CHARACTERISATION AND AVAILABILTY OF ORGANIC NITROGEN WITHIN THE MARINE ENVIRONMENT

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ABSTRACT

Dissolved organic nitrogen (DON) is largely uncharacterised, despite the fact that its concentrations often exceed those of dissolved inorganic nitrogen (DIN) species by several orders of magnitude, within estuaries and rivers (Seitzinger & Sanders, 1997; Perakis & Hedin, 2002). This is mainly due to analytical challenges associated with the qualitative determination of DON, with its array of molecules of different sizes and functionality. However, its characterisation is key to a complete understanding the aquatic nitrogen cycle.

The release of DON by phytoplankton, after assimilation of DIN, is potentially a very important process in the marine nitrogen cycle. Recent studies on the composition of marine DON suggest that most of the higher molecular weight fraction is in the amide form. However, little is known regarding the composition of the quantitatively dominant low molecular weight (LMW) fraction of DON and, therefore, its reactivity and bioavailability.

This project aims to characterise the LMW fraction (< 1000 Da) of DON released by a phytoplankton monoculture during its growth cycle under semi-axenic conditions. Aqueous culture samples were extracted at key stages during the growth cycle and bulk DON and its fractions were determined using high temperature catalytic oxidation and LC-MS. Provisional results and ideas for future work are presented.

FIGURE 1

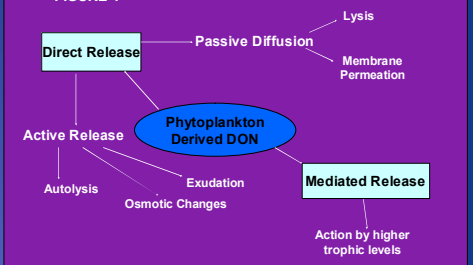
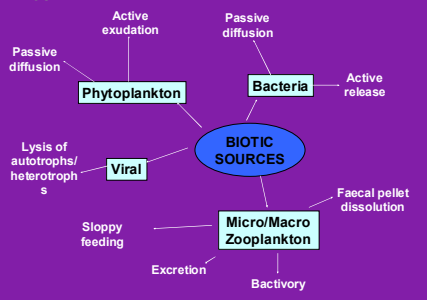


FIGURE 2



1) INTRODUCTION

Dissolved organic nitrogen (DON) is largely uncharacterised, despite it often exceeding dissolved inorganic nitrogen (DIN) species by several orders of magnitude (Seitzinger & Sanders, 1997; Perakis & Hedin, 2002), within estuaries and rivers. This is mainly due to analytical challenges associated with the determination of this fraction, with its array of molecules of different sizes and functionality. However to gain a full understanding of the aquatic nitrogen cycle an understanding of this largely un-investigated fraction must be gained.

Molecules such as combined amino acids, free amino acids, urea, vitamins etc. enter the water column via excretion, exudation, cell lysis, autolysis and decomposition upon the natural death of cells, see figure 1. If these compounds are labile then further degradation is possible subsequent to release (Libes, S.M., 1992). This release of DON by organisms such as phytoplankton after the assimilation of DIN species, is an important process in the marine organic nitrogen cycle, see figure 2. However, our knowledge of DON compounds released during the algal growth cycle is limited. A recently developed technique for the determination of amino acids using liquid chromatography–mass spectrometry (LC-MS) may also be applicable to other amino nitrogen compounds within the low molecular weight (LMW) fraction (<1000 Daltons) of organic nitrogen, whose importance has been identified by Bronk, 2002. The amino acid pool has been chosen as the starting point of the research and method development because the pool of dissolved combined amino acid compounds (containing proteins, oligopeptides, polypeptides and humic bound amino acids, Hubberton *et al.*, 1995) often comprises the largest identifiable portion of DON pools (Berman and Bronk, 2003). For example up to 50% of algal biomass can comprise of amino acids in both free and combined forms (Lee, 1988)

2) AIMS

•***To determine and quantify the release and fate of dissolved organic nitrogen (DON) in an algal monoculture.***

•***To develop a simple and sensitive analytical method for characterising low molecular weight DON compounds using LC-MS.***

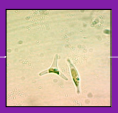
3) Materials and Methods

Four litre cultures are prepared in acid washed, autoclaved containers (17‰ Aquil medium containing 1.00 x 10^{-4} M $NaNO_3$, 1.5 x 10^{-9} M $(NH_4)Mo_7O_{24}.4H_2O$ and 1.0105 x 10^{-4} g/l of vitamins B_{12}, Biotin and Thiamine HCl, as the nitrogen containing nutrients).

At each sampling interval 5ml of sample is removed to determine cell count and pH, 10 ml is removed for nutrient determination and 7ml is removed for total nitrogen determination.
Cell counts are determined using a hemacytometer and microscope which will yield cells/L.
pH is measured using a bench top monitor.
The nutrient concentrations (nitrate, nitrate + nitrite, ammonia) are determined by using both manual and air-segmented-flow analysis (Skalar), both techniques rely on similar chemical reactions and quantification via spectroscopy.
Total nitrogen concentrations are determined using a high temperature catalytic oxidation method. (Shimazdu corporation technology)

4) ALGAL CULTURING

Mono-algal culture

INORGANIC NITROGEN → assimilation →  → release → DISSOLVED ORGANIC NITROGEN

Phaeodactylum tricornutum

Assumptions:

•Semi-axenic conditions i.e the low concentrations of bacteria present will have little to no effect.

•Only growth on inorganic nutrients is considered.

•Inorganic nitrogen concentrations will increase with time.

•Total nitrogen concentrations will remain approximately constant

5) CALCULATION OF DON

$$DON = \text{Total dissolved nitrogen} - \text{Ammonia} + \underbrace{\text{Dissolved oxidised nitrogen}}_{\text{Dissolved inorganic nitrogen}}$$

This approach is problematic because estimates of DON concentrations have the combined analytical error and uncertainty of three analyses. Therefore careful analyses are required.

6) Qualitative Analysis

The qualitative analysis of the samples will determine the period of maximum DON release from the algae. The proportion of amino acids and peptides will be determined. Sample taken during this period will then be analysed for amino acids (figure 3) and peptides via liquid chromatography-mass spectrometry (figure 4).

7) RESULTS

During the growth cycle of the *Phaeodactylum tricornutum* it can be seen that there three clearly defined stages (figure 6), the lag, growth and stationary phases. It has been noted that the point of maximum DON release usually occurs at the end of the growth phase and into the beginning of the stationary phase. It is at this point where the investigation will begin.

The nitrate concentrations are observed to decrease significantly and fell below the limit of detection using the, Skalar, automated analysis technique (figure 7). The falling below the limit of detection corresponded with entry into the growth phase.

FIGURE 6

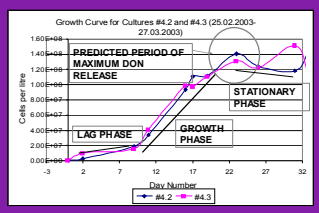
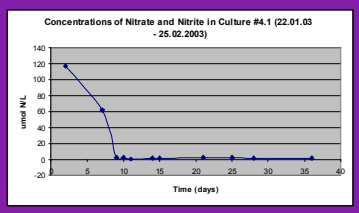


FIGURE 7



8) AMINO ACID ANALYSIS VIA LC/MS

L-Amino acids and peptides are highly polar compounds with low volatilities. Only tyrosine, phenylalanine and tryptophan contain the chromophore groups necessary for underivatised determination. (Petritis *et al.*, 2000). The resolution and detection of these compounds is difficult. With detection remaining the most difficult part of he analysis. Traditionally these compounds have been derivatised in order to analyse them to a high resolution. Derivatisation was designed to increase volatility or create a strong chromophore group. However there are a certain number of disadvantages associated with derivatisation.(Petritis *et al.*, 1999; Adoubel *et al.*, 2000; Chaimbault *et al.*, 2000).
A method is therefore being developed using reverse phase-liquid chromatography as a separation method and electrospray ionisation mass spectrometry as a detector for the direct analysis of underivatised amino acids and peptides derived from an algal source.

Amino acids are ionisable, thermolabile and polar in character (Adoubel *et al.*, 2000), they are therefore good candidates for ESI-MS. The initial method using RPLC/ESI-MS is based upon that of Petritis *et al.*, 2000, where good limits of detection are noted (down to Pico molar) and no derivatisation or pre-concentration is required. This starting point will hopefully lead to method which requires short preparation and analysis times.

See Figure 8, which shows the preliminary run carried out to ascertain, the purity of solvents, the efficiency of separation etc

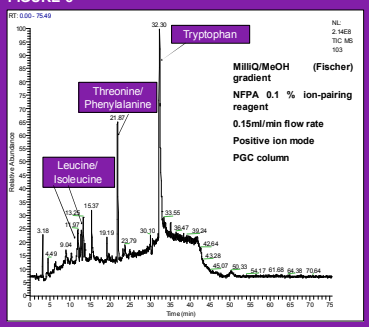
9) RPLC/ESI-MS

The method incorporates the use of a porous graphitic carbon column (Hypercarb PGC column, 2.0 x 150mm length, Shandon HPLC, UK.) as the tool of separation.

Perfluorinated carboxylic acids (specifically nonapentafluoric acid, 97%, Sigma Aldrich) as the ion pairing reagent (In both organic and MilliQ mobile eluent at concentration of 0.1%)

Methanol (Fischer, ROMIL, Chromos and Riedel-de Haen) and Acetonitrile (as before) are used as the organic modifiers, for the gradient elution.

FIGURE 8



11) DISCUSSION

The method development for the detection, characterisation and concentration determination of L-amino acids via an LC/MS technique will be completed, including the problems associated with the saline matrix. This will allow actual culture samples to be assessed.

Algae are known to be involved in the turnover of pools of dissolved free amino acids (DFAA) and dissolved combined amino acids (DCAA) in seawater either by their excretion or by the uptake of these rapidly cycled compounds. It is assumed that microalgae play an important role as producers of DFAA by their exudation or by the decay of senescent cells (Admiraal, 1984).

Previous laboratory cultures have shown that the composition and concentration of intra- and extra-cellular amino acids vary as functions of the growth phase (Marsot, 1991). Work will be carried out to identify and quantify changing amino acids and their associated concentrations throughout the growth phase.

Concentrations of nutrients within the cultures will be varied in order to simulate controlled stresses upon the cultures.

Bacteria will be added in order to ascertain microbial influences on varying amino acid concentrations. Bacteria are assumed to be the main consumers of amino acids and responsible for there very fast cycling.

L-amino acids are being investigated initially, whereas in natural habitats the contribution of D-amino acids can be significant. (The two enantiomeric forms of amino acids may be utilised with differing efficiencies by algae and bacteria (Admiraal, 1984). Therefore the exclusive use of only L-forms in laboratory experiments is a potential source of error.

The LCQ at Plymouth University

SUMMARY

•The knowledge of DON compounds released during the growth cycle is limited.
•The study of the growth cycle using algal monocultures in contrdled conditions will be considered.
•Amino acids, both free and combined, will be characterised via an LC/MS technique.
•**COMPOUNDS WILL ONLY BE FOUND IF LOOKED FOR!**

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