituent behaves non-conservatively there will be deviations above, (for addition), or below, (for removal), the theoretical dilution line (Fig. 7.2). Morris (1985) gives more complex pictures of addition and/or removal of a constituent during its passage down the estuary. (Fig. 7.3).

A map showing the Tamar estuary and the two tributary rivers the R. Lynher and the R. Tavy can be seen in Fig. 7.4 from Morris et al. (1982), which emphasizes the complex nature of this estuarine system.

During the present investigation of estuarine waters, samples were collected from the Tamar estuary at approximately 0.5 m depths along the salinity gradient from 0 to 32. The samples were filtered into plastic bottles and kept cool in an insulating container on board

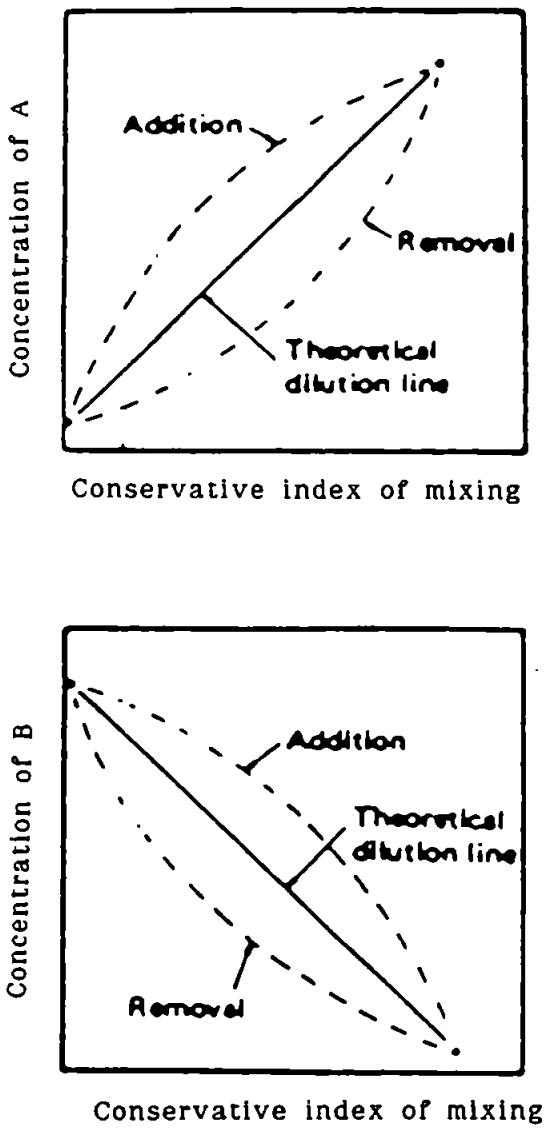


Fig 7.2 Diagram Showing A Simple Model For Conservative And Non-Conservative Behaviour, (from Head 1985).

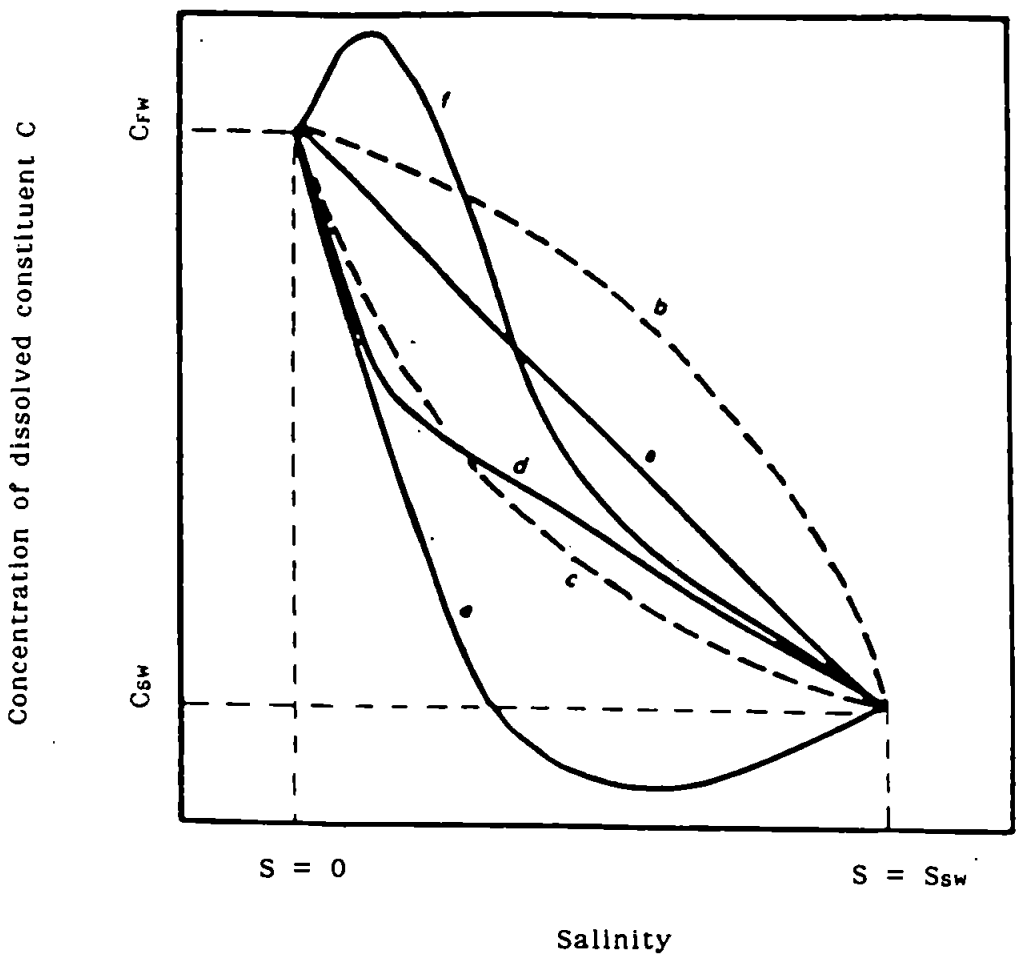


Fig 7.3 Diagram Showing Complex Pictures Of Production And Removal For Dissolved Constituents, (from Morris 1985).

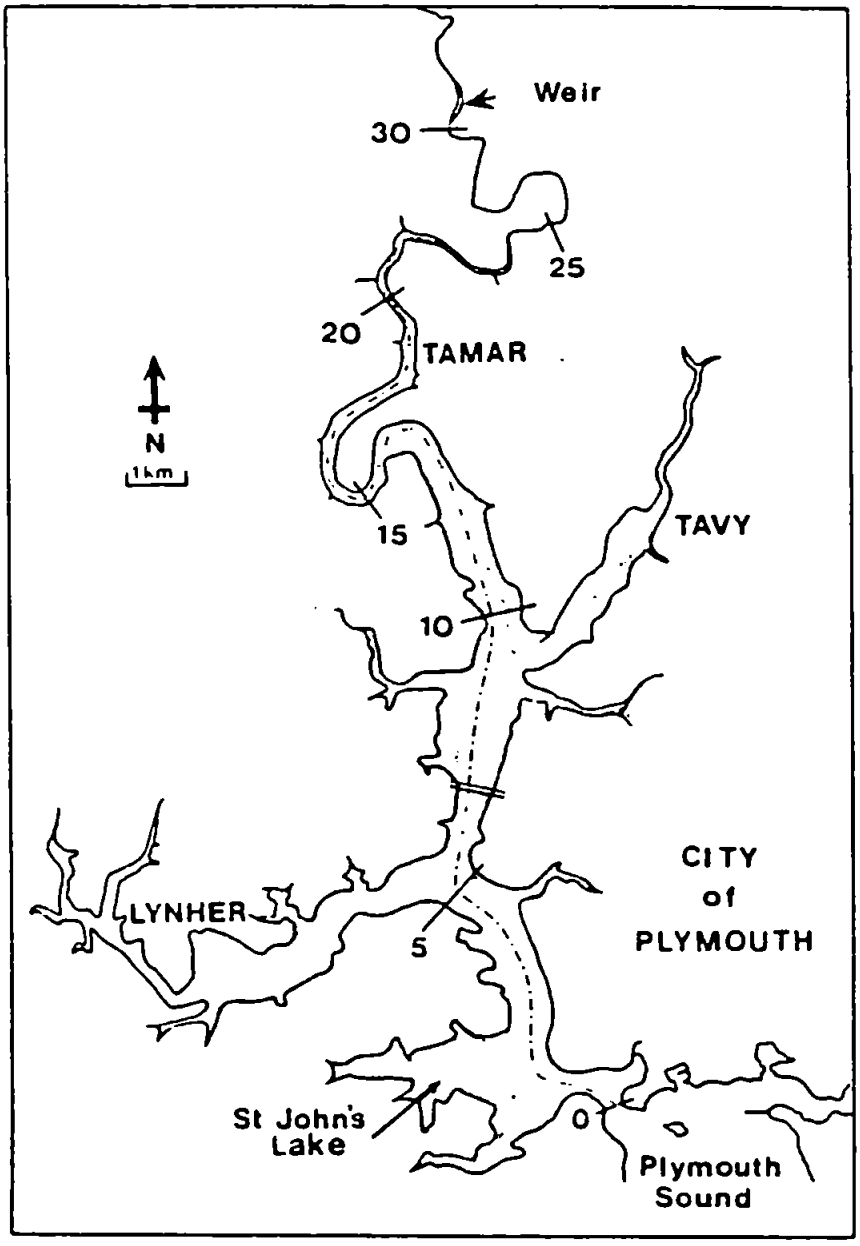


Fig 7.4 Map Showing The Complexity Of The Tamar Estuary, (from Morris 1982).

ship until they could be placed in a refrigerator prior to analysis. All samples were analysed within seven hours of arrival at the laboratory, (See chapter 2). Due to the problem of oxidative losses, occurring during acid hydrolysis in the presence of high nitrate levels as outlined in chapter 3, only dissolved free amino acid analyses were carried out for estuary samples.

The results of the dissolved free amino acid analyses are presented as tables of individual and total dissolved free amino acid levels, and graphs of the total free amino acid levels versus salinity.

7.2 Results.

Date:24.3.86 Dissolved free amino acids.(uM).

	Salinity.							
a.a.	0	2.5	7	10	15	20	25	32
Asp	0.037	0.177	0.289	0.126	0.174	0.155	0.047	0.096
Glu	-----	0.065	0.115	0.063	0.078	0.051	0.024	0.057
Ser	0.388	0.828	1.266	0.490	0.858	0.672	0.156	0.464
His	-----	0.092	0.199	-----	0.134	0.069	----------	
Gly	0.269	0.533	0.790	0.288	0.597	0.463	0.050	0.316
Thr	0.080	0.160	0.274	0.067	0.165	0.126	-----	0.032
Arg	-----	0.062	0.113	0.023	0.091	0.027	-----	0.067
Ala	0.123	0.230	0.318	0.153	0.223	0.190	0.066	0.120
Tyr	0.049	0.074	0.140	0.055	0.090	0.080	0.021	0.025
Abu	----------							
Val	0.108	0.360	0.156	0.137	0.215	0.165	0.036	0.067
Met	----------							
Trp/Ile	-----	0.097	0.167	-----	0.083	0.095	----------	
Phe	-----	0.061	0.099	----------				
Leu	0.033	0.093	0.121	----------		0.073	-----	0.038
Orn	0.188	0.317	0.490	0.189	0.327	0.227	-----	0.151
Lys	-----	0.115	0.167	-----	0.086	----------		
TOTAL(uM)	1.275	3.264	4.704	1.591	3.121	2.393	0.400	1.433

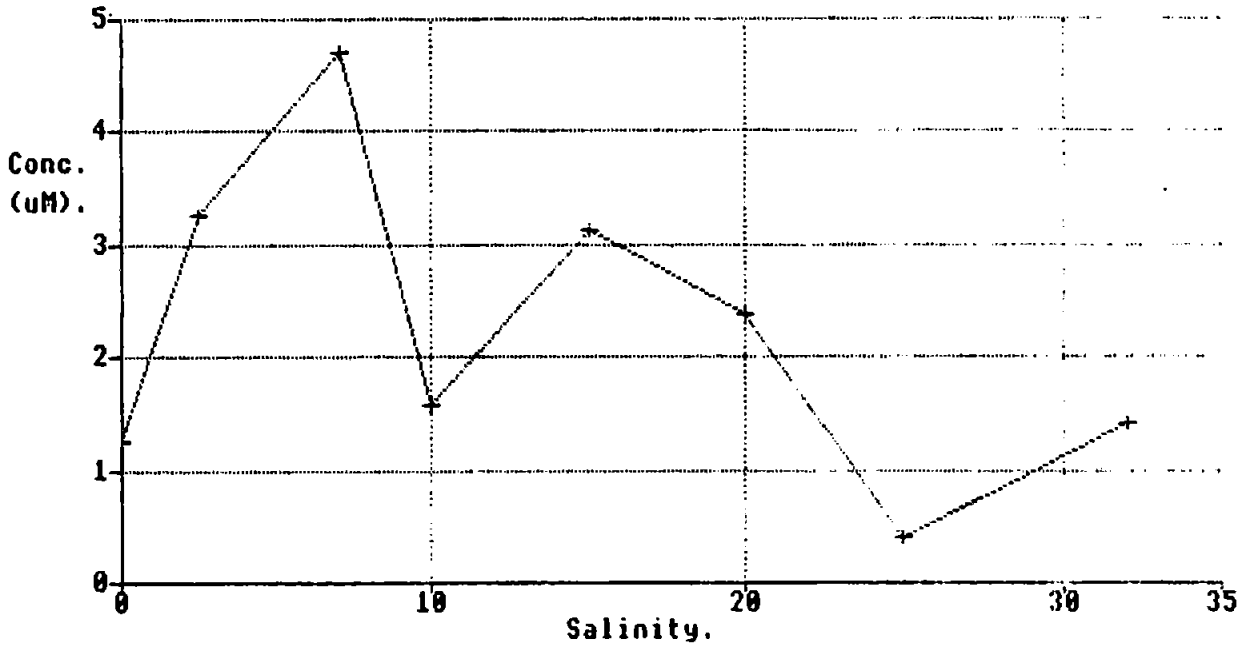


Fig 7.5 Dissolved Free Amino Acid Levels From An Estuary Sweep, 24.3.86.

Date: 21.5.86 Dissolved free amino acids.

Salinity.

a.a.	0	3	5	8	12	15	18	25	29
Asp	0.046	0.027	0.048	0.055	0.118	0.055	0.043	0.047	0.114
Glu	0.047	0.019	-----	0.027	0.049	0.032	0.037	0.037	0.059
Ser	0.155	0.079	0.088	0.235	0.428	0.215	0.063	0.178	0.314
His	----------								
Gly	0.139	-----	0.084	0.196	0.277	0.209	0.094	0.183	0.327
Thr	----------			0.030	----------				0.049
Arg	----------				0.038	0.061	----------		
Ala	0.075	0.033	0.060	0.078	0.166	----------		0.093	0.146
Tyr	----------			0.030	0.024	----------			0.020
Abu	----------								
Val	----------			0.039	0.058	----------			0.085
Met	----------								
Trp/Ile	----------								
Phe	----------								
Leu	----------								
Orn	----------				0.183	----------			
Lys	----------								
TOTAL(uM)	0.462	0.158	0.280	0.690	1.341	0.572	0.237	0.538	1.114

Date: 25.6.86 Dissolved free amino acid levels.

Salinity.

a.a.	0	3	5	8	12	25
Asp	0.070	0.458	0.302	0.161	0.111	0.171
Glu	0.042	0.136	0.092	0.057	0.042	0.086
Ser	0.291	2.035	1.119	0.575	0.332	0.738
His	-----	0.228	0.157	----------		0.107
Gly	0.154	1.038	0.759	0.296	0.196	0.447
Thr	0.048	0.380	0.269	0.105	0.043	0.141
Arg	-----	0.088	-----	0.065	-----	0.065
Ala	0.107	0.642	0.372	0.203	0.149	0.255
Tyr	0.044	0.179	0.099	0.064	0.015	0.069
Abu	----------					
Val	------	0.383	0.196	0.146	0.046	0.112
Met	----------					
Trp/Ile	------	0.195	0.070	0.033	-----	0.067
Phe	------	0.123	0.071	----------		
Leu	------	0.172	0.094	0.046	-----	0.074
Orn	------	0.504	0.424	0.276	-----	0.207
Lys	----------		0.118	----------		
TOTAL(uM)	0.756	6.561	4.142	2.028	0.934	2.539

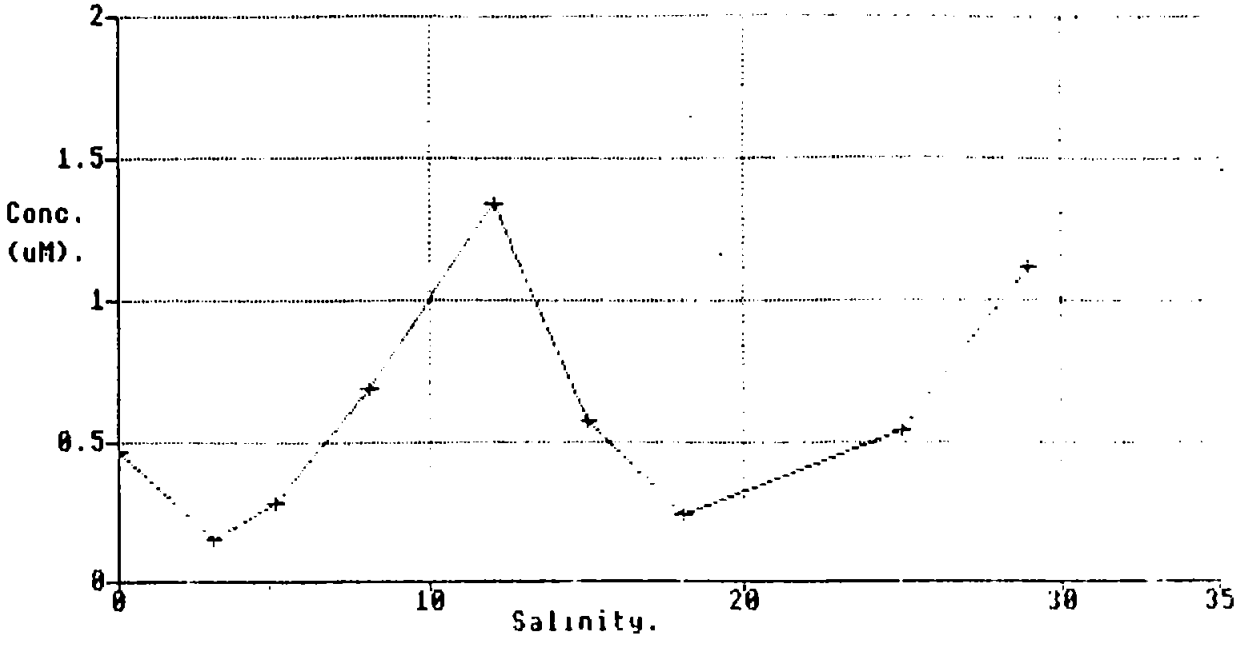


Fig 7.6 Dissolved Free Amino Acid Levels From An Estuary Sweep, 21.5.86.

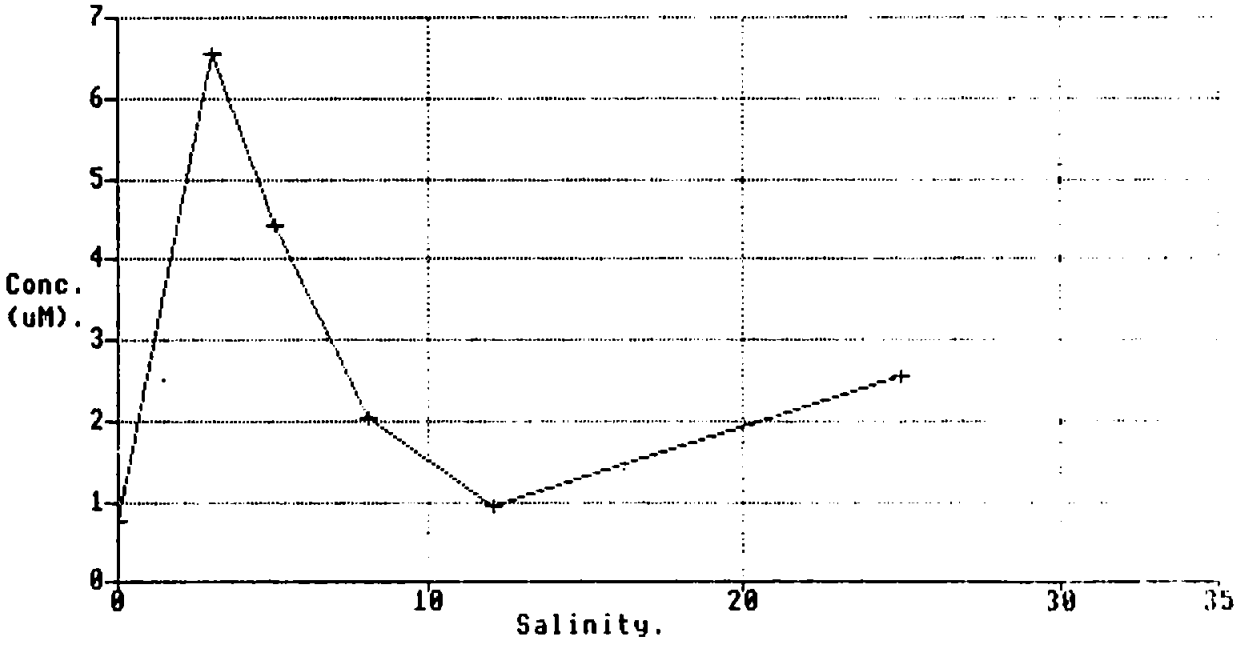


Fig 7.7 Dissolved Free Amino Acid Levels From An Estuary Sweep, 25.6.86.

Date: 18.9.86. Dissolved free amino acid levels.

	Salinity.					
a.a.	0	3	5	12	18	25
Asp	--------	0.026	0.046	-----	0.052	0.037
Glu	--------	0.036	0.071	0.026	-----	0.058
Ser	0.086	----------		0.098	0.114	0.087
His	----------					
Gly	--------	0.056	----------			
Thr	----------					
Arg	----------					
Ala	----------		0.044	0.032	0.051	0.046
Tyr	----------					
Abu	----------					
Val	----------					0.044
Met	----------					
Trp/Ile	----------					
Phe	----------					
Leu	----------			0.100	0.128	------
Orn	----------					
Lys	----------					
TOTAL(uM)	0.086	0.118	0.161	0.256	0.345	0.272

Date: 12.3.87

	Salinity.			
a.a.	12	18	25	29
Asp	0.039	0.041	0.046	0.046
Glu	0.035	0.047	0.054	0.047
Ser	0.048	0.132	0.095	0.155
His	----------			
Gly	------	0.097	0.125	0.124
Thr	----------			
Arg	----------			
Ala	----------			0.044
Tyr	----------			
Abu	----------			
Val	----------			
Met	----------			
Trp/Ile	----------			
Phe	----------			
Leu	----------			
Orn	----------			
Lys	----------			
TOTAL(uM)	0.122	0.317	0.320	0.416

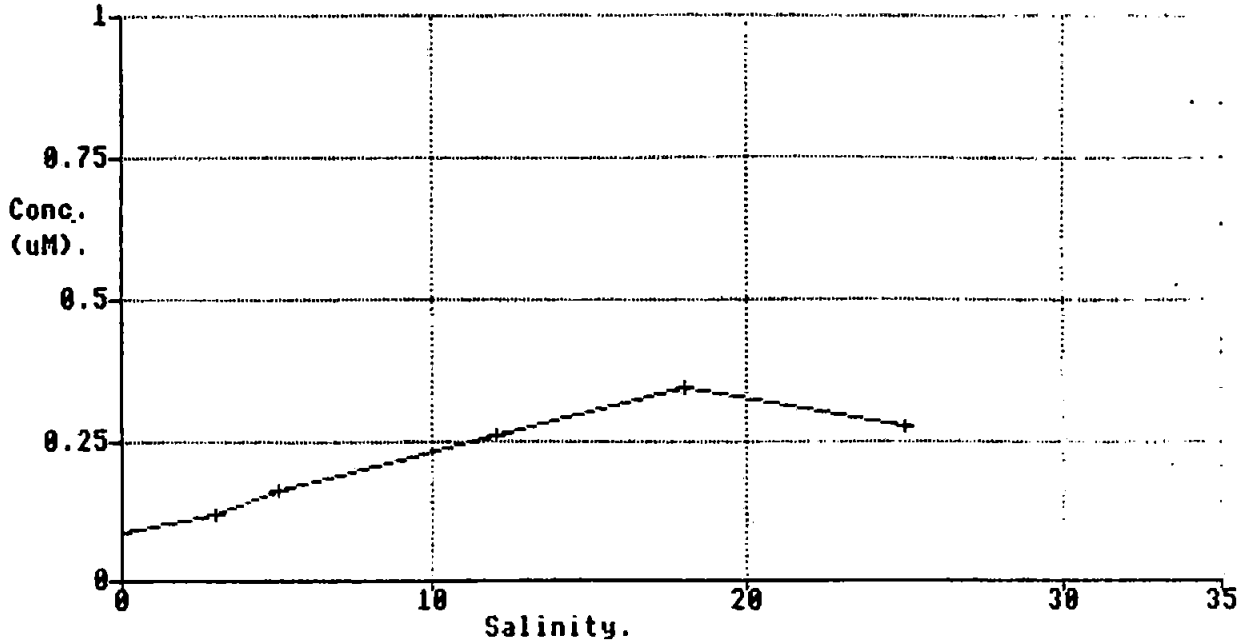


Fig 7.8 Dissolved Free Amino Acid Levels From An Estuary Sweep, 18.9.86.

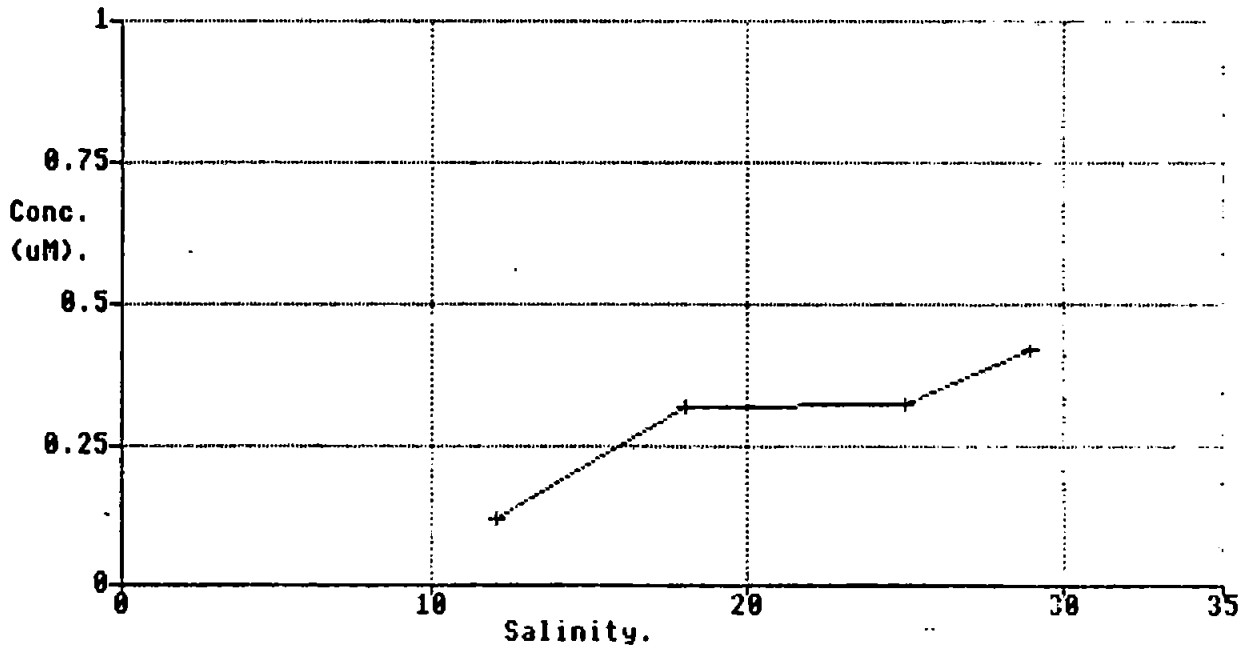


Fig 7.9 Dissolved Free Amino Acid Levels From An Estuary Sweep, 12.3.87.

7.3 Discussion.

The dissolved free amino acid levels, found in this small scale study for the Tamar estuary, are approximately three times those found for coastal waters reported in chapter 5. The dissolved free amino acid versus salinity profiles depicted in Fig. 7.5 to 7.7 show low levels of dissolved free amino acids in the upper fluvial area of the river, followed by amino acid maxima in the salinity region 3 to 12. There is then a fall in the levels of amino acids in the mid estuary, followed by a small increase in levels at the lower seaward end of the estuary. This indicates that dissolved free amino acids do not behave conservatively in the estuarine waters of the Tamar. There is an enrichment in the area of the fresh water/salt water interface and the turbidity maximum. This is followed by removal of acids in mid estuary and enrichment in the lower estuary. The total iron content for Tamar waters can reach levels of 2 mg/l from the humics and associated iron. Under laboratory conditions, it is known that iron can remove some dissolved free amino acids. However, there is little evidence that this happens in the Tamar under natural conditions. Investigations into the removal of dissolved free amino acids in natural systems is hampered by the complex nature of the speciation of iron in the estuarine waters. (Personal communication E.I.Butler).

Flynn & Butler (1986) conclude that the use of dissolved free amino acids by the phytoplankton is likely

to be most significant in the absence of photosynthesis e.g. at night or in areas of high turbidity. It would be expected therefore that the area of the turbidity maximum (salinity 1 to 5) would have low dissolved free amino acid levels. However the position is further complicated by the presence of the fresh water/salt water interface in the same area as the turbidity maximum. Fig. 7.8 shows a dissolved free amino acid versus salinity profile taken at a time when there was no turbidity maximum present in the water body. (Personal communication E. I. Butler). The high dissolved free amino acid levels in the area of low salinity were absent. This may indicate that the fresh water/salt water interface causes release of dissolved free amino acids from the particulate materials in the turbidity maximum as suggested by Morris (1978).

Examination of the dissolved free amino acid spectrum shows more acids present in samples with high free acid levels. An example of this can be seen in the percentage composition data for the 25.6.86, (Fig. 7.10). It can be seen from Fig. 7.10 that samples a and c are similar in both levels and composition whereas sample b differs markedly in both level and the number of acids present. These results would seem to support the suggestion by Morris (1978) that the fresh water/salt water interface found in the area of the turbidity maximum would show a different free acid composition from other areas of the estuary.

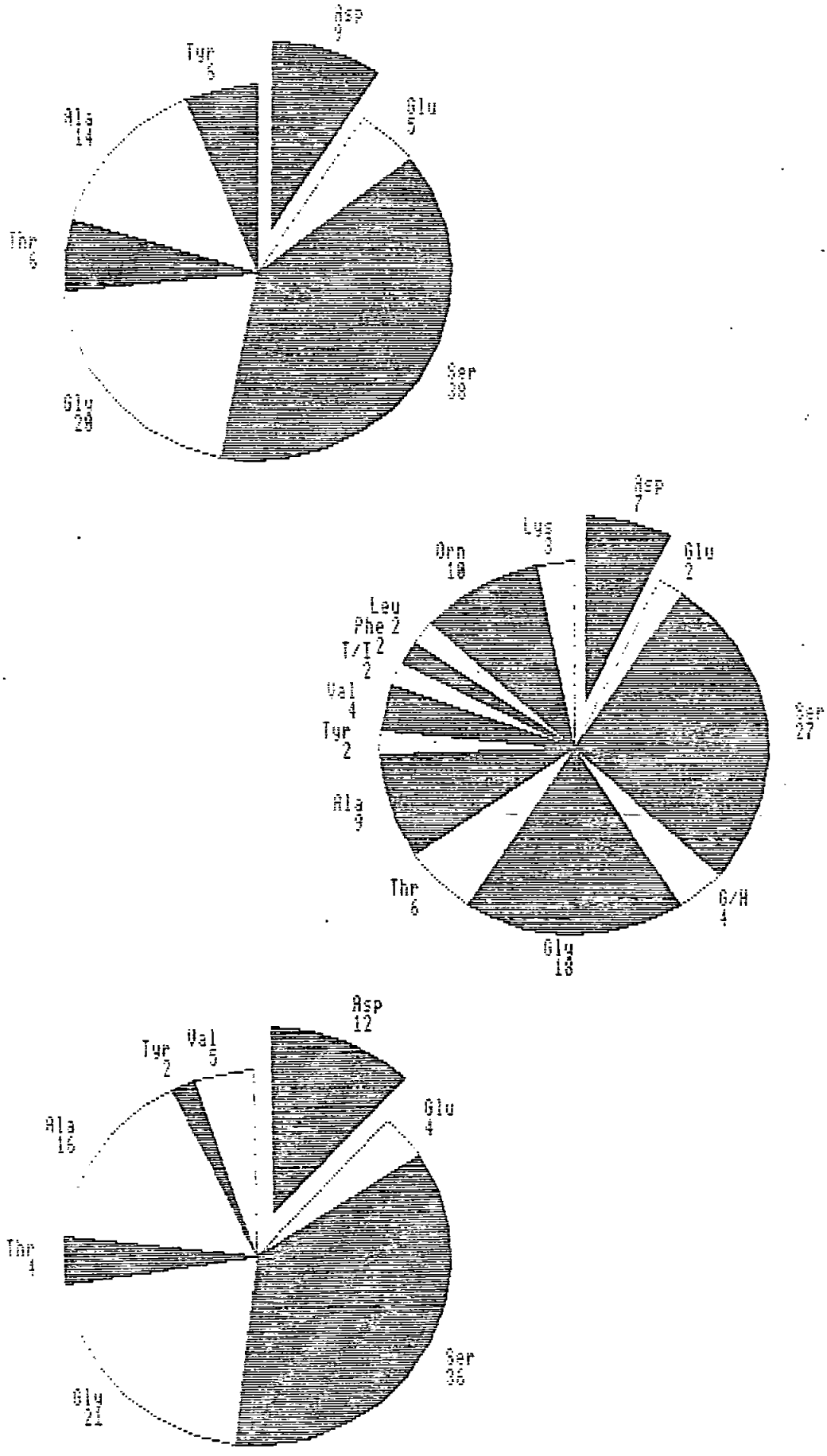


Fig 7.10 Percentage Composition Of The Dissolved Free Amino Acids In An Estuary Sample, 25.6.86. a) Salinity 0. b) Salinity 5. c) Salinity 12.

CHAPTER 8.

PRELIMINARY STUDIES OF DISSOLVED FREE AMINO ACID UPTAKE AND RELEASE BY PHYTOPLANKTON.

8.1 Introduction.

The seasonal results for the coastal dissolved free amino acid concentrations presented in Chapter 5, show a number of pronounced increases in the dissolved free amino acid levels followed by a return to lower concentrations. Samples with high dissolved free amino acid values showed a greater number of acids present than samples with low amino acid levels. The possible causes of the rise in free amino acid levels with the concomitant increase in the acid spectrum are: extracellular release by the boita, sloppy feeding of zooplankton, cell lysis and decay (Keller et al. 1982 Flynn & Butler 1986). The return to lower dissolved free amino acid levels accompanied by a reduction in the acid spectrum may be due to uptake by the biota or absorption onto particles.

A series of experiments was carried out as a preliminary investigation to determine whether phytoplankton uptake and release could be responsible for the increases and decreases in dissolved free amino acids found in the environmental samples. Much work on dissolved free amino acid release and uptake has been carried out using laboratory studies of phytoplankton cultures. As the rise and fall in dissolved free amino

acid levels found in the environment were from natural conditions, it was felt that any work on phytoplankton should be with as natural a population as possible. It was therefore decided, to use a skimming technique to concentrate the phytoplankton occurring naturally in the seawater, in order to determine if these populations exhibited extracellular release. The picture of uptake and release of organic material by phytoplankton has been shown to be more complex. In work with cultures, it has been shown that, a mechanism exists whereby phytoplankton store amino acids for use when nitrogen containing nutrients are at low levels. (Personal communication E.I.Butler, K.J.Flynn).

8.2 Skimming Experiments.

Phytoplankton were removed from the coastal waters using a fine tow net, obtained from the botany department of the Marine Biological Association of the U.K. (MBA), the plankton were then placed in approximately 5 cm3 of sea water in a glass vial. This skimming process effectively concentrates the phytoplankton. The phytoplankton sample was then added to approximately 20cm3 of sea water which had previously been analysed for for dissolved free amino acid content. The 20 cm3 of seawater was from the MBA research tanks. The phytoplankton were left in the MBA water in a covered plastic bottle for one hour. After that time 8 cm3 of water plus plankton were removed from the plastic bottle using an all glass syringe. A 0.22 um Millex-GV filter

was attached to the syringe and 1 cm3 of water filtered, 480 ul of which was analysed for dissolved free amino acid content. (The amount of water filtered compared to that removed was small, also, low pressures where employed during the filtering process to minimize the possibility of cell damage). The amino acid content of the two samples i.e. seawater only and seawater plus phytoplankton were compared to determine the effect of phytoplankton on the dissolved free amino acid levels and composition of the seawater sample.

The experiment outlined above was undertaken using samples of Coscinodiscus, Halosphera and diatoms which were green and buoyant, these were designated "healthy" cells. The experimnet was also carried out using cells of Halosphera and Coscinodicus which were brown in colour and were no longer buoyant, these were designated "dead" cells. The true physiological state of the cells was however unknown.

It should be noted that any experimentation on phytoplankton cells may lead to cell shock. It is difficult therefore to interpret whether the results of an experiment are similar to processes occuring in the environment or are merely a reflect the experiments impact on the phytoplankton cells.

8.3 Results.

The results of the skimming experiments are given in tables 8.1 to 8.8, and detail the changes in dissolved free amino acid levels between the seawater, and the

seawater plus phytoplankton, for the identifiable amino acids. The position of asparagine as a shoulder on the serine peak was known from the analyses of standard samples. However, extensive analyses of coastal seawater samples showed asparagine to be absent from the spectrum, and it was therefore removed from the standard calibration mixture. The presence of asparagine (Asn) in the skimmed phytoplankton samples is, therefore, reported without quantification. Also included in tables 8.1 to 8.8. are the retention times of unidentified dissolved free amino acids present in the seawater containing the phytoplankton. The peak at approximately 24.6 minutes, corresponding to an unknown compound, was seen occasionally in the results of the analyses of environmental samples. The other unknown compounds were only observed in the seawater containing concentrations of phytoplankton cells. Figures 8.1 to 8.8 show the chromatograms of the analyses from which tables 8.1 to 8.8 were derived.

Table 8.1 The Effect On The Dissolved Free Amino Acid Levels Of "Healthy" Coscinodiscus Added To Seawater.
Date: 10.3.87

Amino acids identi-fied.	Amino acids in seawater. (uM)	Amino acids in seawater plus plankton. (uM).	Increase in amino acids. (uM).	Retention time of unidenti-fied compounds. (minutes).
Asp	0.062	0.175	0.113	13.33
Glu	0.050	0.162	0.112	24.62
Asn				27.11
Ser	0.312	1.312	1.000	
His	-----	0.312	0.312	
Gly	0.188	1.575	1.378	
Thr	-----	0.300	0.300	
Arg	-----	0.575	0.575	
Ala	0.075	0.850	0.775	
Tyr	-----	0.188	0.188	
Val	0.100	0.562	0.462	
Met	-----	0.188	0.188	
Trp/Ile	-----	0.500	0.500	
Phe	-----	0.138	0.138	
Leu	-----	0.800	0.800	
Orn	0.125	0.250	0.125	
Lys	-----	0.475	0.475	

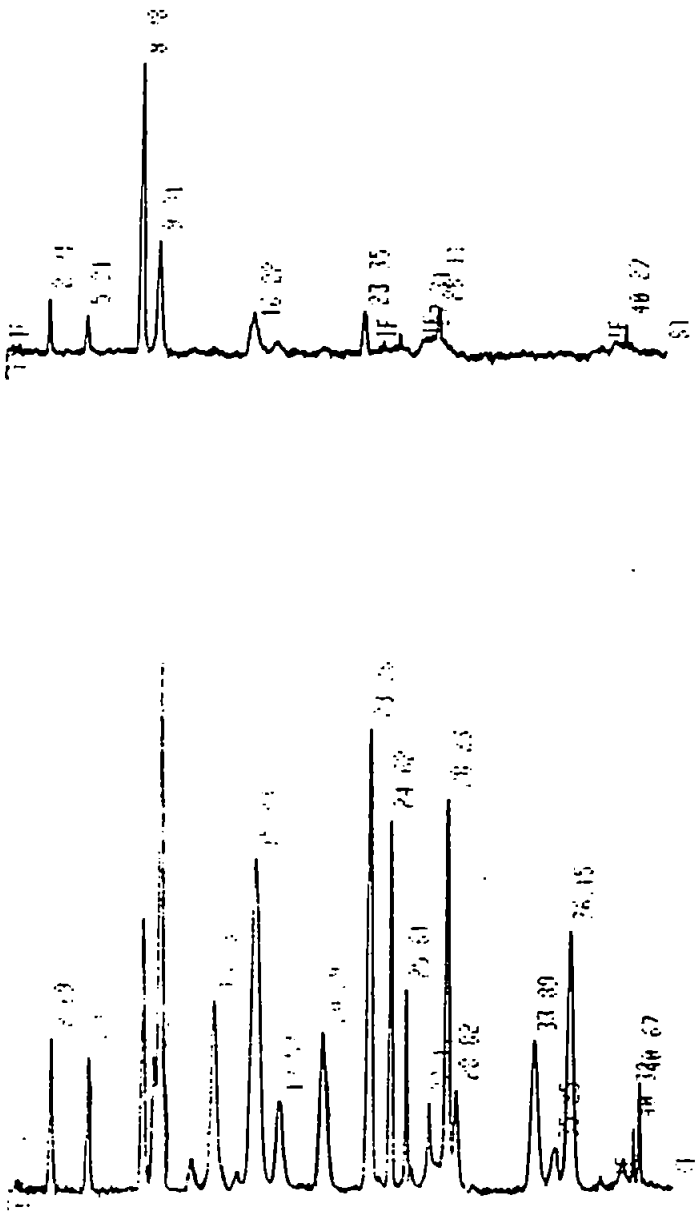


Fig 8.1 DFAA Content Of: a) MBA Seawater. b) The Same MBA Seawater + Healthy Coscinodiscus.

Table 8.2. The Effect On Dissolved Free Amino Acid Levels Of "Healthy" Coscinodiscus And Halosphera Added To Seawater. Date: 20.3.87.

Amino acid identi-fied.	Amino acids in seawater (uM).	Amino acids in seawater plus plankton. (uM).	Increase in amino acids. (uM).	Retention times of unident-fied compounds. (mins).
Asp	0.032	0.646	0.614	0.40
Glu	0.038	0.075	0.037	0.55
Ser	0.161	-----	-----	0.99
Gly	0.079	-----	-----	1.82
Arg	-----	0.356	0.356	6.06
Ala	0.053	0.074	0.021	14.66
Tyr	-----	0.862	0.862	18.98
Trp/Ile	-----	0.047	0.047	20.39
Leu	-----	0.842	0.842	22.34
Lys.	-----	0.336	0.336	24.63
				25.05
				25.85
				27.08
				39.55

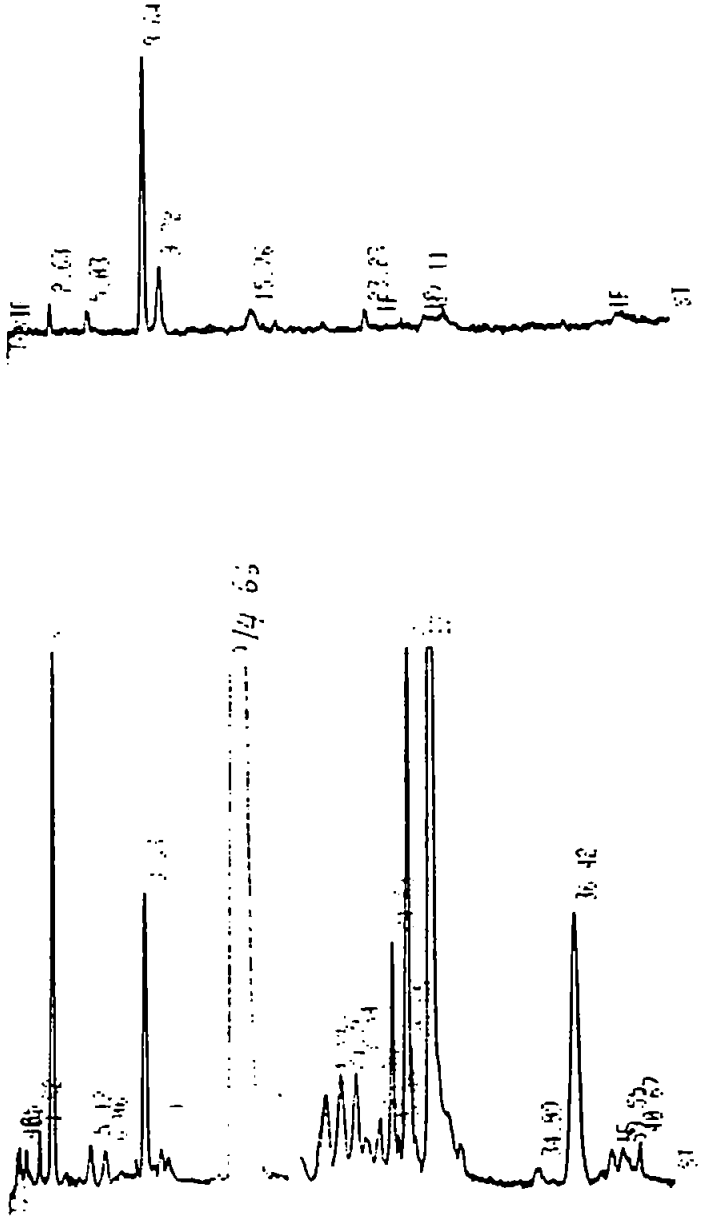


Fig 8.2 DFAA Content Of: a) MBA Seawater. b) The Same MBA Seawater + Healthy Coscinodiscus and Halosphera.

Table 8.3. The Effect On Dissolved Free Amino Acid Levels Of "Healthy" Halosphera Added To Seawater.
Date: 25.3.87

Amino acids identi-fied.	Amino acids in seawater (uM).	Amino acids in seawater plus plankton. (uM).	Increase in amino acids (uM).	Retention time of unidenti-fied compounds. (minutes).
Asp	0.025	0.162	0.137	6.55
Glu		0.325	0.325	12.93
Ser		0.500	0.500	14.63
Gly		1.100	1.100	22.11
Thr		0.287	0.287	24.46
Arg		0.300	0.300	25.88
Ala		0.625	0.625	26.98
Tyr		0.125	0.125	
Val		0.225	0.225	
Met		0.088	0.088	
Trp/Ile		0.225	0.225	
Phe		0.125	0.125	
Leu		0.225	0.225	
Lys		0.275	0.275	

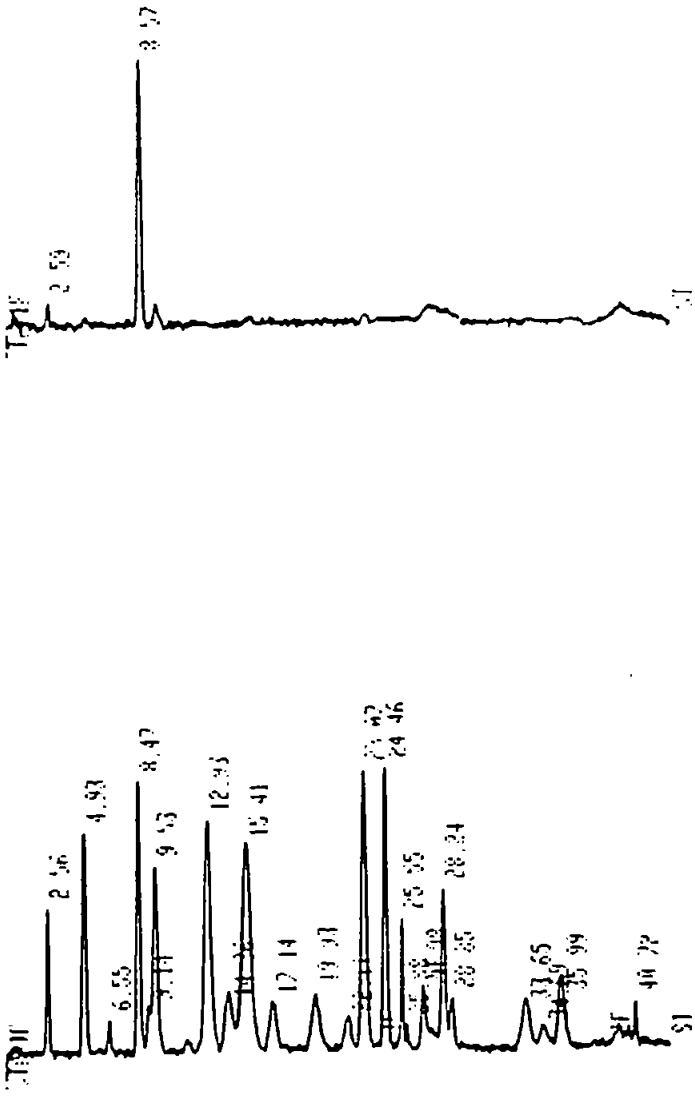


Fig 8.3 DFAA Content Of: a) MBA Seawater. b) The Same MBA Seawater + Healthy Halosphera.

Table 8.4. The Effect On Dissolved Free Amino Acid Levels Of "Healthy" Diatoms Added To Seawater.
Date: 25.3.87

Amino acids identi-fied.	Amino acids in seawater (uM).	Amino acids in seawater plus plankton. (uM).	Increase in amino acids. (uM).	Retention times of unidenti-fied compounds. (minutes).
Asp	0.062	0.200	0.138	6.48
Glu	0.037	0.325	0.288	12.82
Asn				14.22
Ser	0.300	0.775	0.475	21.94
Gly	-----	1.487	1.487	24.42
Thr	-----	0.337	0.337	26.95
Arg	-----	0.375	0.375	39.66
Ala	0.112	0.688	0.576	
Tyr	0.025	0.150	0.125	
Val	0.037	0.225	0.188	
Met	-----	0.088	0.088	
Trp/Ile	-----	0.250	0.250	
Phe	-----	0.138	0.138	
Leu	-----	0.250	0.250	
Orn	0.112	0.125	0.013	
Lys	-----	0.262	0.262	

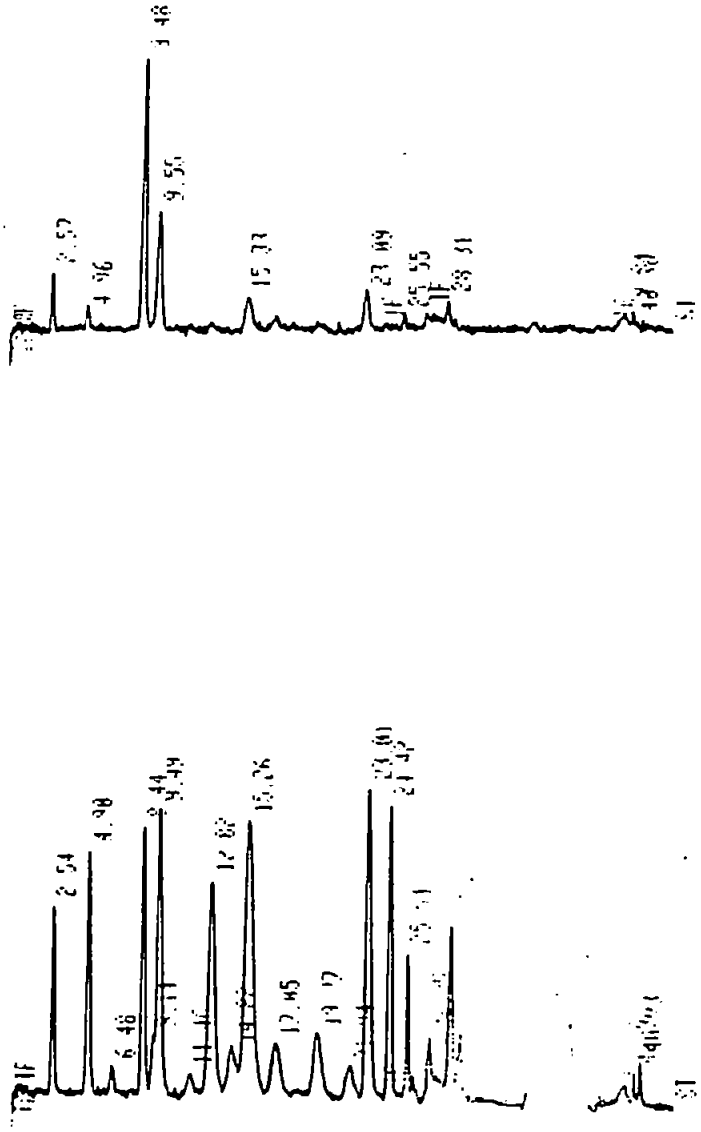


Fig 8.4 DFAA Content Of: a) MBA Seawater. b) The Same MBA Seawater + Healthy Diatoms.

Table 8.5. The Effect On Dissolved Free Amino Acid Levels Of "Healthy" Halosphera Added To Seawater.
Date: 26.3.87

Amino acids identified.	Amino acids in seawater (uM).	Amino acids in seawater plus plankton. (uM).	Increase in amino acids. (uM).	Retention times of unidentified compounds. (minutes).
Asp	0.200	0.362	0.162	12.85
Glu	0.075	0.475	0.400	21.09
Ser	1.250	2.675	1.425	24.39
Gly	0.600	2.700	2.100	25.88
Thr	0.138	0.825	0.687	26.97
Arg	-----	1.012	1.012	39.67
Ala	0.275	2.800	2.525	
Tyr	0.050	0.675	0.625	
Val	0.088	1.775	1.687	
Met	-----	0.587	0.587	
Trp/Ile	-----	1.250	1.250	
Phe	-----	0.600	0.600	
Leu	0.038	2.113	2.075	
Orn	0.250	0.388	0.138	
Lys	-----	1.187	1.187	

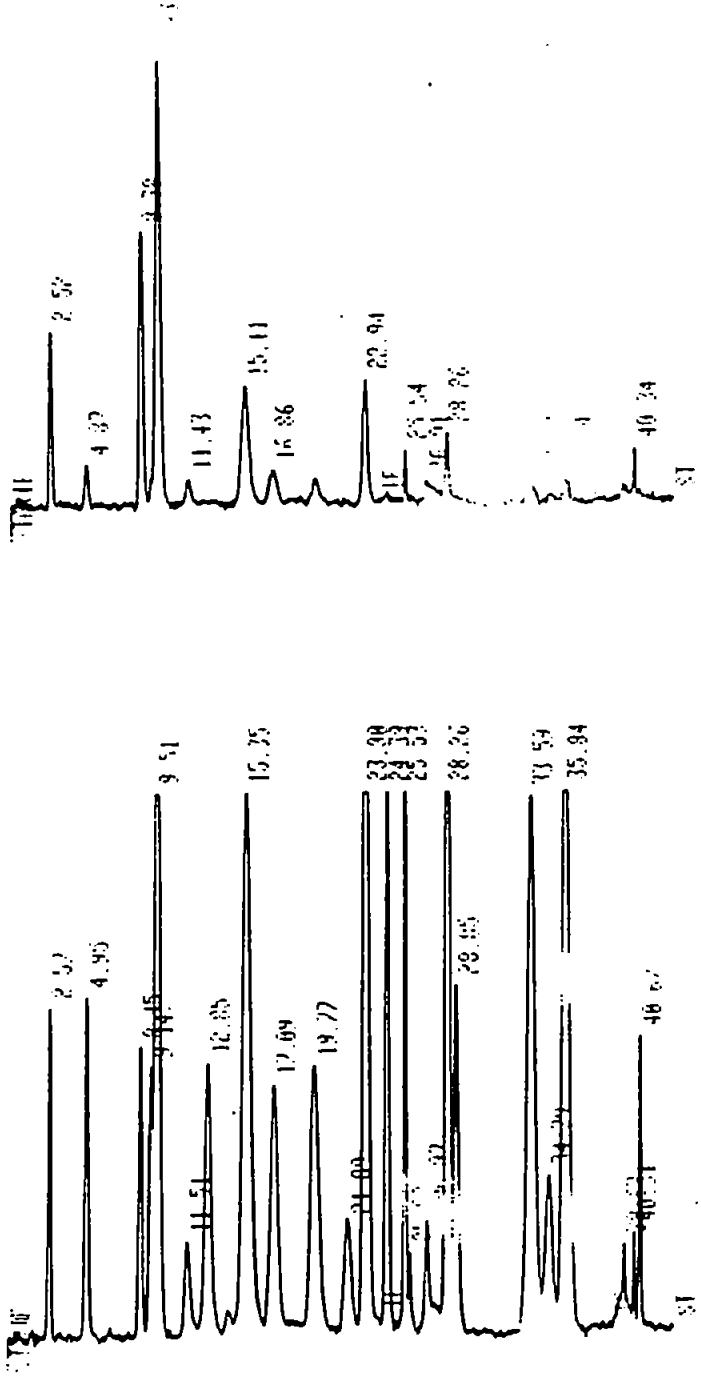


Fig 8.5 DFAA Content Of: a) MBA Seawater. b) The Same MBA Seawater + Healthy Halosphera.

Table 8.6. The Effect On Dissolved Free Amino Acid Levels Of "Healthy" Halosphera Added To The Seawater. Date: 8.4.87

Amino acids identi-fied.	Amino acids in seawater (uM).	Amino acids in seawater plus plankton. (uM).	Increase in amino acids (uM).	Retention times of unident-ified compounds. (minutes).
Asp		0.112	0.112	0.39
Glu	0.050	0.212	0.162	13.21
Asn				22.18
Ser	0.062	3.375	3.313	24.54
His		0.975	0.975	25.91
Gly		1.462	1.462	27.05
Thr		1.265	1.265	38.74
Arg		1.387	1.387	39.66
Ala		3.762	3.762	
Tyr		0.787	0.787	
Val		2.500	2.500	
Met		0.775	0.775	
Trp/Ile		2.887	2.887	
Phe		0.775	0.775	
Leu		2.500	2.500	
Orn		0.125	0.125	
Lys		2.875	2.875	

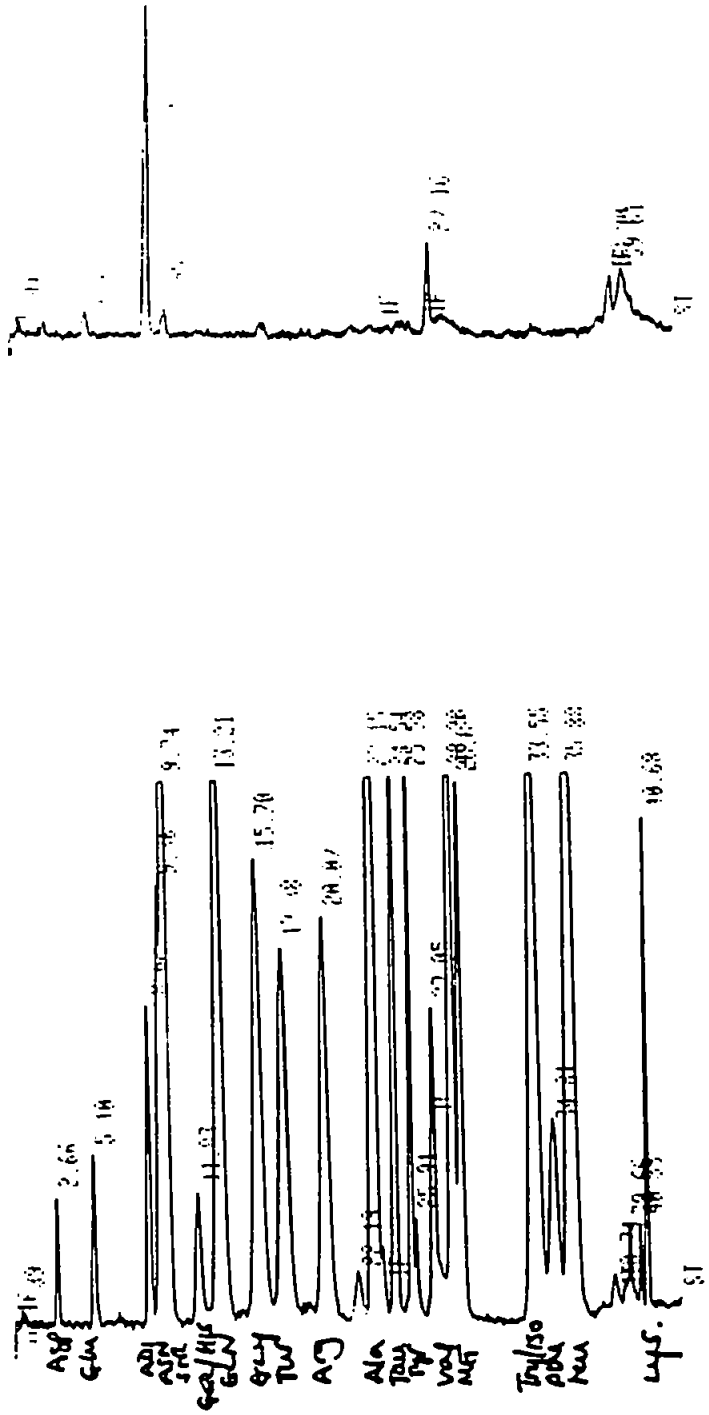


Fig 8.6 DFAA Content Of: a) MBA Seawater. b) The Same MBA Seawater + Healthy Halosphera.

Table 8.7. The Effect On Dissolved Free Amino Acids Of "Dead" Coscinodiscus Added To Seawater. Date: 10.3.87.

Amino acids identi-fied.	Amino acids in seawater (uM).	Amino acids in seawater plus plankton. (uM).	Increase in amino acids. (uM).	Retention times of unident-ified compounds. (minutes).
Glu	-----	0.142	0.142	27.15
Tyr	-----	0.051	0.051	

Table 8.8. The Effect On Dissolved Free Amino Acids Of "Dead" Halosphera Added To Seawater. Date: 26.3.87.

Amino acids identi-fied.	Amino acids in seawater (uM).	Amino acids in seawater plus plankton. (uM).	Increase in amino acids. (uM).	Retention times of unident-ified compounds. (minutes).
Asp	0.041	0.089	0.048	6.39
Glu	0.107	0.034	-----	13.92
Ser	0.109	0.144	0.035	24.34
Ala	-----	0.072	0.072	26.81
Val	-----	0.067	0.067	
Trp/Ile	-----	0.133	0.133	
Leu	-----	0.080	0.080	

8.4. Discussion.

The skimming experiments produced several interesting results. Firstly under the experimental conditions used there is a radical difference in amino acid content of water containing "healthy" phytoplankton cells compared to water containing "dead" phytoplankton. The large increases in free8 amino acid levels were associated with water containing "healthy" buoyant cells.

The number of amino acids for the samples containing "healthy" phytoplankton, were greater than those observed for dissolved free amino acids measured in coastal seawater samples at any time of the year. Thus,

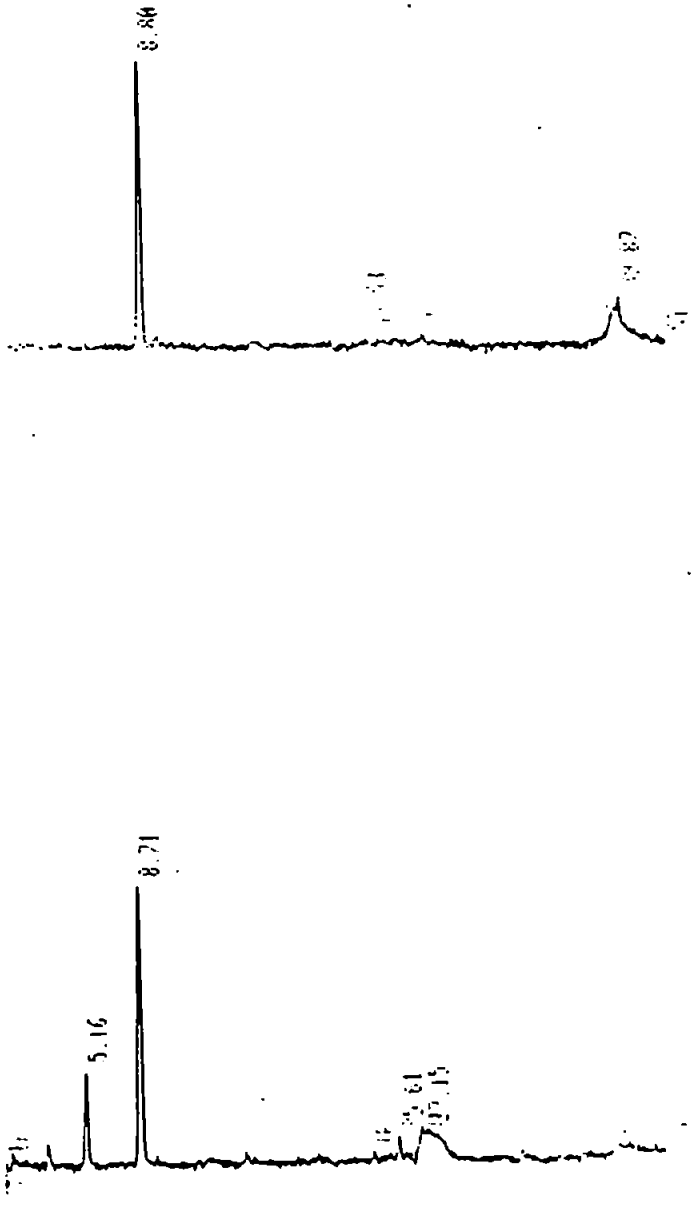


Fig 8.7 DFAA Content Of: a) MBA Seawater. b) The Same MBA Seawater + Dead Coscinodiscus.

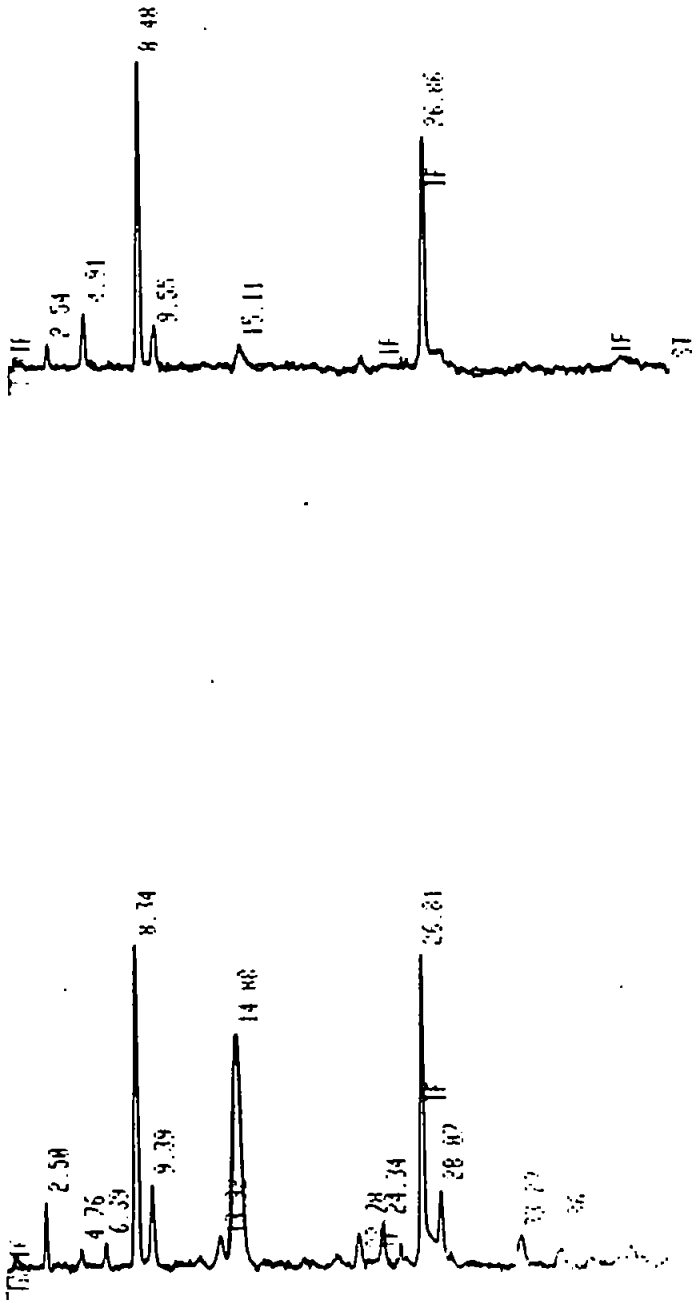


Fig 8.8 DFAA Content Of: a) MBA Seawater. b) The Same MBA Seawater + Dead Halosphera.

lysine, arginine and phenylalanine were present in the samples containing phytoplankton, whereas these three acids were found only infrequently in the environmental samples. Methionine and asparagine are present in the water containing "healthy" phytoplankton, but are not seen at all in coastal seawater samples. Threonine, valine, methionine, tryptophan, isoleucine, leucine, lysine and phenylalanine are the eight amino acids classed as essential; this classification being based on mammalian biochemistry. A similar list is known for ciliate, insect and bird species (Abercrombie et al. 1980). The term essential means that heterotrophs cannot produce these acids by modification of other compounds in the diet and therefore must obtain them from the environment. These essential acids are present in significant amounts in the water containing concentrations of phytoplankton but are absent much of the time from coastal samples. This may indicate a preferential usage by marine heterotrophs.

Four of the five samples of seawater plus "healthy" phytoplankton contain large amounts of serine, glycine and alanine. These are low molecular weight, non-essential acids, with medium carbon to nitrogen ratios; (glycine,5 alanine,6 and serine,7).The seasonal study of free amino acids in seawater, conducted as part of this study, shows these three acids to be major constituents of the dissolved free amino acid fraction throughout the year. Their presence may be explained by a preferential excretion of these acids by phytoplankton.

The possiblity of uptake of dissolved free amino acids by natural populations of phytoplankton were investigated during the July 1987 cruise of the RRS Frederick Russell; the detailed results of these experiments will be reported elsewhere. Phytoplankton were isolated by passing seawater through netting of a size that allowed only the passage of phytoplankton and smaller organisms. The phytoplankton were then kept in the dark for twelve hours, after which the batches of plankton were exposed to different nutrients, including the three amino acids arginine, histidine and glycine. These acids were spiked into the seawater to produce a total added amino acid concentration of 2 ug at. N/l. Added amino acids levels were measured before and after incubation. In one of the four experiments conducted with added amino acids, the levels were reduced significantly, the arginine and histidine falling below the limits of detection of the method. (Chapter 2).

The experiments described above, show the possibilty that the rise and fall of the dissolved free amino acids seen in the seasonal results may be due to excretion and uptake of free amino acids by natural phytoplankton populations. The role of heterotrophs, particularly bacteria, in the uptake of organic compounds was not investigated in these preliminary studies.

Examination of the retention times of unidentified compounds seen in tables 8.1 to 8.8 show that several of these unidentified compounds occur in the majority of the chromatograms e.g. the peak at 12.85 to 12.93 minutes, one at 24.39 to 24.62 and one at 14.66 to 14.88 minutes.

An attempt was made to identify some of these unknown compounds, by selecting acids and determining their retention times, followed by comparison with those of the unidentified compounds in the analyses of the water plus phytoplankton samples.

Several strategies were used to select amino acids to be tested as possible candidates for the unknown compounds. Firstly, compounds related to those acids which were dominant in the dissolved free fraction, e.g. Beta-alanine is a decarboxylation product of aspartic acid, while ethanolamine is a decarboxylation product of serine. Glutamine is the monoamide of glutamic acid. Glucosamine is a simple amino sugar and had been investigated as a possible nutrient source for the growth of phytoplankton. (Antia et al. 1975). Secondly, compounds which are precussors e.g. anthranilic acid which is a precussor of phenylalanine, tyrosine and tryptophan. Also 5-aminolaevulonic acid which is a precussor of chlorophyll and whose presence could possibly indicate a plant source. Thirdly di-aminopimelic acid has been reported as occuring in environmental samples. Finally, the work on identifying unknown compounds provided an opportunity to determine the retention time of taurine which according to Jefferies (1969) is a product of the metabolism of sulphur containing amino acids in animals. This does not occur in plants or detritus and therefore taurine can be used as an index of the relative amounts of plant or animal components. However, Jorgenson (1987), states that taurine is a product of algal exudation as well as

zooplankton excretion. The author also ascribes the presence of beta-alanine and alpha-amino butyric acid to both bacterial and zooplankton metabolism.

Of the 8 compounds tested, 4 had similar retention times to those of the unidentified compounds in the water plus phytoplankton chromatograms. These 4 are; glutamine at approximately 12.9 minutes, Ethanolamine at about 27.2 minutes, taurine at about 24.6 mins and glucosamine which co-elutes with histidine at approximately 12.0 minutes. The presence of a peak with a similar retention time to that of taurine was unexpected, the identification of compounds by co-injection can only be tentative. The only reliable method of positive identification is by high performance liquid chromatography linked to a mass spectrometer. In discussion with Kratos, a manufacturer of mass spectrometer systems, there has currently been no attempt to identify amino acids from the mass spectra of their orthophthalaldehyde derivatives.

Analysis of the macromolecular fraction, showed histidine to be a major component of that fraction. The problem of trying to determine if the peak was in fact histidine or glucosamine was investigated as follows. Glucosamine was subjected to the hydrolysis procedure, and it was found to be stable under the conditions employed in the acid hydrolysis. A sample of glucosamine, which is an amino sugar, was then subjected to 2M alkali, which caused the degradation of the compound. Nevertheless, it also introduced the increased likelihood of contamination. The problem of the coelution of histidine and glucosamine was discovered very near the

end of the practical work on this project. Therefore, due to lack of time, no definitive method has been devised to determine whether the dissolved combined amino acids contains glucosamine, histidine or both.

CHAPTER 9.

CONCLUDING REMARKS.

9.1 A Summary Of The Findings Of The Present Study.

The purpose of this chapter is to summarize the findings of the present study. These can be divided into two broad areas; the hydrolysis methodology for the determination of the dissolved combined amino acid composition and levels in seawater, and the environmental results collected over the season.

It should be emphasized that protein hydrolysis techniques are usually carried out at protein levels of approximately 10^{-3} g ml^{-1}. However the levels of dissolved combined amino acids in seawater are several orders of magnitude lower than this at approximately 10^{-7} g ml^{-1}. The work on hydrolysis methodologies undertaken in this project has shown that the method most commonly used to determine the values for the dissolved combined amino acid fraction i.e. refluxing seawater with 6M hydrochloric acid at atmospheric pressure produces inaccurate results due to a combination of degradative losses and contamination. The method adopted in this work, which employs a low pressure acid hydrolysis, gives an average recovery, of free amino acids from a protein, of 61 percent. The inclusion of both a procedural blank and an internal standard in the hydrolysis procedure are essential if realistic results are to be obtained. The procedural blank monitors contamination levels, while the

internal standard monitors the hydrolysis and analytical techniques.

The seasonal study of the dissolved combined and dissolved free amino acids, in coastal waters, showed the average levels to be 1.7 and 0.403 uM respectively. Therefore the dissolved combined amino acid levels are approximatley 4 times those of the free fraction. This difference in levels can be interpreted in another way: there is a "disappearance or loss" of approximately 1.3 umoles of nitrogen containing molecules from the combined to the free fraction. This loss may be explained by the uptake of parts of the dissolved organic nitrogen by the marine biota. The uptake of dissolved free amino acids by both phytoplankton and heterotrophs is well documented (Chapter 1).

The dissolved free amino acids show a seasonal trend in levels being high in the summer and low in the winter. This pattern is similar to that for dissolved organic nitrogen, (of which the dissolved combined and dissolved free amino acids are a part), but contrasts with that of other nutrients such as nitrate, ammonia and urea. (Flynn & Butler). There is no such pronounced seasonal pattern for the dissolved combined fraction. Both the dissolved free and dissolved combined fractions show fluctuations in levels, which are particularly pronounced for the dissolved free amino acid fraction (Chapter 5). The increase in dissolved free amino acids levels is accompanied by an increase in the number of acids present in the spectrum. Three distinct patterns can be observed for the dissolved free amino acids. Firstly there are

those acids which are present during most of the sampling period. These acids also exhibit a seasonal trend having lower levels in the winter months. Amino acids showing this pattern are aspartate, glutamate, serine, alanine and glycine. All of these acids, with the exception of glutamate, are small molecules (Doolittle 1985). Secondly there are acids which are present only at times of high total dissolved free amino acid levels, these are histidine, threonine, phenylalanine, arginine, leucine, lysine and ornithine, which are generally larger acids. Finally there are those acids never seen in the seawater samples e.g. methionine and aspargine, but which are present in experiments with phytoplankton (Chapter 8).

The dissolved combined amino acid spectrum is far more consistent than that of the free fraction. However, acids that do show a seasonal trend in the dissolved combined amino acid spectrum are aspartate, histidine and threonine which are lower in the winter months, October to January.

The dominant acids in the free fraction are aspartate, glutamate, serine, glycine and alanine. Whereas the dominant acids in the combined fraction are glycine, histidne, aspartate, glutamate, alanine and valine.

Comparison of the composition and levels of acids between the combined and the free fraction allows for an identification of those acids which are present in the combined but not in the free and a quantification of this "disappearance or loss". Acids with a "disappearance" value of 90 percent or greater are histidine, glycine,

threonine, arginine, tyrosine/isoleucine, and phenylalanine. Serine has the lowest "disappearance" value of only 51 percent. These differences in "disappearance" value coupled with the "loss" of even major acids from the dissolved free amino acid fraction may be linked with utilization of the acids by the biota.

Comparison of the amino acid composition of the free and combined fractions with that of phytoplankton cultures shows that both the free and combined fractions do not resemble those of the phytoplankton, which must be their fundamental source. There is therefore no simple picture occurring in the marine environment of the breakdown of phytoplankton, followed by release of dissolved free amino acids. The uptake and release of acids by the biota produces a more complex picture.

The experiments to discover the size of the macromolecular species, carried out as part of this study, show the macromolecules to be of a small size; fifty percent of the material being less than 250 amino acids in length. The material with a molecular weight greater than 25,000 appears to have a high glycine content.

The investigation of the composition and levels of dissolved free amino acids in the Tamar estuary showed that these compounds exhibit non-conservative behaviour. There was an increase of the dissolved free amino acids in the area of the fresh water/salt water interface which overlaps the turbidity maximum. The increase in levels was accompanied by an increase in the number of acids present in the compositional spectrum. These findings

support the suggestion of Morris (1978) that the area of the fresh water/salt water interface would differ in both composition and levels from other areas of the estuary; the difference being a result of the osmotic shock experienced by the biota in that area.

In conclusion, the results of this study indicate that in the natural marine environment there is a complex process of production and breakdown of dissolved combined and dissolved free amino acids. Overall there is a difference of 1.3 umoles of nitrogen containing molecules between the two fractions. The significance of amino acid uptake compared to that for ammonia or nitrate is hard to quantify, but in the absence of other nitrogen sources it appears likely that some amino acids supply nitrogen for growth. Therefore the results of the seasonal study of nitrogen containing macromolecules in coastal waters supports the view that nutrient limitation along classical lines, as explained in chapter 1, is not valid.

9.2 Further Work.

Areas of further work are:

1. The hydrolysis technique, to reduce the degradative losses of free acids in waters with high nitrate levels. Robertson et al. (1987). suggest the use of ascorbic acid to reduce these losses; initial work would be to verify this approach. Once the methodology has been developed an investigation of the macromolecular nitrogen containing

species in the estuarine environment could be undertaken. It would be expected that in the estuary the amino acids would play an even more important role than in coastal waters. Flynn & Butler (1986) state that dissolved free amino acids are more likely to be used as a nutrient source where photosynthesis is limited, for example, in the dark or in turbid waters. As stated in chapter 7 the Tamar estuary can have very high turbidity levels and therefore the amino acids may be an important nutrient/energy source.

2. The environmental data collected during the course of this study was correlated with figures for primary productivity and phytoplankton numbers from past papers. The correlations can therefore only be tentative. Further work matching levels of dissolved free and dissolved combined acids with environmental data measured at the same time will be necessary to determine how these compounds are related to environmental factors. Further study in this area may also confirm that different parts of the season are controlled by different factors or as the 'Gaia Hypothesis' suggests (Lovelock 1979), the environment is in fact controlled by the biota.

3. In this study a list of acids and their "disappearance" values has been produced. Further work with natural populations of phytoplankton on uptake of dissolved free amino acids may confirm if those acids with "disappearance" values greater than 90 percent do in fact serve as a nutrient source.

4. The role of heterotrophs i.e. bacteria and zooplankton in the uptake and production of dissolved organic nitrogen.

5. An investigation into the possibility that the breakdown of glycine in the combined fraction provides ammonia as a nutrient source.

6. The seasonal change found in the dissolved combined amino acid fraction merits further work. Does this occur in other seasons and other locations? If there is a difference, a study of two differing systems can lead to an understanding of the processes and factors controlling those systems.

REFERENCES.

Abercrombie, M.; Hickman, C.J. & Johnson, M.L. (1980). *The Penguin Dictionary of Biology, 7th ed.*

Admiraal, W.; Laane, R.W.P.M. & Peletier, H. (1984). Participation of diatoms in the amino acid cycle of coastal waters; uptake and excretion in cultures. *Mar. Ecol. Prog. Ser.*, 15, 303-306.

Admiraal, W.; Peletier, H. & Laane, R.W.P.M. (1986). nitrogen metabolism of marine planktonic diatoms: excretion, assimilation and cellular process of free amino acids in seven species with different cell size. *J. Expt. Mar. Biol. Ecol.*, 98, 241-263 .

Allen, S.E.; Grimshaw, H.M.; Parkinson, J.A. & Quarmby, C. (1974). In: Allen, S.E. (ed). *Chemical Analysis of Ecological Materials.*

Amano, M.; Hara, S. & Taga, N. (1982). Utilization of dissolved amino acids in seawater by marine bacteria. *Marine Biology*, 68, 31-36.

Andrews, P. & Williams, P.J. (1971). Heterotrophic utilization of dissolved organic compounds in the sea. *J. Mar. Biol. Ass. UK.*, 51, 111-125.

Antia, N.J.; Berland, B.R.; Bonini, D.J. & Maestrini, S.Y. (1975). Comparative evaluation of certain organic and inorganic sources of nitrogen for phototrophic growth of marine microalgae. *J. Mar. Biol. Ass. UK*, 55, 519-539.

Azam, F.; Fenchel, T.; Field, J.G.; Gray, J.S.; Meyer-Reil, L.A. & Thingstad, F. (1983). The ecological role of water-column microbes in the sea. *Mar. Ecol. Prog. Ser.*, 10, 251-263.

Bada, J.L. & Lee, C. (1977). Decomposition and alteration of organic compounds dissolved in seawater. *Mar. Chem.*, 5, 523-534.

Bada, J.L.; Hoopes, E. & Ho, M. (1982). Combined amino acids in Pacific Ocean waters. *Earth Planet. Sci. Lett.*, 58, 276-284.

Bauerfeind, S. (1985). Degradation of phytoplankton detritus by bacteria: estimation of bacterial consumption and respiration in an oxygen chamber. *Marine Ecology Progress Series*, 21, 27-36.

Benson J.R. & Hare P.E. (1975). Orthopthaldialdehyde: fluorogenic detection of primary mines in the picomole range. Comparison with fluorescamine and ninhydrin. *Proc. Nat. Acad. Sci. USA.*, 72, 2, 619-622.

Beyermann, K. (1984). *Organic Trace Analysis.*

Bidigare, R.R. (1983). Nitrogen excretion by marine zooplankton. In: Carpenter, E.J. & Capone, D.G. (eds). *Nitrogen in the Marine Environment.*

Bishop, A.D. & Loudon, L.R. (1965). Separation and identification of amino acids in Galveston and Baffin Bay Texas. In: *Ocean Sci. and Engineering*, 2, 1104-1108.

Boalch, G.T.; Harbour, D.J. & Butler, E.I. (1978). Seasonal phytoplankton production in the Western English Channel, 1964-1974. *J. Mar. Biol. Ass. UK.*, 58, 943-953.

Bohling, H. (1970). Dissolved amino acids in seawater of the North Sea near Helegoland: Concentration changes during the summer 1970. *Mar. Biol.*, 6, 213-225.

Bolter, M. & Dawson, R. (1982). Heterotrophic utilisation of biochemical compounds in antarctic water. *Neth. Jnl. Sea Res.*, 16, 315-332.

Boney, A.D. (1975). *Phytoplankton.*

Braven, J.; Evens, R. & Butler, E.I. (1984). Amino acids in sea water. *Chemistry in Ecology*, 2, 11-21.

Britton, H.T.S. (1955). In: *Hydrogen Ions. Their determination and importance in pure and industrial chemistry*, 1, 319.

Butler, E.I. & Tibbits, S. (1972). Chemical survey of the Tamar Estuary. *Jnl. Mar. Biol. Ass. UK*, 52, 681-699.

Butler, E.I.; Knox, S. & Liddicoat, M.I. (1979). The relationship between inorganic and organic nutrients in seawater. *Jnl. Mar. Biol. Ass. UK*, 59, 239-250.

Carlson, D.J.; Brann, M.L.; Mague, T.H. & Mayer, L.M. (1985). Molecular weight distribution of dissolved organic materials in seawater determined by ultrafiltration. A re-examination. *Mar. Chem.*, 16, 155-171.

Carlucci, A.F.; Craven, D.B.; Robertson, K.J. & Williams, P.M. (1986a). Surface-film microbial populations: free amio acid metabolism, carbon utilization and growth rates. *Mar. Biol.*, 92, 289-297.

Carlucci, A.F.; Craven, D.B.; Robertson, K.J. & Henrichs, S.M. (1986b). Microheterotrophic utilization of dissolved free amino acids in depth profiles of Southern California borderland basin waters. *Oceanologica Acta.*, 9, 1, 88-96.

Casey, H. & Walker, S.M. (1983). Comparison of filtration methods using GF/C and 0.45 um membrane filters for reactive phosphate, dissolved silicon and iron concentrations in chalk water samples. *Arch. Hydrobiol.*, 96, 4, 515-520.

Caswell, F. (1982). *Success in Statistics.*

Chau Y.K. & Riley, J.P. (1966). The determination of amino acids in sea water. *Deep Sea Research*, 13,

1115-1124.

Clegg, C.A. (1980). *Biology*, chapter 5, 104-123.

Crawford, C.C.; Hobbie, J.E. & Webb, K.L. (1974). The utilization of dissolved free amino acids by estuarine microorganisms. *Ecology*, 55, 551-563.

Dagg, M.J.; Vidal, J.; Whitledge, T.E.; Iverson, R.L. & Goering, J.J. (1982). The feeding, respiration and excretion of zooplankton in the Bering Sea during a spring bloom. *Deep Sea Research*, 29, 1A, 45-63.

Darley, W.M. (1982). In: Wilkinson, J.F. (ed). *Algal Biology: a physiological approach.*

Dawson, R. & Goche, K. (1978). Heterotrophic activity in comparison to the free amino acid concentrations in the Baltic sea water. *Oceanol. Acta.*, 1, 45-54.

Dawson, R. & Mopper, K. (1978). A note on the losses of monosaccharides, amino sugars and amino acids from extracts during concentration procedures. *Anal. Biochem.*, 84, 186-190.

Dawson, R. & Pritchard, R.G. (1978). The determination of alpha amino acids in sea water using a fluorimetric analyser. *Mar. Chem.*, 6, 27-40.

Daumas, R.A. (1976). Variations of particulate proteins and dissolved amino acids in coastal sea water. *Mar. Chem.*, 4, 225-242.

Degens, E.T.; Reuter, J.H. & Shaw, K.N.F. (1964). Biochemical compounds in offshore California sediments and seawaters. *Geochimica et Cosmochimica Acta.*, 26, 45-66.

Degens. E.T. (1970). Molecular nature of nitrogenous compounds in seawater and recent marine sediments. In: Woods D.W. (ed). *Institute of Marine Science, University of Alaska, Occasional Publication No 1.*

Doolittle, R.F. (1985). Proteins. *Scientific American.* 235, 4, 74-83.

Dring, M.J. (1982). *The Biology of Marine Plants.* 199.

Duursma, E.K. (1965). The dissolved organic constituents of seawater. In: Riley, J.P. & Skerrow, G. (eds).*Chemical Oceanography*, 1, 433-475.

Eberlein, K.; Leal, M.T.; Hammer, K.D. & Hickel, W. (1985). Dissolved organic substances during a Phaeocystis pouchetii bloom in the German Bight (North Sea). *Mar. Biol.*, 89, 311-316.

Eppley, R.W.; Horroigan, S.G.; Fuhrman, J.A.; Brooks, E.R.; Price, C.C. & Sellner, K. (1981). Origins of

dissolved organic matter in Southern Californian coastal waters. Experiment on the role of zooplankton. *Mar. Ecol. Prog. Ser.*, 6, 149-159.

Evens, R.; Braven, J.; Brown, L. & Butler, E.I. (1982). A high performance liquid chromatographic determination of free amino acids in natural waters in the picomolar (M x 10-12) range suitable for shipboard use. *Chem. Ecol.*, 1, 99-106.

Evens, R. (1986). *A Seasonal Study of Amino Acids in Marine and Estuarine Waters.* Ph.D Thesis, Plymouth Polytechnic, UK.

Evens, R.; Braven, J. & Butler, E.I. (1988). A seasonal comparison of the dissolved free amino acid levels in estuarine and English Channel waters. In Press.

Fisher, N.S. & Cowdell, R.A. (1982). Growth of marine planktonic diatoms on inorganic and organic nitrogen. *Mar. Biol.*, 72, 147-155.

Flynn, K.J. & Butler, E.I. (1986). Nitrogen sources for the growth of marine microalgae: the role of dissolved free amino acids. *Mar. Ecol. Prog. Ser.*, 34, 281-304.

Flynn, K.J. & Syrett, P.J. (1986). Characteristics of the uptake system for L-lysine and L-arginine in Phaeodactylum tricornutum. *Mar.Biol.*, 90, 151-158.

Flynn, K.J. & Wright, C.R.N. (1986). The simultaneous assimilation of ammonia and L-arginine by the marine diatom Phaeodactylum Tricornutum. *J. Exp. Mar. Biol. Ecol.*, 95, 257-269.

Fogg, G.E. (1983). The ecological significance of extracellular products of phytoplankton photosynthesis. *Botanica Marina*, 26, 3-14.

Fuhrman, J.A. & Bell, T.M. (1985). Biological considerations in the measurement of dissolved free amino acids in seawater and implications for chemical and microbiological studies. *Mar. Ecol. Prog. Ser.*, 25, 13-21.

Fuhrman, J.A. & Ferguson, R.L. (1986). Nanomolar concentrations and rapid turnover of dissolved free amino acids in seawater: agreement between chemical and microbiological measurements. *Mar. Ecol. Prog. Ser.*, 33, 237-242.

Gagosian, R.B. & Lee, C. (1981). Processes controlling the diet of biogenic organic compounds in seawater. In: Duursma, E.K & Dawson, R. (eds). *Marine Organic Chemistry, Series 3*, 91-115.

Garrasi, C.; Degens, E.T. & Mopper, K. (1979). The free amino acid composition of sea water obtained without desalting and preconcentration. *Mar. Chem.*, 8, 71-85.

Gocke, V.K. (1970). Investigations on release and uptake of amino acids and polypeptides by plankton organisms. *Acta. Hydrobiol.*, 67, 3, 285-367.

Grasshoff, K. (1986). Filtration and storage. In: *Method of Seawater Analysis*, Chapter 2.

Hagstrom, A.; Ammerman, J.W.; Heinrich, S. & Azam, F. (1984). Bacterioplankton growth in seawater. II. Organic matter utilization during steady-state growth in seawater cultures. *Mar. Ecol. Prog. Ser.*, 18, 41-48.

Hamilton, P.B. (1965). Amino acids on hands. *Nature*, 205, 284-285.

Hammer, K.D. & Kattner, G. (1986). Dissolved free amino acids in the marine environment: A C:N ratio shift during diatom blooms. *Marine Ecol. Prog.Ser.*, 31, 35-45.

Haurowitz, F. (1963) In: *The Chemistry and Function of Proteins*, 131.

Head, P.C. (1985). *Practical Estuarine Chemistry: A Handbook.*

Hellebust, J.A. & Guillard, R.R.L. (1967). Uptake specifity for organic substrates by the marine diatom Melosira numunsloides. *J. Physiol.*, 3, 132-136.

Henrichs, S.M. & Williams, P.M., (1985). Dissolved and particulate amino acids and carbohydrates in the sea surface microlayer. *Mar. Chem.*, 17, 141-163.

Hobbie, J.E.; Crawford, C.C. & Webb, K.L. (1968). Amino acid flux in an estuary. *Science*, 159, 1463-1464.

Hollibaugh, J.T. & Azam, F. (1983). Microbial degradation of dissolved proteins in seawater. *Limnol. Oceangr.*, 28, 6, 1104-1116.

Hunt, S. (1985). Chemistry and biochemistry of the amino acids. In: Barrett, G.C. (ed). *Amino Acids.*

Ittekkot, V. (1982). Variation of dissolved organic matter during a plankton bloom. Qualitative aspects based on sugar and amino acid analyses. *Mar. Chem.*, 11, 143-158.

Jefferies, H.P. (1969). Seasonal composition of temperate plankton communities: free amino acids. *Limnol.Oceanogr.*, 14, 41-52.

Johannes, R.E. & Webb, K.L. (1970). Release of dissolved organic compounds by marine and fresh water invertebrates. In: Woods D.W. (ed). *Institution of Marine Science, University of Alaska, Occasional Publication No 1.*

Jorgensen, E.G. (1968). The adaptation of plankton algae. II. Aspects of the temperate adaptation of Skeletonema costatum. *Physiologia Plantarum*, 21, 423-427.

Jorgenson, N.O.G. & Sondergaard, M. (1984). Are dissolved free amino acids free? *Microbial Ecology*, 10, 301-316.

Josefsson, B.; Lindroth, P. & Ostling, G. (1977). An automated fluorescence method for the determination of total amino acids in natural waters. *Analytical Chimica Acta.*, 89, 21-28.

Kawahara, H. & Maita, Y. (1971). Gas-liquid chromatographic determination of amino acids and vertical distribution of proteinaceous substances in seawater. *Jnl. of the Oceanographical Society of Japan*, 27, 1, 27-33.

Keller, M.D.; Mague, T.H.; Badenhausen, M. & Glover, H.E. (1982). Seasonal variations in the production and consumption of amino acids by coastal microplankton. *Estuarine, Coastal and Shelf Sci.*, 15, 301-315.

Kirchman,D.L.; Newell, S.Y. & Hodson, R.E. (1986). Incorporation vs. biosynthesis of leucine: Implications for measuring rates of protein synthesis and biomass production by bacteria in marine systems. *Mar. Ecol. Prog. Ser.*, 32, 47-59.

Krstulovic, A.M. & Brown, P.R. (1982). In: *Reversed Phase High Performance Liquid Chromatography. Theory, Practice and Biomedical Applications.*

Laanbreok, H.J.; Verplanke, J.C.; de Visscher, P.R.M. & de Vuyst, R. (1985). Distribution of phytoplankton and bacterioplankton: growth and biomass parameters, dissolved inorganic nutrients, and free amino acids during a spring bloom in the Oosterschelcke Basin the Netherlands. *Mar. Ecol. Prog. Ser.*, 25, 1-11.

Laane, R.W.P.M. (1983). Seasonal distribution of dissolved and particulate amino acids in the EMS - Dollart estuary. *Oceanol. Acta.*, 6, 1, 105-109.

Lancelot, C. (1984). Extracellular release of small and large molecules by phytoplankton in the Southern Bight of the North Sea. *Est. Coastal Shelf Sci.*, 18, 65-77.

Lancelot, C. & Billen, G. (1984). Activity of heterotrophic bacteria and its coupling to primary production during the spring phytoplankton bloom in the Southern Bight of the North Sea. *Limnol. Oceanogr.*, 29, 4, 721-730.

Lea, P.J.; Wallsgrove, R.M. & Miflin, B.J. (1985). The biosynthesis of amino acids in plants. In: Barrett, G.C. (ed). *Amino Acids.*

Lee, C. & Bada, J.L. (1975). Amino acids in equatorial

Pacific Ocean water. *Earth Planet. Sci. Lett.*, 2, 61-68.

Lee, C. & Bada, J.L. (1977). Dissolved amino acids in the equitorial Pacific, the Sargasso Sea and Biscayne Bay. *Limnol. Oceano.*, 22, 3, 502-510.

Lee, C. & Cronin, C. (1982). The vertical flux of particulate organic nitrogen in the sea: decomposition of amino acids in the Peru upwelling area and equatorial Atlantic. *J. Mar. Res.*, 40, 227-251.

Lee, C. (1988). Amino acid and amine biogeochemistry in marine particulate matter and sediments. In: Blackburn, T.H. & Sorenson, J. (eds). *Nitrogen Cycling in Coastal Environments*. 125-141.

Liebezeit, G. & Velimikov, B. (1984). Distributio of inorganic and organic nutrients in a sandy beach at Ischia, Bay of Naples. *Oceanis.*, 10, 437-447.

Liebezeit, G. (1985). Residual amino acid fluxes in the upper water column of the Bransfield Strait. *Oceanologica Acta.*, 8, 1, 59-65.

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

Liu, M.S. & Hellebust, J.A. (1974a). Uptake of amino acids by the marine centric diatom Cyclotella cryptica. *Can. J. Microbiol.*, 20, 1109-1118.

Liu, M.S. & Hellebust, J.A. (1974b). Utilization of amino acids as nitrogen sources, and their effects on nitrate reductase in the marine diaton Cyclotella cryptica. *Can. J. Microbiol.*, 20, 1119-1125.

Lovelock, J.E. (1979). *Gaia. A New Look At Life On Earth.*

Lu, M. & Stephens, G.C. (1984). Demonstration of net influx of free amino acids in Phaeodactylum Triconutum using HPLC. *J. Physiol.*, 20, 584-589.

Maddock, L.; Boalch, G.T. & Harbour, D.S. (1981). Populations of phytoplankton in the Western English Channel between 1964 and 1974. *J. Mar. Biol. Ass. UK.* 61, 565-583.

Maurer, L.G. (1976). Organic polymers in seawater: changes with depth in the Gulf of Mexico. *Deep Sea Res.*, 23, 1059-1064.

Menzel. D.W. & Ryther, J.H. (1970). Distribution and cycling of organic matter in the oceans. In: Wood, D.W. (ed). *Institute of Marine Science, University of Alaska, Occasional Publication No 1.*

Miller, J.C. & Miller, J.N. (1985). *Statistics for*

Analytical Chemistry.

Ming, L. & Stephens, G.C. (1985). Uptake of free amino acids by the diatom Melosira mediocris. *Hydrobiologia.*, 128, 187-191.

Minghou, J.; Shuzhu, P.; Yongyao, P. & Hong, N. (1986). Amino acid content of marine phytoplankton. *Acta. Oceanol. Sinica.*, 5, 3, 457-464.

Monkhouse, F.J. & Small, J. (1978). In: *A Dictionary of the Natural Environment.*

Mopper, K. & Lindroth, P. (1982). Diel and depth variations in dissolved free amino acid and ammonium in the Baltic Sea determined by shipboard analysis. *Limnol. Oceanogr.*, 27, 2, 336-347.

Mopper, K. & Zika, R.G. (1987). Free amino acids in marine rains: evidence for oxidation and potential role in nitrogen cycling. *Nature*. 325, 246-249.

Morris, A.W.; Mantoura, R.C.F.; Bale, A.J. & Howland, R.J.M. (1978). very low salinity regions of estuaries: important sites for chemical and biological reactions. *Nature*. 274, 678-680.

Morris, A.W.; Bale, A.J. & Howard, R.J.M. (1982). Chemical variability in the Tamar Estuary, South-West England. *Estuarine, Coastal & Shelf Science*, 14, 649-661.

Morris A.W. (1985). Estuarine chemistry and general survey strategy. In: Head, P.C. (ed). *Practical Estuarine Chemistry*. 1-60.

Morris, G.J.; Clarke, K.; Leeson, E.; Winters, L. & Coulson, G. (1985). Cryobiology. *NERC Jnl.*, March. 10-13.

Muller, P.J.; Suess, E. & Lingerer, C.A. (1986). Amino acids and amino sugars of particulate and sediment trap material from waters of the Scotia Sea. *Deep Sea Research*, 33, 6, 819-838.

McCarthy, J.J.; Taylor, W.R. & Taft, J.L. (1977). Nitrogenous nutrition of plankton in the Chesapeake Bay. I. Nutrient availability and phytoplankton preferences. *Limnol. Oceanogr.*, 22, 6, 996-1011.

McCarthy, J.J. (1980). Nitrogen. In: Morris, I. (ed). *The Physiological Ecology of Phytoplankton.*

Nagel, K. & Liemann, F. (1987). Automated analysis for the quantification of dissolved proteins in natural seawater samples. *Oceanologica Acta.*, 10, 2, 181-186.

Naletova, I.A. (1979). Dissolved amino acids in Alantic Ocean waters. *Oceano.*, 19, 163-166.

North, B.B. (1975). Primary amines in California coastal waters; utilization by phytoplankton. *Limnol. Oceanogr.*, 20, 20-27.

Ogura, N. (1977). High molecular weight organic matter in seawater. *Mar. Chem.*, 5, 535-549.

O'Neill, P. (1985). *Environmental Chemistry.*

Park, P.K.; Williams, W.T.; Prescott, J.M. & Hood, D. W. (1963). Amino acids in Redfish Bay Texas. *Pub. Instit. Mar. Sci. Univ. Texas.* 9, 59-63.

Parsons,T.R.; Takahashi, M. & Hargrave, B. (1984). In: *Biological Oceanographic Processes, 3rd Ed.*

Paul, J.H. (1983). Uptake of organic nitrogen. In: Carpenter, E.J. & Capone, D.G. (eds). *Nitrogen in the Marine Environment,* 275-308.

Poulet, S.A.; Martin-Jezequel, V. & Head, R.N. (1984). Distribution of dissolved free amino acids in the Ushaut Front region. *Marine Ecology - Progress Series,* 18, 49-55.

Price, N.M.; Cochlan, W.P. & Harrison, P.J. (1985). Time course of uptake of inorganic and organic nitrogen by phytoplankton in the Strait of Georgia: comparison of frontal and stratified communities. *Mar. Ecol. Prog. Ser.*, 27, 39-53.

Reid, E. (1981). *Trace Organic Sample Handling.*

Ridder T.B.; Buishand, T.A.; Reijnders, H.F.R.; Hart, M.J. & Slanina, J. (1985). Effects of storage on the composition of main components in rainwater samples. *Atmos. Environ.*, 19, 5, 759-762.

Riley, J.P. & Segar, D.A. (1970). The seasonal variation of the free and combined dissolved amino acids in the Irish Sea. *Jnl. Mar. Biol. Assoc. U.K.*, 50, 713-720.

Robertson, K.J.; Williams, P.M. & Bada, J.L. (1987). Acid hydrolysisof dissolved combined amino acids in seawater. A precautionary note. *Limnol. Oceanogr.*, 32, 4, 996-997.

Roth, M. (1971). Fluorescence reaction for amino acids. *Anal. Chem.*, 43, 7, 880-882.

Roth, M. & Hampai, A. (1973). Column chromatography of amino acids with fluorescence detection. *Jnl. Chrom.*, 83, 353-356.

Royal Society, The. (1983). *The Nitrogen Cycle of the United Kingdom. A Study Group Report.*

Samata, T. & Matsuda, M. (1986). Contaminating peptides widely present in ion-exchanged water, reagents, experimental instruments and natural samples. *Comp.*

Biochem. Physiol., 84B, 4, 531-535.

Schell, D.M. (1974). Uptake and regeneration of free amino acids in marine waters of South East Alaska. *Limnol. Oceanogr.*, 19,260-270.

Sharp, J.H. (1973). Size classes of organic carbon in seawater. *Limnol. Oceanog.* 18, 3, 441-447.

Sharp, J.H. (1975). Gross analyses of organic matter in seawater; why, how and from where? In: Church, T.M. (ed). *Marine Chemistry in the Coastal Environment.*

Shoji, S.; Iyiye, T. & Satoh, Y. (1984). Selective entrapment of organic molecules by altrafiltration. *Jap. Jnl. Limnol.*, 45, 2, 165-168.

Siegel, A. & Degens, E.T. (1966). Concentration of dissolved amino acids from saline waters by ligand exchange chromatography. *Science*, 151, 1098-1101.

Siezen, R.J. & Mague, T.H. (1978). Amino acids in suspended particulate matter from oceanic and coastal waters of the Pacific. *Mar. Chem.*, 6, 215-231.

Simons, S.S. & Johnson, D.F. (1973). The structure of the fluorescent adduct formed in the reaction of o-phthaldialdhyde and thiols with amines. *Jnl. Am. Chem. Soc.*, 98, 7098 -7112.

Solomon, M.E. (1969). In: *Population Dynamics.*

Spackman, D.H.; Stein, W.H. & Moore, S. (1958). Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.*, 30, 1190-1205.

Suzuki, Y. & Sugumura, Y. (1985). A catalytic oxidation method for the determination of total nitrogen dissolved in seawater. *Mar. Chem.*, 16, 83-97.

Syrett, P.J.; Flynnn, K.J.; Molloy, C.J.; Dixon, G.K.; Peplinska, A.M. & Cresswell ?. (1986). Effects of nitrogen deprivation on rates of uptake of nitrogenous compounds by the diatom Phaeodactylum Tricornutum Bohlin. *New Physiol.*, 102, 39-44.

Tatsumoto, M.; Williams, W.T.; Prescott; J.M. & Woods, D.M. (1961). Amino acids in samples of surface sea water. *Jnl. Mar. Res.*, 19, 89-95.

Thurman, E.M. (1986). In: *Organic Geochemistry of Natural Waters. Developments in Biogeochemistry.* chapter 6, 151-169.

Viera, A.A.H. & Klaveness, D. (1986). The utilization of organic nitrogen compounds as sole nitrogen source by some freshwater phytoplanktons. *Nord?. J. Bot. - Section of Phycology*, 6, 93-97.

Webb, K.L. & Wood, L. (1966). Improved techniques for the analysis of free amino acids in seawater. In: Scova N.B. (ed). *Automation in Analytical Chemistry*, 1, 440-444.

Wheeler, P.A.; North, B.B. & Stephens, G.C. (1974). Amino acid uptake by marine phytoplankton. *Limnol. Oceanogr.*, 19, 2, 249-259.

Wheeler, P.A. & Kirchman, D.L. (1986). Utilization of inorganic and organic nitrogen by bacteria in marine systems. *Limnol. Oceanogr.*, 31, 5, 988-1009.

Wiebe, W.J. & Smith, D.F. (1977). Direct measurement of dissolved organic carbon release by phytoplankton and incorporation by microheterotrophs. *Mar. Biol. (Berlin)*, 42, 3, 213-223.

Williams, P.J. LeB. & Gray, R.W. (1970). Heterotrophic utilization of dissolved organic compounds in the sea. II. Observations on the responses of heterotrophic marine populations to abrupt increases in amino acid concentration. *J. Mar. Biol. Ass. UK.*, 50, 871-881.

Williams, P.J. LeB. (1985). Biological and chemical aspects of dissolved organic matter in seawater. In: Riley, J.P. & Skerrow. G. (eds). *Chemical Oceanography*. 2, 301-363.

Williams, R. & Poulet, S.A. (1986). Relationship between the zooplankton, phytoplankton, particulate matter and dissolved free amino acids in the Celtic Sea. I. Unstratified water conditions. *Mar. Biol.*, 90, 274-284.

APPENDIX I.

The Batch Average For Coastal Waters Collected Over A Season And How These Relate To DON and TDN.
(DON & TDN Figures Were Not Directly Measured And Are Taken From Butler et al. 1979)›

Date: 27.11.85 Days. 27.

No.	DFAA (uM).
1	0.180
2	0.279
Average Values.	.229
No. of Samples.	2
CV's.	30

Date: 11.12.85 Days. 41

No.	DFAA (uM).
1	BLD

Date: 17.12.85 Days.47

No.	DFAA (uM).
1	0.121
2	BLD
Average Values.	0.060
No. of Samples.	2
CV's.	141

Date: 23.12.85 Days. 53

No.	DFAA (uM)›
1	0.420

Date: 8.1.86 Days. 69

No.	DFAA (uM).
1	0.058

Date: 21.1.86	Days. 82
No.	DFAA (uM).
1	0.223

Date: 23.1.86	Days. 84
No.	DFAA (uM).
1	0.199

Date: 28.1.86	Days. 89
No.	DFAA (uM).
1	BLD

Date: 3.2.86	Days. 95
No.	DFAA (uM).
1	0.101

Date: 18.2.86	Days. 110
No.	DFAA (uM).
1	0.579
2	0.555
Average Value.	0.567
No. of Samples.	2
CV's.	3

Date: 20.2.86	Days. 112
No.	DFAA (uM).
1	0.089

Date: 4.3.86	Days. 124
No.	DFAA (uM).
1	0.161
2	0.200
Average Value.	0.180
No. of Samples.	2
CV's.	15

Date: 25.4.86	Days. 177
No. Location.	DFAA (uM)
1	0.090
2	0.097
3	0.022
4	0.130
5	0.162
6	0.036
7	0.314
Average Value.	0.121
No. of Samples.	7
CV's.	80

Date: 1.5.86 Days. 183

No.	Location	DFAA (uM).
1		0.115
2		0.084
3		0.146
4		0.311
5		0.162
6		0.318
7		0.146
Average Value.		0.183
No. of Samples.		7
CV's.		51

Date: 8.5.86 Days. 190

No.	Location	DFAA (uM).
1		0.235
2		0.233
3		0.289
4		0.188
5		0.420
6		0.576
7		0.229
Average Value.		0.310
No. of Samples.		7
CV's.		45

Date: 30.5.86 Days. 212

No.	Location	DFAA (uM).
1		0.085
2		0.217
3		5.200
4		0.416
5		0.171
6		0.379
7		0.126
Average Value.		0.942
No. of Samples.		7
CV's.		199

Date: 5.6.86. Days. 218

No.	Location	DFAA	DCAA	TAA	Ratio	%TN	%DON
		‹--------uM----›				8.75	7.7
						‹-----uM ----›	
1	E.end BW.	0.226	2.395	2.621	11	30	34
2	Shagstone	0.364	0.585	0.949	2	11	12
3	Mewstone	0.230	----------				
4	Yealm	0.153	----------				
5	B36 A74	BLD	----------				
6	B33 A71.5	0.082	----------				
7	B30 A69	0.272	----------				
8	A41.5 A65	0.091	----------				
9	A47 A68	0.498	----------				
Average Values		0.212	1.49			21	23
No. of samples.		9	2			2	2
CVs		72	86				

Date: 12.6.86 Days. 225

No.	Location	DFAA	DCAA	TAA	Ratio	%TN(uM) (8.5)	%DON(uM) (7.7)
		‹--------uM--------›				‹-----uM--------›	
1	Melampus B	0.254	1.444	1.698	6	19	22
2	New Grnd.B	0.185	-----	-----	-	--	--
3	W. End BW.	1.358	-----	-----	-	--	--
4	Penlee Pt.	0.167	0.800	0.967	5	11	13
5	Half way to Rame	0.125	-----	-----	-	--	--
6	A2.2 C39	0.153	1.637	1.790	11	20	23
7	Knapp B.	0.145	0.916	1.061	6	12	14
8	A2.35 C41	0.210	-----	-----	--	--	--
9	A2.55 C43	0.185	0.633	0.818	3	9	11
Average Values		0.309	1.086			14	17
No. of Samples		9	5			5	5
CVs		127	40				

Date: 2.7.86 Days. 245

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.0)	%DON (8.8)
		‹-------uM-------›				‹----uM-----›	
1	W. end BW.	1.474	1.822	3.296	1	33	38
2	Knapp B.	1.049	-----	-----	-	--	--
3	Penlee Pt.	0.678	1.159	1.837	2	18	21
4	Half way to Rame	0.592	-----	-----	-	--	--
5	A1.8 C39	0.593	1.105	1.698	2	17	19
6	Whitesands	1.018	-----	------	-	--	--
7	Whitesands nr. Wreck	3.527	1.964	5.491	0.6	55	63
8	A2.5 C40	2.270	2.646	4.916	1	49	56
9	Cawsand Bay	0.967	----------	----------	----------	----------	----------
Average Values		1.352	1.739			34	39
No. of Samples		9	5			5	5
CVs		32	37				

Date: 8.7.86 Days. 251

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.0)	%DON (8.8)
		‹-------uM--------›				‹----uM-----›	
1	Mallard B.	1.511	1.179	2.690	1	27	31
2	New Grnd.B.	0.402	-----	-----	-	--	--
3	W.End BW.	0.462	2.198	2.660	5	27	30
4	Knapp B.	0.560	-----	-----	-	--	--
5	Penlee B.	0.817	1.687	2.504	2	25	34
6	Melampus B.	0.502	-----	-----	-	--	--
7	A2 C36	1.037	-----	-----	-	--	--
8	A2.5 C37.5	0.488	1.995	2.483	4	25	28
12	Cawsand Bay (middle)	2.239	2.611	4.850	1	48	55
Average Values.		0.890	1.934			30	34
No. of Samples.		9	5			5	5
CVs		69	30				

Date: 15.7.86 Days. 258

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.0)	%DON (8.8)
		‹-------uM--------›				‹----uM-----›	
1	Mallard B.	0.197	------	------	-	--	--
2	Melampus B	0.192	1.647	1.839	9	18	21
4	W. End BW.	0.166	------	------	-	--	--
5	Knapp B.	0.217	------	------	-	--	--
8	A2.5 C34.5	BLD	1.760	1.760	› 46	18	20
11	E. End BW.	0.193	1.530	1.723	8	17	20
Average Values.		0.161	1.646			18	20
No. of Samples.		6	3			3	3
CVs		50	7				

Date: 23.7.86 Days. 266

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.0)	%DON (8.8)
		‹---------uM--------›				‹----uM-----›	
1	Mallard B.	0.094	2.796	2.890	30	29	33
2	E. End BW.	0.125	2.260	2.385	18	24	27
3	Shagstone.	0.270	-----	-----	--	--	--
4	Mewstone.	0.177	2.627	2.804	14	28	32
5	HillSea Pt.	0.095	-----	-----	--	--	--
6	B34 A72.6	0.242	3.654	3.896	15	39	44
7	B30 A70.7	0.089	-----	----	--	--	--
8	A44 A66.2	0.105	1.569	1.674	15	17	19
9	A40.6 A63	0.255	-----	-----	--	--	--
10	A38.5 A61	0.099	-----	-----	--	--	--
Average Values.		0.155	2.581			27	31
No. of Samples.		10	5			5	5
CVs		48	29				

Date: 30.7.86 Days. 273

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.0)	%DON (8.8)
		‹---------uM--------›				‹----uM----›	
1	Mallard B	0.105	1.630	1.735	16	17	20
2	E.End BW.	0.146	3.108	3.254	22	33	37
3	Shagstone	1.327	-----	-----	--	--	--
4	Tinker B.	0.101	-----	-----	--	--	--
5	A1 C31	BLD	-----	-----	--	--	--
6	A1.4 C34	0.056	1.725	1.781	30	18	20
7	A1.7 C37.	0.185	-----	-----	--	--	--
8	off Rame.	0.493	2.588	3.081	5	31	35
Average Values		0.302	2.263			25	28
No. of Samples		8	4			4	4
CVs		145	31				

Date: 6.8.86 Days. 280

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (12.3)	%DON (10.0)
		‹-------uM---------›				‹----uM-----›	
1	Mallard B.	0.420	0.863	1.283	2	10	13
2	Duke RockB.	0.163	1.420	1.583	9	13	15
3	E.End BW.	0.024	1.594	1.618	66	13	16
4	Shagstone	0.152	1.525	1.677	10	14	17
5	B46.8 B54.6	0.186	-----	-----	--	--	--
8	C30 B54.2	0.406	1.487	1.887	4	15	19
11	Knapp B.	2.326	0.287	2.613	0.1	21	26
Average Values.		0.525	1.196			14	18
No. of Samples.		7	6			6	6
CVs		153	43				

Date: 4.9.86 Days. 309

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.3)	%DON (6.9)
		‹--------uM-------›				‹----uM----›	
1	Mallard B.	0.311	5.695	6.006	18	58	87
2	Malampus B	1.352	-----	-----	--	--	--
3	BW. Fort	0.329	-----	-----	--	--	--
4	E.End BW.	2.788	0.827	3.615	0.3	35	52
5	Shagstone	2.004	1.685	3.689	0.8	36	53
6	Mewstone	0.889	-----	-----	---	--	--
7	Mouth of R.Yealm	0.650	-----	-----	---	--	--
8	A1.5 B44.3	1.125	0.601	1.726	0.5	17	25
11	Knapp B.	0.717	1.639	2.356	2	22	33
Average Values.		1.129	2.089			34	50
No. of Samples.		9	5			5	5
CVs		72	100				

Date: 24.9.86 Days. 329

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.3)	%DON (6.9)
		‹--------uM-------›				‹----uM-----›	
1	A18.3 C47	2.376	1.381	3.757	0.6	36	54
2	A16.5 C45.5	1.738	1.593	3.331	1.1	32	48
4	A13.5 C43	0.364	3.485	3.849	8	34	51
5	A12 C41.8	0.766	4.002	4.846	5	47	70
7	A9 C39.3	0.888	1.444	2.332	2	22	34
8	A7.5 C38.2	0.706	1.060	1.766	2	17	26
Average Values.		1.139	2.161			31	47
No. of Samples.		6	6			6	6
CVs		66	58				

Date: 1.10.86 Days. 336

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (11.0)	%DON (6.9)
		‹--------uM------›				‹----uM----›	
1	Mallard B.	0.042	1.239	1.281	30	12	19
2	Melumpas B.	0.287	3.448	3.735	12	34	54
3	New Grnd.	0.346	-----	-----	--	--	--
4	W.End BW.	0.321	0.835	1.156	3	11	17
5	Knapp B.	0.939	0.694	1.633	1	15	24
6	C31.4B57.3	0.503	-----	-----	--	--	--
7	C33.8B55.8	1.746	-----	-----	--	--	--
8	C34.1 B34	0.187	1.241	1.428	6	13	21
Average Values.		0.546	1.491			17	27
No.of Samples.		8	5			5	5
CVs		101	75				

Date: 16.10.86 Days. 351

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (11.0)	%DON (6.9)
		‹-------uM--------›				‹-----uM-----›	
1	Ash B.	0.141	0.705	0.846	5	8	12
2	Bridge B.	0.062	-----	-----	-	-	--
3	Queens B.	BLD	1.359	1.359	›36	12	20
4	Penlee Pt.	0.028	1.309	1.337	47	12	19
5	Rame Hd.	BLD	-----	-----	-	--	--
6	Pelawn A1.6C39.5	0.037	0.851	0.888	23	8	13
7	A1.5C42.7	BLD	-----	-----	--	-	--
8	A2.6C39.6	BLD	-----	-----	--	-	--
Average Values.		0.034	1.056			10	16
No. of Samples.		8	4			4	4
CVs		147	31				

Date 23.10.86 Days. 358

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (11.0)	%DON (6.9)
		‹-------uM-------›				‹-----uM----›	
1	Sound	BLD	1.690	1.690	›44	15	24
2	E.End BW.	BLD	6.080	6.080	›160	55	88
3	Tinker B.	0.083	2.358	2.441	28	22	35
4	A1 B46.7	0.063	3.115	3.178	49	29	46
5		BLD	-----	-----	--	--	--
6		0.023	-----	-----	--	--	--
7		0.126	-----	-----	--	--	--
8		0.304	-----	-----	--	--	--
Average Values.		0.075	3.311			30	48
No. of Samples.		8	4			4	4
CVs		138	58				

Date: 8.11.86 Days. 371

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.5)	%DON (5.4)
		‹--------uM-------›				‹-----uM----›	
1	Sound.	0.862	0.486	1.348	1	14	26
2	Penlee Pt.	0.242	0.784	1.026	3	10	19
3	Off Rame.	0.025	-----	-----	--	--	--
4	Rame.	0.151	1.333	1.484	9	14	27
5	Whitesand	0.115	-----	-----	--	--	--
6	Looe	0.067	0.709	0.776	11	7	14
7	A4.7 D38	0.231	1.004	1.235	4	12	23
Average Values.		0.242	0.863			11	22
No. of Samples.		7	5			5	5
CVs		117	37				

Date: 13.11.86 Days. 379

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.5)	%DON (5.4)
		‹-------uM--------›				‹-----uM-----›	
1	A2 B47	0.120	1.249	1.369	10	13	25
2	4 min to BW.	BLD	1.436	1.436	›38	14	26
3	8 min to BW.	0.052	-----	-----	--	--	--
4	12 min to BW.	BLD	1.861	1.861	›49	18	34
5	16 min to BW.	BLD	-----	------	--	--	--
6	20 min to BW.	0.202	-----	------	--	--	--
9	Knapp B.	0.189	1.308	1.497	7	14	28
10	W.End BW.	0.070	1.623	1.693	23	16	31
Average Values.		0.079	1.495			15	29
No. of Samples.		8	5			5	5
CVs		105	17				

Date: 20.11.86 Days. 386

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.5)	%DON (5.4)
		‹-------uM--------›				‹----uM----›	
1	A3 C34	BLD	1.175	1.175	›31	11	22
2	A6.5 C35	BLD	1.315	1.315	›35	13	24
3	A8.5 C38	0.343	0.991	1.334	3	13	25
4	A9 C40	0.25	0.812	1.062	3	10	20
5	A11 D31	0.356	-----	-----	-	---	--
Average Values.		0.190	1.073			12	23
No. of Samples.		5	4			4	4
CVs		94	20				

Date: 27.11.86 Days. 393

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.5)	%DON (5.4)
		‹---------uM-----›				‹----uM----›	
1	A3 C37	BLD	1.09	1.09	›29	10	20
2	A5 C40.5	BLD	2.161	2.161	›56	20	40
3	A4.7 C43	0.044	2.015	2.059	46	20	38
4	A9 C46.5	0.074	1.633	1.707	22	16	32
5	A11 D32	0.058	0.641	0.699	11	7	13
6	A12 D35.5	BLD	-----	-----	--	--	--
Average Values.		0.029	1.508			15	29
No. of Samples.		6	5			5	5
CVs		114	42				

Date: 10.12.86 Days. 406

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.0)	%DON (4.5)
		‹-------uM-------›				‹----uM------›	
1	A2.7C41.5	1.766	0.246	2.012	0.1	20	44
2	A4.6D33.2	BLD	1.938	1.938	>51	19	43
3	A4.7D36.8	BLD	1.233	1.233	>32	12	27
4	A5.1D40.3	BLD	1.475	1.475	>25	9	21
5	A6.5D31.3	0.031	-----	-----	--	--	--
6	A5.5D30	BLD	-----	-----	--	--	--
10	Knapp B.	0.362	0.922	1.284	2	8	19
Average Values.		0.308	1.163			14	31
No. of Samples		7	5			5	5
CVs		213	54				

Date: 18.12.86 Days. 414

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.0)	%DON (4.5)
		‹--------uM-------›				‹----uM----›	
1	A4 C43.5	0.256	-----	------	--	---	---
2	A5 C46.5	0.028	0.976	1.004	36	10	22
3	A5.3C47.2	BLD	-----	------	--	---	---
4	A6.1D32.7	BLD	1.894	1.894	>53	19	42
5	A5.5 D36	0.114	-----	-----	---	---	---
6	A4.8D35.2	0.241	-----	-----	---	---	---
10	Penlee B.	BLD	3.367	3.367	>94	35	75
Average Values.		0.091	2.076			21	46
No. of Samples.		7	3			3	3
CVs		126	58				

Date: 8.1.87 Days. 435

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (11.6)	%DON (4.7)
		‹------uM--------›				‹----uM----›	
1	Mount-Batten	0.035	3.055	3.09	87	26	66
2	Duke Rock	0.344	2.402	2.736	7	23	58
3	Shagstone	BLD	----	-----	--	--	--
4	Mewstone	BLD	1.223	1.233	›32	11	26
5	A1 B42	BLD	0.808	0.808	›21	7	17
6	A0.9B39	BLD	2.474	2.474	›65	21	52
7	A0.9B34.5	BLD	-----	-----	---	--	--
8	A0.85B30.	0.048	-----	-----	---	--	--
Average Values.		0.053	1.992			18	43
No. of Samples.		8	5			5	5
CVs		223	47				

Date: 15.1.87 Days. 442

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (11.6)	%DON (4.7)
		‹-------uM-------›				‹----uM-----›	
1	E.End BW.	1.202	0.492	1.694	0.4	15	36
2	Shagstone	1.089	1.005	2.094	1	18	45
3	Mewstone	1.276	0.547	1.823	0.4	16	39
6	Knapp B.	0.733	1.264	1.997	2	17	42
8	W.End BW.	0.607	1.189	1.796	2	15	38
10	Malampus	2.460	0.901	3.370	0.4	29	71
Average Values.		1.228	0.901			18	45
No. of Samples.		6	6			6	6
CVs		54	35				

Date: 30.1.87 Days. 457

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (11.6)	%DON (4.7)
		‹--------uM------›				‹----uM-----›	
1	Shagstone	BLD	0.943	0.943	›25	8	20
2	Mewstone	BLD	-----	-----	---	--	--
3	A1.1B43.3	BLD	1.199	1.199	›32	10	26
4	A1.1 B41	0.076	0.486	0.562	6	4	12
5	A1 B38.6	BLD	1.117	1.117	›31	10	24
6	A1 B36	BLD	1.110	1.110	›29	10	24
7	A1 B32.6	BLD	-----	-----	---	--	--
Average Value.		0.011	0.971			8	21
No. of Samples.		7	5			5	5
CVs		264	30				

Date: 5.2.87 Days. 463

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (12.4)	%DON (5.5)
		‹-------uM-------›				‹----uM-----›	
1	Malampus	1.456	0.883	2.339	1	19	42
2	New Grnd.	1.423	-----	----	--	--	--
3	W.End BW.	0.270	1.849	2.119	7	17	39
4	Knapp B.	0.317	1.039	1.356	3	11	25
5	Cawsand	0.026	3.861	3.887	148	31	71
6	Penlee.	0.500	-----	-----	---	--	--
7	2 miles S.Rame	0.162	-----	-----	---	--	--
8	2 miles S.BW.	BLD	-----	-----	---	--	--
Average Values.		0.519	1.908			20	44
No. of Samples.		8	4			4	4
CVs		114	72				

Date: 12.2.87 Days. 470

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (12.4)	%DON (5.5)
		‹--------uM--------›				‹----uM-----›	
1	Mallard B.	0.052	2.788	2.84	54	23	52
2	New Grnd.	0.158	2.001	2.159	13	17	39
3	W.End BW.	BLD	-----	-----	--	--	--
4	Knapp B.	BLD	2.004	2.004	›53	16	36
5	Penlee Pt.	BLD	2.552	2.552	›67	21	46
6	2 miles S.Rame	0.071	2.005	2.076	28	17	38
7	4 miles S.Rame	BLD	-----	-----	--	--	--
Average Values.		0.04	2.27			19	42
No. of Samples.		7	5			5	5
CVs		149	17				

Date: 19.2.87 Days. 477

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (12.4)	%DON (5.5)
		‹-------uM-------›				‹----uM-----›	
10	E. End BW.	BLD	1.130	1.13	›30	9	21
9	Tinker B.	0.072	0.678	0.75	9	6	14
8	5 miles S.Rame	0.745	2.48	3.225	3	26	59
7	Station L4.	BLD	0.906	0.906	›24	7	16
6	Penlee	BLD	1.677	1.677	›44	14	30
5	Knapp	BLD	-----	-----	---	---	--
4	W.End BW.	0.062	-----	-----	---	---	--
Average Value.		0.125	1.374			12	28
No. of Samples.		7	5			5	5
CVs		218	52				

Date: 26.2.87 Days. 484

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (12.4)	%DON (5.5)
		‹--------uM-------›				‹----uM------›	
1	Mallard B.	0.963	1.586	2.549	3	21	46
2	Melampus	0.825	-----	-----	--	--	--
3	New Grnds	1.234	1.075	2.309	2	19	42
4	W.End BW.	0.919	1.577	2.496	3	20	45
5	Knapp B.	0.754	6.741	7.495	10	60	136
6	2 miles S.E.Rame	0.934	8.298	9.232	10	74	168
7	Penlee Pt.	2.094	-----	-----	--	--	---
Average Value.		1.103	3.855			39	87
No. of Samples.		7	5			5	5
CVs		42	88				

Date: 5.3.87 Days. 491

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (12.4)	%DON (5.9)
		‹--------uM-------›				‹----uM-----›	
1	Mallard B.	1.487	3.020	4.507	2	36	76
2	MelampusB.	2.624	3.493	6.117	1	49	103
3	New Grnd.	3.458	3.581	7.093	1	56	119
4	W.End BW.	1.792	4.167	5.959	2	48	101
5	Knapp B.	1.771	2.615	4.386	1	35	74
6	Penlee Pt.	2.548	-----	-----	--	---	---
7	L3.	4.534	-----	-----	--	---	---
Average Value.		2.602	3.375			45	95
No. of Samples.		7	5			5	5
CV's		42	17				

Date: 12.3.87 Days. 498

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (12.4)	%DON (5.9)
		‹--------uM------›				‹----uM------›	
9	Melampus B.	0.687	2.023	2.71	3	22	46
8	Knapp B.	0.761	2.184	2.945	3	23	50
7	W. End BW.	1.153	2.609	3.762	2	30	63
6	Barn Pool	0.418	1.835	2.253	4	18	38
5		* 0.320	-----	-----	--	--	--
4		* 0.317	-----	-----	--	--	--
3	Saltash	* 0.121	-----	-----	--	--	--
Average Value		0.755	2.162			21	49
No. of Samples.		4	4			4	4
CV's		40	15				

* These samples not used for average DFAA as they are estuarine samples.

Date: 19.3.87 Days. 505

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (12.4)	%DON (5.9)
		‹--------uM-------›				‹----uM-----›	
1	Mallard B.	2.184	1.497	3.681	1	30	62
2	MelampusB.	1.784	0.751	2.535	0.5	20	43
3	New Grnds.	9.370	-----	-----	---	--	--
4	W End BW.	0.629	1.551	2.18	2	18	37
5	Knapp B.	1.910	1.778	3.688	1	30	63
6	Penlee Pt.	1.670	-----	------	---	--	--
Average Values		2.924	1.394			25	51
No. of Samples		6	4			4	4
CV's		109	32				

Date: 1.4.87 Days. 518

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.5)	%DON (6.1)
		‹------uM--------›				‹----uM------›	
1	A9.3 C46	BLD	1.655	1.655	›43	16	27
2	A8 C44.5	BLD	1.338	1.338	48	13	22
3	A7 C43.2	0.757	1.660	2.417	2	23	40
4	A6 C41.5	BLD	1.752	1.752	›62	17	29
5	A5 C40	0.048	----------				
Average Values.		0.161	1.601			17	30
No. of Samples.		5	4			4	4
CV's.		207	11				

Date: 15.4.87 Days. 532

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.5)	%DON (6.1)
		‹-------uM-------›				‹---uM-----›	
1	New Grnd.	BLD	----------				
2	A1.2 C34	BLD	3.645	3.645	› 96	34	60
3	A2.4 B36	0.109	1.809	1.915	17	18	31
4	A3.8 C39	0.062	0.896	0.958	14	9	16
5	A5.5 C42	0.148	0.692	0.777	5	8	15
6	A7.5 C45	0.133	----------				
Average Values.		0.075	1.760			17	31
No. of Samples.		6	4			4	4
CV's.		86	76				

Date: 23.4.87 Days. 540

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.5)	%DON (6.1)
		‹-------uM-------›				‹----uM----›	
1	Draystone	BLD	1.042	1.042	›29	10	17
2	Off Rame.	0.028	1.989	2.017	71	19	33
3	Rame	0.095	1.670	1.765	18	17	30
4	C41 B61	0.097	0.867	0.964	9	9	16
5	C42 B65	0.083	----------				
6	C39 B56	0.056	----------				
7	C36 B50	0.201	----------				
Average Values.		0.080	1.392			14	24
No. of Samples.		7	4			4	4
CV's.		80	38				

Date: 30.4.87 Days. 547

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.5)	%DON (6.1)
		‹--------uM-------›				‹----uM----›	
1	A3 C36	0.027	1.660	1.687	61	16	27
2	A4 C37	0.033	0.852	0.885	26	8	14
3	A5 C38	0.054	2.063	2.117	38	20	35
4	A5 C38	0.048	0.948	0.996	21	10	17
5	A6 C38	BLD	----------				
Average Values.		0.032	1.380			13	23
No. of Samples.		5	4			4	4
CV's.		77	42				

Date: 7.5.87 Days. 554

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.0)	%DON (7.4)
		‹--------uM-------›				‹---uM-----›	
1	Penlee Pt	BLD	1.332	1.332	› 35	13	18
2	Off Rame	BLD	1.792	1.792	› 47	18	24
3	Rame.	0.092	1.359	1.451	14	14	19
4	Whitesand	0.027	1.335	1.362	49	13	18
5	Mewstone	BLD	----------				
6	Yealm	0.219	----------				
Average Value.		0.056	1.454			14	20
No. of Samples.		6	4			4	4
CV's.		154	15				

Date: 14.5.87 Days. 561

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.0)	%DON (7.4)
		‹-------uM-------›				‹---uM-----›	
1	W End BW	BLD	----------				
2	Penlee Pt	BLD	3.278	3.278	› 86	33	44
3	Off Rame	BLD	1.600	1.600	› 42	16	21
4	Rame	BLD	2.213	2.213	› 58	22	30
5	Whitesand	0.042	----------				
Average Values.		0.001	2.363			23	31
No. of Samples.		5	3			3	3
CV's.		223	36				

Date: 21.5.87. Days. 568

No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.0)	%DON (7.4)
		‹--------uM-------›				‹---uM-----›	
1	B46 B55	0.668	1.432	2.100	2	21	28
2	C30 B55	0.041	----------				
3	C30 B54	0.149	0.773	0.922	5	9	12
4	C31 B53	0.256	1.228	1.484	5	15	20
5	C32 B52	0.209	0.816	1.025	4	10	14
Average Value.		0.264	1.062			14	19
No. of Samples.		5	4			4	4
CV's.		90	30				

APPENDIX II.

The Data For Individual Amino Acid Samples Relating To The Batch Data Presented In Appendix I.

Date: 27.11.85 Day: 27.
DFAA's

	Sample No.	
a.a.	1	2
Asp	-----	0.035
Glu	0.025	0.113
Ser	0.066	-----
His	----------	
Gly	0.059	0.094
Thr	----------	
Arg	----------	
Ala	0.036	0.037
Tyr	----------	
Abu	----------	
Val	----------	
Met	----------	
Trp/Ile	----------	
Phe	----------	
Leu	----------	
Orn	----------	
Lys	----------	
TOTAL	0.180	0.279

Date: 11.12.85 Day: 41.
DFAA's.

	Sample No.
a.a.	1
Asp	-----
Glu	-----
Ser	-----
His	-----
Gly	-----
Thr	-----
Arg	-----
Ala	-----
Tyr	-----
Abu	-----
Val	-----
Met	-----
Trp/Ile	-----
Phe	-----
Leu	-----
Orn	-----
Lys	-----
TOTAL	BLD

Date: 17.12.85 Day: 47.
DFAA's.

a.a.	Sample No. 1	2
Asp	----------	
Glu	----------	
Ser	0.073	-----
His	----------	
Gly	----------	
Thr	----------	
Arg	----------	
Ala	0.048	------
Tyr	----------	
Abu	----------	
Val	----------	
Met	----------	
Trp/Ile	----------	
Phe	----------	
Leu	----------	
Orn	----------	
Lys	----------	
TOTAL	0.121	BLD

Date: 23.12.85 Day: 53.
DFAA's.

a.a.	Sample No. 1
Asp	0.060
Glu	-----
Ser	0.200
His	-----
Gly	0.090
Thr	-----
Arg	-----
Ala	0.053
Tyr	0.017
Abu	-----
Val	-----
Met	-----
Trp/Ile	-----
Phe	-----
Leu	-----
Orn	-----
Lys	-----
TOTAL	0.420

Date: 8.1.86 Day: 69.
DFAA's.

	Sample No.
a.a.	1
Asp	0.013
Glu	0.030
Ser	0.015
His	-----
Gly	-----
Thr	-----
Arg	-----
Ala	-----
Tyr	-----
Abu	-----
Val	-----
Met	-----
Trp/Ile	-----
Phe	-----
Leu	-----
Orn	-----
Lys	-----
TOTAL	0.058

Date: 21.1.86 Day:82.
DFAA's.

	Sample No.
a.a.	1
Asp	0.028
Glu	0.137
Ser	0.058
His	-----
Gly	-----
Thr	-----
Arg	-----
Ala	-----
Tyr	-----
Abu	-----
Val	-----
Met	-----
Trp/Ile	-----
Phe	-----
Leu	-----
Orn	-----
Lys	-----
TOTAL	0.239

Date: 23.1.86 Day:84.
DFAA's.

	Sample No.
a.a.	1
Asp	0.137
Glu	-----
Ser	0.046
His	-----
Gly	-----
Thr	-----
Arg	-----
Ala	0.016
Tyr	-----
Abu	-----
Val	-----
Met	-----
Trp/Ile	-----
Phe	-----
Leu	-----
Orn	-----
Lys	-----
TOTAL	0.199

Date: 28.1.86 Day:89.
DFAA's.

	Sample No.
a.a.	1
Asp	------
Glu	------
Ser	------
His	------
Gly	------
Thr	------
Arg	------
Ala	------
Tyr	------
Abu	------
Val	------
Met	------
Trp/Ile	------
Phe	------
Leu	------
Orn	------
Lys	------
TOTAL	BLD

Date: 3.2.86 Day:95.
DFAA's.

a.a.	Sample No. 1
Asp	0.016
Glu	0.048
Ser	0.020
His	-----
Gly	-----
Thr	-----
Arg	-----
Ala	0.017
Tyr	-----
Abu	-----
Val	-----
Met	-----
Trp/Ile	-----
Phe	-----
Leu	-----
Orn	-----
Lys	-----
TOTAL	0.101

Date: 18.2.86 Day:110.
DFAA's.

a.a.	Sample No. 1	2
Asp	0.052	0.062
Glu	0.058	0.035
Ser	0.222	0.200
His	----------	
Gly	0.123	0.113
Thr	----------	
Arg	----------	
Ala	0.082	0.070
Tyr	0.020	0.018
Abu	----------	
Val	0.022	0.057
Met	----------	
Trp/Ile	----------	
Phe	----------	
Leu	----------	
Orn	----------	
Lys	----------	
TOTAL	0.579	0.555

Date: 20.2.86 Day:112.
DFAA's.

	Sample No.
a.a.	1
Asp	-----
Glu	0.026
Ser	0.063
His	-----
Gly	-----
Thr	-----
Arg	-----
Ala	-----
Tyr	-----
Abu	-----
Val	-----
Met	-----
Trp/Ile	-----
Phe	-----
Leu	-----
Orn	-----
Lys	-----
TOTAL	0.089

Date: 4.3.86 Day:124.
DFAA's.

	Sample No.	
a.a.	1	2
Asp	0.027	-----
Glu	0.022	0.017
Ser	0.040	0.073
His	----------	
Gly	----------	
Thr	----------	
Arg	----------	
Ala	0.060	0.110
Tyr	0.012	-----
Abu	----------	
Val	----------	
Met	----------	
Trp/Ile	----------	
Phe	----------	
Leu	----------	
Orn	----------	
Lys	----------	
TOTAL	0.161	0.200

Date: 25.4.86 Day:177. DFAA's.

	Sample No.						
a.a.	1	2	3	4	5	6	7
Asp	----------	----------	----------	0.024	0.028	-----	0.027
Glu	0.045	0.056	0.022	0.065	0.049	0.023	0.048
Ser	----------	----------	----------	----------	0.034	-----	0.103
His	----------	----------	----------	----------	----------	----------	----------
Gly	----------	----------	----------	----------	----------	----------	0.076
Thr	----------	----------	----------	----------	----------	----------	----------
Arg	----------	----------	----------	----------	----------	----------	----------
Ala	0.045	0.041	-----	0.041	0.051	0.013	0.060
Tyr	----------	----------	----------	----------	----------	----------	----------
Abu	----------	----------	----------	----------	----------	----------	----------
Val	----------	----------	----------	----------	----------	----------	----------
Met	----------	----------	----------	----------	----------	----------	----------
Trp/Ile	----------	----------	----------	----------	----------	----------	----------
Phe	----------	----------	----------	----------	----------	----------	----------
Leu	----------	----------	----------	----------	----------	----------	----------
Orn	----------	----------	----------	----------	----------	----------	----------
Lys	----------	----------	----------	----------	----------	----------	----------
TOTAL	0.090	0.097	0.022	0.130	0.162	0.036	0.314

Date: 1.5.86 Day:183. DFAA's.

	Sample No.						
a.a.	1	2	3	4	5	6	7
Asp	----------	0.015	0.065	0.039	0.032	0.022	
Glu	0.032	0.027	0.080	0.054	0.055	0.089	0.052
Ser	0.047	0.057	0.031	0.126	0.078	0.103	0.036
His	----------	----------	----------	----------	----------	----------	----------
Gly	0.036	----------	----------	----------	----------	----------	----------
Thr	----------	----------	----------	----------	----------	----------	----------
Arg	----------	----------	----------	----------	----------	----------	----------
Ala	----------	0.019	0.066	-----	0.079	0.036	
Tyr	----------	----------	----------	----------	0.015	-----	
Abu	----------	----------	----------	----------	----------	----------	----------
Val	----------	----------	----------	----------	----------	----------	----------
Met	----------	----------	----------	----------	----------	----------	----------
Trp/Ile	----------	----------	----------	----------	----------	----------	----------
Phe	----------	----------	----------	----------	----------	----------	----------
Leu	----------	----------	----------	----------	----------	----------	----------
Orn	----------	----------	----------	----------	----------	----------	----------
Lys	----------	----------	----------	----------	----------	----------	----------
TOTAL	0.115	0.084	0.145	0.311	0.162	0.318	0.146

Date: 8.5.86 Day:190. DFAA's.

	Sample No.						
a.a.	1	2	3	4	5	6	7
Asp	0.020	0.027	0.041	0.029	0.032	0.038	0.040
Glu	0.041	0.047	0.062	0.068	0.098	0.163	0.048
Ser	0.042	0.079	0.078	0.055	0.074	0.120	0.064
His	----------						
Gly	0.097	0.116	0.041	-----	0.150	0.131	0.077
Thr	----------						
Arg	----------						
Ala	0.035	-----	0.039	0.036	0.031	0.073	-----
Tyr	-----	0.014	0.028	-----	0.035	0.018	-----
Abu	----------						
Val	----------					0.033	-----
Met	----------						
T/Ile	----------						
Phe	----------						
Leu	----------						
Orn	----------						
Lys	----------						
TOTAL	0.235	0.233	0.289	0.188	0.420	0.576	0.229

Date: 30.5.86 Day:212. DFAA's.

	Sample No.						
a.a.	1	2	3	4	5	6	7
Asp	-----	0.020	0.165	0.062	0.035	0.034	0.040
Glu	0.045	0.050	0.137	0.050	0.053	0.056	0.054
Ser	0.040	0.117	1.437	0.139	0.046	0.139	0.032
His	----------						
Gly	-----	0.040	1.089	----------		0.061	-----
Thr	----------		0.154	----------			
Arg	----------		0.254	----------			
Ala	----------		0.513	0.049	-----	0.058	-----
Tyr	----------		0.117	0.020	0.037	0.031	-----
Abu	----------						
Val	----------		0.189	0.096	----------		
Met	----------						
Trp/Ile	----------						
Phe	----------		0.080	----------			
Leu	----------		0.123	----------			
Orn	----------		0.653	----------			
Lys	----------		0.289	----------			
TOTAL	0.085	0.217	5.200	0.416	0.171	0.379	0.126

Date: 5.6.86 Day:218. DFAA's.

	Sample no.								
a.a.	1	2	3	4	5	6	7	8	9
Asp	0.017	0.062	0.035	-----	-----	0.021	0.040	-----	0.059
Glu	0.083	0.093	0.065	0.055	-----	0.023	0.027	0.055	0.067
Ser	0.067	0.062	0.094	0.098	-----	0.038	0.126	0.036	0.145
His	----------								
Gly	------	0.063	0.036	----------					0.112
Thr	----------								
Arg	----------								
Ala	0.059	0.067	----------				0.079	-----	0.074
Tyr	-----	0.017	----------						
Abu	----------								
Val	----------								0.041
Met	----------								
Trp/Ile	----------								
Phe	----------								
Leu	----------								
Orn	----------								
Lys	----------								
TOTAL	0.226	0.364	0.23	0.153	BLD	0.082	0.272	0.091	0.498

Date: 5.6.86 DCAA's.

	Sample No.	
a.a.	1	2
Asp	0.120	0.050
Glu	0.092	0.050
Ser	0.017	0.060
His	0.192	0.124
Gly	1.455	0.225
Thr	-----	0.015
Arg	0.075	-----
Ala	0.049	-----
Tyr	0.036	0.006
Abu	----------	
Val	0.312	0.005
Met	----------	
Trp/Ile	----------	
Phe	----------	
Leu	0.037	0.05
Orn	----------	
Lys	----------	
TOTAL	2.395	0.585

Date: 12.6.86 Day:225. DFAA's.

	Sample No.								
a.a.	1	2	3	4	5	6	7	8	9
Asp	0.023	0.036	0.070	0.031	0.031	0.029	0.032	0.022	0.032
Glu	0.146	0.099	0.143	0.047	0.050	0.039	0.071	0.069	0.064
Ser	----------		0.472	0.089	0.044	0.085	0.042	0.069	0.056
His	----------		0.111	----------					
Gly	----------		0.371	----------					
Thr	----------								
Arg	----------		0.038	----------					
Ala	0.085	0.050	----------					0.050	0.033
Tyr	----------		0.040	----------					
Abu	----------								
Val	----------		0.047	----------					
Met	----------								
Trp/Ile	----------								
Phe	----------								
Leu	----------		0.066	----------					
Orn	----------								
Lys	----------								
TOTAL	0.254	0.185	1.358	0.167	0.125	0.153	0.145	0.210	0.185

Date:12.6.86 DCAA's.

	Sample No.				
a.a.	1	4	6	7	9
Asp	0.179	0.102	0.184	0.089	0.058
Glu	0.156	0.036	0.239	0.086	0.062
Ser	0.146	-----	0.206	0.057	0.042
His	0.257	0.120	0.166	0.137	0.063
Gly	0.298	0.405	0.488	0.317	0.236
Thr	0.039	0.029	0.022	0.057	0.045
Arg	0.016	-----	0.052	-----	0.036
Ala	0.083	0.016	0.106	0.041	-----
Tyr	0.052	0.018	0.032	0.011	0.014
Abu	----------				
Val	0.148	0.074	0.142	0.121	0.077
Met	----------				
Trp/Ile	----------				
Phe	0.053	----------			
Leu	0.017	----------			
Orn	----------				
Lys	----------				
TOTAL	1.444	0.800	1.637	0.916	0.633

Date: 2.7.86 Day:245. DFAA's.

	Sample No.								
a.a.	1	2	3	4	5	6	7	8	9
Asp	0.113	0.086	0.068	0.056	0.052	0.123	0.264	0.176	0.077
Glu	0.060	0.043	0.071	0.042	0.066	0.063	0.097	0.059	0.111
Ser	0.443	0.312	0.236	0.223	0.216	0.445	1.174	0.721	0.305
His	---	---	---	---	---	---	---	---	---
Gly	0.325	0.195	0.134	0.147	0.119	0.145	0.728	0.473	0.217
Thr	0.040	0.067	---	---	---	0.053	0.238	0.106	---
Arg	---	---	---	---	---	---	0.104	---	---
Ala	0.182	0.102	0.106	0.074	0.090	0.109	0.393	0.262	0.151
Tyr	0.055	0.029	0.016	0.018	0.050	0.038	0.089	0.046	0.035
Abu	---	---	---	---	---	---	---	---	---
Val	0.043	0.063	0.047	0.032	---	0.042	0.135	0.075	0.071
Met	---	---	---	---	---	---	---	---	---
Trp/Ile	---	---	---	---	---	---	0.017	0.064	---
Phe	---	---	---	---	---	---	---	---	---
Leu	0.062	---	---	---	---	---	0.083	0.031	---
Orn	0.151	0.152	---	---	---	---	0.203	0.257	---
Lys	---	---	---	---	---	---	---	---	---
TOTAL	1.474	1.049	0.678	0.592	0.593	1.018	3.527	2.270	0.967

Date: 2.7.86 DCAA's.

	Sample No.				
a.a.	1	3	5	7	8
Asp	0.204	0.098	0.132	0.161	0.226
Glu	0.354	0.132	0.136	0.560	0.460
Ser	0.040	---	---	---	0.209
His	0.275	0.153	0.156	0.276	0.419
Gly	0.280	0.280	0.335	0.370	0.490
Thr	0.162	0.085	0.106	0.093	0.163
Arg	0.108	---	0.024	---	0.050
Ala	0.107	0.034	0.068	0.054	0.156
Tyr	---	---	---	0.050	---
Abu	---	---	---	---	---
Val	0.080	0.062	0.059	0.061	0.194
Met	---	---	---	---	---
Trp/Ile	0.051	0.059	---	0.079	0.018
Phe	0.051	0.067	---	0.079	0.060
Leu	0.020	0.076	---	0.058	0.060
Orn	0.090	0.113	0.089	0.054	0.141
Lys	---	---	---	0.069	---
TOTAL	1.822	1.159	1.105	1.964	2.646

Date: 8.7.86 Day:251 DFAA's.

	Sample No.								
a.a.	1	2	3	4	5	6	7	8	9
Asp	0.159	0.051	0.044	0.061	0.079	0.074	0.086	0.045	0.158
Glu	0.083	0.058	0.051	0.060	0.065	0.066	0.056	0.057	0.086
Ser	0.436	0.141	0.150	0.248	0.317	0.187	0.393	0.193	0.678
His	---	---	---	---	---	---	---	---	---
Gly	0.245	0.060	0.111	-----	0.186	0.098	0.243	0.126	0.483
Thr	0.056	----------	0.047	---	---	---	---	---	0.116
Arg	---	---	---	---	---	---	---	---	---
Ala	0.183	0.092	0.106	0.093	0.109	0.028	0.115	0.067	0.291
Tyr	0.023	----------	---	---	0.030	-----	0.058	-----	0.041
Abu	---	---	---	---	---	---	---	---	---
Val	0.082	----------	0.051	0.031	0.049	0.086	-----		0.080
Met	---	---	---	---	---	---	---	---	---
Trp/Ile	0.038	---	---	---	---	---	---	---	---
Phe	---	---	---	---	---	---	---	---	---
Leu	0.036	---	---	---	---	---	---	---	0.071
Orn	0.170	---	---	---	---	---	---	---	0.235
Lys	---	---	---	---	---	---	---	---	---
TOTAL	1.511	0.402	0.462	0.560	0.817	0.502	1.037	0.488	2.239

Date: 8.7.86 DCAA's.

	Sample No.				
a.a.	1	3	5	8	12
Asp	0.073	0.217	0.182	0.225	0.256
Glu	0.227	0.199	0.268	0.248	0.436
Ser	-----	0.244	0.102	0.129	0.193
His	0.252	0.330	0.182	0.105	0.214
Gly	0.235	0.492	0.380	0.442	0.522
Thr	0.062	0.098	0.113	0.102	0.204
Arg	-----	0.096	0.112	-----	0.152
Ala	0.041	0.141	0.069	0.149	0.124
Tyr	0.002	0.048	0.016	0.056	0.033
Abu	---	---	---	---	---
Val	0.249	0.208	0.263	0.445	0.352
Met	---	---	---	---	---
Trp/Ile	0.012	0.032	---	---	---
Phe	---	---	---	---	0.056
Leu	0.026	0.093	------	0.094	0.037
Orn	---	---	---	---	0.032
Lys	---	---	---	---	---
TOTAL	1.179	2.198	1.687	1.995	2.611

Date: 15.7.86 Day:258 DFAA's.

	Sample No.					
a.a.	1	2	4	5	8	11
Asp	0.039	0.023	0.008	0.034	-----	0.026
Glu	0.080	0.045	-----	0.049	-----	0.043
Ser	-----	0.081	0.123	0.093	-----	0.066
His	----------					
Gly	0.078	----------				
Thr	----------					
Arg	----------					
Ala	-----	0.043	0.035	----------		0.058
Tyr	----------					
Abu	----------					
Val	----------			0.041	----------	
Met	----------					
Trp/Ile	----------					
Phe	----------					
Leu	----------					
Orn	----------					
Lys	----------					
TOTAL	0.197	0.192	0.166	0.217	BLD	0.193

Date: 15.7.86 DCAA's.

	Sample No.		
a.a.	2	8	11
Asp	0.132	0.227	0.211
Glu	0.088	0.168	0.138
Ser	0.117	0.239	0.168
His	0.262	0.233	0.229
Gly	0.502	0.454	0.444
Thr	0.165	0.138	0.176
Arg	0.093	----------	
Ala	0.111	0.268	0.086
Tyr	0.022	0.033	0.032
Abu	----------		
Val	----------		0.013
Met	----------		
Trp/Ile	0.101	----------	
Phe	----------		
Leu	0.054	------	0.033
Orn	----------		
Lys	----------		
TOTAL	1.647	1.760	1.530

Date: 23.7.86 Day:266 DFAA's.

	Sample No.									
a.a.	1	2	3	4	5	6	7	8	9	10
Asp	0.034	0.016	0.028	0.037	0.025	0.046	0.027	0.022	0.027	0.018
Glu	0.042	0.054	0.065	-----	0.043	0.047	0.045	0.026	0.037	-----
Ser	----------		0.135	0.098	0.027	0.114	0.017	-----	0.143	0.081
His	----------									
Gly	----------									
Thr	----------									
Arg	----------									
Ala	0.018	0.055	0.042	0.042	------	0.035	------	0.057	0.048	------
Tyr	----------									
Abu	----------									
Val	----------									
Met	----------									
Trp/Ile	----------									
Phe	----------									
Leu	----------									
Orn	----------									
Lys	----------									
TOTAL	0.094	0.125	0.270	0.177	0.095	0.242	0.089	0.105	0.255	0.099

Date: 23.7.86 DCAA's.

	Sample No.				
a.a.	1	2	4	6	8
Asp	0.291	0.193	0.209	0.369	0.145
Glu	0.266	0.183	0.261	0.414	0.166
Ser	0.368	0.281	0.258	0.565	0.228
His	0.189	0.130	0.164	0.191	0.199
Gly	0.716	0.868	1.019	0.522	0.522
Thr	0.241	0.018	0.167	0.334	0.125
Arg	----------			0.257	0.029
Ala	0.322	0.188	0.223	0.323	0.129
Tyr	0.045	0.045	0.043	0.146	0.026
Abu	----------				
Val	0.358	0.214	0.283	0.206	-----
Met	----------				
Trp/Ile	-----	0.066	-----	0.063	-----
Phe	----------			0.062	-----
Leu	-----	0.074	-----	0.078	-----
Orn	----------				
Lys	----------			0.124	-----
TOTAL	2.796	2.260	2.627	3.655	1.569

Date: 30.7.86 Day:273 DFAA's.

	Sample No.							
a.a.	1	2	3	4	5	6	7	8
Asp	0.030	0.062	0.107	0.035	-----	0.020	0.028	0.044
Glu	0.075	-----	0.065	0.066	-----	0.036	0.046	0.064
Ser	----------		0.346	----------			0.078	0.159
His	----------							
Gly	------	0.037	0.203	----------				0.114
Thr	----------		0.030	----------				
Arg	----------							
Ala	------	0.047	0.178	----------			0.033	0.083
Tyr	----------		0.016	----------				0.029
Abu	----------							
Val	----------		0.049	----------				
Met	----------							
Trp/Ile	----------							
Phe	----------							
Leu	----------							
Orn	----------		0.333	----------				
Lys	----------							
TOTAL	0.105	0.146	1.327	0.101	BLD	0.056	0.185	0.493

Date: 30.7.86 DCAA's

	Sample No.			
a.a.	1	2	6	8
Asp	0.168	0.256	0.152	0.203
Glu	0.162	0.323	0.146	0.242
Ser	0.093	0.426	0.163	0.415
His	0.302	0.116	0.113	0.083
Gly	0.416	0.818	0.612	0.681
Thr	0.083	0.144	0.103	0.184
Arg	----------			0.064
Ala	0.164	0.225	0.142	0.234
Tyr	0.033	0.059	0.027	0.022
Abu	----------			
Val	0.209	0.152	0.221	0.295
Met	----------			
Trp/Ile	------	0.083	----------	
Phe	----------			
Leu	------	0.084	0.046	0.020
Orn	------	0.176	-----	0.145
Lys	------	0.246	----------	
TOTAL	1.630	3.108	1.752	2.588

Date: 6.8.86 Day:280 DFAA's.

	Sample No.						
a.a.	1	2	3	4	5	6	7
Asp	0.032	0.023	0.024	0.024	-----	0.035	0.126
Glu	0.059	0.057	-----	0.065	0.064	0.072	0.139
Ser	0.133	0.083	-----	0.042	0.028	0.109	0.688
His	----------						
Gly	0.128	----------			0.039	0.021	0.447
Thr	----------						
Arg	----------						0.096
Ala	0.068	----------		0.021	0.024	0.054	0.324
Tyr	----------				0.031	0.066	0.062
Abu	----------						
Val	----------					0.049	0.062
Met	----------						
Trp/Ile	----------						
Phe	----------						
Leu	----------						0.067
Orn	----------						0.315
Lys	----------						
TOTAL	0.420	0.163	0.024	0.152	0.186	0.406	2.326

Date: 6.8.86 DCAA's

	Sample No.					
a.a.	1	2	3	4	8	11
Asp	0.084	0.105	0.153	0.138	0.121	0.036
Glu	0.039	0.027	0.156	0.123	0.108	-----
Ser	----------		0.099	0.083	0.028	-----
His	0.116	0.131	0.136	0.146	0.213	0.112
Gly	0.268	0.882	0.639	0.689	0.532	0.139
Thr	0.134	-----	0.056	0.068	----------	
Arg	0.033	----------				
Ala	0.034	0.084	0.128	0.076	0.072	-----
Tyr	0.028	-----	0.034	----------		
Abu	----------					
Val	0.099	0.191	0.193	0.202	0.149	-----
Met	----------					
Trp/Ile	----------					
Phe	----------					
Leu	0.028	----------				
Orn	----------					
Lys	----------				0.264	-----
TOTAL	0.863	1.420	1.594	1.525	1.487	0.287

Date: 4.9.86 Day:309 DFAA's.

	Sample No.								
a.a.	1	2	3	4	5	6	7	8	9
Asp	0.035	0.115	0.049	0.178	0.116	0.084	0.065	0.083	0.087
Glu	0.064	0.075	0.029	0.128	0.094	0.049	0.052	0.093	0.062
Ser	0.094	0.409	0.107	0.713	0.581	0.317	0.254	0.398	0.298
His	----------			0.084	----------				
Gly	0.074	0.235	0.088	0.449	0.407	0.204	0.182	0.198	0.111
Thr	----------			0.158	0.174	----------			
Arg	----------			0.111	0.067	----------			
Ala	0.044	0.137	0.056	0.295	0.204	0.128	0.097	0.154	0.107
Tyr	-----	0.044	-----	0.064	0.039	0.056	-----	0.015	-----
Abu	----------								
Val	-----	0.086	------	0.192	0.149	0.051	-----	0.068	0.052
Met	----------								
Trp/Ile	----------								
Phe	----------			0.068	----------				
Leu	----------			0.044	0.044	----------			
Orn	------	0.251	------	0.304	0.129	----------		0.116	------
Lys	----------								
TOTAL	0.311	1.352	0.329	2.788	2.004	0.889	0.650	1.125	0.717

Date: 4.9.86 DCAA's.

	Sample No.				
a.a.	1	4	5	8	11
Asp	0.578	0.083	0.148	0.033	0.199
Glu	0.522	0.102	0.156	0.052	0.166
Ser	0.783	-----	0.138	0.028	0.132
His	0.444	0.106	0.150	0.105	0.116
Gly	1.135	0.228	0.382	0.257	0.547
Thr	0.406	0.068	0.058	0.050	0.084
Arg	0.188	-----	0.048	-----	0.073
Ala	0.640	0.074	0.128	0.033	0.130
Tyr	0.043	----------			0.015
Abu	----------				
Val	0.200	-----	0.092	0.043	0.058
Met	----------				
Trp/Ile	0.142	0.012	----------		
Phe	0.066	0.038	0.064	----------	
Leu	0.139	-----	0.080	----------	
Orn	0.252	-----	0.126	-----	0.119
Lys	0.157	0.116	0.115	----------	
TOTAL	5.695	0.827	1.685	0.611	1.639

Date: 24.9.86 Day:329 DFAA's.

	Sample No.					
a.a.	1	2	4	5	7	8
Asp	0.225	0.201	0.063	0.107	0.092	-----
Glu	0.073	0.077	0.052	0.067	0.044	-----
Ser	0.815	0.410	0.113	0.247	0.316	0.312
His	----------					
Gly	0.375	0.343	----------		0.160	0.153
Thr	0.075	0.113	----------			
Arg	0.125	----------		0.111	----------	
Ala	0.325	0.217	0.114	0.146	0.154	0.159
Tyr	0.029	0.071	----------		0.033	------
Abu	----------					
Val	0.185	0.136	0.022	0.088	0.089	0.082
Met	----------					
Trp/Ile	----------					
Phe	----------					
Leu	----------					
Orn	0.149	0.169	----------			
Lys	----------					
TOTAL	2.376	1.738	0.364	0.766	0.888	0.706

Date: 24.9.86 DCAA's

	Sample No.					
a.a	1	2	4	5	7	8
Asp	0.088	0.268	0.482	1.109	0.266	0.338
Glu	0.146	0.208	0.335	0.489	0.190	0.224
Ser	-----	0.025	0.365	0.608	0.066	-----
His	0.152	0.283	0.249	0.268	----------	
Gly	0.741	0.508	0.855	0.718	0.692	0.383
Thr	0.121	-----	0.103	0.112	0.051	0.045
Arg	0.012	0.125	0.264	0.083	----------	
Ala	0.008	0.074	0.182	0.253	0.057	0.030
Tyr	0.019	-----	0.084	0.068	-----	0.040
Abu	----------					
Val	0.026	0.090	0.263	-----	0.122	-----
Met	----------					
Trp/Ile	----------			0.108	----------	
Phe	0.016	0.012	0.099	0.044	----------	
Leu	----------			0.142	----------	
Orn	0.052	-----	0.204	----------		
Lys	----------					
TOTAL	1.381	1.593	3.485	4.002	1.444	1.060

Date: 1.10.86 Day:336 DFAA's.

	Sample No.							
a.a.	1	2	3	4	5	6	7	8
Asp	-----	0.078	0.051	-----	0.102	0.046	0.097	0.048
Glu	0.042	0.037	0.015	0.034	-----	0.033	0.044	-----
Ser	-----	0.121	0.130	0.153	0.373	0.218	0.663	0.125
His	----------							
Gly	----------		0.107	0.070	0.235	0.136	0.293	-----
Thr	----------						0.058	-----
Arg	----------							
Ala	-----	0.051	-----	0.064	0.145	0.070	0.275	0.014
Tyr	----------				0.023	-----	0.058	-----
Abu	----------							
Val	----------		0.043	------	0.061	------	0.137	------
Met	----------							
Trp/Ile	----------							
Phe	----------							
Leu	----------							
Orn	----------						0.121	------
Lys	----------							
TOTAL	0.042	0.287	0.346	0.321	0.939	0.503	1.746	0.187

Date: 1.10.86 DCAA's

	Sample No.				
a.a.	1	2	4	5	8
Asp	0.165	0.196	0.108	0.037	0.118
Glu	0.081	0.209	0.007	0.091	0.095
Ser	0.012	0.234	----------		
His	0.513	0.484	0.202	0.206	-----
Gly	0.322	1.374	0.467	0.252	0.509
Thr	0.066	0.213	----------		0.111
Arg	-----	0.205	-----	0.087	-----
Ala	0.112	0.209	----------		0.121
Tyr	----------				0.042
Abu	----------				
Val	0.022	0.278	0.051	0.021	0.245
Met	----------				
Trp/Ile	----------				
Phe	----------				
Leu	-----	0.046	----------		
Orn	----------				
Lys	----------				
TOTAL	1.293	3.448	0.835	0.694	1.241

Date: 16.10.86 Day:351 DFAA's.

a.a.	Sample No. 1	2	3	4	5	6	7	8
Asp	0.032	----------						
Glu	0.032	0.062	-----	0.028	-----	0.037	----------	
Ser	0.077	----------						
His	----------							
Gly	----------							
Thr	----------							
Arg	----------							
Ala	----------							
Tyr	----------							
Abu	----------							
Val	----------							
Met	----------							
Trp/Ile	----------						----------	
Phe	----------							
Leu	----------							
Orn	----------							
Lys	----------							
TOTAL	0.141	0.062	BLD	0.028	BLD	0.037	BLD	BLD

Date: 16.10.86 DCAA's

a.a.	Sample No. 1	3	4	6
Asp	0.115	0.183	0.155	0.094
Glu	0.125	0.152	0.162	0.072
Ser	----------			0.046
His	0.232	0.176	----------	
Gly	0.183	0.442	0.766	0.460
Thr	-----	0.124	----------	
Arg	----------			
Ala	0.046	0.100	0.116	0.100
Tyr	-----	0.054	0.042	-----
Abu	----------			
Val	0.004	0.128	0.068	0.079
Met	----------			
Trp/Ile	----------			
Phe	----------			
Leu	----------			
Orn	----------			
Lys	----------			
TOTAL	0.705	1.359	1.309	0.851

Date: 23.10.86 Day:358 DFAA's.

	Sample No.							
a.a.	1	2	3	4	5	6	7	8
Asp	----------					0.023	------	0.062
Glu	----------							
Ser	---------		0.083	0.063	----------		0.101	0.186
His	----------							
Gly	----------							
Thr	----------							
Arg	----------							
Ala	----------						0.025	0.056
Tyr	----------							
Abu	----------							
Val	----------							
Met	----------							
Trp/Ile	----------							
Phe	----------							
Leu	----------							
Orn	----------							
Lys	----------							
TOTAL	BLD	BLD	0.083	0.063	BLD	0.023	0.126	0.304

Date: 23.10.86 DCAA's

	Sample No.			
a.a.	1	2	3	4
Asp	0.214	0.588	0.251	0.280
Glu	0.149	0.606	0.155	0.260
Ser	0.043	0.548	0.146	0.189
His	-----	0.576	0.364	-----
Gly	0.708	1.214	1.044	1.463
Thr	-----	0.391	----------	
Arg	-----	0.465	-----	0.139
Ala	0.204	0.502	0.103	0.256
Tyr	-----	0.181	-----	0.056
Abu	----------			
Val	0.372	0.394	0.295	0.248
Met	----------			
Trp/Ile	----------			
Phe	----------			
Leu	-----	0.209	------	0.086
Orn	----------			0.138
Lys	----------			
TOTAL	1.690	6.080	2.358	3.115

Date: 8.11.86 Day:371 DFAA's.

	Sample No.						
a.a.	1	2	3	4	5	6	7
Asp	0.109						0.048
Glu	0.062		0.025				0.026
Ser	0.223	0.096			0.050		0.118
His							
Gly	0.166	0.057		0.069			
Thr							
Arg							
Ala	0.121	0.087		0.082		0.067	
Tyr	0.041						
Abu							
Val	0.140				0.065		0.039
Met							
T/Ile							
Phe							
Leu							
Orn							
Lys							
TOTAL	0.862	0.242	0.025	0.151	0.115	0.067	0.231

Date: 8.11.86 DCAA's

	Sample No.				
a.a.	1	2	4	6	7
Asp	0.026	0.130	0.101	0.104	0.027
Glu	0.052	0.102	0.112	0.071	0.073
Ser					
His		0.174	0.191		0.183
Gly	0.290	0.358	0.781	0.348	0.509
Thr					
Arg					
Ala		0.007		0.063	0.100
Tyr					
Abu					
Val	0.050	0.013	0.148	0.123	0.112
Met					
Trp/Ile	0.068				
Phe					
Orn					
Lys					
TOTAL	0.486	0.784	1.333	0.709	1.004

Date: 13.11.86 Day:379 DFAA's.

	Sample No.							
a.a.	1	2	3	4	5	6	9	10
Asp	0.030	----------	----------	----------	----------	----------	0.030	-------
Glu	----------	----------	----------	----------	----------	----------	0.045	-------
Ser	0.090	-------	0.052	----------	----------	0.202	0.048	0.070
His	----------	----------	----------	----------	----------	----------	----------	----------
Gly	----------	----------	----------	----------	----------	----------	----------	----------
Thr	----------	----------	----------	----------	----------	----------	----------	----------
Arg	----------	----------	----------	----------	----------	----------	----------	----------
Ala	----------	----------	----------	----------	----------	----------	0.066	-------
Tyr	----------	----------	----------	----------	----------	----------	----------	----------
Abu	----------	----------	----------	----------	----------	----------	----------	----------
Val	----------	----------	----------	----------	----------	----------	----------	----------
Met	----------	----------	----------	----------	----------	----------	----------	----------
Trp/Ile	----------	----------	----------	----------	----------	----------	----------	----------
Phe	----------	----------	----------	----------	----------	----------	----------	----------
Leu	----------	----------	----------	----------	----------	----------	----------	----------
Orn	----------	----------	----------	----------	----------	----------	----------	----------
Lys	----------	----------	----------	----------	----------	----------	----------	----------
TOTAL	0.120	BLD	0.052	BLD	BLD	0.202	0.189	0.070

Date: 13.11.86 DCAA's

	Sample No.				
a.a.	1	2	4	9	10
Asp	0.090	0.201	0.218	0.172	0.227
Glu	0.090	0.155	0.121	0.069	0.141
Ser	-----	0.098	0.108	0.074	0.007
His	----------	----------	----------	----------	0.402
Gly	0.777	0.683	1.029	0.635	0.423
Thr	----------	----------	----------	0.126	0.122
Arg	----------	----------	----------	0.059	-----
Ala	0.099	0.154	0.149	0.096	0.192
Tyr	0.052	----------	----------	----------	----------
Abu	----------	----------	----------	----------	----------
Val	0.103	0.104	0.152	0.076	0.109
Met	----------	----------	----------	----------	----------
Trp/Ile	0.038	----------	----------	----------	----------
Phe	----------	----------	----------	----------	----------
Leu	-----	0.041	0.084	----------	----------
Orn	----------	----------	----------	----------	----------
Lys	----------	----------	----------	----------	----------
TOTAL	1.249	1.436	1.861	1.308	1.623

Date: 20.11.86 Day:386 DFAA's.

	Sample No.				
a.a.	1	2	3	4	5
Asp	----------			0.045	0.044
Glu	----------		0.131	0.044	------
Ser	----------		0.042	0.097	0.147
His	----------				
Gly	----------		0.097	-----	0.080
Thr	----------				
Arg	----------				
Ala	----------			0.045	0.085
Tyr	----------		0.073	0.019	------
Abu	----------				
Val	----------				
Met	----------				
Trp/Ile	----------				
Phe	----------				
Leu	----------				
Orn	----------				
Lys	----------				
TOTAL	BLD	BLD	0.343	0.250	0.356

Date: 20.11.86 DCAA's

	Sample No.			
a.a.	1	2	3	4
Asp	0.148	0.102	0.107	0.080
Glu	0.120	0.007	-----	0.008
Ser	0.032	----------		
His	0.271	0.373	-----	0.220
Gly	0.390	0.721	0.643	0.432
Thr	----------			
Arg	----------			
Ala	0.105	0.032	0.090	-----
Tyr	-----	0.080	----------	
Abu	----------			
Val	0.109	-----	0.101	0.072
Met	----------			
Trp/Ile	----------			
Phe	----------			
Leu	----------		0.050	-----
Orn	----------			
Lys	----------			
TOTAL	1.175	1.315	0.991	0.812

Date: 27.11.86 Day:393 DFAA's.

	Sample No.					
a.a.	1	2	3	4	5	6
Asp	----------					
Glu	----------					
Ser	----------		0.044	0.074	0.058	-----
His	----------					
Gly	----------					
Thr	----------					
Arg	----------					
Ala	----------					
Tyr	----------					
Abu	----------					
Val	----------					
Met	----------					
Trp/Ile	----------					
Phe	----------					
Leu	----------					
Orn	----------					
Lys	----------					
TOTAL	BLD	BLD	0.044	0.074	0.058	BLD

Date: 27.11.86 DCAA's

	Sample No.				
a.a.	1	2	3	4	5
Asp	0.082	0.247	0.234	0.165	0.089
Glu	0.072	0.311	0.212	0.146	0.086
Ser	0.123	0.167	0.185	0.212	0.073
His	-----	0.311	0.190	----------	
Gly	0.310	0.409	0.380	0.390	0.242
Thr	-----	0.141	0.157	0.120	-----
Arg	-----	0.109	0.102	0.116	0.054
Ala	0.294	0.184	0.224	0.192	0.084
Tyr	-----	0.056	0.048	0.056	-----
Abu	----------				
Val	0.106	0.056	0.082	0.055	0.013
Met	----------				
Trp/Ile	----------				
Phe	-----	0.056	----------		
Leu	0.103	0.114	0.076	0.063	-----
Orn	----------			0.118	-----
Lys	----------		0.125	----------	
TOTAL	1.090	2.161	2.015	1.633	0.641

Date: 10.12.86 Day:406 DFAA's.

	Sample No.					
a.a.	1	2	3	4	5	6	10
Asp	0.144	----------			0.031	----------	
Glu	0.066	----------					
Ser	0.453	----------					0.104
His	----------						
Gly	0.353	----------					0.107
Thr	0.109	----------					
Arg	----------						
Ala	0.178	----------					0.060
Tyr	0.049	----------					
Abu	----------						
Val	0.059	----------					
Met	----------						
Trp/Ile	0.079	----------					0.091
Phe	----------						
Leu	----------						
Orn	0.276	----------					
Lys	----------						
TOTAL	1.766	BLD	BLD	BLD	0.031	BLD	0.362

Date: 10.12.86 DCAA's

	Sample No.				
a.a.	1	2	3	4	10
Asp	0.019	0.122	0.101	0.058	0.108
Glu	0.019	0.149	0.116	0.138	0.166
Ser	-----	0.090	0.102	0.098	-----
His	----------			0.293	-----
Gly	0.171	0.864	0.647	0.888	0.551
Thr	----------				
Arg	----------				0.051
Ala	0.023	0.093	----------		
Tyr	-----	0.001	----------		
Abu	----------				
Val	-----	0.092	0.063	-----	0.046
Met	----------				
Trp/Ile	0.014	0.270	0.138	----------	
Phe	-----	0.079	0.024	----------	
Leu	-----	0.178	0.042	----------	
Orn	----------				
Lys	----------				
TOTAL	0.246	1.938	1.233	1.475	0.922

Date: 18.12.86 Day:414 DFAA's.

	Sample No.						
a.a.	1	2	3	4	5	6	10
Asp	----------	----------	----------	----------	----------	0.032	------
Glu	----------	----------	----------	----------	----------	----------	----------
Ser	0.152	0.028	----------	----------	0.114	0.127	------
His	----------	----------	----------	----------	----------	----------	----------
Gly	0.057	----------	----------	----------	----------	0.082	------
Thr	----------	----------	----------	----------	----------	----------	----------
Arg	----------	----------	----------	----------	----------	----------	----------
Ala	0.047	----------	----------	----------	----------	----------	----------
Tyr	----------	----------	----------	----------	----------	----------	----------
Abu	----------	----------	----------	----------	----------	----------	----------
Val	----------	----------	----------	----------	----------	----------	----------
Met	----------	----------	----------	----------	----------	----------	----------
Trp/Ile	----------	----------	----------	----------	----------	----------	----------
Phe	----------	----------	----------	----------	----------	----------	----------
Leu	----------	----------	----------	----------	----------	----------	----------
Orn	----------	----------	----------	----------	----------	----------	----------
Lys	----------	----------	----------	----------	----------	----------	----------
TOTAL	0.256	0.028	BLD	BLD	0.114	0.241	BLD

Date: 18.12.86 DCAA's

	Sample No.		
a.a.	2	4	10
Asp	0.092	0.067	0.162
Glu	0.074	0.076	0.248
Ser	-----	0.074	0.096
His	0.117	0.235	0.324
Gly	0.636	1.193	2.008
Thr	----------	----------	----------
Arg	-----	0.052	-----
Ala	-----	0.071	0.183
Tyr	----------	----------	----------
Abu	----------	----------	----------
Val	0.048	0.046	0.237
Met	----------	----------	----------
Trp/Ile	-----	0.026	-----
Phe	----------	----------	----------
Leu	----------	----------	0.059
Orn	----------	----------	----------
Lys	-----	0.054	-----
TOTAL	0.967	1.894	3.367

Date: 8.1.87 Day:435

	Sample No.							
a.a.	1	2	3	4	5	6	7	8
Asp	0.035	0.033	----------					
Glu	----------							
Ser	-------	0.173	----------					0.048
His	----------							
Gly	-------	0.096	----------					
Thr	----------							
Arg	----------							
Ala	-------	0.042	----------					
Tyr	----------							
Abu	----------							
Val	----------							
Met	----------							
Trp/Ile	----------							
Phe	----------							
Leu	----------							
Orn	----------							
Lys	----------							
TOTAL	0.035	0.344	BLD	BLD	BLD	BLD	BLD	0.048

Date: 8.1.87 DCAA's

	Sample No.				
a.a.	1	2	4	5	6
Asp	0.034	----------			0.016
Glu	-----	0.062	----------		
Ser	0.190	0.137	----------		0.260
His	0.115	----------			
Gly	2.062	1.544	0.958	0.503	1.694
Thr	0.042	----------			
Arg	0.113	0.122	0.046	0.084	0.126
Ala	0.076	0.116	----------		0.060
Tyr	----------				
Abu	----------				
Val	0.299	0.149	0.042	0.054	0.123
Met	----------				
Trp/Il	----------				0.071
Phe	----------				
Leu	----------				
Orn	0.124	0.086	----------		
Lys	----	0.186	0.187	0.106	0.124
TOTAL	3.055	2.402	1.233	0 808	2.474

Date: 15.1.87 Day:442 DFAA's-

	Sample No.					
a.a.	1	2	3	6	8	10
Asp	0.071	-----	0.064	0.055	0.045	0.133
Glu	0.022	0.044	----------		0.041	0.008
Ser	0.476	0.464	0.548	0.387	0.283	0.947
His	----------					
Gly	0.260	0.288	0.269	----------		
Thr	----------					
Arg	----------					
Ala	0.113	0.093	0.106	0.075	0.083	0.208
Tyr	0.017	-----	0.045	0.041	0.032	0.082
Abu	----------					
Val	0.047	0.056	0.052	0.047	------	0.085
Met	----------					
Trp/Ile	----------					
Phe	----------					
Leu	----------					0.080
Orn	0.196	0.144	0.192	0.128	0.123	0.790
Lys	----------					0.127
TOTAL	1.202	1.089	1.276	0.733	0.607	2.460

Date 15.1.87 DCAA's

	Sample No.					
a.a.	1	2	3	6	8	10
Asp	-----	0.038	----------		0.066	0.074
Glu	0.090	0.062	0.075	0.099	0.065	0.193
Ser	0.049	----------		0.096	0.185	-----
His	----------					
Gly	0.100	0.652	0.287	0.594	0.620	0.420
Thr	----------					0.114
Arg	0.034	----------		0.096	----------	
Ala	0.013	0.061	0.029	0.052	-----	0.012
Tyr	0.206	0.190	0.156	0.301	0.220	0.098
Abu	----------					
Val	-----	0.002	----------			
Met	----------					
Trp/Ile	----------				0.033	------
Phe	----------					
Leu	----------			0.026	----------	
Orn	----------					
Lys	----------					
TOTAL	0.492	1.005	0.547	1.264	1.189	0.910

Date: 30.1.87 Day:457 DFAA's

	Sample No.						
a.a.	1	2	3	4	5	6	7
Asp							
Glu							
Ser				0.076			
His							
Gly							
Thr							
Arg							
Ala							
Tyr							
Abu							
Val							
Met							
Trp/Ile							
Phe							
Leu							
Orn							
Lys							
TOTAL	BLD	BLD	BLD	0.076	BLD	BLD	BLD

Date: 30.1.87 DCAA's

	Sample No.				
a.a.	1	3	4	5	6
Asp	0.078	0.109	0.109	0.075	0.057
Glu	0.049	0.076	0.036	0.043	0.080
Ser					0.017
His					
Gly	0.650	0.843	0.276	0.898	0.886
Thr					
Arg					
Ala	0.112	0.149	0.008	0.101	
Tyr					
Abu					
Val	0.002	0.022	0.057		0.070
Met					
Trp/Ile					
Phe					
Leu	0.052				
Orn					
Lys					
TOTAL	0.943	1.199	0.486	1.117	1.110

Date: 5.2.87 Day:463 DFAA's.

	Sample No.							
a.a.	1	2	3	4	5	6	7	8
Asp	0.104	0.114	-----	0.064	0.026	0.059	0.022	-----
Glu	0.063	0.064	----------			0.042	----------	
Ser	0.752	0.638	0.206	0.186	-----	0.328	0.140	-----
His	----------							
Gly	0.320	0.344	0.064	0.067	-----	0.046	----------	
Thr	----------							
Arg	----------							
Ala	0.175	0.098	----------					
Tyr	0.042	0.061	----------			0.025	----------	
Abu	----------							
Val	----------							
Met	----------							
Trp/Ile	----------							
Phe	----------							
Leu	----------							
Orn	------	0.104	----------					
Lys	----------							
TOTAL	1.456	1.423	0.270	0.317	0.026	0.500	0.162	BLD

Date: 5.2.87 DCAA's

	Sample No.			
a.a.	1	3	4	5
Asp	0.055	0.163	0.052	0.164
Glu	0.268	0.437	0.058	0.264
Ser	-----	0.063	-----	0.236
His	----------			0.420
Gly	0.504	0.790	0.772	1.443
Thr	----------			0.168
Arg	----------			0.198
Ala	0.056	0.118	0.088	0.189
Tyr	----------		0.007	0.034
Abu	----------			
Val	----------		0.062	0.153
Met	----------			
Trp/Ile	----------			0.126
Phe	----------			0.123
Leu	----------			0.166
Orn	------	0.278	----------	
Lys	----------			0.186
TOTAL	0.883	1.849	1.039	3.861

Date: 12.2.87 Day:470

	Sample No.						
a.a.	1	2	3	4	5	6	7
Asp	-----	0.024	----------			0.032	------
Glu	0.052	0.033	----------				
Ser	-----	0.043	----------			0.039	------
His	----------						
Gly	-----	0.058	----------				
Thr	----------						
Arg	----------						
Ala	----------						
Tyr	----------						
Abu	----------						
Val	----------						
Met	----------						
Trp/Ile	----------						
Phe	----------						
Leu	----------						
Orn	----------						
Lys	----------						
TOTAL	0.052	0.158	BLD	BLD	BLD	0.071	BLD

Date: 12.2.87 DCAA's

	Sample No.				
a.a.	1	2	4	5	6
Asp	0.198	0.116	0.097	0.147	0.042
Glu	0.112	0.057	0.086	0.060	0.038
Ser	0.208	0.072	0.100	0.220	-----
His	----------				
Gly	1.352	1.480	1.285	1.677	1.618
Thr	----------			0.053	0.106
Arg	----------				
Ala	0.155	0.141	0.092	0.124	0.046
Tyr	0.113	----------		0.035	0.019
Abu	----------				
Val	0.275	0.083	0.176	0.236	0.136
Met	----------				
Trp/Ile	0.133	0.052	----------		
Phe	0.126	----------			
Leu	0.116	----------			
Orn	----------				
Lys	----------		0.168	----------	
TOTAL	2.788	2.001	2.004	2.552	2.005

Date: 19.2.87 Day:477 DFAA's.

	Sample No.					
a.a	10	9	8	7	6	5	4
Asp	----------		0.056	----------			0.032
Glu	----------		0.033	----------			----------
Ser	-----	0.072	0.291	----------			0.030
His	----------		----------	----------			----------
Gly	----------		0.223	----------			----------
Thr	----------		0.036	----------			----------
Arg	----------		0.065	----------			----------
Ala	----------		----------	----------			----------
Tyr	----------		0.041	----------			----------
Abu	----------		----------	----------			----------
Val	----------		----------	----------			----------
Met	----------		----------	----------			----------
Trp/Ile	----------		----------	----------			----------
Phe	----------		----------	----------			----------
Leu	----------		----------	----------			----------
Orn	----------		----------	----------			----------
Lys	----------		----------	----------			----------
TOTAL	BLD	0.072	0.745	BLD	BLD	BLD	0.062

Date: 19.2.87 DCAA's

	Sample No.				
a.a.	10	9	8	7	6
Asp	0.100	0.108	0.212	0.135	0.148
Glu	0.066	0.081	0.352	0.081	0.118
Ser	0.070	-----	0.280	0.131	0.151
His	----------		----------	----------	----------
Gly	0.710	0.355	0.576	0.272	0.382
Thr	0.056	----------	----------	----------	0.114
Arg	----------		----------	----------	----------
Ala	0.057	0.083	0.257	0.089	0.136
Tyr	----------		----------	----------	0.021
Abu	----------		----------	----------	----------
Val	0.071	0.051	0.221	0.066	0.328
Met	----------		----------	----------	----------
Trp/Ile	----------		0.117	-----	0.084
Phe	----------		0.063	----------	
Leu	----------		0.043	-----	0.070
Orn	----------		0.243	----------	
Lys	----------		0.116	0.132	0.125
TOTAL	1.130	0.678	2.480	0.906	1.677

Date: 26.2.87 Day:484 DFAA's.

	Sample No.						
a.a.	1	2	3	4	5	6	7
Asp	0.131	0.057	0.089	0.068	0.082	0.055	0.125
Glu	0.058	0.037	0.045	-----	0.033	-----	0.049
Ser	0.268	0.239	0.429	0.341	0.383	0.290	0.740
His	----------						
Gly	0.207	0.214	0.327	0.266	-----	0.290	0.497
Thr	----------			0.063	0.078	-----	0.067
Arg	----------						
Ala	0.081	0.054	0.079	0.105	0.102	0.088	0.193
Tyr	0.036	0.028	0.034	0.042	0.034	0.043	0.053
Abu	----------						
Val	0.041	0.035	0.043	0.034	0.042	0.040	0.071
Met	----------						
Trp/Ile	----------						0.068
Phe	----------						
Leu	0.048	-----	0.044	----------			0.035
Orn	0.093	0.161	0.144	----------		0.128	0.196
Lys	----------						
TOTAL	0.963	0.825	1.234	0.919	0.754	0.934	2.094

Date: 26.2.87 DCAA's

	Sample No.				
a.a.	1	3	4	5	6
Asp	0.088	0.062	0.122	0.576	1.142
Glu	0.252	0.232	0.308	1.083	1.368
Ser	0.144	-----	0.098	1.047	1.383
His	----------				0.276
Gly	0.653	0.406	0.516	1.830	0.996
Thr	0.123	0.103	0.123	0.162	0.436
Arg	----------			0.192	0.458
Ala	0.134	0.106	0.116	0.422	0.443
Tyr	----------				0.301
Abu	----------				
Val	0.139	0.122	0.083	0.256	0.485
Met	----------				
Trp/Ile	----------			0.175	-----
Phe	----------			0.208	0.274
Leu	0.053	0.044	0.068	0.276	0.428
Orn	----------		0.143	0.286	-----
Lys	----------			0.228	0.338
TOTAL	1.586	1.075	1.577	6.741	8.298

Date: 5.3.87 Day:491 DFAA's.

	Sample No.						
a.a.	1	2	3	4	5	6	7
Asp	0.122	0.196	0.204	0.113	0.108	0.164	0.256
Glu	0.083	0.108	0.087	0.054	0.064	0.090	0.068
Ser	0.585	0.935	1.115	0.608	0.652	0.953	1.538
His	----------		0.180	----------			0.249
Gly	0.275	0.437	0.517	0.366	0.424	0.482	0.965
Thr	0.073	0.146	0.128	0.053	0.119	0.138	0.242
Arg	----------		0.084	----------			
Ala	0.122	0.186	0.257	0.152	0.152	0.219	0.352
Tyr	0.038	0.094	0.092	0.067	0.056	0.064	0.100
Abu	----------						
Val	-----	0.094	0.102	0.076	0.057	0.115	0.121
Met	----------						
Trp/Ile	0.032	-----	0.080	----------			
Phe	-----	0.071	----------				
Leu	-----	0.028	0.092	0.074	----------		0.102
Orn	0.157	0.329	0.392	0.229	0.139	0.323	0.443
Lys	----------		0.128	----------			0.098
TOTAL	1.487	2.624	3.458	1.792	1.771	2.548	4.534

Date: 5.3.87 DCAA's

	Sample No.				
a.a.	1	2	3	4	5
Asp	0.155	0.218	0.243	0.269	0.306
Glu	0.469	0.655	0.745	0.573	0.453
Ser	0.047	0.073	-----	0.293	0.097
His	-----	0.362	0.266	0.479	-----
Gly	1.424	1.032	1.531	1.339	0.842
Thr	0.197	0.096	0.185	0.175	0.112
Arg	----------		0.004	0.053	-----
Ala	0.225	0.280	0.168	0.213	0.206
Tyr	----------				
Abu	----------				
Val	0.336	0.203	0.223	0.198	0.288
Met	----------				
Trp/Ile	0.105	0.166	-----	0.137	0.064
Phe	-----	0.006	-----	0.112	0.092
Leu	-----	0.126	0.036	0.043	-----
Orn	0.062	0.093	0.132	0.116	0.155
Lys	-----	0.183	0.048	0.166	-----
TOTAL	3.020	3.493	3.581	4.167	2.615

Date: 12.3.87 Day:498 DFAA's.

	Sample No.						
a.a.	9	8	7	6	5	4	3
Asp	0.124	0.087	0.102	0.046	0.046	0.041	0.038
Glu	0.072	-----	0.071	0.047	0.055	0.047	0.035
Ser	0.336	0.328	0.471	0.156	0.093	0.132	0.048
His	----------						
Gly	-----	0.235	0.261	0.124	0.126	0.097	-----
Thr	----------						
Arg	----------						
Ala	-----	0.065	0.122	0.044	----------		
Tyr	0.027	0.019	0.030	----------			
Abu	----------						
Val	0.035	0.027	----------				
Met	----------						
Trp/Ile	----------						
Phe	----------						
Leu	----------						
Orn	0.093	------	0.096	----------			
Lys	----------						
TOTAL	0.687	0.761	1.153	0.418	0.320	0.317	0.121

Date: 12.3.87 DCAA's

	Sample No.			
a.a.	9	8	7	6
Asp	0.082	0.208	0.287	0.226
Glu	0.309	0.430	0.467	0.244
Ser	0.141	0.056	0.092	0.075
His	----------			
Gly	0.616	0.842	0.645	0.288
Thr	----------			
Arg	0.088	-----	0.143	-----
Ala	0.458	0.216	0.124	0.079
Tyr	0.020	0.017	----------	
Abu	----------			
Val	0.113	0.141	0.363	0.428
Met	----------			
Trp/Ile	----------			
Phe	0.052	----------		
Leu	0.144	0.113	0.296	0.494
Orn	-----	0.161	----------	
Lys	----------		0.192	-----
TOTAL	2.023	2.184	2.609	1.835

Date: 19.3.87 Day:505 DFAA's.

	Sample No.					
a.a.	1	2	3	4	5	6
Asp	0.112	0.091	0.199	0.057	0.126	0.117
Glu	0.079	0.059	0.235	-----	0.060	0.032
Ser	0.668	0.543	0.637	0.201	0.735	0.632
His	-----	0.291	0.210	----------		
Gly	0.392	0.322	5.033	0.161	0.378	0.323
Thr	0.114	0.096	0.170	-----	0.128	0.104
Arg	0.097	-----	0.920	----------		
Ala	0.152	0.138	0.589	0.051	0.159	0.168
Tyr	0.052	0.039	0.203	0.019	0.062	0.036
Abu	----------		0.050	----------		
Val	0.103	0.048	0.179	------	0.058	0.056
Met	----------		0.082	----------		
Trp/Ile	----------		0.197	----------		
Phe	0.055	-----	0.143	----------		
Leu	0.079	-----	0.322	------	0.045	0.028
Orn	0.289	0.157	----------		0.168	0.174
Lys	----------		0.203	0.140	----------	
TOTAL	2.184	1.784	9.370	0.629	1.910	1.670

Date: 19.3.87 DCAA's

	Sample No.			
a.a.	1	2	4	5
Asp	0.222	0.052	0.149	0.245
Glu	0.496	0.236	0.238	0.497
Ser	----------		0.018	-----
His	----------		----------	0.137
Gly	0.374	0.134	0.563	0.492
Thr	0.010	0.040	0.168	-----
Arg	----------		----------	
Ala	0.082	-----	0.106	0.182
Tyr	----------		----------	
Abu	----------		----------	
Val	0.133	0.060	0.236	0.139
Met	----------		----------	
Trp/Ile	0.099	0.047	-----	0.070
Phe	----------		----------	
Leu	0.081	0.108	0.073	-----
Orn	-----	0.074	-----	0.016
Lys	----------		----------	
TOTAL	1.497	0.751	1.551	1.778

Date: 1.4.87 Day 518. DFAA's.

	Sample No.				
a.a.	1	2	3	4	5
Asp	----------		0.075	----------	
Glu	----------		----------		
Ser	----------		0.216	-----	0.048
His	----------		----------		
Gly	----------		0.052	----------	
Thr	----------		----------		
Arg	----------		----------		
Ala	----------		0.122	----------	
Tyr	----------		----------		
Abu	----------		----------		
Val	----------		----------		
Met	----------		----------		
Trp/Ile	----------		----------		
Phe	----------		----------		
Leu	----------		----------		
Orn	----------		0.292	----------	
Lys	----------		----------		
TOTAL	BLD	BLD	0.757	BLD	0.048

Date: 1.4.87 DCAA

	Sample No.			
a.a.	1	2	3	4
Asp	0.102	0.126	0.038	0.135
Glu	0.088	0.182	0.146	0.120
Ser	0.035	0.127	-----	0.003
His	----------	----------	----------	----------
Gly	0.677	0.575	0.917	0.899
Thr	0.040	0.024	0.096	-----
Arg	----------	----------	----------	----------
Ala	0.079	0.145	-----	0.063
Tyr	0.077	0.107	0.076	0.082
Abu	----------	----------	----------	----------
Val	0.012	0.052	0.167	-----
Met	----------	----------	----------	----------
Trp/Ile	0.145	----------	----------	0.176
Phe	0.082	----------	----------	----------
Leu	----------	----------	----------	----------
Orn	----------	----------	----------	----------
Lys	0.318	-----	0.220	0.274
TOTAL	1.655	1.388	1.660	1.752

Date: 15.4.87 Days.532 DFAA's.

	Sample No.					
a.a.	1	2	3	4	5	6
Asp	----------	0.054	0.028	0.036	0.018	
Glu	----------	0.055	0.034	0.041	0.047	
Ser	----------			0.071	0.068	
His	----------					
Gly	----------					
Thr	----------					
Arg	----------					
Ala	----------					
Tyr	----------					
Abu	----------					
Val	----------					
Met	----------					
Trp/Ile	----------					
Phe	----------					
Leu	----------					
Orn	----------					
Lys	----------					
TOTAL	BLD	BLD	0.109	0.062	0.148	0.133

Date: 15.4.87 DCAA

	Sample No.			
a.a.	2	3	4	5
Asp	0.216	0.074	0.069	0.053
Glu	0.167	0.075	0.065	0.046
Ser	0.536	0.072	0.024	-----
His	0.324	----------		
Gly	1.374	1.087	0.512	0.323
Thr	0.189	0.052	0.075	-----
Arg	----------			
Ala	0.252	0.109	0.054	0.083
Tyr	0.022	0.093	0.021	0.058
Abu	----------			
Val	0.139	0.142	0.076	0.129
Met	----------			
Trp/Ile	----------			
Phe	0.188	----------		
Leu	0.065	0.105	----------	
Orn	0.173	----------		
Lys	----------			
TOTAL	3.645	1.809	0.896	0.692

Date: 23.4.87 Days.540 DFAA's.

	Sample No.						
a.a.	1	2	3	4	5	6	7
Asp	------	0.028	0.036	0.049	0.031	0.022	0.054
Glu	----------	0.059	0.048	0.052	0.034	0.059	
Ser	----------						
His	----------						
Gly	----------						
Thr	----------						
Arg	----------						
Ala	----------						0.088
Tyr	----------						
Abu	----------						
Val	----------						
Met	----------						
Trp/Ile	----------						
Phe	----------						
Leu	----------						
Orn	----------						
Lys	----------						
TOTAL	BLD	0.028	0.095	0.097	0.083	0.056	0.201

Date: 23.4.87 DCAA

	Sample No.			
a.a.	1	2	3	4
Asp	0.063	0.038	0.116	0.062
Glu	-----	0.065	0.095	0.034
Ser	----------		0.052	0.082
His	0.236	-----	0.227	-----
Gly	0.708	1.173	0.728	0.396
Thr	----------			0.102
Arg	----------			
Ala	0.035	0.058	0.063	0.080
Tyr	-----	0.034	0.009	-----
Abu	----------			
Val	-----	0.204	0.146	0.111
Met	----------			
Trp/Ile	-----	0.362	----------	
Phe	----------			
Leu	-----	0.055	----------	
Orn	----------			
Lys	----------		0.234	-----
TOTAL	1.042	1.989	1.670	0.867

Date: 30.4.87 Days.547 DFAA's.

	Sample No.				
a.a.	1	2	3	4	5
Asp	0.027	----------		0.048	------
Glu	-----	0.033	----------		
Ser	----------		0.054	----------	
His	----------				
Gly	----------				
Thr	----------				
Arg	----------				
Ala	----------				
Tyr	----------				
Abu	----------				
Val	----------				
Met	----------				
Trp/Ile	----------				
Phe	----------				
Leu	----------				
Orn	----------				
Lys	----------				
TOTAL	0.027	0.033	0.054	0.048	BLD

Date: 30.4.87 DCAA

	Sample No.			
a.a.	1	2	3	4
Asp	0.109	0.118	0.118	0.076
Glu	0.198	0.099	0.163	0.207
Ser	0.104	0.082	0.007	0.044
His	0.282	-----	0.274	-----
Gly	0.813	0.356	1.169	0.474
Thr	-----	0.082	-----	0.045
Arg	----------			
Ala	0.074	0.052	0.084	0.094
Tyr	0.030	0.045	0.034	-----
Abu	----------			
Val	0.050	0.018	0.214	0.008
Met	----------			
Trp/Ile	----------			
Phe	----------			
Leu	----------			
Orn	----------			
Lys	----------			
TOTAL	1.660	0.852	2.063	0.948

Date: 7.5.87 Days.554 DFAA's.

	Sample No.					
a.a.	1	2	3	4	5	6
Asp	----------		0.028	----------		0.029
Glu	----------		----------	0.027	-----	0.032
Ser	----------		0.064	----------		0.097
His	----------					
Gly	----------					
Thr	----------					
Arg	----------					
Ala	----------					
Tyr	----------					
Abu	----------					
Val	----------					0.061
Met	----------					
Trp/Ile	----------					
Phe	----------					
Leu	----------					
Orn	----------					
Lys	----------					
TOTAL	BLD	BLD	0.092	0.027	BLD	0.219

Date: 7.5.87 DCAA

	Sample No.			
a.a.	1	2	3	4
Asp	0.174	0.096	0.101	0.173
Glu	0.158	0.102	0.170	0.112
Ser	0.204	0.128	0.084	0.135
His	-----	0.147	0.184	0.221
Gly	0.406	0.996	0.588	0.456
Thr	----------			0.045
Arg	----------			0.072
Ala	0.156	0.101	0.152	------
Tyr	----------			
Abu	----------			
Val	0.234	0.222	0.080	0.121
Met	----------			
Trp/Ile	----------			
Phe	----------			
Leu	----------			
Orn	----------			
Lys	----------			
TOTAL	1.332	1.792	1.359	1.335

Date: 14.5.87 Days.561 DFAA's.

	Sample No.				
a.a.	1	2	3	4	5
Asp	----------				
Glu	----------				
Ser	----------				0.042
His	----------				
Gly	----------				
Thr	----------				
Arg	----------				
Ala	----------				
Tyr	----------				
Abu	----------				
Val	----------				
Met	----------				
Trp/Ile	----------				
Phe	----------				
Leu	----------				
Orn	----------				
Lys	----------				
TOTAL	BLD	BLD	BLD	BLD	0.042

Date: 14.5.87 DCAA

	Sample No.		
a.a.	2	3	4
Asp	0.208	0.124	0.148
Glu	0.126	0.062	0.073
Ser	0.634	0.140	0.308
His	----------		0.163
Gly	1.459	0.968	0.938
Thr	----------		
Arg	----------		
Ala	0.258	0.088	0.163
Tyr	----------		0.045
Abu	----------		
Val	0.274	0.218	0.119
Met	----------		
Trp/Ile	----------		
Phe	----------		
Leu	----------		
Orn	0.319	------	0.256
Lys	----------		
TOTAL	3.278	1.600	2.213

Date: 21.5.87. Days.568 DFAA's.

	Sample No.				
a.a.	1	2	3	4	5
Asp	0.075	-----	0.039	0.069	0.031
Glu	-----	0.041	----------		
Ser	0.312	-----	0.110	0.187	0.109
His	----------				
Gly	0.164	----------			
Thr	----------				
Arg	----------				
Ala	0.117	----------			0.069
Tyr	----------				
Abu	----------				
Val	----------				
Met	----------				
Trp/Ile	----------				
Phe	----------				
Leu	----------				
Orn	----------				
Lys	----------				
TOTAL	0.668	0.041	0.149	0.256	0.209

Date: 21.5.87 DCAA

	Sample No.			
a.a.	1	3	4	5
Asp	-----	0.039	0.085	0.021
Glu	0.093	0.045	0.023	0.067
Ser	----------		0.006	-----
His	0.181	-----	0.284	0.192
Gly	0.700	0.402	0.466	0.448
Thr	----------			
Arg	----------			
Ala	0.088	0.115	0.166	0.045
Tyr	0.049	0.054	0.036	0.043
Abu	----------			
Val	0.321	0.118	0.162	-----
Met	----------			
Trp/Ile	----------			
Phe	----------			
Leu	----------			
Orn	----------			
Lys	----------			
TOTAL	1.432	0.773	1.228	0.816

APPENDIX III.

The Calculations Used to Produce the Graphs and Tables in Chapter 5 from the Raw Data in Appendices I and II.

i/. Table 1 below is a data set taken from Appendix I. Column 3 headed DFAA (dissolved free amino acids) is the sum of the individual amino acids present in that sample. The six DFAA values for this data set have been averaged. The average values of each data set over the sampling period have been plotted producing a graph of average total dissolved free amino acids levels per batch of samples. (Fig 5.3a, Chapter 5). The same process was repeated for the dissolved combined amino acids,(column 4 DCAA), and the average values for each batch of samples can be seen in Fig. 5.3b, Chapter 5. These batch averages were then grouped in months and averaged to give the monthly average levels of the 2 fractions (Figs. 5.4 a+b, Chapter 5).

ii/. The calculation of the ratio of dissolved combined to dissolved free amino acids was performed as follows; Column 6 of appendix I is the DCAA level divided by the DFAA of that sample. These values are then averaged for the month giving rise to Fig. 20, Chapter 5.

iii/. Use of data in appendix I to determine what percentage of Total dissolved nitrogen (TN) and dissolved organic nitrogen (DON) was present as amino acids. Column

5 of appendix I is the sum of columns 3 and 4 and is the total dissolved amino acid level of the sample (TAA). TAA is divided by total dissolved nitrogen or dissolved organic nitrogen levels and multiplied by 100. Columns 7 and 8 respectively. The data for TN and DON values are taken from Butler et al 1979. Again, the values were grouped into month and averaged to produce Figs. 5.21b and 5.22b.

Table 1. Taken from Appendix 1.

Date: 15.7.86 Days. 258

		Column number.					
1	2	3	4	5	6	7	8
No.	Location	DFAA	DCAA	TAA	Ratio	%TN (10.0)	%DON (8.8)
		<-------uM-------->				<----uM----->	
1	Mallard B.	0.197	------	------	-	--	--
2	Melampus B	0.192	1.647	1.839	9	18	21
4	W. End BW.	0.166	------	------	-	--	--
5	Knapp B.	0.217	------	------	-	--	--
8	A2.5 C34.5	BLD	1.760	1.760	> 46	18	20
11	E. End BW.	0.193	1.530	1.723	8	17	20
Average Values..		0.161	1.646			18	20
No. of Samples.		6	3			3	3
CVs		50	7				

Figures for the concentration of individual amino acids in each sample of the data set can be found in Appendix II an example of such a sheet of data which relates to Table 1 above, can be seen in Table 2 below.

TABLE 2. A data sheet from Appendix II. All figures quoted are in umoles/l.

Date: 15.7.86 Day:258 DFAA's.

	Sample No.							
a.a.	1	2	4	5	8	11	Av	TOTAL
Asp	0.039	0.023	0.008	0.034	-----	0.026	0.022	0.130
Glu	0.080	0.045	-----	0.049	-----	0.043	0.036	0.217
Ser	-----	0.081	0.123	0.093	-----	0.066	0.060	0.363
His	----------							
Gly	0.078	----------					0.013	0.078
Thr	----------							
Arg	----------							
Ala	-----	0.043	0.035	----------		0.058	0.022	0.136
Tyr	----------							
Abu	----------							
Val	----------			0.041	----------		0.006	0.041
Met	----------							
Trp/Ile	----------							
Phe	----------							
Leu	----------							
Orn	----------							
Lys	----------							
TOTAL	0.197	0.192	0.166	0.217	BLD	0.193		0.965

Date: 15.7.86 DCAA's.

	Sample No.			Av	TOTAL
a.a.	2	8	11		
Asp	0.132	0.227	0.211	0.190	0.570
Glu	0.088	0.168	0.138	0.131	0.394
Ser	0.117	0.239	0.168	0.174	0.524
Glc/His	0.262	0.233	0.229	0.241	0.724
Gly	0.502	0.454	0.444	0.466	1.400
Thr	0.165	0.138	0.176	0.159	0.479
Arg	0.093	----------		0.031	0.093
Ala	0.111	0.268	0.086	0.155	0.465
Tyr	0.022	0.033	0.032	0.029	0.087
Abu	----------				
Val	----------		0.013	0.004	0.013
Met	----------				
Trp/Ile	0.101	----------		0.033	0.101
Phe	----------				
Leu	0.054	------	0.033	0.029	0.087
Orn	----------				
Lys	----------				
TOTAL	1.647	1.760	1.530		4.937

iv/. Use of appendix II to determine individual amino acid levels over sampling period. The columns labelled Average (Av) and TOTAL in Table 5.2 above are not specified in Appendix II but are shown here to illustrate how data has been processed. The total concentration of each acid is found by summing the values in the data row corresponding to that acid. The total is then divided by the number of samples to produce an average value for the acid. These average values are plotted to give a graph of the individual amino acid levels over the sampling period (Figs.5.5 to 5.19 a+b).

v/. How the data in appendix II was used to calculate percentage composition figures. The total concentration of each acid is calculated as shown in iv/. above i.e. the row corresponding to the acid is summed. To determine the percentage composition of an acid e.g. aspartic acid (Asp) the total level of (Asp) is divided by the grand total (sum of all the amino acids) and multiplied by 100. These results were grouped into monthly figures and averaged to produce Figs. 5.38 to 5.56.

vi. How the data in appendix II was used to calculate the "disappearence" values quoted in chapter 5. The average values for the free amino acids was added to the average value for the same acid in the combined fraction giving a total amino acid value. e.g. if aspartate had an average value of 5 umoles/l in the free fraction and 15 umoles/l

in the combined fraction the total amino acid content for that acid would be 20 umoles/l. The disappearance value was calculated by dividing the 5 by the 20 and multiplying by 100. i.e. 25 percent of the aspartate was left (assuming the total amino acid content to be 100 percent). Therefore 75 percent of the aspartate had "disappeared". This process was repeated throughout Appendix II and a list of "disappearance" values was obtained. The range and average values were then calculated for the acid and are reported in chapter 5. The process was repeated for all the acids.

APPENDIX IV.

Publications.

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OPTIMISATION OF THE REACTION CONDITIONS FOR THE DIRECT DETERMINATION OF THE DISSOLVED COMBINED AMINO ACID LEVELS IN SEA WATER

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ABSTRACT

An investigation into the conditions necessary to determine accurately the amino acid composition of the dissolved combined amino acid (DCAA) fraction of sea water was undertaken. Maximum recoveries were obtained by hydrolysing sea water samples in 6 *M* HCl in tubes evacuated to 0.05 mm Hg pressure and heated at 110°C for 16 h.

Full details of the method are reported, together with environmental data indicating the range of total DCAA found in the coastal waters off Plymouth, U.K. The need for further studies of this fraction in the context of phytoplankton nutrition is commented upon.

INTRODUCTION

In recent years it has become apparent that in the marine environment, studies of primary production should take account of not only the dissolved inorganic nitrogen nutrients but also the dissolved organic nitrogen compounds (Butler et al., 1979).

The importance of the biota-related organic nitrogen compounds which are present in relatively high concentrations in the waters of the euphotic zone, particularly during the summer months, was emphasized in the report of the Royal Society Study Group on the Nitrogen Cycle of the United Kingdom (The Royal Society 1983). Until recently little was known about the nature of these compounds but the development of HPLC has enabled a number of them, such as the dissolved free amino acids (DFAA) to be quantified (Lindroth and Mopper, 1979). The role of DFAA in the growth of phytoplankton has recently been reviewed (Flynn and Butler, 1986). A significant portion of the remaining unidentified organic nitrogen is contained in larger molecules with molecular weights ranging from 500 to more than 50 000 (Ogura, 1977). This high molecular weight fraction contains compounds such as polypeptides and

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TABLE 1

Reported free and combined amino nitrogen levels in sea water

Levels (μM)		Ratio of DFAA to DCAA	Ref.	Hydrolysis method
Dissolved free amino acids (DFAA)	Dissolved combined amino acids (DCAA)			
0.56	4.01	7	Siegels and Degens (1966)	6 M HCl, 22 h Reflux
0.72	1.85	3		
0.77	1.87	2		
0.50	1.29	3		
0.58	2.40	4		
0.04	0.40	10	Lee and Bada (1975)	6 M HCl, 24 h Reflux
0.12	1.20	10		
0.306	0.93	3	Daumas (1976)	No details
0.306	0.654	2		
0.079	1.571	20		
0.468	0.472	1		
0.561	1.051	2		
0.015	0.175	12	Lee and Bada (1977)	6 M HCl, 24 h Reflux
0.015	0.180	12		
0.386	0.619	2	Garrasi et al. (1979)	5 M HCl, 22 h, 110°C Glass ampoule flushed with N_2 and sealed
0.514	0.857	2		
0.362	0.633	2		
0.318	0.372	1		
1.067	4.534	4	Bolter and Dawson (1982)	8 M HCl mixed 1:1 with seawater 110°C, 22 h
0.017	0.49	29	Henrichs and Williams (1985)	6 M HCl, 110°C 24 h. Glass ampoule flushed with N_2 and sealed
0.013	0.48	37		
0.094	1.06	11		

proteins and although it is improbable that such compounds are directly utilised by phytoplankton, the natural processes in the marine ecosystems results in their breakdown to simpler molecules such as amino acids which can be utilised. The nitrogen content of the high molecular weight fraction is therefore of importance when all the possible nutrient nitrogen sources are being considered in studies of primary productivity. A number of workers have measured the level of amino-nitrogen in this fraction and examples of the results found are given in Table 1. As Table 1 shows, the method most commonly used to determine combined amino-N levels has been to hydrolyse

the filtered sample with concentrated hydrochloric acid and then determine the total amino acid (TAA) level. The dissolved combined amino acid (DCAA) level is then given by the difference between the TAA and the dissolved free amino acids (DFAA). This difference method is used since the levels of DCAA compounds found in the sea water are far below the limits of detection of the methods currently used to measure such compounds directly, e.g. protein determination by biuret. Also, a knowledge of the amino acid composition of the macromolecules is required for environmental studies.

In our own studies we have used the hydrolysis method to investigate the level of nitrogen contained in the unidentified organic N fraction in the English Channel, but experienced a variety of difficulties when using these published methods. We therefore carried out a series of experiments with the objective of developing a reliable method and in this paper we describe the results of our studies.

The main difficulty in method development was due to the low concentrations of naturally occurring free and combined amino acids in sea water, which are often at picomole levels. It is not possible to use a concentration step prior to an analysis as this can cause losses or contamination of the analyte (Garrasi et al., 1979).

EXPERIMENTAL

Development of the method

Dissolved free amino acids

Amino acid analysis was carried out by the method of Evens et al. (1982) with the following modification. A Waters 420 C detector was converted to a 420 AC detector with a resultant increase in sensitivity. The limits of detection and precision at two levels are shown in Table 2.

Dissolved combined amino acids

Initially the samples for estimating the combined amino acids were hydrolysed at atmospheric pressure in a semi-sealed reflux apparatus using 6 *M* hydrochloric acid at its boiling point. This procedure gave unreliable results, one of the main problems being high blanks. Further studies showed that these high blanks could be partly accounted for by a contaminant in the deionized double distilled water used in the blank hydrolysis procedure. The contaminant was found to be an amino-acid-containing macromolecular species which could be removed by filtration through a 0.45 μm filter. Similar contaminants in deionized double distilled water have been reported by other workers (Samata and Matsuda, 1986). However, even when this particular contaminant had been removed by filtration the blank values obtained were still of the same order as the naturally occurring levels of dissolved free amino acids in seawater and furthermore the blank level results were not reproducible (Table 3). It was concluded that the high blank values were due to an

TABLE 2

Limits of detection (LOD) and precision [Coefficient of variation (CV)] at the levels of 6.6 pmol (0.08 μM) and 19.8 pmol (0.25 μM) for each amino acid in seawater

Amino acid	LOD (pmol)	CV at 6.6 pmol level (%)	CV at 19.8 pmol level (%)
Asp	2	10	4
Glu	1	11	13
Ser	4	17	9
His	9	BLD	16
Gly	6	38	35
Thr	6	97	3
Arg	6	39	11
Ala	2	4	4
Tyr	1	6	6
Abu	2	15	7
Val	1	7	4
Met	4	4	1
Trp/Ile	4	110	7
Phe	6	90	9
Leu	4	94	7
Orn	10	BLD	5
Lys	10	BLD	15

BLD = below the limits of detection.
pmol values refer to the amount chromatographed. μM values refer to the concentration in the sample solution. These two values are related by a factor derived from the experimental procedure.

aerial contaminant.

Another problem with this hydrolysis technique was that when it was carried out using water spiked with free amino acids, recovery of some of these acids was very poor. These low yields are probably due to decomposition under the harsh conditions of the acid hydrolysis (Hunt, 1985) (Table 4). As far as we

TABLE 3

Duplicate procedural blanks (PB) obtained using 0.45 μm filtered deionized double distilled water which had been hydrolysed in a semi-sealed reflux apparatus

Date	Blanks:	TAA (μM)
8 Jan 86	2.203.	2.118
21 Jan 86	1.230.	2.590
23 Jan 86	0.952.	1.234
28 Jan 86	0.250.	0.164
3 Feb 86	0.066.	0.053
11 Feb 86	1.251.	0.906
25 Feb 86	0.125.	0.106
6 Mar 86	0.645.	1.633

TABLE 4

Percent recoveries of free amino acids (29 pmol, 0.36 μM) which have been subjected to hydrolysis conditions using different methodologies

Amino acids	Reflux	Sealed tube	Evacuated sealed tube (0.05 mm Hg)
Asp	29	0	112
Glu	24	0	118
Ser	18	0	102
His	0	0	116
Gly	40	0	89
Thr	0	0	125
Arg	0	0	105
Ala	91	0	111
Tyr	0	0	89
Abu	104	0	79
Val	70	0	97
Met	0	0	0
Trp/Ile	18	0	90
Phe	0	0	68
Leu	48	0	76
Orn	0	0	57
Lys	0	0	52

are aware detailed experimental studies of the hydrolytic losses of amino acids in sea water have not been reported.

It is possible that some of the reported results obtained by this often-used technique may be influenced by these problems, especially those with the picomolar levels found in natural sea waters.

Due to the problems with blanks and losses on hydrolysis outlined above, attention was then turned to hydrolysis using a sealed-tube method which eliminated exposure to the air. Initial results were encouraging as apparently amino acid-free blanks were obtained. However, under these conditions added free amino acids at the 29 pmol (0.36 μmolar) level gave zero recoveries, indicating extensive degradation. (Table 4, column 3). Degassing of the sample with helium or flushing with nitrogen were without effect.

The main difference between the reflux and the sealed-tube methodologies is the pressure developed in the apparatus during the hydrolysis process. Therefore, determination of blanks (Table 5) and recoveries of free amino acids (Table 4, column 4), were repeated using a technique where pressure was reduced to 0.05 mm Hg before the tubes were sealed.

The problems of contamination in trace level work have been emphasized earlier in this paper. For environmental results to be meaningful the natural levels must be at least twice that of the blank. For DFAA work the blanks obtained are below the limits of detection of the method (Table 2). The increase in operations necessary to hydrolyse a protein results in a higher PB for TAA.

TABLE 5

Within batch (column 1) and between batch (column 2) means and coefficients of variation (CV) for procedural blanks obtained by evacuated sealed tube hydrolysis

Column 1		Column 2	
Amino acids	Mean of three analyses (μM)	Amino acids	Mean of 20 analyses (μM)
Asp	0.083	Asp	0.084
Glu	0.073	Glu	0.090
Ser	0.192	Ser	0.175
Gly	0.087	Gly	0.122
Ala	0.102	Thr	0.014
Total	0.537	Ala	0.096
CV	3.2%	Tyr	0.004
		Val	0.034
		Phe	0.002
		Leu	0.019
		Total	0.644
		CV	33%

The evacuated sealed-tube hydrolysis method gives low reproducible blanks and high recovery of added free amino acids. The next stage of the investigation was to evaluate the hydrolysis procedure with respect to the percentage recovery of amino acids from a sample containing known amounts of dissolved combined amino acids. This was achieved by hydrolysing a protein with known amino acid composition (Bovine Serum Albumin, BSA) under various conditions (Table 6). An aqueous solution of BSA at a concentration of 3×10^{-7} g ml^{-1} was selected for these studies since it contains DCAAs at approximately the same order of magnitude as expected to be present in the environment. Bovine Serum Albumin was hydrolysed in UV-irradiated seawater to examine the effect of the nitrate on combined amino acid recoveries. Pure water, seawater and UV-irradiated seawater all gave similar results. Addition of nitrate to 36 μmolar, the highest level normally encountered in estuarine waters and approximately three times greater than normal seawater, resulted in negligible recovery of amino acids from added protein. Both the reflux method and the sealed tube method gave recoveries of ~90% at the 1 mg ml^{-1} level. However, this is several thousand times greater than that present in sea water. Further pressure reduction or changes in the sample volume were without effect on the results of PBs. the recoveries of amino acids at the added 29 pmol level or the recovery of a.a. from BSA hydrolysed at the 3×10^{-7} g ml^{-1} level. Hydrolysis of BSA at a level of 3×10^{-7} g ml^{-1} yields a.a. at a theoretical level approximately equal to that of the added 29 pmol level (Table 6, column 1). A comparison of Table 4 with column 5 of Table 6 shows that at this level some amino acid losses occur during the degradation of the protein, since recoveries of added amino acid are generally better than the

TABLE 6

Percent recoveries of amino acids obtained by acid hydrolysis of BSA (at different levels) in sealed tubes under various conditions

Amino acid	Column				
	1	2	3	4	5
Asp	23.0	0	62	57	67
Glu	31.4	0	51	51	65
Ser	11.3	0	114	43	64
His	7.2	0	83	54	80
Gly	6.8	0	142	110	62
Thr	13.7	0	62	48	70
Arg	11.1	0	53	73	84
Ala	19.6	0	65	63	65
Tyr	7.5	0	59	69	69
Val	14.2	0	74	77	59
Phe	11.2	0	44	50	61
Leu	26.0	0	48	50	56
Lys	24.6	0	51	50	66

Column 1: Theoretical level of amino acid (pmol) obtainable from a 3×10^{-3} g ml^{-1} aqueous solution of BSA (calculated from data given by Haurowitz 1963).
Column 2: Percent recovery of amino acid from BSA. Hydrolysed in ultrapure water at 4×10^{-3} g ml^{-1}, 760 mm Hg.
Column 3: Percent recovery of amino acid from BSA. Hydrolysed in ultrapure water at 3×10^{-3} g ml^{-1}, 0.05 mm Hg.
Column 4: Percent recovery of amino acid from BSA. Hydrolysed in seawater at 3×10^{-3} g ml^{-1}, 0.05 mm Hg.
Column 5: Percent recovery of amino acid from BSA. Hydrolysed in UV-irradiated seawater at 3×10^{-3} g ml^{-1}, 0.05 mm Hg.

recoveries from the hydrolysed protein. We were unable to improve upon these results. The full experimental detail of the hydrolysis procedure are given below.

Recommended procedures

Materials

The water for preparing all solutions, blanks, washing glassware, etc. was obtained from a Milli-Q ultrapure water unit.

Aristar Hydrochloric acid and reference amino acids were obtained from BDH Ltd. Poole, England.

Bovine Serum alumin and Alpha Amino Adipic Acid were obtained from SIGMA Ltd. Poole, England.

Hydrolysis procedure

(i) Pyrex test tubes measuring 150 × 18 mm were cleaned by immersion in 10% HCl overnight. They were then rinsed many times in MilliQ water and

allowed to drain in an inverted position.

(ii) A constriction ~ 3 mm in diameter was made 2 cm from the rim of the tube using a glass lathe.

(iii) The batch of constricted tubes was then placed in an inverted position in a beaker. The beaker was covered with aluminium foil to prevent contamination and placed in an oven. The oven temperature was raised to 560°C over a period of 30 min and was maintained at that level for 20 min and then reduced to 500°C for 6 h. The oven was then turned off. The tubes remained overnight in the oven to cool. This pyrolysis was necessary to ensure complete removal of traces of contaminating amino acids.

(iv) The next stages of the hydrolysis procedure were carried out in a fume cupboard. Clean gloves were worn at all times to prevent contamination of glassware or sample.

(v) Samples with appropriate blanks were hydrolysed as follows: Aristar hydrochloric acid (1 ml) was added to the hydrolysis tube using a washed, all-glass syringe, followed by the water sample (or MilliQ water in the case of blanks) (1 ml) using an all-glass syringe with a Millex GV (0.22 μm) filter attached. Amino adipic acid (100 μl of a $1 \times 10^{-5} M$ solution) was added, as internal standard, using a micropipette with a pre-rinsed plastic tip. The solution was degassed by bubbling helium through it (2 min) using a previously cleaned glass capillary tube. After degassing, the samples were frozed by immersion in liquid nitrogen. The sample tubes were then evacuated to a pressure of 0.05 mm Hg and sealed at the constriction with a flame. Hydrolysis was carried out in an oven at 100°C for 16 h.

(vi) After hydrolysis the tubes were immersed in liquid nitrogen and opened. The contents were carefully poured into a clean 10 ml round-bottomed flask and the hydrolysate evaporated to dryness on a rotary film evaporator. Final traces of moisture and hydrogen chloride were removed by vacuum desiccation over potassium hydroxide for at least 15 h. Following release of the vacuum, 1 ml of ultrapure water was added to each round-bottomed flask and a 500 μl aliquot

TABLE 7

Results of duplicate sea water hydrolyses and range and average levels for both DFAA and DCAA over sampling period

Date	Duplicate DCAA analysis (μM)	Average	CV (%)
5 Jun 86	2.395		
	2.200	2.297	6
12 Jun 86	1.520		
	1.367	1.443	8
	DFAA	DCAA	
Range:	BLD–9.372	0.246–6.741	
Average values:	0.333	1.579	

TABLE 8

Results of DF and DCAA data with other measurements for depth samples for E1. Date 21 Jan 87

Depth (m)	Temp. (°C)	Salinity	Si	TDN (μg-at l^{-1})	NO_3	NH_4	DFAA (μmol l^{-1})	DCAA
0	8.90	35.34	2.96	13.71	3.51	1.40	0.364	0.167
5	8.92	35.35	2.91	10.52	3.33	1.33	0.191	0.358
10	8.92	35.40	2.77	10.21	4.15	1.20	0.910	1.261
20	8.94	35.36	2.82	10.98	4.06	1.52	0.241	0.952
70	8.92	35.35	2.79	10.46	3.26	1.14	1.11	1.143

Abbreviations used: Si, reactive silicon; NO_3, nitrate; TDN, total dissolved nitrogen; NH_4, ammonia.

analysed for amino acid content.

DISCUSSION

Levels of both DCAA and DFAA have now been regularly measured throughout the year in the sea off Plymouth and the full results will be published in a later issue of this journal. Table 7 gives a selection of duplicate analyses of environmental samples to indicate precision and the range of levels obtained.

Table 8 shows the depth profile from International Hydrographic station E1, 22 miles off Plymouth in the English Channel. It is clear that, at least on this occasion, the levels of DCAA were similar to those found for ammonia which is a normal routine measurement in productivity studies. It also clearly indicates that both the dissolved free and combined amino acids contribute significantly to the dissolved organic nitrogen content of sea water. This emphasizes the need for further studies of the role played by such compounds as alternative planktonic nutrients.

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REFERENCES

Bolter, M. and R. Dawson, 1982. Heterotrophic utilization of biochemical compounds in antartic water. Neth. J. Sea Res., 16: 315–332.

Butler, E.I., S. Knox and M.I. Liddicoat, 1979. The relationship between inorganic and organic nutrients in sea water. J. Mar. Biol. Assoc. U.K., 59: 239–250.

Daumas, R.A., 1976. Variations of particulate proteins and dissolved amino acids in coastal sea water. Mar. Chem., 4: 225–245.

Evens, R. J. Braven, L. Brown and E.I. Butler, 1982. An High Performance Liquid Chromatographic determination of free amino acids in natural waters in the picomolar range. Chem. Ecol., 1: 99–106.

Flynn, K. and E.I. Butler. 1986. Nitrogen sources for the growth of marine microalgae: the role of dissolved free amino acids. Mar. Ecol. Prog. Ser., 34: 281-304.

Garrasi, C., E.T. Degens and K. Mopper. 1979. The free amino acid composition of sea water obtained without desalting and preconcentration. Mar. Chem., 8: 71-75.

Haurowitz, F., 1963. Chemistry and Function of Proteins. Academic Press Inc., London. p. 131.

Henrichs, S.M. and P.M. Williams. 1985. Dissolved and particulate amino acids and carbohydrates in the sea surface microlayer. Mar. Chem., 17: 141-163.

Hunt, S., 1985. In: G.C. Barrett (Ed.), Chemistry and Biochemistry of the Amino Acids. Chapman and Hall, London. Ch. 12, p. 376.

Lee, C.L. and J.L. Bada. 1975. Dissolved amino acids in the equitorial pacific ocean waters. Earth Planet. Sci. Lett., 26: 61-68.

Lee, C.L. and J.L. Bada. 1977. Dissolved amino acids in the equitorial pacific, the Sargasso sea and Biscoyne Bay. Limnol. Oceanogr., 22: 502-510.

Lindroth, P. and K. Mopper. 1979. HPLC determination of subpicomole amounts of amino acids by precolumn fluorescence derivitisation with o-phthaldialdehyde. Anal. Chem., 51: 1667-1674.

Ogura, N., 1977. High molecular weight organic matter in seawater. Mar. Chem., 5: 535-549.

Samata, T. and M. Matsuda. 1986. Contaminating peptides widely present in ion-exchanged water, reagents, experimental implements and natural samples. Comp. Biochem. Physiol., 84B (4): 531-535.

Siegel, A. and E.T. Degens. 1966. Concentration of dissolved amino acids from saline waters by Ligand exchange chromatography. Science, 151: 1098-1101.

The Royal Society. 1983. A study group report. The Nitrogen cycle of the United Kingdom. The Royal Society, London. pp. 178-204.