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# Characterisation and quantification of the fluxes of particulate and dissolved nitrogen in the unicellular diazotroph *crocospaera watsonii*: towards a closed nitrogen budget

Benjamin Randall

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Sorbonne Université

Sciences de l'Environnement: ED129

*Laboratoire d'Océanographie de Villefranche-sur-Mer*

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**Characterisation and Quantification of the Fluxes of  
Particulate and Dissolved Nitrogen in the Unicellular  
Diazotroph *Crocosphaera watsonii*  
*Towards a Closed Nitrogen Budget***

Par Benjamin Randall

Thèse de doctorat d'Océanographie

Dirigée par Sophie Rabouille et Andreas Oschlies

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Présentée et soutenue publiquement le 8 Novembre 2019

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## Dedications

This thesis is dedicated to my family, who helped shape me to be the person I am today,

To my best friends Ephraim and Nicole, who talked to me every day and kept me sane despite being  
a thousand miles apart,

**And most of all to Djihane, my ever supporting, ever loving, ever understanding fiancée,  
without whom I never would have had the strength to finish this PhD.**

## Acknowledgements

First of all, we would like to acknowledge INSU, LEFE-CYBER program as well as the Regional PACA government and GEOMAR, for without their support, this project would not be possible.

I would personally like to thank my supervisors Sophie Rabouille and Andreas Oeschies, whose expertise and ideas helped guide me through the challenges of this PhD. I would especially like to thank Sophie, because despite our differences at times, your passion and dedication has always inspired me, and I've especially enjoyed exploring science with you over the past few years. I would also like to thank Eric Achterburg and Christophe Migon for the wisdom and support they have shared in their roles as my thesis committee.

As well as Eric, I'd like to thank Ames Latifi for kindly agreeing to review this thesis, and the jury members Nathalie Vigier and Aurelien Paulmier for agreeing to attend and judge my defence.

I would also like to highlight the immense help of Patrick Raimbault, whose insight and expertise of methodology has transformed the project in many ways impossible without him. I would also like to thank Patrick for the many samples he has kindly processed for us over the course of the project.

A special thanks to all the engineers at the LOV, Thierry Blasco, Nathalie Leblond, and Amélie Talec for their assistance, advice, and kindness over the years. I would especially like to thank Amélie, whose ability to solve problems in the lab i'm convinced is magic. Seriously, thank you Amélie for all you do to keep the team and lab running, it doesn't go unappreciated from me.

Finally I would like to extend my gratitude to all those who made me feel welcome at the lab when I moved to France. Thank you Margaux Caïa, Manon Gachelin, Martin Laviale, Richard Copley, Mandela Prünster, Eric Pruvost, Fabio Benedetti, David Demory, Anne Sophie Pavaux, Simon Ramondenc, Kévin Druet, Sarah Perché, Lauralie Tournier, Antoine Sciandra and more and more and more...

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## List of Symbols

<b><sup>12</sup>C</b>	Normal isotope of carbon (98.9%)	<b>N<sub>2</sub></b>	Dinitrogen (atmospheric)
<b><sup>13</sup>C</b>	<sup>13</sup> isotope of carbon (1.1%)	<b>NaHCO<sub>3</sub></b>	Sodium bicarbonate
<b><sup>14</sup>N</b>	Normal isotope of nitrogen (>99%)	<b>NaNO<sub>3</sub></b>	Sodium nitrate
<b><sup>15</sup>N</b>	<sup>15</sup> isotope of nitrogen (~0.367%); molecular symbols with <sup>15</sup> N (e.g. <sup>15</sup> N <sub>2</sub> ) denote that the nitrogen atoms in the molecule are <sup>15</sup> N	<b>NH<sub>3</sub></b>	Ammonia
<b>ARA</b>	Acetylene reduction assay; sometimes 'acetylene reduction activity'	<b>NH<sub>4</sub><sup>+</sup></b>	Ammonium
<b>C</b>	Carbon	<b>nif</b>	Genes which code for enzymes related to N fixation e.g. nifH
<b>C<sub>2</sub>H<sub>2</sub></b>	Acetylene	<b>NO<sub>2</sub><sup>-</sup></b>	Nitrite
<b>C<sub>2</sub>H<sub>4</sub></b>	Ethylene	<b>NO<sub>3</sub><sup>-</sup></b>	Nitrate
<b>CHN</b>	Carbon, hydrogen, nitrogen (elemental analysis)	<b>O<sub>2</sub></b>	Oxygen
<b>CO<sub>2</sub></b>	Carbon dioxide	<b>OMZ</b>	Oxygen minimum zone
<b>DIN</b>	Dissolved inorganic nitrogen (e.g. NO <sub>3</sub> <sup>-</sup> , NH <sub>4</sub> <sup>+</sup> )	<b>OPA</b>	ortho-phthalaldehyde
<b>DOM</b>	dissolved organo material	<b>P</b>	Phosphorous
<b>DON</b>	Dissolved organic nitrogen	<b>P*</b>	Geochemical parameter to signify anomaly (excess or lack) of phosphorous with respect to Redfield ratio of nitrogen : phosphorous of 16:1
<b>F/x</b>	A nutrient rich medium often used for culturing algae; /x denotes the dilution of the medium (i.e. /2 = 50% dilution)	<b>PBR</b>	Photobioreactor
<b>Fe</b>	Iron	<b>PO<sub>4</sub><sup>3+</sup></b>	Phosphate
<b>fixed-N</b>	Biologically available nitrogen; Nitrogen not in the form of N <sub>2</sub>	<b>POC</b>	Particulate organic carbon
<b>GC</b>	Gas chromatograph	<b>PON</b>	Particulate organic nitrogen
<b>GF</b>	Glass fibre (filters)	<b>T<sub>0</sub></b>	Time point at the start of an assay
<b>h</b>	Hours	<b>TDN</b>	Total dissolved nitrogen (NO <sub>3</sub> <sup>-</sup> + NH <sub>4</sub> <sup>+</sup> + DON + ...)
<b>H<sub>2</sub>O</b>	Water	<b>V</b>	Specific uptake rate
<b>H<sub>2</sub>SO<sub>4</sub></b>	Sulphuric acid	<b>WCO</b>	Wet chemical oxidation; a chemical technique for measuring the concentration of TDN (and therefore DON) in a solution
<b>HCl</b>	Hydrochloric acid	<b>YBCII</b>	A DIN free version of F/2 medium commonly used for diazotroph culturing
<b>HgCl</b>	Mercuric chloride	<b>ρN<sub>2</sub></b>	Incorporation of N into cells from nitrogen fixation (derived by <sup>15</sup> N <sub>2</sub> incubations)
<b>IRMS</b>	Isotope ratio mass spectroscopy	<b>ρNO<sub>3</sub></b>	Incorporation of N into cells from NO <sub>3</sub> <sup>-</sup> uptake (derived by <sup>15</sup> NO <sub>3</sub> incubations)
<b>K<sub>2</sub>S<sub>2</sub>O<sub>8</sub></b>	Potassium peroxydisulphate or just potassium persulphate		
<b>Ln/Dn</b>	nth hour of the light (L) phase or dark (D) phase of a 12:12 L:D experiment		
<b>MgO</b>	Magnesium Oxide		
<b>N</b>	Nitrogen		
<b>N*</b>	Geochemical parameter to signify anomaly (excess or lack) of nitrogen with respect to Redfield ratio of nitrogen : phosphorous of 16:1		

## Summary

Nitrogen (N) fixation, or diazotrophy, exists as a major source of biologically available N in the marine environment, affecting important processes such as the marine food web and the biological carbon (C) pump. Despite this, important fluxes into and out of the dissolved N phase, which inform the overall N budgets of individual diazotrophs, are poorly understood. This is especially true of recently discovered diazotrophs which may not function to previously established paradigms. One such diazotroph is the unicellular cyanobacteria *Crocosphaera watsonii* (UCYN-B), which is easily culturable and widespread throughout sub-tropical oceans. Here we attempt to characterise N fluxes consequent to diazotrophy for *Crocosphaera*, including the importance of fluxes of nitrate ( $\text{NO}_3^-$ ), ammonium ( $\text{NH}_4^+$ ), and dissolved organic N (DON). Furthermore, we aim to use our results to assess the accuracy of commonly used biological  $\text{N}_2$  fixation assays. We first assessed the use of  $\text{NO}_3^-$  by *Crocosphaera*. To do this, batch cultures of *Crocosphaera* were grown long term with  $\text{NO}_3^-$ , with measurements made to quantify the uptake  $\text{NO}_3^-$  and its effect on diazotrophy, culture growth and other important physiological parameters. In short, we found little uptake with no effect on the assessed parameters, including diazotrophy. We next focussed on flux of DON and  $\text{NH}_4^+$  in continuous cultures regulated for culture conditions such as temperature, turbidity, and pH. In addition we targeted light availability as an important parameter. Between two culture conditions of sub-saturating and saturating light, we found an important increase in N acquisition, C acquisition and cell population growth rate in the saturating light condition. Furthermore, we found a significant uptake of DON over a 24h period, with a far more important uptake in the sub saturating light scenario (42% v.s. 7%). We also found greater flux of  $\text{NH}_4^+$  into the medium in said scenario. When using an isotopic approach, we found roughly 2.5-3.5% of recently fixed N was present in the medium after 24h. Our assessment of the accuracy of commonly used methodology found that  $^{15}\text{N}_2$  incubation consistently underestimated  $\text{N}_2$  fixation while the acetylene reduction assay overestimated. Overall however,  $^{15}\text{N}_2$  incubations were more reliable, especially when factoring in fluxes of dissolved N, which is often uncontrolled for. Our findings represent new perspectives into the importance of fluxes of dissolved N to N budgets of *Crocosphaera watsonii*.

## Sommaire en Français

La fixation biologique du diazote, ou diazotrophie, représente une source majeure d'azote (N) disponible dans l'environnement marin, qui contribue à d'importants processus tel que le réseau trophique marin et la pompe biologique à carbone (C). Cependant, d'importants flux d'azote dissout, liés à l'activité d'organismes diazotrophes unicellulaires, sont mal compris. Ceci est particulièrement vrai en ce qui concerne les diazotrophes récemment découverts, qui pourraient ne pas fonctionner selon des modèles précédemment établis. L'un de ces diazotrophes est la cyanobactérie unicellulaire *Crocospaera watsonii* (UCYN- B), très répandue dans les océans subtropicaux et facilement cultivable. Ici, nous faisons une tentative de caractérisation des flux d'azote (N) résultant en la diazotrophie pour *Crocospaera*, y compris l'importance des flux de nitrate ( $\text{NO}_3^-$ ), d'ammonium ( $\text{NH}_4^+$ ) et de N organique dissout (DON). De plus, nous voulons utiliser nos résultats afin d'évaluer l'exactitude des essais de fixation biologique de  $\text{N}_2$  fréquemment utilisés. D'abord, nous avons évalué l'usage de  $\text{NO}_3^-$  par *Crocospaera*. Pour ce faire, des lots de culture de *Crocospaera* ont été crûs sur le long terme avec  $\text{NO}_3^-$ , avec des mesures faites pour quantifier l'absorption de  $\text{NO}_3^-$  et son effet sur la diazotrophie, la croissance de la culture et d'autres paramètres physiologiques importants. En somme, nous avons constaté peu d'absorption sans effet sur les paramètres évalués, y compris la diazotrophie. Nous nous sommes ensuite concentrés sur le flux de DON et de  $\text{NH}_4^+$  dans des cultures régulées continues pour des conditions de culture comme la température, la turbidité et le pH. En outre, nous avons visé la disponibilité de lumière comme paramètre important. Entre deux conditions de culture de sous-saturation et de saturation de lumière, nous avons trouvé une hausse importante en acquisition de N, en acquisition de C et en taux de croissance de la population de cellules dans la condition de saturation de lumière. De plus, nous avons trouvé une absorption significative de DON lors d'une période de 24 h, avec une absorption bien plus importante dans le scénario de sous-saturation de lumière (42% v.s. 7%). Nous avons également trouvé un plus grand flux de  $\text{NH}_4^+$  vers le milieu dans ce scénario. Lorsqu'une approche isotopique est employée, nous avons trouvé qu'environ 2,5%-3,5% d'azote (N) récemment fixé était présent dans le milieu après 24 h. Notre évaluation de l'exactitude de méthodologies fréquemment utilisées constate qu'une incubation  $^{15}\text{N}_2$  a systématiquement sous-estimé la fixation de  $\text{N}_2$  alors que l'essai de réduction d'acétylène l'a sur-estimée. Mais globalement, les incubations  $^{15}\text{N}_2$  ont été plus fiables, surtout lorsqu'on inclut les flux de N dissout, ce qui est souvent tenu en compte. Nos conclusions représentent de nouvelles perspectives dans l'importance de flux de N dissout vers les budgets N de *Crocospaera watsonii*.

# Introduction

For the proliferation of life, be it through structure or metabolism, nitrogen (N) is undoubtedly among the most important elements. It is therefore not surprising that within marine biogeochemical cycles, N plays a significant role, especially considering N's position as a limiting element to marine primary productivity. The complexity of N chemistry (Fig. 1.1) makes the marine N-cycle among the most interesting, yet difficult to study. At the root of almost every N chemical transformation is the metabolism of specific marine organisms, and this inextricably links the study of N in the ocean with the studies of marine biogeochemistry, biogeography, and ecology at the macro-scale, as well as the study of marine microbiology at the micro-scale.

N is among the most abundant elements in the ocean. The vast majority of N exists however in the form of dissolved dinitrogen ( $N_2$ ), which is biologically unavailable to most organisms. In contrast, most other chemical forms of N are biologically available, and these forms are termed collectively as 'fixed N'. Biologically available N exists primarily in the forms of nitrate ( $NO_3^-$ ) and ammonium ( $NH_4^+$ ), although  $NO_3^-$  exists approximately at concentrations three orders of magnitude greater than  $NH_4^+$  [Capone et al., 2008]. However, the majority of the euphotic layer experiences below detection level concentrations of these N forms. This commonly leads to N limitation for primary producers which are spatially locked to the upper ocean due to the need for light [Zehr, 2011]. As marine primary productivity is paramount for many ecologically and anthropologically important processes, such as the marine food web and the biological carbon sink, N availability is therefore an important process to study and understand.

The majority of the N demand for primary productivity is supplied through short term recycling of N, via the shallow remineralisation of organic matter to  $NH_4^+$  ('regenerated production') or through longer term process of input via deep remineralisation of organic matter (dissolved organic N; DON  $\rightarrow NH_4^+$ ), nitrification in the aphotic zone ( $NH_4^+ \rightarrow NO_3^-$ ), and then upwelling of  $NO_3^-$  to the euphotic zone ('new production') [e.g. Dugdale and Goering, 1967; Voss et al., 2013]. However, despite the names 'new' and 'regenerated' production, these processes are both notably 'regenerative' processes when the marine N cycle is considered as a whole, as the same fixed-N is

utilised and recycled over time. This is an important distinction, as these processes cannot globally mediate the size of the biologically available N pool in the ocean, nor consequent interconnected processes: only locally to the euphotic zone. This is instead controlled by the inputs and outputs of N to the ocean, for which we return to the aforementioned greatest source of N in the ocean:  $N_2$ .

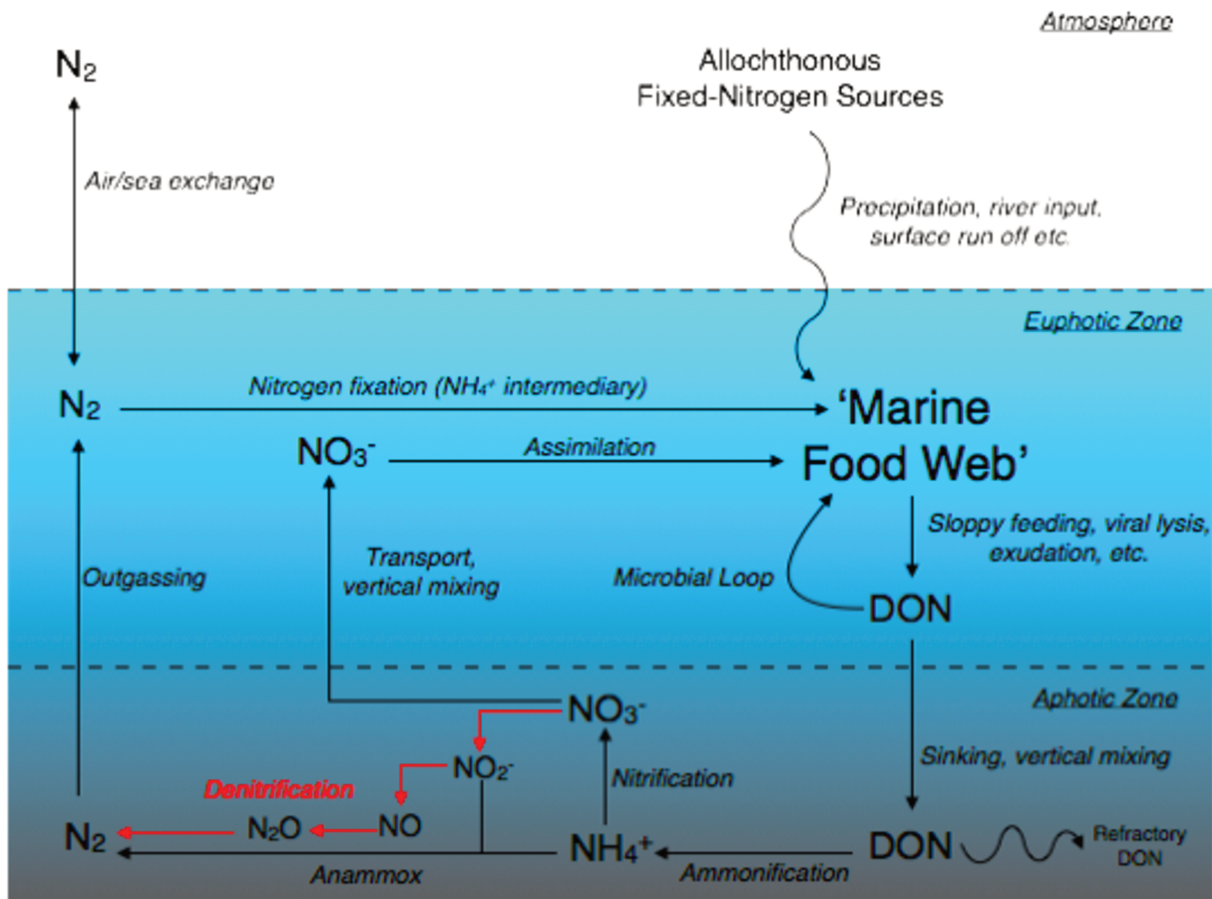


Figure 1.1: Diagram of marine nitrogen cycle

### Nitrogen Input into the Ocean

Input of 'new' fixed N into the system can be roughly divided into the allochthonous processes of riverine and atmospheric deposition, and the autochthonous process of  $N_2$  fixation. While the former two processes are certainly significant [e.g. Hansell et al., 2007],  $N_2$  fixation has often been considered the most important process inputting fixed N into the marine environment.

As previously established,  $N_2$  is largely inaccessible to the majority of marine organisms. This is due to the strength of the covalent triple bond which holds the N atoms together. There does, however, exist a polyphyletic group of marine microorganisms which are capable of ‘fixing’  $N_2$ , a process referred to as ‘ $N_2$  fixation’ or ‘diazotrophy’. This process is performed using an enzyme called ‘nitrogenase’, which acts to split the triple bond in  $N_2$  to form  $NH_4^+$ . This process is therefore notably significant to marine primary productivity, firstly because it links normally unavailable  $N_2$  to the marine N pool; biologically mediating the size of the marine fixed N pool, and secondly because it introduces a group of marine photoautotrophs (of which many diazotrophs are) which are not limited by N availability.

Determining the size of the marine fixed N budget, and the relative sizes of its individual components, has been an often tackled, yet poorly resolved issue. When considering inputs, most consider  $N_2$  fixation the primary source of ‘new’ nitrogen to the system, with estimates suggesting a global input of ~ 50-200 (Table 1.1). While many attempts have been made to establish a global  $N_2$  fixation budget, there is only vague agreement between estimates, with only a generally agreed scale but difficulty establishing a precise estimate. This is in part due to a constantly evolving paradigms regarding the ecology and metabolism of diazotrophs, as well the methodological and analytical differences in the many ways of measuring and estimating  $N_2$  fixation employed by marine biogeochemical researchers. Furthermore, recent evidence has placed doubt on the established scale for which we expect global  $N_2$  fixation to operate on, as denitrification, the antithetical process to  $N_2$  fixation, seems to be removing fixed N at a rate inconsolable with our understanding and expectations of N cycle equilibria.

### *Denitrification vs $N_2$ fixation: Resolving the Fixed Nitrogen Budget*

While  $N_2$  fixation is able to bring ‘new’ N into the system, at the opposite end are processes capable of reducing fixed N back into  $N_2$ , essentially making N once again biologically unavailable. The term denitrification has classically been used as an umbrella term for processes which chemically reduce  $NO_3^-$  into subsequent N oxides ( $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O$ ; Fig. 1.1) until finally reaching  $N_2$ . These are biologically mediated processes which occur in anoxic environments such as sediments and pelagic oxygen minimum zones (OMZs). In addition to classical denitrification, non-

canonical routes of fixed N  $\rightarrow$  N<sub>2</sub> have recently become more appreciated as significant contributors [Codispoti et al., 2005], most notably the ‘anammox’ reaction which uses both NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> to produce N<sub>2</sub> (NH<sub>4</sub><sup>+</sup> + NO<sub>2</sub><sup>-</sup>  $\rightarrow$  N<sub>2</sub> + 2H<sub>2</sub>O).

While it is true that estimates of N<sub>2</sub> fixation suggest a large annual input of N into the marine fixed N pool, it must be noted that recent studies have cast aspersions on whether or not current N<sub>2</sub> fixation estimates are accurate. This is mostly due to the assertion that rates of denitrification outstrip rates of N<sub>2</sub> fixation severely, suggesting a loss of between 188 [Codispoti et al., 2001] and 234 Tg N y<sup>-1</sup> [Codispoti, 2007], although there is by no means a consensus [Eugster and Gruber, 2012; Wang et al., 2019]. This is a surprising result, as a number of lines of evidence suggest that the marine fixed nitrogen budget should be balanced. These include Holocene carbon dioxide records [Gruber and Sarmiento, 1997] and sedimentary δ<sup>15</sup>N records [e.g. Altabet et al., 2002; 2006]. In addition, the presence of a nitrogen to phosphorous (P) ratio in the ocean close to the canonical Redfield ratio of biological uptake of 16N:1P suggests a strong feedback mechanism which pushes the system towards balance. Simply from a logical perspective, a strongly overbalanced sink of N in the ocean would not be sustainable: according to the budget presented in Capone et al., [2008], there is roughly 6.6E+05 Tg of fixed N in the ocean. Firstly, if fixed N is truly so abundant, how could it possibly have accumulated to such a size if denitrification was consistently so much more active than N<sub>2</sub> fixation? Secondly, for a sink of around ~200 Tg N y<sup>-1</sup>, an estimate roughly between Codispoti et al. [2001] and Codispoti [2007], the marine fixed N inventory would be depleted in only ~3300 years. These lines of reasoning lead us to three possible conclusions: a) recent denitrification estimates have been overestimated, b) recent N<sub>2</sub> fixation estimates have been underestimated, or c) the balance between these two processes is not homogeneously balanced in real time, and we are currently experiencing an imbalance in favour of denitrification. Whilst options a) and c) are intriguing questions in themselves, most work has focussed on the idea that N<sub>2</sub> fixation has been underestimated. For the purpose of this thesis, only this aspect shall be discussed in any detail.

## Underestimation of N<sub>2</sub> Fixation: Routes of Study

Arguably, two major factors play into the underestimation of N<sub>2</sub> fixation. These issues are: a) underestimation derived from issues with methodology and subsequent computation and analysis, and b) issues derived from inaccurate paradigms of understanding of the ecology, physiology, and metabolism of diazotrophs, with some cases where these issues are inextricably linked. These issues are difficult to outline summarily, however column a) can be quickly defined as ‘methodologies, through issues formerly or currently unbeknownst to investigators, that lead to significant underestimations in the rate of N<sub>2</sub> fixation calculated’. Potentially the most notorious recent method within the *Zeitgeist* of N<sub>2</sub> fixation research is the <sup>15</sup>N<sub>2</sub> incubation method, although other methods have also been found to suffer from similar deficiencies. The most discussed issue of late with the aforementioned method has been questions surrounding the efficacy of <sup>15</sup>N<sub>2</sub> delivery methods and gas equilibration [Mohr et al., 2010]. In short, the <sup>15</sup>N<sub>2</sub> incubation method functions by aliquoting <sup>15</sup>N<sub>2</sub> tracer into an incubation bottle containing diazotrophs. These diazotrophs take up <sup>15</sup>N<sub>2</sub> as well as <sup>14</sup>N<sub>2</sub> via N<sub>2</sub> fixation, and incorporate it into their biomass. This can then be analysed to estimate the rate of N<sub>2</sub> fixation. This calculation requires an accurate estimation of the initial enrichment of <sup>15</sup>N<sub>2</sub> when first aliquoted. Traditionally, this aliquot has been delivered via gas tight syringe and gas bottle, with equilibration of the gas assumed to be rapid and complete. However, a recent study placed doubt on this assumption, suggesting that equilibration is instead slow and incomplete. These findings imply that N<sub>2</sub> fixation estimates using this method are underestimates.

While the aforementioned issues have been discussed at length in the literature, another issue exists with this method that has gone relatively understudied. To resume with the description of the protocol of the typical <sup>15</sup>N<sub>2</sub> incubation, once incubations are complete, their contents are usually filtered using glass fibre (GF) filters in order to harvest and analyse the biomass. However, the filtrate is often discarded with the assumption that no significant exudation, excretion or release of fixed N has occurred. This, has however been challenged recently, with certain results showing significant exudation in multiple species of diazotroph. Assuming there is indeed a consistent and significant release of N into the filtrate, that would necessitate that many results derived from <sup>15</sup>N<sub>2</sub> incubations have also underestimated in this way.

Column b) issues instead are out-of-date assumptions which inform paradigms of understanding of every facet of diazotrophy, be it the ecological distribution of diazotrophic organisms, their nutrient



**Table 1.1:** Various global estimates of N<sub>2</sub> fixation from the last 20 years (Non-exhaustive)

Method	Value (10 <sup>11</sup> mol N yr <sup>-1</sup> )	Reference
Derived from global N* distribution	94 ± 30	Gruber and Sarmiento, 2002
As above, with added uncertainty	96 ± 36	Gruber, 2004
P* Flux convergence	~100	Deutsch et al. 2007
Geometric mean of measured biological rates of N <sub>2</sub> fixation	37 - 52	Luo et al. 2012
Arithmetic mean of measured biological rates of N <sub>2</sub> fixation	97 ± 7	Luo et al. 2012
Global integrated N <sub>2</sub> fixation (open ocean)	36 - 72	Luo et al. 2014
Inverse biogeochemical model/ Prognostic Ocean model	~170	Wang et al. 2019

limitations and subsequent metabolic behaviour, their physiology *etc.* Possibly the largest paradigm shift of recent times in this field has been the discovery of unicellular diazotrophic cyanobacteria. Formerly, understanding of marine diazotrophy was centred around macroscopic, colonial diazotrophs which were easy to study. However, environmental genomics has somewhat expanded this limited world, with *nif* genes, the genes which encode N<sub>2</sub> fixation enzymes such as nitrogenase, being found far more abundantly than once thought. Possibly the most well known and most studied of these macroscopic, colonial diazotrophs is *Trichodesmium* spp., and as such, many of the paradigms surrounding the study of diazotrophic research have been born of assumptions of uniformity with this characteristic and (relatively) famous diazotroph. Among the most important paradigms about diazotrophs wrought from studying *Trichodesmium* spp. are distribution in warm, oligotrophic regions, with limitation of N<sub>2</sub> fixation by energetically less costly forms of N (i.e. NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>). The discovery of an abundance of diazotrophs fundamentally different in physiology and metabolism has raised incredibly important questions in the field of marine diazotrophic research. These questions must be addressed if our collective understanding of marine diazotrophy is to reach a respectably complete level (to the extent that is possible in science).

## The Aims of this Thesis

Originally, the aim of this PhD project was to attempt to characterise a complete metabolic N budget for the unicellular diazotroph *Crocospaera watsonii* (WH8501). This was done with emphasis on shedding light on the aforementioned questions regarding the release of N from the biomass and into the medium. This was to be done with a method outlined in Slawyk and Raimbault [1995] which is capable of chemically extracting different species of dissolved N ( $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , and DON) from a filtrate. This can be therefore used on filtrates from  $^{15}\text{N}_2$  incubations to not only quantify the rate of exudation of dissolved fixed N from *Crocospaera watsonii* cells, but also the relative sizes of the different pools.

As is typical with PhD projects, this one evolved significantly over the process. While the original goal remained, the perspectives and direction of the project changed significantly over its course. While fundamentally a project on methodology, in order to bring the research in line with wider perspectives and questions within the field, the project's direction was driven towards posing questions with respect to the question touched on above: how can we justify upwardly revising  $\text{N}_2$  fixation estimates to be in line with denitrification? This has therefore shaped the content and context of this thesis and has been expressed thusly: first, in chapter one, we performed an extensive 'state of the art' literature review. Here the two factors driving global  $\text{N}_2$  fixation rate underestimation mentioned above are discussed at length, attempting to encompass modern findings and directions in the literature. From this, two conditions for experimentation were chosen. The first was verification of  $\text{NO}_3^-$  utilisation and its effect on  $\text{N}_2$  fixation in *Crocospaera watsonii* (WH8501). While generally it is considered that  $\text{NO}_3^-$  in significant concentrations will inhibit  $\text{N}_2$  fixation, first order culture results at this lab (Laboratoire d'Océanographie de Villefranche-sur-Mer) have fomented doubt that the particular strain of *Crocospaera watsonii* in use does in fact utilise  $\text{NO}_3^-$ . It was therefore necessary to verify this before starting longer term continuous cultures. As such, a collection of batch culture experiments were designed in order to test  $\text{NO}_3^-$  utilisation and its effect on  $\text{N}_2$  fixation using a wide range of methods. The results of these culture experiments are presented in chapter three. The second condition tested was light availability. Given that logically, a higher abundance of light should provide cultures with more energy to fix  $\text{N}_2$ ; a notably energetically costly process, more  $\text{N}_2$  should therefore be fixed. It is therefore interesting to observe what effect this change has on the N budget of *Crocospaera watsonii* WH8501, and how this may

affect fluxes of dissolved N into our out of the biomass. For this purpose, two semi-continuous, turbidostat culture experiments were performed at disparate light intensities: one saturating ( $\sim 400 \mu\text{Einsteins m}^{-2} \text{ s}^{-1}$ ), and one sub-saturating ( $\sim 60 \mu\text{Einsteins m}^{-2} \text{ s}^{-1}$ ). The N budget was studied in these cultures using a wide array of measurement types, including the  $^{15}\text{N}_2$  incubation method, the acetylene reduction assay (ARA), elemental analysis (CHN) for  $\text{N}_2$  fixation, and methods including the diffusion method outlined in Slawyk and Raimbault [1995] to analyse N release into the biomass. The results of these experiments are presented in chapter five. Finally, with the high number of measurements of N acquisition generated in our experiments, with multiple methodologies used to derive said values, we present a look into the accuracy of commonly used  $\text{N}_2$  fixation assays (ARA and  $^{15}\text{N}_2$  incubations). This was done by comparing estimates of  $\text{N}_2$  fixation with N acquisition rates calculated via changes in PON concentration over time, which were corrected with rates of DON and  $\text{NH}_4^+$  flux to estimate the net  $\text{N}_2$  fixed. These results are presented in chapter four.

# Chapter One: State of the Art

Current understanding of the marine fixed N budget has brought under contention our global estimations of  $N_2$  fixation. As suggested in the above section, recent studies defining the global scale of denitrification far outstrip that of  $N_2$  fixation [Codispoti et al., 2001; Codispoti 2007] despite multiple lines of evidence that these processes should probably be in equilibrium. This has led to the general conclusion that it is likely that  $N_2$  fixation is being underestimated. The factors which contribute to this underestimation seem to be roughly sorted into two categories of inaccuracy: methodological (systematic measurement errors) and paradigmatic (issues with communal understanding of how diazotrophs distribute and function). Here we first explain classical understanding and then outline current literature on both categories, including highlighting interesting routes of study which may help to solve the aforementioned budget issue.

## Methods of Study

Here we outline the methods which are used to estimate the rate of  $N_2$  fixation, from the scale of one experiment to the global scale. Methods can be roughly divided into three types: i) Biological; which measure the actual or a proxy of the rate of  $N_2$  fixation by living diazotrophs, ii) Geochemical; which use geochemical proxies to infer rates of  $N_2$  fixation over larger spatial and temporal scales, and iii) Models; which use modelling approaches in conjunction with information from the former two method types in order to infer global rates.

### *i) Biological Methods*

#### **$^{15}N_2$ incubations**

One of the most commonly used methods for the observation of  $N_2$  fixation is the  $^{15}N_2$  tracer method. The basis of this method involves the addition of  $^{15}N_2$  (a stable isotope of N) to a sample of containing diazotrophs, the incubation of this sample over the diazotrophic period, and then the filtration of biomass on glass fibre filters. This ‘particulate organic nitrogen’ (PON) sample can then be analysed using a isotope ratio mass spectrometry (IRMS) to observe the increase of  $^{15}N$  in the biomass over the incubation period. By knowing the initial enrichment of  $^{15}N$  in the total N pool of the sample, one can easily infer a rate of  $N_2$  fixation by multiplying the total  $^{15}N$  incorporated by the

ratio of  $^{14}\text{N}$  to  $^{15}\text{N}$  in the incubation. This is assuming there is no significant isotope fractionation during  $\text{N}_2$  fixation, which has been confirmed to be small (-2-0‰) [Hoering and Ford, 1960; Minagawa and Wada, 1986; Carpenter et al., 1997]. This method has been available for decades, but it has only gained popularity within the community over the last ~20 years with improvements of high sensitivity IRMS [Montoya et al., 1996], which has allowed for detection of environmentally relevant small scale incorporation. The use of  $^{15}\text{N}_2$  incubations is certainly important, as it is the only assay available which directly measures  $\text{N}_2$  fixation qualitatively, as it is the only way in which  $^{15}\text{N}$  may enter the biomass from  $^{15}\text{N}_2$  is through  $\text{N}_2$  fixation. It must be noted, however, that quantitatively,  $^{15}\text{N}_2$  incubations does *not* measure  $\text{N}_2$  fixation directly, as the total  $\text{N}_2$  fixation calculation requires the correction using the initial enrichment value, something which is difficult to measure and is thus normally estimated.

### **Issues with $^{15}\text{N}_2$ incubations**

Despite the rise in popularity of this method, recently, many important issues with this method have been raised in the literature. This has been an intense point of debate within the community. The first point of contention to raise is the recent finding that a number of commercial  $^{15}\text{N}_2$  gas bottles have been found to be contaminated by assimilable fixed  $^{15}\text{N}$  species, such as  $^{15}\text{NO}_3^-$ ,  $^{15}\text{NO}_2^-$ , and  $^{15}\text{NH}_4^+$ . Introduction and uptake of these labelled species of N would certainly cause false positive results in  $^{15}\text{N}_2$  incubations of diazotrophs, which could cause methodological overestimations of  $\text{N}_2$  fixation rates [Dabundo et al. 2014]. Depending on experimental conditions, these contaminations were shown to infer rates of  $\text{N}_2$  fixation ranging from undetectable, to  $530 \text{ nmoles N L}^{-1} \text{ d}^{-1}$ , rates which often compare to or outstrip commonly detected rates in field assays. Contamination is, thankfully brand specific, and companies are now more rigorously quality testing their products to ensure there is no contamination of labelled fixed N.

Possibly the most far reaching criticism has come from a recent review by Mohr et al. [2010]. Classically, the introduction of  $^{15}\text{N}_2$  into incubation bottles has been performed by using a gas tight syringe to sample and inject a bubble of  $^{15}\text{N}_2$  gas into the incubation bottle through a septum. When shaken vigorously, the gas is then assumed to equilibrate quickly with  $^{14}\text{N}_2$  gas in the liquid across the bubble-water interface. By estimating the amount of  $^{14}\text{N}_2$  and  $^{15}\text{N}_2$  gas in the bubble *and* the water, the ratio of  $^{15}\text{N}$  to  $^{14}\text{N}$  in the liquid can be estimated and this enrichment value can be used to

estimate the rate of N<sub>2</sub> fixation after the enriched biomass has been processed using an IRMS. However, this is contingent on the assumption that equilibration is rapid. This assumption was brought into doubt by the aforementioned study, in which after a number of experiments, the results indicated that equilibration was neither rapid *nor* complete. As such, this led to the conclusion that there was a methodological underestimation of N<sub>2</sub> fixation when introducing <sup>15</sup>N<sub>2</sub> as a bubble.

In response to the purported deficiencies in this method, the authors suggested an alternative: the use of seawater pre-enriched with <sup>15</sup>N<sub>2</sub>. This method involves degassing nutrient enriched artificial seawater (e.g. YBCII medium; [Chen et al., 1996]), verification of degassing via e.g. Winkler titrations to check the oxygen concentration, transfer of this degassed medium to a septum capped glass bottle until overflow, and then injection of a <sup>15</sup>N<sub>2</sub> gas bubble. This bottle is then shaken vigorously until the bubble disappeared. Additionally, helium is sometimes added so as to not add air to the mixture once it has been sampled. Aliquots of this solution can then be used to enrich incubations with <sup>15</sup>N<sub>2</sub>. This method appears to give larger estimations of N<sub>2</sub> fixation compared with the N<sub>2</sub> fixation method, as well as appearing to instantaneously equilibrate with the medium.

Likely with the aforementioned discrepancies in the marine fixed N budget in mind [Codispoti et al. 2001,; Codispoti 2007; Eugster and Gruber, 2012, Wang et al., 2019], the above revelation was fervently adopted by the community, with seemingly immediate and almost dogmatic adoption of the pre-enriched method. However, recently, this position has been challenged. A meta-analysis along with complementary experimental and theoretical results by Wannicke et al. [2018] has added certain points of contention to the conclusions outlined by Mohr et al. [2010]. The meta-analytical portion of this study compared a swathe of published and unpublished comparisons of the bubble addition and enriched seawater methods. The result of the meta-analysis was the revelation that, whilst there was a 61% chance that results from the enriched seawater method were likely to be above associated incubations using the bubble method, 84% of the measurements between the two groups overlapped, suggesting that, statistically, there is little difference between the two methods. The other portion of this study dealt with calculating an error estimate for the bubble method during isotopic equilibration. The results of this analysis demonstrated a striking disparity between incubation times when using the bubble method. Short incubations (~1h) were shown to have an underestimation error of up to -72%. On the other hand, longer incubations (12h - 24h) showed a

negligible error of -0.2%. This was, however contingent on the  $^{15}\text{N}_2$  having sufficient time to equilibrate (~6h) before the beginning of the diazotrophic period. These results cast doubt on the absolute superiority of the enriched seawater method, instead suggesting that results from the two are reasonable alternatives when performed correctly.

In addition, while the bubble addition method's deficiencies have been well discussed, the same cannot be said for the enriched seawater method. A number of drawbacks with the pre-enriched seawater method are clear. Firstly, pre-preparation of enriched seawater to aliquot into incubation bottles may introduce unwanted compounds into the incubation bottle, be it nutrients, organic compounds, trace metals, etc. Given that incubations sometimes require a replacement of up to 10% of the volume by pre-enriched seawater, the introduction of unwanted materials may bias the incubation results by way of altering the stoichiometry of the medium. This is further compounded by the requirement of degassing the seawater used for the pre-enrichment, which may for example, alter dissolved inorganic carbon concentrations, and therefore the pH. Ideally, seawater matching the incubation would be used to best avoid these issues. This quickly becomes limiting, however, when performing field assays when preparation of pre-enriched seawater for each new station on a cruise is required. This is due to the additional steps and equipment required (filtration of seawater, degassing and verification of successful degassing, addition of helium etc.), making this process somewhat laborious. While less trivial for use on culture studies, if studying batch cultures over time, the use of pre-enriched medium may bias the results by altering nutrient concentrations or dissolved organic material (DOM) in an unwanted way. These considerations are all in comparison with simply injecting a  $^{15}\text{N}_2$  bubble into an incubation bottle and shaking sufficiently. It must also be stated that, often, a larger comparative estimate of  $\text{N}_2$  fixation rates is used to verify the pre-enriched seawater's efficacy. This is making the implicit assumption that larger results = more accurate results, which is a logical fallacy as it is presumptive of the truth. An increased value may well indeed be inaccurate as well. For example, to play Devil's advocate, the use of the pre-enriched method may also stimulate  $\text{N}_2$  fixation in some way, thus biasing the results.. A result which is not expected is not always incorrect.

In summary, it would appear that neither method is explicitly superior to one another; both have their advantages and disadvantages. Choice of method should therefore be down to personal

preference, as long as best practice is used to cover for their respective deficiencies, and results are interpreted with these deficiencies in mind.

In addition to the methodological issues suggested, another potential bias has can be highlighted with the  $^{15}\text{N}_2$  incubation method. Normally, when processing incubations, the particulate fraction is isolated by filtration onto glass fibre filters, whereas the dissolved fraction is disposed of. This is due to the assumption that there is no significant exudation of recently fixed N into the total dissolved nitrogen (TDN) pool. Recent evidence has however suggested this may not strictly be the case. The TDN is normally divided into three pools:  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , and dissolved organic N (DON). While  $\text{NO}_3^-$  is generally not considered a potential exudate of diazotrophs,  $\text{NH}_4^+$  is the natural product of  $\text{N}_2$  fixation and could therefore potentially be released by diazotrophs. DON is an umbrella term for any organic N that is found in the dissolved fraction. Through multiple mechanisms, organic N originating from the biomass of diazotrophs could potentially be introduced to the surrounding waters. As such, when tracking the fluxes of N *into* diazotrophs by way of  $^{15}\text{N}_2$  incubations, not tracking the fluxes of N *out* of diazotrophs may cause significant biases in measurements.

Previous field studies which aimed to measure  $^{15}\text{N}$  incorporation in both the particulate and dissolved phases have demonstrated that fixed N released into the dissolved phase may account for 10 - 50+% of the total fixed N [Glibert and Bronk, 1994; Konno et al., 2010; Benavides et al., 2013b], a fraction which may be shunted into the marine food web and promote significant primary production. In field studies, this release is usually related to senescence [Berman-Frank et al., 2004] or microbial interactions such as sloppy feeding [O'Neil and Roman, 1992] or viral lysis [Fuhrman, 1999]. However unialgal cultures of diazotrophs such as *Trichodesmium* spp, have shown significant overproduction (relative to cell growth) and release of fixed N into the surrounding medium [Mulholland et al., 2004; Mulholland and Bernhardt, 2005; Benavides et al., 2013a]. Parallel studies in unicellular diazotrophs are rare, although a study by Berthelot et al. [2015] compared nitrogen release in the colony forming *Trichodesmium* (IMS1010) with multiple species of unicellular diazotrophs, including two strains of *Crocospaera watsonii* (WH0003 and WH8501) and a strain of *Cyanothece* (ATCC51142). The results of this study indicated that N release by diazotrophs could account for up to 1% of fixed N production when analysing the dissolved phase



for isotopic enrichment, and interestingly 1-20% when comparing 'net' N<sub>2</sub> fixation (from <sup>15</sup>N<sub>2</sub> incubations) with 'gross' N<sub>2</sub> fixation (from total accumulation of fixed N in the various N pools).

Whilst this could potentially be seen as counter intuitive due to the high cost of N<sub>2</sub> fixation [Falkowski, 1983], potential explanations for this behaviour in colonial diazotrophs such as *Trichodesmium* spp. and *Gloeotheca* range from sharing of N with non-nitrogen fixing cells in colonies [Mulholland and Capone, 2000; Mulholland et al., 2004], excretion of amino acids [Carpenter et al., 1992; Capone et al., 1994; Mulholland and Capone, 1999], or extracellular storage of N in the mucilage [Flynn and Gallon, 1990]. Explanation for release in unicellular strains is however less obvious.

## **ARA**

N<sub>2</sub> fixation has been studied in great detail for the last half century. Arguably the first method used to observe N<sub>2</sub> fixation (the biological process) with decent sensitivity was the acetylene reduction assay (ARA). A useful quirk of the nitrogenase enzyme is that in addition to reducing N<sub>2</sub>, it can also reduce acetylene (C<sub>2</sub>H<sub>2</sub>) to ethylene (C<sub>2</sub>H<sub>4</sub>), gases which are both easily quantified on a gas chromatograph (GC). As such, it is possible to incubate samples containing diazotrophs with C<sub>2</sub>H<sub>2</sub> gas and measure the production of C<sub>2</sub>H<sub>4</sub> gas in order to quantify the rate of nitrogenase activity by said diazotrophs. This is usually a theoretical factor of three or four, which is based on the electron stoichiometry of the two chemical equations [Capone, 1993; Staal et al., 2001]. Using this conversion factor, it is therefore possible to use this to estimate the rate of N<sub>2</sub> fixation by proxy of nitrogenase activity. Advanced utilisation can allow for inline collection and analysis by GC of produced C<sub>2</sub>H<sub>4</sub> gas for high temporal sensitivity of nitrogenase enzymatic activity and this method is therefore highly useful for tracking the diazotrophic behaviour of diazotrophs over time.

## **Issues with ARA**

As with <sup>15</sup>N<sub>2</sub> incubations, and realistically the majority of complicated assays which involve biological processes, the ARA is not without its flaws. While questions such as the effect of incubating a diazotroph in conditions which differ significantly from its natural or even cultured environment are ever present [e.g. Flett et al., 1976, Fulweiler et al., 2015], maybe the most pressing issue with the ARA is the the accuracy of conversion ratios used.

When comparing results gathered from the two methods for quantifying N<sub>2</sub> fixation, there is usually a clear disparity, with larger values when using the ARA. Methodological differences between the two have often been attributed to the difference in what these assays measure. The ARA measures C<sub>2</sub>H<sub>4</sub> production which has been produced as a by product of nitrogenase activity, not fixed N production directly. It is therefore a proxy of nitrogenase activity rather than explicitly a quantification of N<sub>2</sub> fixation. When C<sub>2</sub>H<sub>4</sub> production rates are converted to give an estimate of N<sub>2</sub> fixation, it is often described as ‘gross’ N<sub>2</sub> fixation, i.e. the total amount of N fixed. On the other hand, <sup>15</sup>N<sub>2</sub> incubations do directly observe and measure the production of fixed N through N<sub>2</sub> fixation. However, not all N fixed remains in the biomass after the incubation, and as previously discussed, a fraction of the produced fixed N may significantly find its way into the dissolved phase. Given that most investigators discard this fraction, it has often been reasoned that estimates made using the <sup>15</sup>N<sub>2</sub> incubation method are ‘net’ N<sub>2</sub> fixation. As such, the differences in values are often explained away using this terminology reasoning that estimates from PO<sup>15</sup>N + TD<sup>15</sup>N = ARA. But has this been verified?

To return, utilisation of the ARA requires a conversion factor. The factor used is often theoretical. However, multiple studies have demonstrated that C<sub>2</sub>H<sub>4</sub>/<sup>15</sup>N<sub>2</sub> assimilation ratios can sometimes vary greatly from this presumed ratio, ranging from as little as 0.96 to as much as 56 [Mague et al., 1974; Graham et al., 1980; Capone, 1993; Montoya et al., 1996; Wilson et al., 2012], with little agreement as to the relevant ranges. As such, while it is clear that <sup>15</sup>N<sub>2</sub> incubations may indeed be justifiably described as ‘net’ N<sub>2</sub> fixation, because a significant fraction has been shown to enter the dissolved phase, it is not clear that the activity measured by ARA can accurately be considered ‘gross’ N<sub>2</sub> fixation, as it would appear that there is a large potential for ARA measurements to be overestimating.

### **Culture Studies vs Field Measurements**

While the techniques for studying N<sub>2</sub> fixation have been discussed at length, it is important to briefly mention the advantages and disadvantages of the different ways to study diazotrophs, specifically the differences between in vitro laboratory and in situ field study.

Culture studies are important because they allow for study of an organism with more highly defined controls. It is possible to perform study of the biology of an organism, including the growth, physiology, behaviour, and metabolism, in response to a wide array of controllable stimuli, in order to observe how an organism may react in the environment in similar circumstance. It is also possible to repeat or replicate your experiments or the experiments of others accurately to allow for a greater degree of confidence in your results. Precisely controlling the environment of an organism is arguably a more rigorous application of the scientific method in comparison to study of an organism in the environment, and technology is ever improving to allow for more and more accurate control.

Environmental studies are important because they allow for the examination of how organisms *actually* behave in the environment they live in. Whilst culture studies offer the ability to tightly control the environment of an organism, in situ, the environment of an organism is in fact controlled by a multitude of factors that are impossible to control in a laboratory. While a culture study may accurately control the irradiance delivered, the nutrient concentrations, the pH (etc.), a culture study cannot properly simulate stochastic conditions found in nature. These include variability in light or nutrient availability from vertical or horizontal ocean currents, competition, grazing, commensalism, or symbiosis from a consortia of other phytoplankton or heterotrophic bacteria, genetic variability in strains etc. In addition, the vast majority of organisms are currently uncultivable, and many undiscovered. As such, all of our laboratory studies are based on 'model' organisms which are easily cultured. Applicability to the environment is therefore contingent on how representative the behaviour of said organisms are to the natural consortia. Given that significant variability can be found even at the species level, this assumption is not always wise. As such, the applicability of lab studies is sometimes questionable.

The variability in the environment does however affect in situ studies also. Extreme stochasticity means that behaviour may be variable over even small timescales. In order to properly understand how these organisms behave over time and space, time series analyses over a wide range of geographic locations should be performed. As most diazotrophic activity is expected to be performed in oligotrophic regions, such as in the middle of subtropical gyres, studying N<sub>2</sub> fixation in the environment requires significant investment in the form of long cruises. Given the limiting

cost of said cruises, environmental studies of N<sub>2</sub> fixation do not generally have good spatial or temporal resolution. This makes drawing wide ranging conclusions from environmental studies potentially unwise. In the case of studying how certain variabilities may affect diazotrophy, laboratory studies may be superior as long as those variabilities can be accurately studied.

Despite this, oftentimes in order to study processes efficiently, investigators often take advantage of conditions potentially unrepresentative of the environment for use in laboratory studies. For example, in order to study some processes, the sensitivity limitations may require high density cultures. In order to reach high density cultures, significant concentrations of nutrients are required, concentrations which are rarely or never reached in the natural environment. This brings into question how reliable studies such as these are.

Given these considerations, it is clear that many considerations must be made when drawing conclusions from any study. While many of their deficiencies are highlighted, it is important to recognise their strengths also. In reality, neither approach is perfect, but do cover for each other in certain respects and thus must be considered together and inform each other when building our understanding of processes and organisms such as N<sub>2</sub> fixation and diazotrophs.

## *ii) Geochemical Methods*

While the topic of this thesis more expediently requires an understanding of the biological methods of measuring N<sub>2</sub> fixation, to properly understand how N<sub>2</sub> fixation has been studied on a global scale, it would be improper to not to include the geochemical methods used.

In-situ biological assays for measuring N<sub>2</sub> fixation have historically focussed on shipboard measurements. Given the impossibility of gathering a sufficiently exhaustive amount of data on the spatial and temporal variability inherent for *any* biological process in the ocean, the ‘expensive snapshots in time’ that are generated from shipboard assays are generally not robust enough to provide global applications without vast extrapolation. The relevance of these measurements are temporally and geographically locked to their sampling time and place and as such, cannot give much about N<sub>2</sub> fixation over longer periods of time or larger regions of the ocean. As such,

geochemical methods have risen as popular alternatives to compliment those data. These usually involve analysing the imprints left by diazotrophic activity on the geochemistry (nutrients, stable isotopes, etc.) of the ocean.

### **N\* and Derivatives**

A popular geochemical approach to studying N<sub>2</sub> fixation has been the use of observed divergences from the classical NO<sub>3</sub><sup>-</sup>: phosphate (PO<sub>4</sub><sup>3-</sup>) Redfield ratio of 16:1 [Redfield, 1958] expected from the remineralisation of biomass in the ocean. Specifically, it was reasoned that while diazotrophs continue to consume PO<sub>4</sub><sup>3-</sup>, they also introduce newly fixed N due to their ability to fix atmospheric N<sub>2</sub>. As such, the ratio of NO<sub>3</sub><sup>-</sup>:PO<sub>4</sub><sup>3-</sup> of remineralised biological material sourced in waters where N<sub>2</sub> fixation has occurred should be significantly higher than material remineralised from waters without N<sub>2</sub> fixation (>16:1) [Gruber and Sarmiento, 1997; Deutsch et al., 2001]. Additionally, denitrification leaves the opposite signal by way of removing N relative to Redfield (<16:1). This parameter is called N\*. The abundance of high quality nutrient data from global surveys (e.g. JGOFs/WOCE; Conkright et al., 2002) allows for the employment of this method over good spatial and temporal resolutions of N\*. When combined with ventilation ages of the ocean derived from from stable, abiotic tracers in the ocean, it is possible to infer N<sub>2</sub> fixation rates over large spatial and temporal ranges. Another approach similar to N\* is P<sub>t</sub>\* [Deutsch et al., 2007], which estimates N<sub>2</sub> fixation by assessing total P consumption. P<sub>t</sub>\* is defined by the excess P in relation to Redfield equivalent amounts of N. It differs from N\* in that it accounts for organic nutrients in its estimate, giving a global estimate ~25% greater than N\*. Another approach by Landolfi et al., [2008] also took into account organic N was TN<sub>xs</sub> (total N excess), and this estimate further doubled estimates of N<sub>2</sub> fixation compared with similar estimates by N\* in the subtropical North Atlantic.

While an important contribution to the field, N\* is not without its inherent deficiencies. It has been noted that N\* may underestimate the total excess NO<sub>3</sub><sup>-</sup> (relative to PO<sub>4</sub><sup>3-</sup>) which is introduced to the ocean via atmospheric deposition [Hansell et al., 2004; 2007; Zamora et al., 2010]. This would inflate N\*, and thus overestimate the contribution which N<sub>2</sub> fixation provides to N\*. Furthermore, non-Redfield consumption of nutrients by Phytoplankton have also suggested that N\* estimates should be approached with caution [Mills and Arrigo, 2010; Weber and Deutsch, 2014]. Further issues include inaccuracies with current understandings of ocean circulation [Dietze and Loptien,

2013; Cabré et al., 2015] as well as water age uncertainties derived from tracers [Koeve and Kähler, 2016].

### **Stable Isotope Approaches**

The inherent weaknesses in the  $N^*$  approach and its derivatives highlight the need for complimentary approaches to estimating  $N_2$  fixation by geochemical methods. One such method which has recently been described, is the use of stable isotope ratios of N ( $\delta^{15}N$  [‰]) in the ocean [Knapp et al., 2008].  $\delta^{15}N$  is defined as:

$$\delta^{15}N (\text{‰}) = \left\{ \left[ \frac{(^{15}N/^{14}N)_{\text{sample}}}{(^{15}N/^{14}N)_{\text{reference}}} \right] - 1 \right\} \times 1000 \quad (\text{Eq. 1.1})$$

The  $\delta^{15}N$  of atmospheric  $N_2$  is light ( $\sim -2\text{‰}$ ) [Hoering and Ford, 1960; Minagawa and Wada, 1986; Carpenter et al., 1997] in relation to the mean  $\delta^{15}N$  of marine  $NO_3^-$  ( $\sim 5\text{‰}$ ) [Sigman et al., 2000].  $N_2$  fixation has little to no isotope fractionation effect (the preference for one isotope over another) and as such, introduction of recently fixed N into the ocean should introduce isotopically light  $NO_3^-$  once remineralised. It can therefore be reasoned that  $\delta^{15}N\text{-}NO_3^-$  observed below the global average has been diluted with ‘recently fixed’  $NO_3^-$ . There are however other N cycle processes that have notable isotope fractionation effects that can act to alter the  $\delta^{15}N$  of different N pools in the ocean. Denitrification is generally considered to contribute the most to values of  $\delta^{15}N\text{-}NO_3^-$  elevated above the global average of  $\sim 5\text{‰}$  [Deutsch et al., 2004], and can fractionate marine  $NO_3^-$   $\delta^{15}N$  to  $\sim 20\text{-}30\text{‰}$  [Sigman et al., 2009]. Assimilation by phytoplankton is another source of isotopically ‘heavy’ N, as they often preferentially incorporate  $^{14}N\text{-}NO_3^-$ , thus leaving a relatively higher proportion of  $^{15}N$  in the  $NO_3^-$  pool [Waser et al., 1998; Needoba et al., 2003].  $N_2$  fixation is not the only process which introduces isotopically light N, however. Atmospheric deposition of fixed N has been shown to introduce isotopically light N to the ocean, and considering the larger than assumed amount of atmospheric deposition of fixed N reported recently [Hansell et al., 2004; 2007; Zamora et al., 2010], this deposition may be important to the isotopic composition of  $NO_3^-$  in the ocean. By accounting for potential isotopic deviations from the global average  $\delta^{15}N\text{-}NO_3^-$ , and by it’s therefore possible to make an estimate of the fraction of  $NO_3^-$  which has been recently fixed. When combined with ventilation ages of the ocean, as with  $N^*$ , it is possible to make an integrated areal rate of  $N_2$  fixation [e.g. Knapp et al., 2018].

### *iii) Modelling Approaches*

The final approach used to study N<sub>2</sub> fixation in the oceans is the use of models. Models vary in their design and approach, with use of both implicit and explicit parameterisations of N<sub>2</sub> fixation [Landolfi et al., 2018]. Implicit models tend to employ methods which restore upper ocean NO<sub>3</sub>:PO<sub>4</sub><sup>3-</sup> ratios towards the Redfield ratio [Ilyina et al., 2013]. Approaches such as this tend to ignore the ecophysiological complexities that distribute diazotrophs and determine the intensity of N<sub>2</sub> fixation, instead opting to use the prognostic approach of using ocean biogeochemistry to imply N<sub>2</sub> fixation. This method is advantageous as the factors which control diazotroph ecology are not well constrained. Explicit methods are those which do attempt to constrain diazotroph distribution by ecophysiological factors. These tend to make the assumption that diazotrophs are slow growing organisms which are photosynthetic and capable of fixing N [Landolfi et al., 2018], which are outcompeted by larger, faster growing phytoplankton [Tyrrell, 1999]. Both methods tend to distribute diazotrophs in some way by low N:P ratios relative to Redfield, although for different reasons. Implicit models do so as they reason that, because Redfield ratios of N:P in the ocean have been so prevalently observed, there must be strong feedback mechanisms controlling it as such. N<sub>2</sub> fixation adds more fixed N to the ocean relative to P, thus acting as a feedback mechanism to restore depressed N:P ratios. This therefore suggests that N<sub>2</sub> fixation should be found in areas where N:P ratios are low. Explicit models instead suggest exclusion in areas of high N:P ratios due to out competition for nutrients by larger phytoplankton [Tyrrell, 1999]. This would thus limit them to areas where competing phytoplankton would be limited by N. Additionally, exclusion or inhibition of diazotrophs by high concentrations of DIN has also been reasoned due to assumed inhibition of N<sub>2</sub> fixation by DIN [Zahariev et al., 2008; Dunne et al., 2013; Aumont et al., 2015]. This is predicated on the idea that N<sub>2</sub> fixation is energetically expensive in comparison to utilisation of DIN such as NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup> [Falkowski, 1983; Grosskopf and LaRoche, 2012], suggesting that when DIN is available, diazotrophy will not occur. Additional factors which have been used to parameterise N<sub>2</sub> fixation have been light, temperature, and iron (Fe) limitation [Moore et al., 2013].

Interestingly, most models have not managed to replicate the high rates N<sub>2</sub> fixation observed in the subtropical North Atlantic [Landolfi et al., 2015; Landolfi et al., 2018]. This is usually attributed to the low P\* observed in this region. A modelling attempt by Landolfi et al., [2015] noted that even when accounting for a number of factors, such as preferential P remineralisation, utilisation of

alternative P sources (i.e. dissolved organic P; DOP), or iron limited growth, they were not able to improve simulations of P\* supply to expand the predicted niche of diazotrophs in this region [summary from Landolfi et al., 2018]. Given these deficiencies, it is becoming apparent that modellers may need to rethink the assumptions used when attempting to model N<sub>2</sub> fixation.

### Paradigms of N<sub>2</sub> fixation

Paradigms are a set of concepts which can be used to define a distinct model of how something may function or behave. In the case of diazotrophs, there are a number of factors which have classically led discussion, many of which are now proving defunct. Much of this understanding was built around research surrounding macroscopic, easily observable diazotrophs, such as *Trichodesmium* spp., however, our understanding on the diversity of diazotrophs has recently been expanded thanks to developments in environmental genomics [e.g. Zehr, 2011]. As such, if our understanding of diazotrophy is to become more complete, our understanding of paradigms of diazotrophy must expand to accommodate these ‘new’ diazotrophs. Here we outline the classical understanding of diazotrophy, as well as highlighting how newer findings are bringing doubt to the universality of these paradigms. These are organised by factors which are thought to ecologically control diazotroph distribution as well as the rate of N<sub>2</sub> fixation itself.

#### *i) Temperature Control*

Classically, an important control of N<sub>2</sub> fixation has been temperature. Marine diazotrophs have most often been observed in warm sub-tropical and tropical waters above 15-20°C [Capone and Carpenter, 1982; Capone et al., 1997; Needoba et al., 2007; Boyd et al., 2010]. This was originally seen with macroscopic species such as *Trichodesmium* spp., an organism famous for forming large characteristic blooms in subtropical oceans [e.g. Carpenter, 1983]. Molecular techniques have recently done more to characterise this relationship, with nifH copies (the gene responsible for nitrogenase) being found to be distributed by temperature in both the North Atlantic [Langlois et al., 2008] and the North Pacific [Church et al., 2008]. Furthermore, theoretical and culture studies have demonstrated that many diazotrophs tend to have warm optimal temperatures for growth [Staal et al., 2003; Falcón et al., 2005; Breitbarth et al., 2007; Stal, 2009] and modelling approaches



frequently distribute diazotrophy along temperature constraints (although not exclusively) [e.g. Monteiro et al., 2011]. Warm temperatures have been suggested as important for diazotrophs due to the energetic cost of splitting the triple bond of the N<sub>2</sub> molecule. Slow growth rates compared to other marine phytoplankton have been reported consistently and are thought to be a result of this energetic cost [e.g. Falcón et al., 2005; Goebel et al., 2008].

Interestingly, the recent discovery of diazotrophs operating in significantly colder environments has broken down this relationship somewhat. Diazotrophy in the Baltic Sea has been studied for a long time [e.g. Ohlendieck et al., 2000; Larsson et al., 2001; Staal et al., 2003], however colder areas previously thought to be devoid of N<sub>2</sub> fixation have recently been investigated. Small but significant rates of fixation [Blais et al., 2012] as well as abundant instances of the nifH gene [Blais et al. 2012; Díez et al. 2012; Fernández-Méndez et al. 2016; Shiozaki et al., 2018] have been found in the Arctic. Additionally, according to Shiozaki et al., [2018] many of the genes discovered there were ‘dissimilar’ to those from tropical and subtropical oceans; potentially representing ‘indigenous’ communities of diazotrophs. The discovery of marine diazotrophs operating in the cold waters of the Arctic is an important result as it shakes up the commonly believed paradigm that marine diazotrophy will only occur in the warm waters of the sub-tropical and tropical oceans.

#### *ii) Light Availability*

Light is another important distributing factor for diazotrophs. For decades, marine diazotrophy has been attributed to cyanobacteria [Zehr, 2011], which are generally photoautotrophic. Coupling of N<sub>2</sub> fixation with photosynthesis is logical due to the high energetic requirement of N<sub>2</sub> fixation. It therefore follows that significant light availability is a prerequisite for these diazotrophs. Indeed, studies have demonstrated that light is a significant factor in the biogeographic distribution of diazotrophs [e.g. Monteiro et al., 2011]. Furthermore, culture studies have shown certain diazotrophs to be acclimated to high light environments [Carpenter and Roenneberg, 1995; LaRoche and Breitbarth, 2005; Masotti et al., 2007; Goebel et al., 2008].

While the paradigm of light as a requirement for marine diazotrophs has been well defined for decades, recent studies have highlighted the potential for N<sub>2</sub> fixation in the deep ocean, a source that

has traditionally been ignored. The use of molecular techniques have placed a significant presence of *nifH* as deep as the abyssopelagic zone [Hewson et al., 2007], and measurable rates of  $N_2$  fixation have been detected multiple times in the mesopelagic [Bonnet et al., 2013; Rahav et al., 2013; Benavides et al., 2015]. A recent speculative paper [Benavides et al., 2018] has highlighted that, despite a generally low rate of  $N_2$  fixation currently described by the literature (summarised by Moisander et al. [2017]), when the size of the deep ocean is fully accounted for, the input of fixed N by aphotic diazotrophs is potentially huge.  $N_2$  fixation by aphotic, non-cyanobacterial diazotrophs may be an important topic in the coming years of research.

### *iii) Nutrient Availability*

It is reasonable to state that, unlike non-diazotrophs, most diazotrophs cannot be limited in growth by lack of N availability due to their ability to fix abundant dissolved  $N_2$  in their surrounding waters. This therefore shifts the focus of limitation onto other nutrients in the ocean. Of these nutrients, two are often noted as being most important: phosphorous (P), generally in the form of  $PO_4^{3-}$ , and Fe.

P is an essential element for all life, and is used for multiple purposes, such as the construction of DNA and RNA, the construction of cell membranes through phospholipids, and the functioning of all energetic cellular processes through the production and utilisation of ATP. As such, the maintenance of cellular P stoichiometry is essential for the growth of diazotrophs. Fe is important for cyanobacterial diazotrophs as it is an essential element for the construction of nitrogenase [e.g. Georgiadis et al., 1992] as well as PSI and other redox enzymes. Depending on the relative supply, these two elements are thought to be key factors in the distribution of diazotrophs across the marine environment; where one is stoichiometrically less abundant, it will become limiting to diazotroph growth. For example, high levels of Fe deposition from Saharan dust deposits are known to cause important P limitation for diazotrophs in the North Atlantic. Conversely, in the South Atlantic and Pacific oceans, there are relatively higher concentrations of dissolved P to dissolved Fe, which pushes diazotrophs in these systems towards Fe limitation [summarised in Sohm et al., 2011b].

Culture studies following Fe limitation in diazotrophs have demonstrated reduced growth and N<sub>2</sub> fixation rates across multiple strains of *Trichodesmium* spp. [e.g. Chappell and Webb, 2009], as well as in the unicellular cyanobacterial diazotroph *Crocospaera watsonii* [Fu et al., 2008]. Given added Fe cost of constructing nitrogenase, the relative Fe requirement for diazotrophs has been demonstrated to be ~5 times that of non-diazotrophs [Berman-Frank et al., 2001; Kustka et al., 2003]. This added cost makes diazotrophs particularly susceptible to Fe stress, and there are many instances of mechanisms which allow diazotrophs cope with, or be alleviated from it.

*Trichodesmium*, for example, has been suggested to be a highly efficient scavenger of Fe, as determined by the high Fe content of cells [Kustka et al., 2003; Whittaker et al., 2011]. Additionally, *Trichodesmium* cells may potentially deal with Fe stress mechanistically by down-regulating N<sub>2</sub> fixation, but maintaining photosynthesis through rearrangement of cellular machinery [Küpper et al., 2009]. While *Trichodesmium* fixes N<sub>2</sub> during daylight hours, which means Fe is competed for by photosynthetic and N<sub>2</sub> fixation enzymes, *Crocospaera watsonii* instead separates these processes by fixing N<sub>2</sub> at night instead. As such, photosynthetic and N<sub>2</sub> fixation related proteins are only produced when needed throughout the day. *Crocospaera watsonii* is therefore able to efficiently scavenge and repurpose Fe from related enzymes produced sequentially throughout the day, and as such, can reduce its Fe requirements by up to ~40% [Saito et al., 2011]. This adaptation potentially extends the niche of *Crocospaera watsonii*, and similar diazotrophs, to areas with low Fe, and may allow for more productive growth and N<sub>2</sub> fixation than had it not developed this adaptation.

Similar studies for P limitation of additionally revealed a number of mechanisms for which diazotrophs may alleviate P stress. These mechanisms have been most well studied in cultures of *Trichodesmium*, which have been shown to employ a dual strategy of firstly increasing P uptake efficiency, and secondly reducing P requirements for the cell. Increasing P uptake efficiency includes increasing uptake of PO<sub>4</sub><sup>3-</sup> [Fu et al., 2005] while also scavenging P from dissolved organic P (DOP) sources by increasing alkaline phosphatase production [Mulholland et al., 2002] in order to enable efficient cleaving of PO<sub>4</sub><sup>3-</sup> groups from phosphomonoesters. *Trichodesmium* has also been shown to be able to take P from phosphonates [Dyrman et al., 2006], which are not usually considered particularly labile. These strategies, while additionally increasing cellular N:P ratios [Krauk et al., 2006; White et al., 2006a], allow for *Trichodesmium* to survive under P stress. This

stress however, does still often come at the cost of inhibited N<sub>2</sub> fixation rates [Krauk et al., 2006]. Vertical migration has been another fairly novel strategy to reducing P stress by *Trichodesmium*. The mechanism proposed for this has been suggested as the usage of stored glycogen and cyanophycin granules [Romans et al., 1994] as a ‘ballast’ which enables colonies to sink below the nutricline and access more abundant P species [Romans et al., 1994; Villareal and Carpenter, 2003; White et al., 2006b]. Metabolism of this ‘ballast’ would then allow for return to the euphotic zone [Bergman et al., 2012]. Work on unicellular cyanobacterial diazotrophs are rarer, but *Crocospaera watsonii* has been shown to deal with P stress similarly to *Trichodesmium*, as both increases PO<sub>4</sub><sup>3-</sup> uptake [Dyhrman and Haley, 2006] while also employing alkaline phosphatase in order to utilise phosphomonoesters [Dyhrman and Haley, 2006]. *Crocospaera watsonii* however cannot utilise phosphonates.

#### iv) DIN inhibition

As previously alluded to in the previous section, a classically important controlling factor considered relevant to diazotroph distribution is the presence of dissolved inorganic N (DIN; NO<sub>3</sub><sup>-</sup> & NH<sub>4</sub><sup>+</sup>). The energetic cost required to incorporate DIN is smaller by ~25% in comparison to the cost of fixing an equivalent amount N<sub>2</sub> [Falkowski, 1983; Grosskopf and LaRoche, 2012]. As such, it is often assumed that for concentrations of DIN >1µM, diazotrophs would have no reason to fix N<sub>2</sub>. This reasoning has had an important effect on the field as it has dominated how modelling approaches in the past have distributed diazotrophs.

Previous experimental and field studies have aimed to assess the accuracy of this idea. Due to its prominence and visibility in previous research, the majority of early studies focussed on *Trichodesmium* spp., and many paradigms to do with diazotrophy were built off of this research. Field manipulations involving fixed N additions (NH<sub>4</sub><sup>+</sup>, chloramphenicol, glutamate, glutamine) to natural populations of *Trichodesmium* spp. show clear instances of significant inhibition of N<sub>2</sub> fixation [Capone et al., 1990; Mulholland et al., 2001]. Culture studies of *Trichodesmium* spp. further describe the relationship, revealing a clear distinction between short term and long term exposure to DIN (NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub>) and urea. Short term exposure to DIN demonstrated high variability for inhibition of N<sub>2</sub> fixation, ranging from 0-100% [Ohki et al., 1991; Mulholland et al., 2001; Fu

and Bell, 2003; Holl and Montoya, 2005; Sandh et al., 2011]. Long term exposures on the other hand showed a clearer relationship [Knapp, 2012], with all incubations showing almost complete inhibition (60-100%) [Ohki et al., 1991; Mulholland et al., 1999; Fu and Bell, 2003; Milligan et al., 2007; Knapp et al., 2012]. Furthermore, these studies also demonstrated a clear difference between  $\text{NH}_4^+$  and  $\text{NO}_3^-$ .  $\text{NH}_4^+$  generally showed a much faster and complete inhibition of  $\text{N}_2$  fixation in comparison with  $\text{NO}_3^-$ , likely due to its lower comparative cost to uptake [Falkowski, 1983; Grosskopf and LaRoche, 2012]. These results have broadly supported the notions of DIN limitation of diazotrophs and caused the community to consider DIN replete zones, such as coastal upwelling regions, to be inhospitable for diazotrophs.

Recent advances have however, brought this concept under scrutiny. While *Trichodesmium* spp. has arguably dominated the  $\text{N}_2$  fixation research spotlight over the last few decades, molecular techniques have revealed a severe underestimation in the number of nifH containing prokaryotes in the ocean [e.g. Zehr, 2011]. This has prompted research as to the applicability of *Trichodesmium* spp. derived paradigms on  $\text{N}_2$  fixation, especially the notion of DIN limitation. A recent review by Knapp [2012] has highlighted recent research efforts attempting to delineate this question. In this review, the author highlights culture efforts to firstly clarify the relationship of DIN with *Trichodesmium* spp. and to do the same with a more recently discovered unicellular cyanobacterial diazotroph *Crocospaera watsonii*. The broad conclusion for *Trichodesmium* spp, by this review highlighted the aforementioned relationship with the length of exposure to DIN, stating that inhibition was generally only important for long term exposures, suggesting only diazotrophs chronically exposed to DIN would be implicated. For *Crocospaera watsonii*, currently there has been fewer studies examining this relationship. However while the same short term-long term relationship has been observed [Dekaezemaker and Bonnet, 2011; Knapp et al., 2012], there was less overall inhibition demonstrated in both short term and long term exposures for this cyanobacteria. A more recent study by Garcia and Hutchins [2014] has however challenged this notion by observing that, when exposed to a greater irradiance,  $\text{NO}_3^-$  did not inhibit  $\text{N}_2$  fixation, but enhanced growth overall, suggesting  $\text{N}_2$  could not support higher growth rates completely. Inhibition was only observed in low light experiments, suggesting an important role for energy in this relationship, but even then it was not complete.

The review by Knapp [2012] additionally pooled examples of diazotrophs being found in DIN replete zones, along with significant rates of N<sub>2</sub> fixation by these diazotrophs (so as to prove they did not drift there). The review reports examples diazotrophic activity in the euphotic zone with ambient NO<sub>3</sub><sup>-</sup> concentrations ranging from 5.5 μM - 21 μM [Voss et al., 2004; Grosse et al., 2010; Bombar et al., 2011; Sohm et al., 2011a; Fernandez et al., 2011]. Furthermore, as previously alluded to, a handful of reports of significant rates in the mesopelagic were demonstrated [Fernandez et al., 2011; Hamersley et al., 2011] where ambient NO<sub>3</sub><sup>-</sup> concentrations were reported up to 32 μM.

With these pieces of evidence in mind, it is becoming clear that the assumption that N<sub>2</sub> fixation would not occur where DIN concentrations exceeded 1 μM is in dire need of revision. While this still may be *somewhat* true for *Trichodesmium* spp., room needs to be made to accommodate the avalanche of evidence which suggests our *Trichodesmium* derived paradigms of the past are not universal to all diazotrophs.

### Conclusions and Suggestions

In this chapter, we highlight clearly the factors which seem to drive supposed inaccuracy in our N<sub>2</sub> fixation estimates. Firstly, we highlight the methodological issues surrounding the estimation of N<sub>2</sub> fixation. Clear issues are highlighted with both the ARA and <sup>15</sup>N<sub>2</sub> incubation methods. For the ARA, the uncertainty appears to be down to poor reliability of the conversion ratio used between C<sub>2</sub>H<sub>2</sub> and fixed N production. For the <sup>15</sup>N<sub>2</sub> incubations, there is of course the issue with <sup>15</sup>N<sub>2</sub> gas equilibration brought to attention by Mohr et al., [2010]. However, we also highlight evidence that N exudation may also be causing methodological bias, and further study may be a potential route to take to alleviate N<sub>2</sub> fixation underestimation.

In addition, we take a look at the paradigms which have been built around the ecology and physiology of diazotrophs. A number of paradigms have pervaded the study of diazotrophy for the last half century or so, including the necessity for warm, low nutrient and low DIN environments. Furthermore, as most diazotrophs were observed to be cyanobacteria, previously, diazotrophy was believed to be limited the euphotic zone. Paradigms built on observable, macroscopic diazotrophs are however beginning to unravel. Firstly, recent work has demonstrated significant active diazotrophy in arctic waters, as well by heterotrophic diazotrophs in the deep ocean. Furthermore,

previous observations as to the down regulation of N<sub>2</sub> fixation under significant concentrations of DIN have been demonstrated to not hold true for all diazotrophs; while there is indeed a down regulation in the presence of NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup> was shown to not always to completely stop diazotrophy in species such as *Crocospaera watsonii*. It is therefore clear that if we are to revise our global estimates of N<sub>2</sub> fixation to be in equilibrium with denitrification, we must look to both the deficiencies in our methodology, as well as ‘new’ diazotrophs which do not conform to old paradigms of N<sub>2</sub> fixation.

## Chapter Two: Sampling and Analytical Methodology

In this chapter, we list the common sampling, sample processing, and data analysis methodology utilised in the three results chapters presented in this thesis. Where individual methodology is deviated from in a specific chapter, it will be noted in said chapter. This chapter will be organised first by sampling and sample processing methods, then data analysis methodology.

### *Sampling and Processing Methodology*

#### Cell counts and cell size

Samples for cell counts were taken once per day, after cell division was complete, and where applicable, additional samples were taken to match with other analyses such as CHN measurements and at the end of  $^{15}\text{N}_2$  incubations. These samples were processed immediately in triplicate using a Beckman Coulter Counter, which presents particle density within specific size bins. Cell counts were derived per sample by integrating the curve presented by the coulter. Average (mean) cell size was additionally derived using these data.

#### Particulate C and N

20ml of culture samples were taken in triplicate from the PBRs or culture flasks using a syringe and immediately filtered on pre-calcinated (400°C for 4 hours) GF/F 25mm filters. These were collected in pre-calcinated glass tubes and placed in drying cupboards set to 70°C until analysis. Once ready, the filters were processed using a CHN elemental autoanalyser (Perkin Elmer) to determine the concentration of C and N in  $\mu\text{moles L}^{-1}$ .

#### Concentration of TDN, $\text{NH}_4^+$ and DON

The filtrate generated from above particulate C and N samples were split in two, and collected in Falcon brand centrifugation tubes, reserving one half for the determination of total dissolved N concentration [TDN] and the other for the determination of  $[\text{NH}_4^+]$ . These tubes were acid washed (10% HCl) prior to the experiment for 24h in order to remove traces of  $\text{NH}_4^+$ . Both filtrate sample types were spiked with a 0.34 $\mu\text{l}$  of 6g/L HgCl to ensure no microbial activity from possible contaminant microbes could alter the concentrations inside the tubes.



To calculate the  $\text{NH}_4^+$  concentration, filtrate samples were analysed using a fluorimeter [Futura, Alliance Instruments], which operates by reacting ammonium within the sample with an ortho-phthalaldehyde (OPA) and sulphite reagent, to form a pink colour.

To calculate the TDN concentration, filtrate samples were processed using wet chemical oxidation (WCO) using the method described in Raimbault and Slawyk [1991]. This notably converts all 'fixed' nitrogen species, including  $\text{NH}_4^+$  and dissolved organic N (DON) to  $\text{NO}_3^-$ . Sample processing for this involves the addition of the WCO reagent to the samples, which contains potassium persulphate (an oxidiser) and boric acid. The sample solutions were then digested by autoclaving them for 30 minutes at  $121^\circ\text{C}$ . Produced  $\text{NO}_3^-$  samples were then analysed using a Technicon Autoanalyser which gives the  $\text{NO}_3^-$  concentration in  $\mu\text{moles N L}^{-1}$ .

By subtracting  $\text{NH}_4^+$  concentrations from parallel TDN concentrations, the DON concentration can be derived. Note, normally this would require derivation and subtraction of  $\text{NO}_3^-$  concentrations, and was performed in the case of the  $\text{NO}_3^-$  utilisation experiments. Most experiments were however prepared  $\text{NO}_3^-$  free (and verified as such), so it was unnecessary to complete this step for these experiments and samples.

#### Acetylene Reduction Assays (ARA)

Nitrogenase enzymatic activity was monitored using the acetylene reduction assay (ARA). A sample was first prepared by filtering ~50ml of culture onto a 47mm Whatman GF/C filter. This filter was then placed in an air tight cell incubator thermoregulated to  $27^\circ\text{C}$  using a cryostat. A gas mixture of  $\text{N}_2$  (70%),  $\text{O}_2$  (20%), and  $\text{C}_2\text{H}_2$  (10%), was channelled through this cell incubator at a rate of  $1 \text{ L h}^{-1}$ . The gas contained in the cell incubator was continuously channelled out of the cell, the outflow being injected into a GC column (Agilent 7890) equipped with a PID detector every 10 minutes, as described in Dron et al. [2012]. This assay was run continuously throughout the dark phase, and into the light phase to cover exhaustively the entirety of the  $\text{N}_2$  fixation period. The conversion factor we used to estimate the rate of  $\text{N}_2$  fixation from ethylene production was a factor of four, derived from the electron stoichiometry of the two equations; it takes four more electrons to reduce acetylene to ethylene than it does to convert  $\text{N}_2$  to fixed N (Capone, 1993; Staal et al., 2001).

#### $^{15}\text{N}_2$ and $^{13}\text{C}$ incubations (particulate)

The incubations were executed as follows. Firstly, at the time points described in each individual experiment, ~170ml of culture volume was taken from the PBRs using a syringe. These were

deposited in sterilised 125ml (true volume ~170ml) Nalgene polycarbonate incubation bottles. Before sealing, each incubation bottle was spiked with 0.1ml of 286 mmoles L<sup>-1</sup> <sup>13</sup>C labelled NaHCO<sub>3</sub><sup>-</sup> to a concentration of 168µmoles L<sup>-1</sup> of labelled <sup>13</sup>C, an enrichment of ~4.375%. Bottles were then filled to the brim, to empty the bottles completely of air, and capped with septum caps. Due to the difficulty in eliminating air bubbles completely, bottles were photographed upside down with the bubble showing to later estimate the volume of this bubble; this is important for the later determination of the initial enrichment of <sup>15</sup>N. After this, 0.5ml of pure <sup>15</sup>N<sub>2</sub> gas (Eurisotop) was sampled from a gas tank and injected directly into the bottles. The end enrichment of <sup>15</sup>N<sub>2</sub> gas of these bottles was estimated to be ~21% (Eq. 2.x), with minor variations due to residual air bubbles in the incubation bottles. These bottles were shaken vigorously for ~3 minutes to equilibrate the <sup>15</sup>N<sub>2</sub> gas bubble with the liquid, placed in an incubator set to the same temperature and light intensity settings as the PBRs, and left for the required amount of time (12h or 24h).

Once these incubations were complete, a small aliquot of the incubation volume was taken to do a post-incubation cell count using the Beckman Coulter Counter. This was to verify that the *Crocospaera watsonii* population either did not alter significantly from the PBR cultures, and in the case of incubations covering the light phase, to compare growth within the incubations and PBR cultures. Following this, a larger aliquot (~20ml) was taken from the incubation bottles and filtered onto a pre-calcinated GF/F filter (25mm), taking care to preserve the filtrate. These filters were preserved in the exact same way as the above particulate C and N samples. Once this sample was secure, the remaining incubation volume was filtered on a larger pre-calcinated GF/F filter (47mm) to collect the rest of the filtrate. As with TDN and NH<sub>4</sub><sup>+</sup> samples, filtrates were collected in acidified falcon tubes (175ml) and spiked with HgCl<sub>2</sub>. Once dried, filters were processed using a CHN-IRMS (Isotope Ratio Mass Spectrometer; INTEGRA CN - SERCON) and productions derived according to Montoya et al 1996.

#### <sup>15</sup>N<sub>2</sub> Incubations: Dissolved Phase (NH<sub>4</sub><sup>+</sup> and DON)

Filtrates gathered from <sup>15</sup>N<sub>2</sub> incubations were processed using the 3-step diffusion method outlined in [Slawyk and Raimbault, 1995]. This method can be used to sequentially remove forms of ‘fixed’ N from a filtrate sample, specifically NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup>, and DON. To prepare the samples, filtrates were decanted into 250ml Duran Schott bottles, which were capped with teflon lined screw caps. Before this, bottles were pre-digested using WCO reagent to remove any trace of organic matter from the insides. Additionally, the teflon lined screw caps were equipped with a glass hook, designed to hold

an acidified filter. These filters were pre-calcinated GF/C filters which were acidified by blotting a 50  $\mu\text{l}$  of 2M  $\text{H}_2\text{SO}_4$ . Filters were added and removed at the start and end of every step respectively, collecting them in pre-calcinated glass tubes for later analysis. Filters were again processed using a CHN-IRMS set-up.

We detail the 3-step diffusion process used as follows:

- 1)  **$\text{NH}_4^+$  removal:** 200 $\mu\text{g}$  of MgO was added to the bottle to create an alkaline environment necessary to convert dissolved  $\text{NH}_4^+$  to  $\text{NH}_3$  gas. In addition to this, a known ‘carrier’ quantity of  $\text{NH}_4^+$  (14 $\mu\text{g}$ ) was added to the bottles to ensure that if the process was successful, it could be detected. The bottle was kept at 70°C in a drying cupboard for a week. This allows the gas to escape solution and be trapped on the filter by the acid.
- 2)  **$\text{NO}_3^-$  removal:** 200 $\mu\text{g}$  of Devarda’s alloy was added to the solution. This acts to convert dissolved  $\text{NO}_3^-$  to  $\text{NH}_4^+$ . For the same reasons as above, a known concentration of  $\text{NO}_3^-$  (28 $\mu\text{g}$ ) was added to the solution. Converted  $\text{NO}_3^-$  (as  $\text{NH}_4^+$ ) can then be removed as in step 1). **NOTE:**  $\text{NO}_3^-$  was *not* found in the filtrate, but this step is routine. However, the Devarda’s alloy can act to remove a fraction of the DON. As such, the results from this step are combined with the DON step when considering the total quantification of DON.
- 3) **DON removal:** All that remains in solution is DON. This is processed using the WCO method, which as specified above, converts DON to  $\text{NO}_3^-$ . Additional Devarda’s alloy was added to the solution to convert this  $\text{NO}_3^-$  to  $\text{NH}_4^+$ , and then this  $\text{NH}_4^+$  is removed as with the previous two steps.

#### Bacterial contamination verification

As it is relatively impossible to grow these cultures axenically in continuous mode, it was necessary to determine the scale of contamination as a large scale growth of bacteria may affect the above results. To do this, at various points of time throughout the experiment, triplicate samples of culture volume were taken from each culture. These were filtered through 0.2 $\mu\text{m}$  pore size syringe filters and placed in cryotubes. These were immediately fixed with glutaraldehyde, chilled in a 4°C fridge for 15 minutes and then stored in a -80°C freezer until they could be processed. These samples were processed using a flow cytometer.

Calculation of isotopic enrichment of incubation bottle medium

Isotopic enrichment of the incubation bottle medium was calculated using the following formula:

$$A_{N_2} (\%) = \left( \frac{M_{15Ntrac} + A_{N_2_0}(M_{TNB} + M_{TNb})}{M_{15Ntrac} + M_{TNB} + M_{TNb}} \cdot 100 \right) - A_{N_2_0} \quad (\text{Eq. 2.1})$$

Where,  $A_{N_2}$  is the percentage enrichment of  $^{15}\text{N}$  in  $\text{N}_2$  in the medium after adding  $^{15}\text{N}_2$  gas,  $M_{15Ntrac}$  is the number of moles of  $^{15}\text{N}$  within added tracer bubble,  $A_{N_2_0}$  is the natural percentage of  $^{15}\text{N}$  in  $\text{N}_2$  (0.367%),  $M_{TNB}$  is the total number of moles of  $\text{N}$  within the incubation bottle,  $M_{TNb}$  is the total number of moles of  $\text{N}$  within residual bubble. Note, this equation is highly sensitive to the size of the residual bubble in the bottles. As such in experiments, some form of estimate should be made to ensure that the volume of this bubble is properly accounted for. For the purpose of these experiments, this was done by taking a photograph of the bubble and comparing this to reference bubbles of known sizes.

Calculation of  $^{15}\text{N}_2$  fixation rate

The rate of  $\text{N}_2$  fixation derived from  $^{15}\text{N}_2$  incubations was calculated using the method described by Montoya et al. [1996]. This was first done by calculating  $V (T^{-1})$ , or the specific uptake rate of  $\text{N}_2$  by particles in the experimental bottle.  $V$  can be calculated in two ways, as follows:

$$V (T^{-1}) = \left( \frac{1}{\Delta t} \right) \left( \frac{[PON]_{\Delta}}{[PON]_f} \right) \quad (\text{Eq. 2.2})$$

$$V (T^{-1}) = \left( \frac{1}{\Delta t} \right) \left( \frac{A_{PON_f} - A_{PON_0}}{A_{N_2} - A_{PON_0}} \right) \quad (\text{Eq. 2.3})$$

Where  $T$  is a time unit,  $\Delta t$  is the duration of the incubation,  $[PON]_{\Delta}$  is the change in PON concentration over the course of the incubation,  $[PON]_f$  is the PON concentration at the end of the incubation,  $A_{PON_f}$  is the isotopic enrichment of the PON at end of the incubation,  $A_{PON_0}$  is the isotopic enrichment of particulate  $\text{N}$  at start of experiment (0.366%), and  $A_{N_2}$  is the isotopic enrichment of  $\text{N}_2$  in medium after the aliquot of  $^{15}\text{N}_2$  is introduced. The first equation (Eq. 3.2) describes  $V$  using the change in PON concentration over the course of the incubation. The second

equation (Eq 3.3), the more traditionally presented equation, instead describes  $V$  using the enrichment of  $^{15}\text{N}_2$  in PON after incubation and the enrichment of  $^{15}\text{N}_2$  in the medium. The time units for  $T$  and  $\Delta t$  used are days. Note that for the purpose of this experiment, 1 day was the length used for all incubations, even for 12h incubations. This is because *Crocospaera watsonii* primarily fixes N in the dark, and as such, most of the light phase will not see any production of N. *Therefore* results are not normalised per hour as this would cause issues with the presentation of results; similar rates of  $\text{N}_2$  fixation observed over a 12h and 24h incubation would effectively be halved in the 24h incubation as compared with the 12h incubation when normalising by hour.

To further calculate the volumetric rate of  $\text{N}_2$  fixation, or  $\rho$  ( $\text{M} \cdot \text{V}^{-1} \cdot \text{T}^{-1}$ ), the following formula was applied:

$$\rho(\text{M} \cdot \text{Vol}^{-1} \cdot \text{T}^{-1}) = V \overline{[\text{PON}]} \quad (\text{Eq. 2.4})$$

The units for which are *Moles (quantity i.e.  $\mu\text{moles}$ )* • *Volume;  $\text{L}^{-1}$*  • *Time (i.e.  $\text{day}^{-1}$ )*. In this experiment the values are  $\mu\text{moles N L}^{-1} \text{ d}^{-1}$ . It is important to note that moles of **N** fixed is used here and not moles of  $\text{N}_2$  fixed. For the term  $\overline{[\text{PON}]}$ , or the average  $[\text{PON}]$  value over the course of an incubation, two or three values of  $[\text{PON}]$  were used depending on the incubation type. For 12h incubations, the  $[\text{PON}]$  at the start and end of the incubation was used. For 24h incubations, the aforementioned were used in addition to a ‘midpoint’ value, which is the mean averaged triplicate  $[\text{PON}]$  values measured at the end of the 12h dark phase incubations which were started at the same time as the associated 24h incubation.

The cellular rate (i.e.  $\rho$  divided by the cell density; femtomoles  $\text{N cell}^{-1} \text{ d}^{-1}$ ), was calculated using an average cell density over the course of the incubation, in order to adjust for the average  $[\text{PON}]$  used in the  $\rho$  calculation. The cell density values used were from the time point at the start of the incubation and an individual cell count sampled **from the incubation bottle after opening**. This was done in case cell density did not evolve in the same way in the incubation bottles as in the culture proper.

#### Calculation of $^{13}\text{C}$ fixation rate

The calculation of C fixation rate was done identically to the above  $\text{N}_2$  fixation rate, substituting the terms involving PON for equivalent terms involving POC, and using the initial enrichment of  $^{13}\text{C}$  after adding an aliquot of  $\text{NaH}^{13}\text{CO}_3$ .

Calculation of NH<sub>4</sub><sup>+</sup> and DON exudation rate

The calculation of the exudation rate of NH<sub>4</sub><sup>+</sup> and DON was done similarly to the above calculations calculating a specific exudation rate ( $V$ ) of NH<sub>4</sub><sup>+</sup> or DON into the medium and then a volumetric rate ( $\rho$ ):

$$V (T^{-1}) = \left( \frac{1}{\Delta t} \right) \left( \frac{[X]_{\Delta}}{[X]_f} \right) \quad (\text{Eq 2.5})$$

$$V (T^{-1}) = \left( \frac{1}{\Delta t} \right) \left( \frac{A_{X_f} - A_{X_0}}{A_{PON}} \right) \quad (\text{Eq 2.6})$$

$$\rho (M \cdot Vol^{-1} \cdot T^{-1}) = V [\overline{X}] \quad (\text{Eq 2.7})$$

Where the terms are mostly the same as before, except  $X$  which is used as a stand-in for either NH<sub>4</sub><sup>+</sup> or DON. One notable difference is the initial <sup>15</sup>N<sub>2</sub> enrichment factor in Eq 3.5 is not the initial <sup>15</sup>N enrichment of the medium, but instead an average of the enrichment in the PON over time, as any <sup>15</sup>N above the natural abundance would necessarily have to pass through *Crocospaera watsonii* cells first before being exuded. Practically speaking, this was the average value between the natural abundance of <sup>15</sup>N in PON (0.367%) and the final enrichment of PON.

## Chapter Three: Assessment of $\text{NO}_3^-$ Utilisation in *Crocospaera watsonii*

### ABSTRACT

Dissolved inorganic N (DIN), including  $\text{NO}_3^-$ , is often considered a preferable nitrogen (N) source to  $\text{N}_2$  fixation when made available at sufficient concentrations. This assumption was verified, however, using characteristic macroscopic diazotrophs such as *Trichodesmium*. With the phylogenetic world of diazotrophs expanding within the last two decades, it has become important to test these assumptions on diazotrophs newer to science. One such diazotroph is *Crocospaera watsonii*, a free living, unicellular cyanobacteria which has become important in the study of marine diazotrophy due to its ease of culturing. While a few studies have been performed with regards to  $\text{NO}_3^-$  interaction with this diazotroph, results from said experiments show variable interaction of *Crocospaera watsonii* with  $\text{NO}_3^-$ . Cursory results from our laboratory however suggest minimal or no interaction of *Crocospaera watsonii* with  $\text{NO}_3^-$ . To assess this, we performed multiple fed batch culture experiments, in which *Crocospaera watsonii* cultures were grown long term (>10 generations) in  $\text{NO}_3^-$  replete conditions. Metabolic function of cells from these cultures were compared to cultures grown without  $\text{NO}_3^-$ . We tested whether  $\text{N}_2$  fixation would be inhibited in cultures with  $\text{NO}_3^-$ , whether  $\text{NO}_3^-$  was taken up by the cells, and whether cellular growth and metabolism (population growth rate, cell size, carbon (C) fixation, C:N ratios and C:N fixation ratios) are affected by  $\text{NO}_3^-$ . The results of this study revealed that our strain of *Crocospaera watsonii* (WH8501) does not interact considerably with  $\text{NO}_3^-$ . Neither  $\text{N}_2$  fixation nor maximal rates nitrogenase activity were significantly affected by  $\text{NO}_3^-$ . Uptake of  $\text{NO}_3^-$  was minuscule (2-4%) within total acquisition of N and thus was not important for cell growth and median cellular N fixation rates were calculated to be ~10 times that of  $\text{NO}_3^-$  uptake rates. Population growth rate, cell size, C fixation, C:N ratios and C:N fixation ratios were all statistically similar between culture conditions. The results of this study suggest the ability for unicellular diazotrophs to fix  $\text{N}_2$  at significant rates despite the presence of  $\text{NO}_3^-$ . This has important implications for diazotroph ecology and models of global marine  $\text{N}_2$  fixation, as diazotrophs tend not to be placed in models where  $\text{NO}_3^-$  is replete.

## Introduction

One of the key parameters classically considered to constrain the distribution of diazotrophs is the availability, or lack thereof, of DIN. The energetic cost of the uptake of DIN, such as  $\text{NH}_4^+$  and  $\text{NO}_3^-$  has been considered less costly than that of nitrogenase activity to convert  $\text{N}_2$  to  $\text{NH}_4^+$  [Falkowski, 1983; Grosskopf and LaRoche, 2012], so logically one might assume that given the choice, a diazotroph would always choose DIN at whenever available. This has been demonstrated as true for *Trichodesmium* spp. A review by Knapp [2012] summarised from multiple culture and field experiments that inhibition occurred infrequently and incompletely in short term exposures [Ohki et al., 1990; Mulholland et al., 2001; Fu and Bell, 2003; Holl and Montoya, 2005; Sandh et al., 2011]. Inhibition in this case was stronger depending on the species of DIN:  $\text{NH}_4^+$  for example was much more likely to induce inhibition over short time scales, whereas  $\text{NO}_3^-$  was less likely to cause high rates of inhibition ( $\sim >20\%$ ) over these timescales. However, over longer timescales and therefore multiple generations of exposure to DIN, *Trichodesmium* tended to switch to DIN utilisation over  $\text{N}_2$  fixation for the majority of its N acquisition needs [Ohki et al., 1990; Mulholland et al., 1999; Fu and Bell, 2003; Milligan et al., 2007; Knapp et al., 2012]. Given this result, this would necessitate an exclusion of diazotrophs, or at least diazotrophy, from eutrophic regions, such as coastal shelves, where DIN is subject to more consistent replenishment over time.

For DIN limitation, most studies are confined to *Trichodesmium* spp. This is due to a significant research bias surrounding this characteristic diazotroph in the earlier years of diazotrophy research. However, with many new species having been revealed operating in environments not previously associated with diazotrophy, it has become apparent in recent years that the basis for our paradigmatic understanding of diazotrophy could and should be challenged. Parallel studies for other diazotrophs are difficult due to the current inability of culturing the vast majority of different cyanobacteria. However, one unicellular cyanobacteria, *Crocospaera watsonii*, has proven to be easily culturable across multiple strains (WH0003 & WH8501). A small number of DIN limitation experiments have been performed on *C. watsonii*, but show variability in results, with some experiments mirroring, albeit to a seemingly lesser degree, the results of *Trichodesmium* spp. experiments [Knapp et al. 2012; long term studies], and others suggesting the ability to assimilate  $\text{NO}_3^-$  and  $\text{NH}_4^+$  efficiently whilst continuing to fix nitrogen with only a small reduction in  $\text{N}_2$  fixation [Dekaezemacker & Bonnet, 2011; short term exposure] or reduction only contingent on low



light conditions [Garcia and Hutchins, 2014; long term exposure]. It is not, however, clear how important DIN is to *Crocospaera watsonii*, especially for  $\text{NO}_3^-$ . The research touched on above is muddied even further by cursory results from this laboratory (LOV): culture experiments of *Crocospaera watsonii* grown on medium enriched with  $\text{NO}_3^-$  did not appear to uptake significant N through  $\text{NO}_3^-$ , with  $\text{NO}_3^-$  not disappearing significantly from the medium.

Given the uncertainty surrounding this relationship, we propose a multifaceted approach to further interrogate the question of DIN limitation in diazotrophs. For the purpose of this experiment, we will use the aforementioned unicellular cyanobacteria *Crocospaera watsonii* WH8501 and focus on  $\text{NO}_3^-$ , whose relationship with diazotrophs is less clear than  $\text{NH}_4^+$ . In order to elucidate this information systematically, the following functional questions were asked: 1) ‘Does *Crocospaera watsonii* WH8501 utilise  $\text{NO}_3^-$  when cultured in enriched medium?’, 2) ‘Is  $\text{N}_2$  fixation inhibited in  $\text{NO}_3^-$  enriched cultures of *C. watsonii* WH8501?’, and 3) ‘Are there any major differences between *C. watsonii* WH8501 grown in  $\text{NO}_3^-$  enriched medium and obligate diazotrophy in terms of growth dynamics, physiology and C and N budget?’

In order to answer these questions, we performed two sets of fed-batch culture experiments in which *Crocospaera watsonii* WH8501 was subjected to significant concentrations of  $\text{NO}_3^-$  in order to be compared against an obligate diazotrophy control. The first set of cultures was performed using a very high concentration of  $\text{NO}_3^-$  (~600  $\mu\text{moles NO}_3^- \text{ L}^{-1}$ ), a concentration implemented in order to allow complete usage of  $\text{NO}_3^-$  for the culture’s N requirements. The second set of cultures used a reduced concentration of  $\text{NO}_3^-$  (~80  $\mu\text{moles NO}_3^- \text{ L}^{-1}$ ) to allow for more precise  $\text{NO}_3^-$  concentration measurements during the experiment. A number of measurements were performed in order to identify and quantify parameters important to the three ‘functional questions’ posed above, with multiple approaches used where possible in order to interrogate the questions from many angles.  $\text{NO}_3^-$  consumption was measured using both colorimetric analysis and isotopic incubations with  $^{15}\text{NO}_3^-$ .  $\text{N}_2$  fixation was measured using both  $^{15}\text{N}_2$  incubations as well as acetylene reduction assays to measure nitrogenase activity. Finally, in order to test for major differences in *Crocospaera watsonii* cell dynamics, cellular growth rate in cultures, cellular C:N ratio, carbon uptake rate via  $^{13}\text{C}$  incubations, and finally ratio of C and  $\text{N}_2$  fixation were examined. All results were agglomerated and compared to shed more light on this understudied question.

## Methods

### Experimental design:

In order to test the utilisation of  $\text{NO}_3^-$  by *Crocospaera watsonii* WH8501, two fed-batch culture experiments were performed. In both, two cultures were grown on YBCII medium [Chen et al., 1996] in triplicate, one with a large  $\text{NO}_3^-$  concentration, the other kept under obligate diazotrophy as a control condition. Medium was prepared from aged Mediterranean Sea surface water, with an initial salinity of  $\sim 38$ . The first culture was operated with the consideration that cultures may utilise significant concentrations of  $\text{NO}_3^-$ . As such, a very large concentration of  $\text{NaNO}_3$  was added ( $\sim 600 \mu\text{moles L}^{-1}$ ), a concentration equivalent to F/4 medium [Guillard and Ryther 1962; Guillard 1975], to support a large scale growth of *Crocospaera watsonii* cells. In the second experiment, the  $\text{NO}_3^-$  enriched culture was spiked with a much lower concentration of  $\text{NaNO}_3$  ( $\sim 80 \mu\text{moles L}^{-1}$ ). To clarify,  $\text{NO}_3^-$  concentrations were chosen for either experiment to first test for any potentially inhibiting concentration (first experiment) and second, to get a better signal to noise ratio when measuring  $\text{NO}_3^-$  concentrations to derive bulk uptake rates (second experiment).

The specific strain chosen was a non-axenic strain of *Crocospaera watsonii* WH8501. Cultures were housed in 2L borosilicate erlenmeyer flasks sealed with a microporous sponge stopper to allow air pressure equilibration without contamination to cultures. Agitation was provided through the use of magnetic stirrers and daily manual stirring of the flasks. Constant air bubbling was maintained through a canule topped with a  $0.2 \mu\text{m}$  hydrophobic air filter, which served to aerate the cultures. Erlenmeyers were kept in an incubator set to maintain the cultures at a constant temperature of  $27^\circ\text{C}$  and 12:12 L:D light conditions with roughly  $200 \mu\text{Einsteins m}^{-2} \text{ s}^{-1}$  (Oxram fluorescent tubes, white light) during the light phase. Cultures were distributed within the incubator in a way to prevent shading effects from one culture to another. When cultures approached a limiting density, they were diluted with YBCII medium before the stationary phase was reached, to maintain cells in an exponential state of growth, and  $\text{NO}_3^-$  cultures were re-spiked with  $\text{NO}_3^-$  to attempt maintain its concentration within the medium.

## Sampling methodology:

During the culture growth stage, cell counts were taken daily at the end of the light phase to monitor culture growth, using methodology described in the methodology chapter. For  $\text{NO}_3^-$  enriched cultures, samples were taken daily also, using methodology described below. Once cultures had reached the exponential phase of their growth, determined by daily analysis of cell counts and growth rates, analyses into the various nitrogen fluxes within the cultures were performed. For the first experiment, a daily sampling was performed on one of the cultures. Cultures chosen for daily nitrogen flux analyses were selected based on their growth rate, with higher concentration cultures chosen first to ensure those were sampled before they reached saturation. Analyses performed during this phase were as follows: assessment of nitrogenase enzyme activity (via ARA),  $^{15}\text{N}_2$  and  $^{13}\text{C}$  combined incubations,  $^{15}\text{NO}_3^-$  and  $^{13}\text{C}$  combined incubations and elemental (CHN) analyses. Incubations were specifically performed for 24h each. Methodology for which can be found in the methodology chapter, aside for  $^{15}\text{NO}_3^-$  incubations, which are unique to this chapter and described below. Specifics for all  $^{15}\text{N}/^{13}\text{C}$  incubations can be found below in Table 3.1.

**Table 2.1:** Table outlining the various particulars for the  $^{15}\text{N}/^{13}\text{C}$  incubations performed on the two experiments

	<b>Experiment 1</b>		<b>Experiment 2</b>	
	$^{15}\text{NO}_3^-$ Incubations	$^{15}\text{N}_2$ Incubations	$^{15}\text{NO}_3^-$ Incubations	$^{15}\text{N}_2$ Incubations
<b>Performed On</b>	$\text{NO}_3^-$ enriched cultures only	Both $\text{NO}_3^-$ enriched and control cultures	$\text{NO}_3^-$ enriched cultures only	Both $\text{NO}_3^-$ enriched and control cultures
<b>Incubation Length</b>			24h	
<b># of Replicates</b>		2		3
<b>Incubation Volume (ml)</b>		~170		~20
<b>Incubation Bottle</b>	125ml Nalgene Bottles (+Septum Cap)		20ml Glass Bottles (+Septum Cap)	
$^{15}\text{N}_2$ Tracer Volume (ml)	0.5	0.5	0.1	0.1
$[\text{Na}^{15}\text{NO}_3]$ ( $\mu\text{mol L}^{-1}$ ) *	NA	~2.9	NA	~2.9
$[\text{NaH}^{13}\text{CO}_3]$ ( $\mu\text{mol L}^{-1}$ ) *			~260	
<b>Filtrate collection</b>		Yes		No

\* Concentration after spiking

### *NO<sub>3</sub><sup>-</sup> concentration:*

For the NO<sub>3</sub><sup>-</sup> enriched cultures, in order to measure the expected consumption of NO<sub>3</sub><sup>-</sup>, cultures were sampled regularly and collected in centrifugation tubes. Samples were collected at the same time as cell counts. These samples were filtered through 0.2µm syringe filters and then stored in a freezer until analysis. Samples were analysed using a Technicon Auto-Analyser.

### *<sup>15</sup>NO<sub>3</sub><sup>-</sup> and <sup>13</sup>C tracer additions*

The above incubations were performed in almost the same way, but instead of <sup>15</sup>N<sub>2</sub> tracer gas, incubation bottles were spiked with Na<sup>15</sup>NO<sub>3</sub>, to a final concentration of 2.9 µmoles <sup>15</sup>NO<sub>3</sub><sup>-</sup>. Post incubation processing was also identical to the above.

### *Adsorption tests*

As a later consideration, we aimed to test the effect of adsorption on the <sup>15</sup>N enrichment of PON post <sup>15</sup>NO<sub>3</sub><sup>-</sup> incubation. We performed batch cultures identical to the second experiment. Once at exponential growth rate we performed two sets of triplicate <sup>15</sup>NO<sub>3</sub><sup>-</sup> incubations per culture per day for two days. Once filtered, filters from one triplicate per culture were rinsed with 200ml of filtered, autoclaved seawater (as above for medium preparation), and processed as normal. We then noted the difference in <sup>15</sup>N enrichment in the PON before and after rinsing.

### Analytical methodology:

Analytical methodology for the calculation of initial <sup>15</sup>N<sub>2</sub> enrichment in the medium ( $A_{N_2}$ ; Eq. 2.1), specific uptake rate of N and C into the PON and POC via isotopic enrichment ( $V_E$ ; Eq. 2.2) and finally the volumetric rates ( $\rho$ ) for all 'V's above (Eq. 2.4), can all be found in the methods chapter of this thesis. Initial enrichment of NO<sub>3</sub><sup>-</sup> ( $A_{NO_3}$ ) was ~0.5% for the first experiment, and was used to calculate  $V_{NO_3}$  and  $\rho^{15}NO_3$  in the same way as N<sub>2</sub>. Note, in some cases an average PON or POC was not available. In this case, for calculation of  $\rho$ , the final [PON] or [POC] was used instead of the average [PON] or [POC] over the time step. Analytical methodology for cellular growth rate can be found below.

### *Cellular growth rate:*

Cellular growth rate ( $\mu$ ) was estimated by fitting linear models to log transformed data for each culture, for each growth phase. This was done during the exponential phase of each experimental phase, for each replicate culture, and in both the obligate diazotrophy and the  $\text{NO}_3^-$  condition cultures. As experiment one had three experimental phases,  $\mu$  was averaged for each experimental condition ( $\text{NO}_3^-$  and obligate diazotrophy).

**A note on data treatment for  $\rho\text{N}_2$  and  $\rho\text{NO}_3^-$ :** accuracy tests for  $^{15}\text{N}_2$  incubations performed similarly to the current experiment, albeit after and in continuous rather than fed-batch cultures, demonstrated a potential underestimation in values. These tests are outlined in chapter 4. Within said tests, we note a relatively consistent linear relationship with  $V_{\text{N}_2}$  and measurement accuracy based on bulk values ( $R^2 = 0.79$ ), with increasing accuracy as  $V_{\text{N}_2}$  increased. Using the equation derived by said regression, we tentatively correct the  $\rho\text{N}_2$  values from the present experiment, with correction factors (division) ranging between  $\sim 0.05$  to  $0.30$ , albeit with a majority of correction factors being between  $0.05$  and  $0.15$ .

We note that this correction strategy may bring a degree of doubt on the values presented here. However, there is clear underestimation of values without correction; it is more likely that false conclusions would be drawn from underestimated values.

### Statistical tests:

Tests of statistical difference between  $\rho\text{N}_2$  for  $\text{NO}_3^-$  enriched and obligate diazotrophy cultures were performed using a Welch two sample T-test. Distribution of  $\rho\text{C}$  and  $\rho\text{NO}_3^-$  was found to be non-parametric with positive skew. However, the positive skew was the result of a few samples and thus for test of statistical difference between  $\rho\text{C}$  for  $\text{NO}_3^-$  enriched and obligate diazotrophy, and between  $\rho\text{N}_2$  and  $\rho\text{NO}_3^-$  we again used a Welch two sample T-test.

## Results

### *Cell counts, Cell size and Growth rates*

There was no significant difference in the average cell diameter between the two conditions in both experiments. In both experiments, there was no significant differences in exponential growth rates between NO<sub>3</sub><sup>-</sup> enriched cultures and associated obligate diazotrophy culture controls. The average fitted growth rate and standard errors over the exponential phase were  $0.25 \pm 0.03 \text{ d}^{-1}$  and  $0.29 \pm 0.02 \text{ d}^{-1}$  for the NO<sub>3</sub><sup>-</sup> enriched cultures (experiment 1 and 2 respectively), and  $0.27 \pm 0.02 \text{ d}^{-1}$  and  $0.26 \pm 0.2 \text{ d}^{-1}$  for the obligate diazotrophy cultures.

### *NO<sub>3</sub><sup>-</sup> concentration over time*

High signal to noise ratio in the NO<sub>3</sub><sup>-</sup> measurements from the first experiment meant that determining uptake was difficult. Despite this, it was clear that cultures of *Crocospaera watsonii* did not use NO<sub>3</sub><sup>-</sup> exclusively, but variations in NO<sub>3</sub><sup>-</sup> concentration could indicate that a small uptake was present. The highest disappearance rate of any one sampling period was 14% d<sup>-1</sup>. However, when averaging out these values over the course of the whole culture, the change in NO<sub>3</sub><sup>-</sup> concentration was <0.1% d<sup>-1</sup>. Despite this, for all cultures, NO<sub>3</sub><sup>-</sup> concentration at the end of the culture was never less than concentration at the start. The second experiment was designed in order to answer more definitively to what order *Crocospaera watsonii* cultures used NO<sub>3</sub><sup>-</sup> to supply their N demand. In this experiment, total average NO<sub>3</sub><sup>-</sup> consumption was  $13.7 \pm 1.2 \text{ } \mu\text{moles L}^{-1}$  over a 15 day period (Fig 3.1). During this period, there was a total growth of  $\sim 1.5\text{E}+09 \text{ cells L}^{-1}$ , constituting an estimated production of  $\sim 840 \mu\text{moles N L}^{-1}$ . This indicates that NO<sub>3</sub><sup>-</sup> uptake constituted <2% of total N incorporation in the cellular biomass.

### *PON and POC*

The average per cell content for PON was  $55.8 \pm 11.70$  and  $52.70 \pm 12.05$  femtomoles N cell<sup>-1</sup> for the NO<sub>3</sub><sup>-</sup> and obligate diazotrophy experiments respectively. Average per cell C content  $371.67 \pm 97.53$  and  $374 \pm 76$  femtomoles C cell<sup>-1</sup> for the NO<sub>3</sub><sup>-</sup> and obligate diazotrophy experiments respectively. The average values of cellular PON and POC between both NO<sub>3</sub><sup>-</sup> enriched and obligate diazotrophy conditions were not significantly different. The majority of associated POC:PON values were between 6.3 and 7.7, mean values of  $6.8 \pm 0.9$  and  $7.2 \pm 0.9$ .

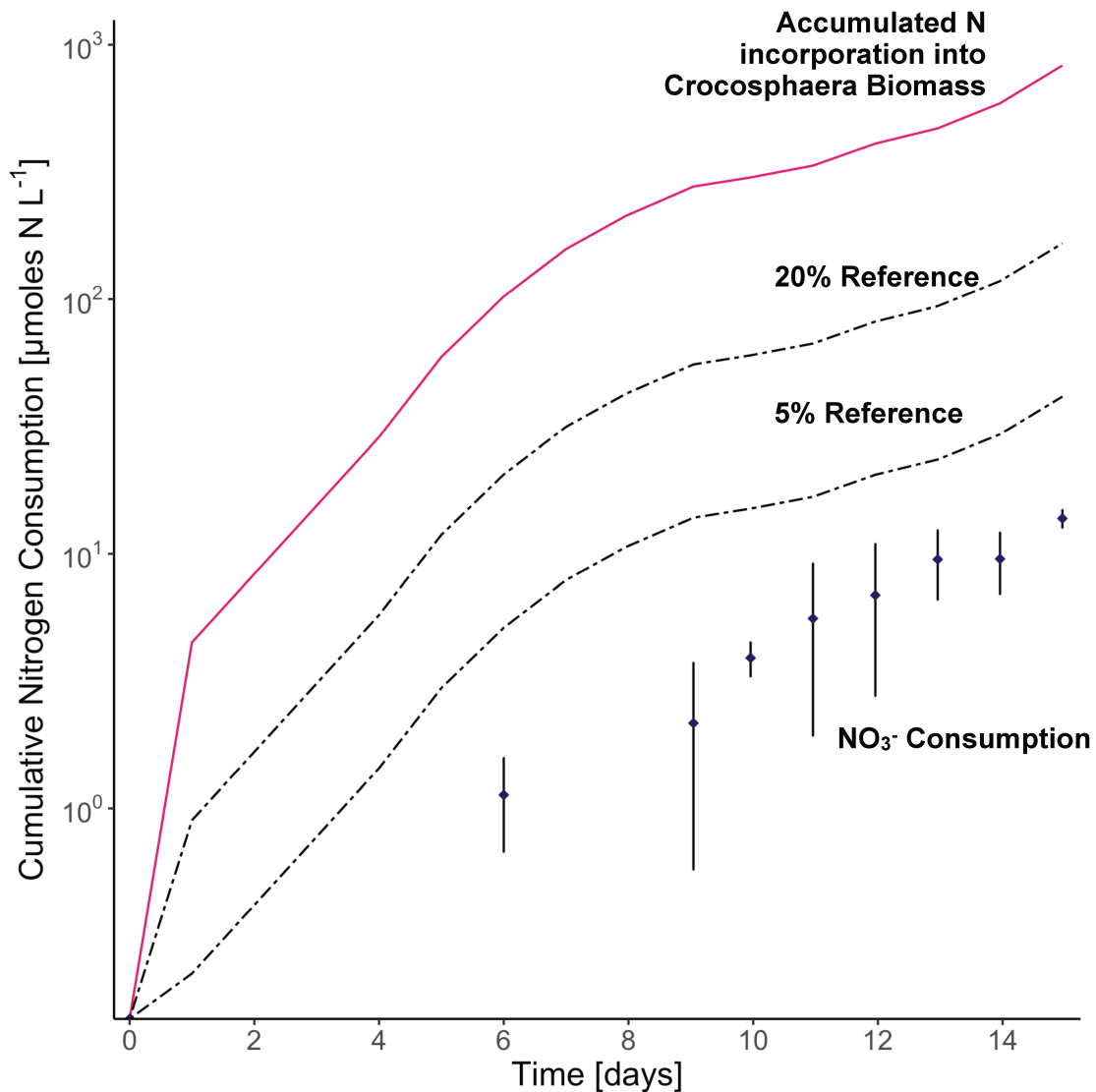


Figure 3.1: Cumulative  $\text{NO}_3^-$  consumption rate (disappearance from medium; blue points) over the course of the second experiment. The pink (solid) line is accumulated total N incorporation calculated from cell growth. The two dot-dash lines represent 20% and 5% of the total consumption rate to allow for comparison with cumulative  $\text{NO}_3^-$  consumption. All values are log transformed to allow for better comparison.

### *<sup>15</sup>N<sub>2</sub> Incubations*

Cellular N incorporation derived from  $^{15}\text{N}_2$  incubations ( $\rho\text{N}_2$ ) from both experimental conditions had similar interquartile ranges, minima and maxima, albeit with a somewhat greater median in the obligate diazotrophy (Fig. 3.2; 11.84 and 9.95 femtomoles N cell<sup>-1</sup> d<sup>-1</sup>) and again for the mean ( $10.84 \pm 3.73$  and  $9.84 \pm 4.61$  femtomoles N cell<sup>-1</sup> d<sup>-1</sup>). A Welch two sample T-test between the two conditions showed no significant difference in  $\rho\text{N}_2$  values between conditions ( $p=0.54$ ).

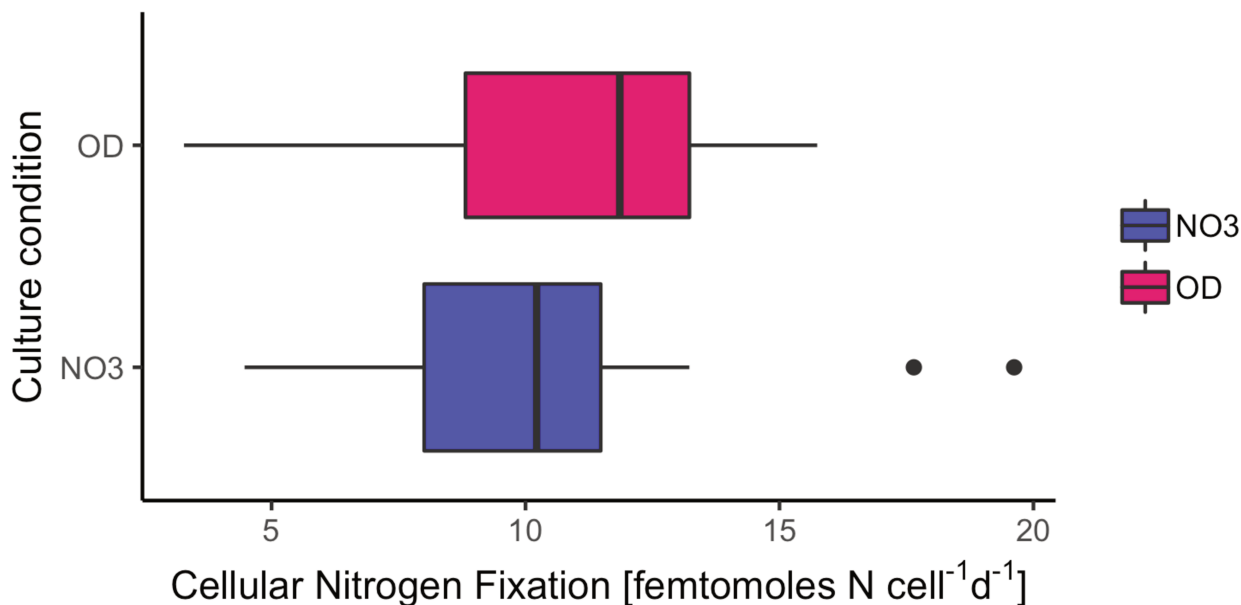


Figure 3.2: Box and whisker plots of  $N_2$  fixation rates from  $^{15}N_2$  incubations. The pink box is rates from the obligate diazotrophy (OD) condition ( $NO_3^-$  free), and the blue box is rates from the  $NO_3^-$  enriched condition. Points are statistical outliers.

### *$^{15}NO_3^-$ Incubations*

The overall range of cellular  $NO_3^-$  incorporation rates estimated from  $^{15}NO_3^-$  incubations ( $\rho NO_3^-$ ) was between 0.59 and 5.30 femtomoles N cell<sup>-1</sup> d<sup>-1</sup>. Data were however, positively skewed, with an interquartile range between 1.03 and 3.33 but a median of 1.16 femtomoles N cell<sup>-1</sup> d<sup>-1</sup>. Comparing medians,  $\rho NO_3^-$  appeared to be ~10 times smaller than  $\rho N_2$  (Fig. 3.3). Despite the overlapping range of values, a Welch two sample T-test between  $\rho NO_3^-$  and  $\rho N_2$  suggests that the latter is indeed significantly larger than the former ( $p < 0.05$ ).

### *$^{13}C$ Incubations*

We note a slightly larger mean and median  $\rho C$  in  $NO_3^-$  enriched cultures compared with the obligate diazotrophy culture (Fig. 3.4) which was not significantly different when compared via T test ( $p = 0.17$ ). Mean and standard deviation of the  $\rho C:\rho N_2$  ratio between the  $NO_3^-$  enriched ( $5.57 \pm 1.72$ ) and obligate diazotrophy cultures ( $5.29 \pm 1.89$ ) were practically identical.



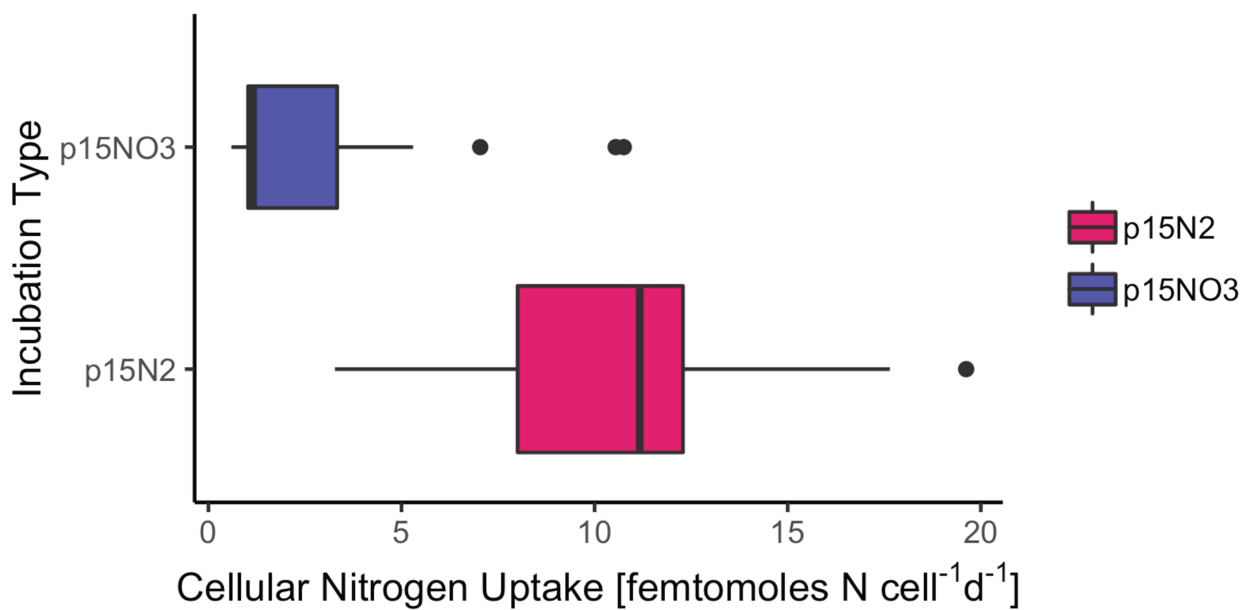


Figure 3.3: Box and whisker plots of nitrogen uptake rates from <sup>15</sup>N<sub>2</sub> (ρN<sub>2</sub>) and <sup>15</sup>NO<sub>3</sub> incubations (ρNO<sub>3</sub><sup>-</sup>). The pink box is ρN<sub>2</sub>, and the blue box is ρNO<sub>3</sub><sup>-</sup>. Points are statistical outliers.

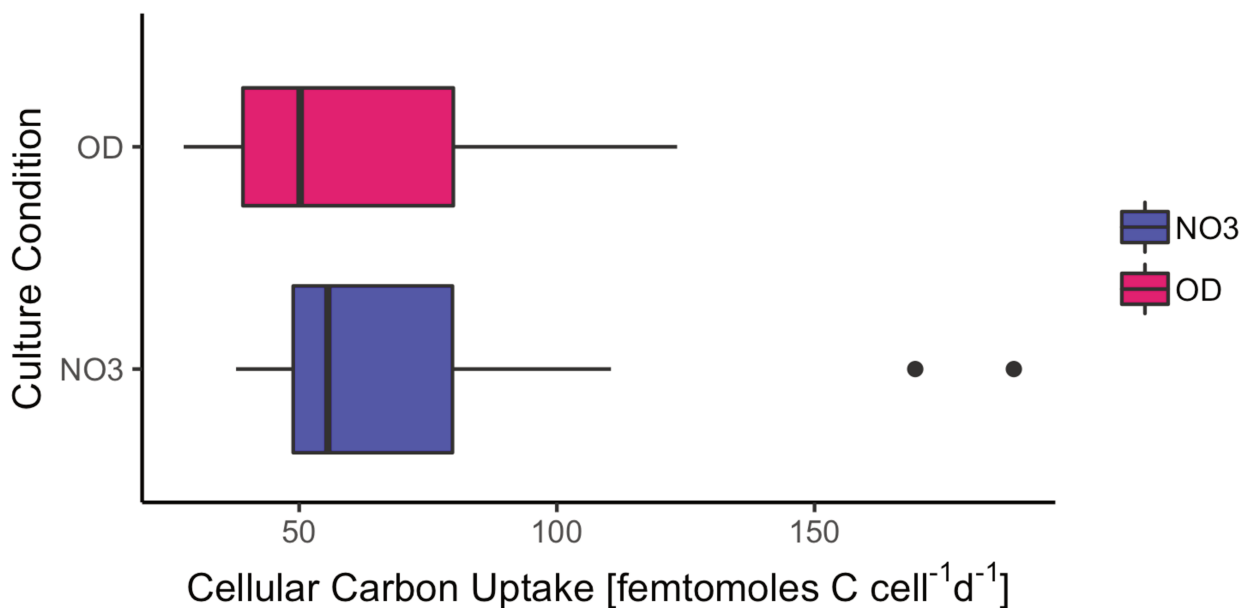


Figure 3.4: Box and whisker plots of C fixation rates from <sup>13</sup>C incubations. The pink box is rates from the obligate diazotrophy (OD) condition (NO<sub>3</sub><sup>-</sup> free), and the blue box is rates from the NO<sub>3</sub><sup>-</sup> enriched condition. Points are statistical outliers.

### *Nitrogenase Activity (ARA)*

Nitrogenase activity was approximated using the ARA. We report here the maximal average and standard deviations of ethylene (C<sub>2</sub>H<sub>2</sub>) production per curve. Cellular maximal gross ethylene production was  $16.09 \pm 7.6$  femtomoles C<sub>2</sub>H<sub>2</sub> cell<sup>-1</sup> h<sup>-1</sup> for the NO<sub>3</sub><sup>-</sup> enriched cultures, and  $17.16 \pm 7.3$  femtomoles C<sub>2</sub>H<sub>2</sub> cell<sup>-1</sup> h<sup>-1</sup> for the obligate diazotrophy cultures. We note that these two values are very similar. We also note that when a conversion factor of 4 is used over the entire curve, that the ARA method estimates impossibly large gross N<sub>2</sub> fixation rates; we therefore limit our interpretation of these values to comparing the nitrogenase activity of these two culture conditions.

### *Adsorption tests*

The results of the adsorption test suggest that post rinsing, <sup>15</sup>N enrichment of particulate N on filters was reduced by an average factor of  $1.80 \pm 0.35$ .

## *Discussion*

Classically, understanding of the occurrence and rate of N<sub>2</sub> fixation has been predicated upon the idea that, in the presence of a more labile nitrogen source such as NO<sub>3</sub><sup>-</sup>, a diazotroph would have no or less reason to fix N<sub>2</sub>. This understanding has driven the study of N<sub>2</sub> fixation significantly, with global models of N<sub>2</sub> fixation often being centred around areas with low NO<sub>3</sub><sup>-</sup>. There is, however, a growing literature which challenges this understanding [summary by Knapp, 2012]. Coupled with the presumed erroneous phenomenon that the global marine fixed N budget is in decline [Codispoti, 2007] due to losses of marine nitrogen (denitrification, anammox) outstripping inputs (N<sub>2</sub> fixation), the need to study how NO<sub>3</sub><sup>-</sup> may affect N<sub>2</sub> fixation is clear. Here we present an attempt to tackle this problem from a variety of different angles, using an important diazotroph, *Crocospaera watsonii*, in order to improve our understanding of how diazotrophs and N<sub>2</sub> fixation are, or are not, affected by NO<sub>3</sub><sup>-</sup>.

*'Does C. watsonii use NO<sub>3</sub><sup>-</sup> when cultured in enriched medium?'*

The primary functional question presented at the onset of this experiment was a simple query as to whether or not *Crocospaera watsonii* uses NO<sub>3</sub><sup>-</sup> when it is present in its medium. Inhibition of N<sub>2</sub> fixation by DIN is predicated upon the idea that DIN is less energetically costly than N<sub>2</sub> fixation, and therefore is preferred as an N acquisition strategy. It logically follows then, that if DIN replaces N<sub>2</sub>, it must also be consumed from the media in which the diazotroph is grown. Examples from parallel DIN inhibition experiments with *Crocospaera watsonii* in the literature are varied as to the extent of this. In the study by Dekaezemacker and Bonnet [2011], NO<sub>3</sub><sup>-</sup> incorporation was observed in very short term exposures, with consumption by strain WH8501 going to completion for NO<sub>3</sub><sup>-</sup> concentrations <5 μmoles L<sup>-1</sup>. Interestingly, however, at their highest concentration of 10 μmoles L<sup>-1</sup>, there was a reduced rate of consumption, and there was NO<sub>3</sub><sup>-</sup> still in the medium after the experimental period (one night). This is in contrast to their parallel observations of strain WH0003, which consumed all NO<sub>3</sub><sup>-</sup> during the experimental period. The result of this experiment appeared to indicate a notable difference between the metabolic functions of these two strains, as there was also an increased inhibition of N<sub>2</sub> fixation observed by NH<sub>4</sub><sup>+</sup> in the WH0003 strain, as compared with the WH8501. In the study by Garcia and Hutchins [2014], *Crocospaera watsonii* WH0003 was exposed to relatively high concentrations of NO<sub>3</sub><sup>-</sup> (~30 μmoles L<sup>-1</sup>) over a longer period of time. The consumption of NO<sub>3</sub><sup>-</sup>, measured by following diminishing NO<sub>3</sub><sup>-</sup> concentrations, was indeed significant although complete consumption was not observed, potentially due to the relatively low culture concentration used in the Garcia and Hutchins [2014] study.

Cursory observations of *Crocospaera watsonii* WH8501 cultures conducted before the current experiment indicated that the consumption of NO<sub>3</sub><sup>-</sup> may indeed be minimal. Consequently, the current experiment was designed with the initial assumption that NO<sub>3</sub><sup>-</sup> consumption would be negligible. However, a very high concentration of NO<sub>3</sub><sup>-</sup> was chosen in order to satisfy the N requirements of a maintained; fed-batch culture of *Crocospaera watsonii* in the event that cells would indeed switch to an obligate NO<sub>3</sub><sup>-</sup> consumption scenario. To select this concentration, an average cellular N concentration (between 25 and 75 femtomoles N cell<sup>-1</sup>) was selected [Dron et al., 2013] and combined with a sensible growth rate to generate a growth curve capable of estimating the N requirements of a culture lasting ~3 weeks. The resultant figure led us to use a NO<sub>3</sub><sup>-</sup> concentration in the first experiment of ~600 μmoles L<sup>-1</sup>. Once the data was available, this concept was used with an actual observed growth of cells using the actual cell count data. Thus, the

estimated N requirement for the cultures was plotted against the observed temporal evolution of the  $\text{NO}_3^-$  concentration within the cultures. This allowed us to reject an obligate  $\text{NO}_3^-$  consumption scenario:  $\text{NO}_3^-$  consumption did not match overall N requirement for growth, and therefore it can be confirmed that when *Crocospaera watsonii* is grown in a  $\text{NO}_3^-$  medium it does not switch its N acquisition strategy to exclusive  $\text{NO}_3^-$  consumption. This confirms previous studies [Dekaezemacker and Bonnet, 2011; Knapp et al. 2012, Garcia and Hutchins 2014] which also showed that  $\text{NO}_3^-$  did not become the sole, exclusive source of N to *Crocospaera watsonii*. Those studies however showed *some* level of  $\text{NO}_3^-$  utilisation, raising the question as to whether or not any consumption was supported by our data.

Due to the high signal to noise ratio in the  $\text{NO}_3^-$  uptake measurements, it was difficult to discern the rate of  $\text{NO}_3^-$  consumption confidently in this experiment. This led to the second experiment, which was conducted with a lower concentration of  $\text{NO}_3^-$  ( $\sim 80 \mu\text{M}$ ). This was a design choice in order to more accurately measure  $\text{NO}_3^-$  concentrations. In this experiment there was an observed total average consumption of  $13.7 \pm 1.2 \mu\text{moles L}^{-1}$  over a 15 day period. During this period, an estimated cumulative  $\sim 840 \mu\text{moles N L}^{-1}$  was incorporated into the biomass. This indicates that consumption of  $\text{NO}_3^-$  again contributed  $<2\%$  of the total biomass over the course of this experiment (Fig 3.1). It must be noted that, in comparison to most parallel studies of  $\text{NO}_3^-$  inhibition in diazotrophs, the concentration of  $\text{NO}_3^-$  used during these two experiments can be considered high to extreme. Given the total repleteness of  $\text{NO}_3^-$ , and the general idea that  $\text{NO}_3^-$  should be a more energetically efficient N substrate than  $\text{N}_2$  for diazotrophs, the fact that hardly any  $\text{NO}_3^-$  was consumed over many cell generation times experiment demonstrates that nitrate is potentially not an important substrate used for growth in *Crocospaera watsonii* WH8501.

In addition to tracking the  $\text{NO}_3^-$  concentration,  $^{15}\text{NO}_3^-$  incubations were employed to assess the consumption of  $\text{NO}_3^-$ . The strength of this analysis over simply tracking  $\text{NO}_3^-$  concentrations is that it allows for the confirmation of incorporation of N from  $\text{NO}_3^-$  in the cell, using  $^{15}\text{N}$  as a tracer. With these analyses, we were able to observe a nominal uptake of  $\text{NO}_3^-$  into *Crocospaera watsonii* cells, as  $^{15}\text{N}$  was indeed found in filtered PON samples following  $^{15}\text{NO}_3^-$  incubation. Interestingly, rates were very similar from incubation to incubation, mostly within the region of 0.5 - 1.5 femtomoles N  $\text{cell}^{-1} \text{d}^{-1}$ , which contrasted parallel observations of incorporation of  $\text{N}_2$  which saw a far larger range of uptake. While the range in uptake of  $\text{N}_2$  is likely due to the large variability in growth from day to day, as is uncontrollable in batch conditions,  $\text{NO}_3^-$  incorporation rates were somewhat more

stable, albeit ~10 times less on average. There were, however a few days with significantly larger incorporation rates, but these were rare. We note that when particulate samples were rinsed using sterilised, filtered seawater, enrichment of  $^{15}\text{N}$  in the PON decreased by a factor of ~1.5 - 2.0, suggesting significant adsorption of  $\text{NO}_3^-$  onto the filters or filtered biomass. This would notably reduce the  $\text{NO}_3^-$  uptake measured using this method.

If it can be assumed that the average, minimal N cost to build a new cell is equal to the average cellular N content, then based on the results of the present experiments, cellular demand for growing a new cell was roughly 56 femtomoles N cell<sup>-1</sup>. Given the observed daily growth rate of ~0.25 - 0.30 d<sup>-1</sup> in the present cultures, the daily N demand for each cell was ~14.25 - 17.10 femtomoles N cell<sup>-1</sup>. This suggests that, based on the median rate of incorporation by  $^{15}\text{NO}_3^-$  incubations in the first experiment (1.18 femtomoles N cell<sup>-1</sup> d<sup>-1</sup>),  $\text{NO}_3^-$  accounted for 6 to 8% of N demand in  $\text{NO}_3^-$  enriched cultures, which is within the same order of magnitude as the observations of  $\text{NO}_3^-$  consumption in the second experiment, albeit higher by a factor of 3-4. Our findings directly contrast findings in the WH0003 strain by Garcia and Hutchins [2014], which indicated  $\text{NO}_3^-$  contributed greater than 40% of the total N to biomass. This disparity potentially suggests that the two strains are metabolically quite different. Their very distinct appearance, with WH0003 producing much more exopolymeric substances rendering it more sticky, was already a first hint towards differences in their carbon metabolism. The present study points to very distinct strategies as for the N metabolism as well.

### *Is Nitrogen fixation inhibited in $\text{NO}_3^-$ enriched cultures of *C. watsonii*?*

The second functional question asked was whether or not the rate of  $\text{N}_2$  fixation was inhibited in *Crocospaera watsonii* cultures enriched with  $\text{NO}_3^-$ . The parallel studies of DIN inhibition of  $\text{N}_2$  fixation discussed in the previous section [Dekaezemaker and Bonnet, 2011; Knapp et al. 2012; Garcia and Hutchins, 2014] are divided in terms of the extent of  $\text{N}_2$  fixation inhibition by  $\text{NO}_3^-$  in *Crocospaera watsonii*. The short term exposure experiment by Dekaezemaker and Bonnet [2011] indicated a 5 - 25% inhibition depending on the strain and the concentration of  $\text{NO}_3^-$ , with higher concentrations yielding lower inhibitions. The long term exposure experiment by Knapp et al. [2012] suggested significant, but not complete inhibition of  $\text{N}_2$  fixation in the WH8501 strain, ranging from 71 - 85% inhibition, again depending on the concentration, this time with higher concentrations of  $\text{NO}_3^-$  yielding higher inhibitions. This study also put forward evidence to suggest

that higher  $\text{PO}_4^{3-}$  concentrations may act to alleviate inhibition. The study by Garcia and Hutchins [2014] on the WH0003 strain suggested that  $\text{NO}_3^-$  was only inhibitive at low light conditions. The results of this study suggested at sufficient light intensities, and therefore with higher provision of energy to the cultures,  $\text{N}_2$  fixation was not inhibited. Instead, they suggest that growth was enhanced by the presence of  $\text{NO}_3^-$ , suggesting that N was not sufficient by itself to support higher growth. The disparity between these three studies is notable, suggesting that there are elements unaccounted for in previous studies. A great need for further elucidation on this subject.

In order to assess this disparity, we measured  $\text{N}_2$  fixation in cultures both exposed to  $\text{NO}_3^-$  and cultures under obligate diazotrophy. We used both ARA and the  $^{15}\text{N}_2$  incubation methods to provide complimentary evidence, the former to compare if there was a significant difference in nitrogenase activity and the latter to measure quantitative uptake of N in the biomass.

Arguably, of the discussed parallel studies, the study by Knapp et al. [2012] is most directly comparable to this study as it is both long term and uses the same strain. In their study, they reported a greater than 70% inhibition of  $\text{N}_2$  fixation in batch cultures replete with  $\text{NO}_3^-$ , this condition being sustained for over 10 generations. Cultures in the current study were exposed to  $\text{NO}_3^-$  over the course of ~6 weeks (10+ generations) suggesting that similar rates of inhibition should occur. In the present study, however, we found similar ranges in per cell  $\text{N}_2$  fixation between both conditions, and there was no significant difference in  $\text{N}_2$  fixation between cultures enriched with  $\text{NO}_3^-$  and cultures under obligate diazotrophy. We also found highly similar average rates of nitrogenase activity between experimental conditions. It therefore cannot be said that there was an inhibitory effect from  $\text{NO}_3^-$  on  $\text{N}_2$  fixation within the current experiments, as *Crocospaera watsonii* not only fixed  $\text{N}_2$  in the presence of high quantities of  $\text{NO}_3^-$ , but did so without *any* significant or obvious inhibition.

The disparity between these results and the results of the Knapp et al. [2012] study is potentially due to the difference in irradiance: our experiment used  $200 \mu\text{Einstein m}^{-2} \text{ s}^{-1}$  whereas the Knapp used  $80 \mu\text{Einstein m}^{-2} \text{ s}^{-1}$ . The study by Garcia and Hutchins [2014] described an inhibition of  $\text{N}_2$  fixation by DIN only when cultures were subjected to low irradiance, suggesting that  $\text{NO}_3^-$  may only inhibit  $\text{N}_2$  fixation when irradiance and growth rate are lower. However, their experiment used the WH0003 strain. It is uncertain as to whether or not the WH8501 would behave in the same way.

## *Do cultures of C. watsonii differ in growth rate or physiology when grown on NO<sub>3</sub><sup>-</sup>?*

The third functional question asked was whether or not NO<sub>3</sub><sup>-</sup> repletion would have an effect on the growth dynamics or physiology of *Crocospaera watsonii* cultures. With regards to the effect of consumption of NO<sub>3</sub><sup>-</sup> on the growth dynamics of *C. watsonii*, it is not unreasonable to consider that the addition of NO<sub>3</sub><sup>-</sup> may increase the growth rate as compared with in obligate diazotrophy scenarios, given the lower energetic cost of consuming NO<sub>3</sub><sup>-</sup> over fixing N<sub>2</sub>. Indeed, when provided with NO<sub>3</sub><sup>-</sup>, *Crocospaera watsonii* WH0003 demonstrated an increased growth rate [Garcia and Hutchins, 2014], attributed to an inability acquire sufficient N using N<sub>2</sub> fixation alone. Furthermore, increasing concentrations of NO<sub>3</sub><sup>-</sup> did demonstrate an increased growth rate in cultures of *Crocospaera watsonii* WH8501 [Knapp et al., 2012], although this was due to alleviation of P limitation accompanying this condition.

In contrast to this, growth rate during the exponential period during all phases of this experiment, including both the first and second experiments, were not significantly different. In fact, there was a consistent growth rate of 0.25 - 0.30 d<sup>-1</sup> across all phases, suggesting good replicability across all experiments. We note that this challenges previous results which aim to suggest that differing NO<sub>3</sub><sup>-</sup> concentrations may in some-way produce differing growth rates. Despite a large disparity in NO<sub>3</sub><sup>-</sup> concentration between the first and second experiments we found *no* difference in average growth rate. This is Furthermore, we observed consistent cellular C and N content at the end of the light phase between conditions, consistent cellular C:N ratios, similar C fixation rates and identical ρC:ρN<sub>2</sub> ratios. The lack of difference between the NO<sub>3</sub><sup>-</sup> enriched and obligate diazotrophy conditions suggest little interaction of NO<sub>3</sub><sup>-</sup> with the growth of the *Crocospaera watsonii* WH8501 cultures in our experiments. The lack of interaction was not unsurprising: with no apparent inhibition of N<sub>2</sub> fixation [Fig. 3.2] plus very little uptake of NO<sub>3</sub><sup>-</sup> [Fig. 3.1 & 3.3], it is clear that during this experiment, NO<sub>3</sub><sup>-</sup> went mostly ignored.

## Conclusions

The overall lack of interaction between these cultures of *Crocospaera watsonii* WH8501 and NO<sub>3</sub><sup>-</sup> is an unprecedented result in this field. While results in the literature vary, they all demonstrate some interaction of *Crocospaera watsonii* with the NO<sub>3</sub><sup>-</sup> in the medium. Knapp et al. [2012]

demonstrates significant inhibition of  $N_2$  fixation in the WH8501 strain when exposed long term with ‘environmentally relevant’ concentrations of  $NO_3^-$ ; the results of our experiments show no inhibition, even when exposed long term to far higher concentrations of  $NO_3^-$ . Despite a significant concentrations of  $NO_3^-$  in cultures, the results of our experiments demonstrate that  $NO_3^-$  consumption could only account for an insignificant portion of the biomass (<2% of total N). Garcia and Hutchins [2014] demonstrated that, under high light conditions, the WH0003 strain would use  $NO_3^-$  to stimulate even higher growth; despite higher light conditions in our experiments, there was no difference in growth between the  $NO_3^-$  enriched treatment and the obligate diazotrophy control. The result of this experiment therefore sets a precedent for the potential for diazotrophs to operate ignorant of  $NO_3^-$  in their environment, even when exposed long term, and is thus an important result. Continuing from the observations in the review by Knapp [2012], reevaluating our understanding of how DIN and diazotrophs interact in the environment is likely to be an important factor in revising the apparent discrepancies with the marine fixed N budget. The existence of diazotrophs which do not appear to interact significantly with the  $NO_3^-$  in their environment could allow for important  $N_2$  fixation to occur even in DIN replete environments. It must be stated though, that despite the clear potential for ‘ $NO_3^-$  blind’ diazotrophs, physiological studies on cultures cannot account for ecological factors that may limit the biogeographical range and function of diazotrophs in the ocean. Competition for nutrients and trace metals by larger, more efficient phytoplankton and heterotrophic bacteria, as well as temperature and light availability, could still be a limiting factor in high  $NO_3^-$  environments. However, the results of our experiments, along with a growing literature, seem to suggest that if our understanding of how  $N_2$  fixation operates within the marine N cycle, our understanding of how diazotrophs distribute through the ocean needs to be revisited. Along with diazotrophs who are capable of significant  $N_2$  fixation within  $NO_3^-$  rich environments, recent studies have placed group A diazotrophs in both cold regions [Blais et al. 2012; Díez et al. 2012; Fernández-Méndez et al. 2016; Shiozaki et al., 2018] as well as the mesopelagic, where light is limiting [Hewson et al., 2007; Bonnet et al., 2013; Rahav et al., 2013; Benavides et al., 2015; Moisaner et al., 2017; Benavides et al., 2018]. These results violate the canonical assumption of the diazotroph living in the euphotic regions of the subtropical oligotrophic gyres, and may be the direction needed to find the significant upward revision required to balance the marine fixed N budget.



## Chapter Four: Verification of Biological Measurements of diazotrophy against Bulk Uptake in Cellular Nitrogen

### *ABSTRACT*

Inaccuracies in biological N<sub>2</sub> fixation methods are often pointed to as one of the causes of methodological underestimation in global marine N<sub>2</sub> fixation estimates. <sup>15</sup>N<sub>2</sub> incubations and the acetylene reduction assay (ARA), common biologic assays used for N<sub>2</sub> fixation measurements, have both been scrutinised for inaccuracies. The former is often plagued by doubts of the rapidity and completion of the equilibration of <sup>15</sup>N<sub>2</sub> gas with the medium, whereas the latter suffers from irregular conversion ratios between acetylene fixation and N<sub>2</sub> fixation. While a number of methods have been used to assess these methods, one simple verification is often overlooked by reporters: the use of bulk nitrogen (N) uptake to verify that <sup>15</sup>N<sub>2</sub> and ARA estimations are accurate and reasonable. Here we describe the results of parallel N<sub>2</sub> fixation estimates of the bulk N method with both the <sup>15</sup>N<sub>2</sub> method and the ARA, using the former, less statistically variable method to assess the accuracy and reasonability of the latter two. Results sourced from two continuous turbidostat cultures demonstrate strong evidence suggesting methodological underestimation of N<sub>2</sub> fixation via the <sup>15</sup>N<sub>2</sub> fixation method between 40-80%. Results also suggest that underestimation may be a result of irregular <sup>15</sup>N<sub>2</sub> gas equilibration, although this seems to be just one component, as inaccuracy surpasses that suggested by previous bubble-addition criticisms. Despite this, gross difference of measured and expected values were not large, suggesting measurements are still somewhat reasonable. ARA on the other hand demonstrated consistent large *overestimation* of N<sub>2</sub> fixation by up to 650%, suggesting larger issues with this method. Verification with bulk N measurements, where possible, may provide a stronger verification for the methods commonly used in contemporary studies of N<sub>2</sub> fixation.

## Introduction

A common question which pervades the literature surrounding marine N<sub>2</sub> fixation is the notion that rates of marine N<sub>2</sub> fixation are potentially underestimated on the global scale [Codispoti, 2007]. Given the general importance of N<sub>2</sub> fixation to many anthropogenically important marine processes, and the above consideration, it is of utmost importance that a solid understanding of marine N<sub>2</sub> fixation is achieved by the marine biogeochemical research community. In order to achieve an understanding of N<sub>2</sub> fixation, a wide range of methods of study and quantification have arisen. Whilst other methods exist, such as modelling and geochemical efforts (outlined in chapter 1), direct measurements of biological parameters are among the most frequently used. The two methods which find most frequent usage are i) the <sup>15</sup>N<sub>2</sub> incubation method, and ii) the acetylene reduction assay (ARA). The <sup>15</sup>N<sub>2</sub> incubation method, simply put, involves the addition of isotopically labelled N<sub>2</sub> to a sample containing diazotrophs. After incubation, the resultant biological material is analysed for incorporation of <sup>15</sup>N, and using this, a rate of N<sub>2</sub> fixation can be inferred. The second method, the ARA, also involves the incubation of diazotrophs. This method however is based on a quirk of the nitrogenase enzyme. Nitrogenase operates by breaking the triple bond in the N<sub>2</sub> molecule. Acetylene also contains a triple bond, and nitrogenase is capable of breaking this also, converting it to ethylene. The rate of N<sub>2</sub> can therefore also be estimated by incubating diazotrophs with acetylene, measuring the concentration of ethylene produced, and then using a conversion factor to produce the final figure.

As with every biological assay, and truthfully every analytical method in science, there are advantages and disadvantages to the use of both of these methods. One aspect of the ARA is that it is a measure of nitrogenase activity rather than of N<sub>2</sub> fixation directly. This has benefits and downfalls. The benefit of this is that by measuring nitrogenase activity, one could infer that ARA measures the gross rate of N<sub>2</sub> fixation. By carrying out this assay online using high frequency measurements of ethylene production via gas chromatograph, it is possible to derive very precise description of the metabolic regulation of nitrogenase dynamics as well as its immediate sensitivity to environmental factors. Accurate rates of N<sub>2</sub> fixation are however contingent on the use of an accurate conversion ratio between ethylene production and fixed N production. The theoretical conversion factor used for this purpose is 3 or 4. This is based on the electron stoichiometry of the two chemical equations [Capone, 1993; Staal et al., 2001]. However, these conversion factors often do not hold up to scrutiny when experimentally verified. A wide array of studies have demonstrated

deviation from the 3-4 conversion factor, with suggested factors ranging anywhere from as little as 0.96 to as much as 56 [Mague et al., 1974; Graham et al., 1980; Capone, 1993; Montoya et al., 1996; Wilson et al., 2012]. This places a large degree of doubt as to the efficacy of the ARA method to estimate N<sub>2</sub> fixation rates.

While the <sup>15</sup>N<sub>2</sub> incubation method is a powerful tool, having been used in both shipboard and laboratory experiments for the last few decades, a number of lines of evidence have recently arisen, challenging our confidence in the accuracy of this method significantly. Firstly, commercial <sup>15</sup>N<sub>2</sub> gas stocks were recently observed with significant contamination from labile <sup>15</sup>N-labelled N species [Dabundo et al., 2014]. The presence of this contamination was found to cause false detection of N<sub>2</sub> fixation when used in incubations due to the misattribution of <sup>15</sup>N uptake into the biomass. The results of the original study by Dabundo et al. [2014] suggested that false detection from contaminated bottles could be on par with in situ rates of N<sub>2</sub> fixation derived from field assays.

The second point of controversy to this method is the uncertainty of rapid gas equilibration when injecting <sup>15</sup>N<sub>2</sub> gas [Mohr et al., 2010]. To infer a rate of N<sub>2</sub> fixation from its results, the <sup>15</sup>N<sub>2</sub> gas method has to be normalised against the initial <sup>15</sup>N<sub>2</sub> enrichment of medium. As this parameter is tedious to accurately measure, it is often simply estimated based on the volume of <sup>15</sup>N<sub>2</sub> gas added, the volume of medium in the incubation bottle, and a number of transiently variable parameters that affect the equilibration of gas in water. The traditional protocol involves the addition of a bubble of <sup>15</sup>N<sub>2</sub> gas, usually via gas tight syringe, done with the explicit assumption that equilibration between the gas bubble and the medium is rapid enough to have a statistically insignificant effect on the estimated initial enrichment of <sup>15</sup>N<sub>2</sub>. A review by Mohr et al. [2010] has, however, attempted to refute this claim, providing evidence to suggest that equilibration may not be as rapid as once thought. A slower equilibration would mean lower enrichment and therefore an underestimation of the final rate. Mohr et al. [2010] instead suggested the use of a potentially more rigorous method; the addition of an aliquot of seawater pre-enriched with <sup>15</sup>N<sub>2</sub> gas. While the impact this review has had on the literature has been large, a recent meta-analysis and review of methods by Wannicke et al. [2018], which compares the rates of N<sub>2</sub> fixation derived using both methods, has demonstrated that while the bubble addition method does appear to underestimate in certain situations, such as shorter incubations, there was rarely a statistical difference between results generated by the two methods. As such, it would appear that the use of either method is acceptable as long as care is taken to minimise the weaknesses inherent to the method of choice.

Despite the above considerations, one method of verification remains apparently untested. Montoya et al. [1996] describes two methods of calculating the uptake rate of N, one using enrichment of  $^{15}\text{N}$  in the particulate organic nitrogen (PON), and another using gross change in the PON concentration of samples. The first was labelled uptake specific to  $\text{N}_2$  fixation, and the other the general uptake rate of all forms of N. However, if other forms of N ( $\text{NO}_3^-$ ,  $\text{NH}_4^+$  and dissolved organic N; DON) are controlled for via either omission or measurement of flux into or out of the medium, it stands to reason that the second method can be used to calculate the gross uptake rate of N via  $\text{N}_2$  fixation, assuming no significant flux of nitrogenous gas species barring  $\text{N}_2$ . While traditionally, the uptake rate of  $^{15}\text{N}_2$  is favoured for the estimation of the net rate of  $\text{N}_2$  fixation, this method depends upon many parameters in order to calculate, which notably increases the variability in estimation a great deal. The use of elemental analysis is far simpler to analyse, requires fewer parameters to calculate and can therefore be considered more accurate. As the traditional method of performing  $^{15}\text{N}_2$  incubations requires the measurement of PON through elemental analysis in parallel to  $^{15}\text{N}$  enrichment, this method can be used to verify, or at least test the reasonability of concurrent measurements of  $\text{N}_2$  fixation via  $^{15}\text{N}$  enrichment, so long as dissolved N fluxes are accounted for. ARA may also be verified using concurrent measurements of flux in the PON and dissolved organic and inorganic N phases. To clarify, we describe the following mass balance equations:

$$\rho E_{\text{N}_2} (M \cdot \text{Vol}^{-1} \cdot T^{-1}) = \rho E_{\text{PON}} + \rho E_{\text{DON}} + \rho E_{\text{NH}_4^+} \quad (\text{Eq. 4.1})$$

$$\rho B_{\text{N}_2} (M \cdot \text{Vol}^{-1} \cdot T^{-1}) = \rho B_{\text{PON}} - \rho B_{\text{DON}} - \rho B_{\text{NH}_4^+} \quad (\text{Eq. 4.2})$$

Where  $\rho$  describes the volumetric rate of N flux. Positive flux is in the direction of the cell for  $\text{N}_2$  and *PON* (Eq. 2.4), but in the in the direction of the medium for *DON*, and  $\text{NH}_4^+$  (Eq. 2.7). *E* and *B* denote that these values were calculated using either *Enriched* N (Eq. 2.2 and 2.5) or *Bulk* (Eq. 2.3 and 2.6) measurements respectively. Note, these calculations are made *from the same incubations*.

Here, we therefore used the method described above in order to test the efficacy and efficiency of the two most commonly used methods of measuring biological  $\text{N}_2$  fixation, the  $^{15}\text{N}_2$  incubation method and the ARA. In addition, we describe a method of approximating the reasonability of an  $\text{N}_2$  fixation measurement by using the observed growth rate and approximate amount of N found in a newly formed diazotroph cell. Finally, we used the data gathered to test the effect of bottle

incubation on cellular growth and cellular carbon and N stoichiometry. Data used to produce the current work were taken directly from two experiments detailed in the companion study Randall et al. [in prep.] or chapter 5 of this thesis. These experiments were designed to test the effect of light on the C and N budgets of *Crocospaera watsonii* WH8501, and as such, the current study also tests the effects of differing light intensities on the accuracy of the two N<sub>2</sub> fixation assays described.

## Methods

### *Acquisition of data*

In the experiments used to gather data for the current study, cultures of *Crocospaera watsonii* WH8501 were grown in continuous 5l turbidostat cultures designed to maintain constant culture conditions over a long period of time (>1 month), and in particular here, regulate at an approximately constant value the light intensity of actually experienced by the culture. The two experiments specifically target saturating light conditions (375 - 425  $\mu\text{Einsteins m}^{-2} \text{s}^{-1}$ ) and sub-saturating light conditions (60-75  $\mu\text{Einsteins m}^{-2} \text{s}^{-1}$ ) within the cultures in order to observe any differences in the N budget due to differing light availability. When this was achieved, high frequency sampling was performed at specific time points throughout the diel metabolic cycle of *Crocospaera watsonii*, over four continuous sampling days. Over the course of this high frequency sampling regime, many parameters were measured, including cellular size and growth rate, elemental analysis (CHN) of cells, DON and NH<sub>4</sub><sup>+</sup> concentrations, the rates of C and N<sub>2</sub> fixation, and finally the rates of exudation or uptake of the aforementioned dissolved fractions of N. Specific methodologies for the measurements of those parameters can be found in the companion piece previously mentioned.

### *Estimating the accuracy of N<sub>2</sub> fixation assays*

#### *Method 1 - Comparison with bulk N uptake*

In order to enumerate N<sub>2</sub> fixation, two discrete methods were employed: i) <sup>15</sup>N<sub>2</sub> incubations over 12h (dark phase and light phase) and 24h periods, and ii) the ARA. For the first method, in addition to quantifying the uptake of <sup>15</sup>N into the biomass and the dissolved phase, elemental analysis was performed on both the filtered biomass and the dissolved N fractions (DON and NH<sub>4</sub><sup>+</sup>), methods for which are detailed in Randall et al. [in prep] or chapter 5 of the current thesis. This allows for a dual

calculation of the specific rate of uptake of N that occurred over the course of the incubations as detailed in Montoya et al. [1996]; use of bulk measurements of N and the rate of uptake of  $^{15}\text{N}_2$  gas specific to diazotrophy. This method notably ignores specificity in the source of N, meaning N uptake could come from a combination of dissolved inorganic N (DIN;  $\text{NH}_4^+$  and  $\text{NO}_3^-$ , DON, and  $\text{N}_2$ ). However, in this experiment, DIN is not included in the medium. Nevertheless, there may be DON; remnants from the aged seawater used to produce the YBCII medium [Chen et al., 1996], or potentially due to exudation or cell lysis from previous generations of *Crocospaera watsonii* in the cultures. During the high frequency sampling period, an average of  $49.7 \pm 4.8$  and  $52.7 \pm 17.9$   $\mu\text{moles DON L}^{-1}$  could be found in the cultures, for the saturating and sub-saturating light experiments respectively. Of this, roughly  $0.464 \mu\text{moles DON L}^{-1}$  were added intentionally to the medium in the form of vitamins. Small concentrations of  $\text{NH}_4^+$  may also potentially exuded (up to  $16 \mu\text{moles N L}^{-1}$ ), as observed by Randall et al. [in prep] or in chapter 5 of the current thesis. However, by measuring and controlling for the rates of exudation and/or uptake of these dissolved fractions of N, we are able to deduce the specific rate of  $\text{N}_2$  fixation from bulk measurements. Given the higher confidence in this method's accuracy, it can therefore be used to validate both the estimations of  $\text{N}_2$  fixation as derived from the uptake of  $^{15}\text{N}_2$ , and as derived from the ARA. For all incubations performed in these experiments ( $n > 130$ ), the  $\text{N}_2$  fixation rate was calculated using the specific uptake rates of the  $^{15}\text{N}$  isotope after enrichment, and compared to the uptake rate as calculated by bulk changes in PON, DON and  $\text{NH}_4^+$  over the course of the incubations. Comparisons were made specifically between measurements derived from the same incubation, and are presented as percentage accuracy. We describe the specific equations below:

$$Accuracy_E (\%) = 100 \left( \frac{\rho E_{N_2}}{\rho B_{N_2}} \right) \quad (\text{Eq. 4.3})$$

$$Accuracy_{ARA} (\%) = 100 \left( \frac{\rho ARA_{N_2}}{\rho B_{N_2}} \right) \quad (\text{Eq. 4.4})$$

Where  $Accuracy_E$  is the accuracy of  $\rho E_{N_2}$  (Eq. 4.1) and  $Accuracy_{ARA}$  is the accuracy of  $\rho ARA_{N_2}$  or the rate of  $\text{N}_2$  fixation as calculated by the ARA method (see chapter 2).

Statistical difference between the two measurement types were further tested using paired T-tests. Further comparisons of the average accuracies were made between the different incubation types (12h light, 12h dark, and 24h) and between the two experimental light conditions in order to verify

if these factors affected the accuracy at all. For the verification of the ARA results, as measurements were limited to one per diel cycle, these values are instead compared to the average 'bulk' measurement of N<sub>2</sub> fixation ( $\rho B_{N_2}$ ) for the same diel cycle.

### *Method 2 - Measurement of reasonability*

The acquisition of N is integral to the building of the necessary cellular machinery, proteins, and DNA for the production of new cells. The production of a new cell therefore comes with an associated N cost. N acquisition must therefore produce sufficient N in order to build a new cell. If a measurement of N<sub>2</sub> fixation produces above the sufficient amount, it can therefore be considered a 'reasonable' estimate. In order to derive the required N cost to produce cells, two values are necessary: the average N cost of a new cell, and the cellular growth rate for the incubation period in question. For the first, a value of 43 femtomoles N cell<sup>-1</sup> was used. This value was derived from the results of the companion study [Randall et al., in prep]. There it was observed that, over both experiments, at the end of the light phase after cellular division was complete, the average N cost was identical:  $42.81 \pm 5.57$  and  $43.34 \pm 2.69$  femtomoles N cell<sup>-1</sup> for the saturating light and sub-saturating light experiment respectively. These values were both the minima for cellular N mass, and also the value for cellular N mass at the end of division, and were thus assumed to be the minimum N requirement of a newly produced cell. The growth rate can therefore be used to estimate an N cost, with reasoning as follows: if the cellular growth rate is 0.2 d<sup>-1</sup>, it can be assumed that 20% of the cost of a new cell will be need to be acquired, *on average*, by each cell per day. Thus, if a measurement of N<sub>2</sub> fixation estimates an N production of close to that number, it may be considered 'reasonable'. Note, this assumes the vast majority of N acquired is used for cellular division, ignoring any cost of cellular upkeep and repair that is lost from the cell. This would suggest that a fixation rate *above* the N cost is required.

For all 24h incubations performed in these experiments (n>45), cellular growth rate over the incubation period was calculated and used to determine the required N to produce new cells observed in said incubation. The cellular N<sub>2</sub> fixation rate determined via <sup>15</sup>N isotopic enrichment was then compared with the parallel estimated N cost to determine a percentage fulfilment of the cost. Values close to and above 100% were considered 'reasonable' estimates. Paired T-tests were again used to measure for significant difference between cellular N requirement and N<sub>2</sub> fixation rate measured via isotopic enrichment. For ARA results, the growth rate used was from the culture proper for the same diel cycle that the ARA was performed.

### *Comparisons of incubations with the culture proper*

When using incubations, such as the  $^{13}\text{C}$  and  $^{15}\text{N}_2$  incubations used in this experiment, there is an implicit assumption that behaviour inside the incubation bottle will be representative of the environment of origin, may that be the natural environment or a culture environment. However, incubations generally involve large deviations from the environment of origin, the effects of which tend to go ignored in many instances of usage. In the case of the  $^{13}\text{C}$  and  $^{15}\text{N}_2$  incubations used in these experiments, while the experimental design intended to minimise perturbing effects, some could not be minimised without jeopardising the assays in question. One in particular is the lack of exposure to the atmosphere. In the cultures, there is open space and continuous input of filtered atmospheric air into the cultures. In addition, to ensure a lack of  $\text{CO}_2$  limitation, pH was regulated consistently at 8.2 by  $\text{CO}_2$  injection. In the incubations, there could not be any mixing with air, as this would cause the  $^{15}\text{N}_2$  in the sample to be diluted or even removed. As such, there is potential for the lack of gas exchange to cause a significant effect on the metabolism of the cultures. In particular  $\text{N}_2$  or  $\text{CO}_2$  may become limiting in the dark or light phases respectively. This could cause a comparative reduction in  $\text{N}_2$  fixation or C fixation. Furthermore, a reduction in the output of these processes may also have a knock on effect of limiting the production of new cells, as there may not be enough material to produce as much. As such, it is necessary to verify the incubations against the culture proper where possible.

To achieve this, we first compared cell densities at the end of the incubations with the cell densities inside the culture at the same time. This demonstrates, in the case of the dark phase incubations, if the *Crocospaera watsonii* population maintained throughout the incubation, and in the case of the light phase and 24h incubations, whether the growth rate stayed the same between the incubation and the culture proper. Note, that for all light phase and 24h comparisons with the culture, the culture concentrations are approximated by reconstituting from the applied turbidostat dilution, which may introduce some error. Further comparisons were then made with POC and PON to ensure that cellular stoichiometry, C and  $\text{N}_2$  fixation remained constant throughout. Comparisons were made by expressing the cellular density, POC and PON concentrations at the end of the incubations as a percentage of the same parameters at the same time as in the cultures, with this parameter being called 'fulfilment'. A one sample T-test was used to measure similarity to mean fulfilment against a  $\mu$  of 100%. This was performed for each parameter (cellular density, POC, and PON) for each incubation type, for each experiment.



## Results

### *Method 1 - Comparison with bulk N uptake*

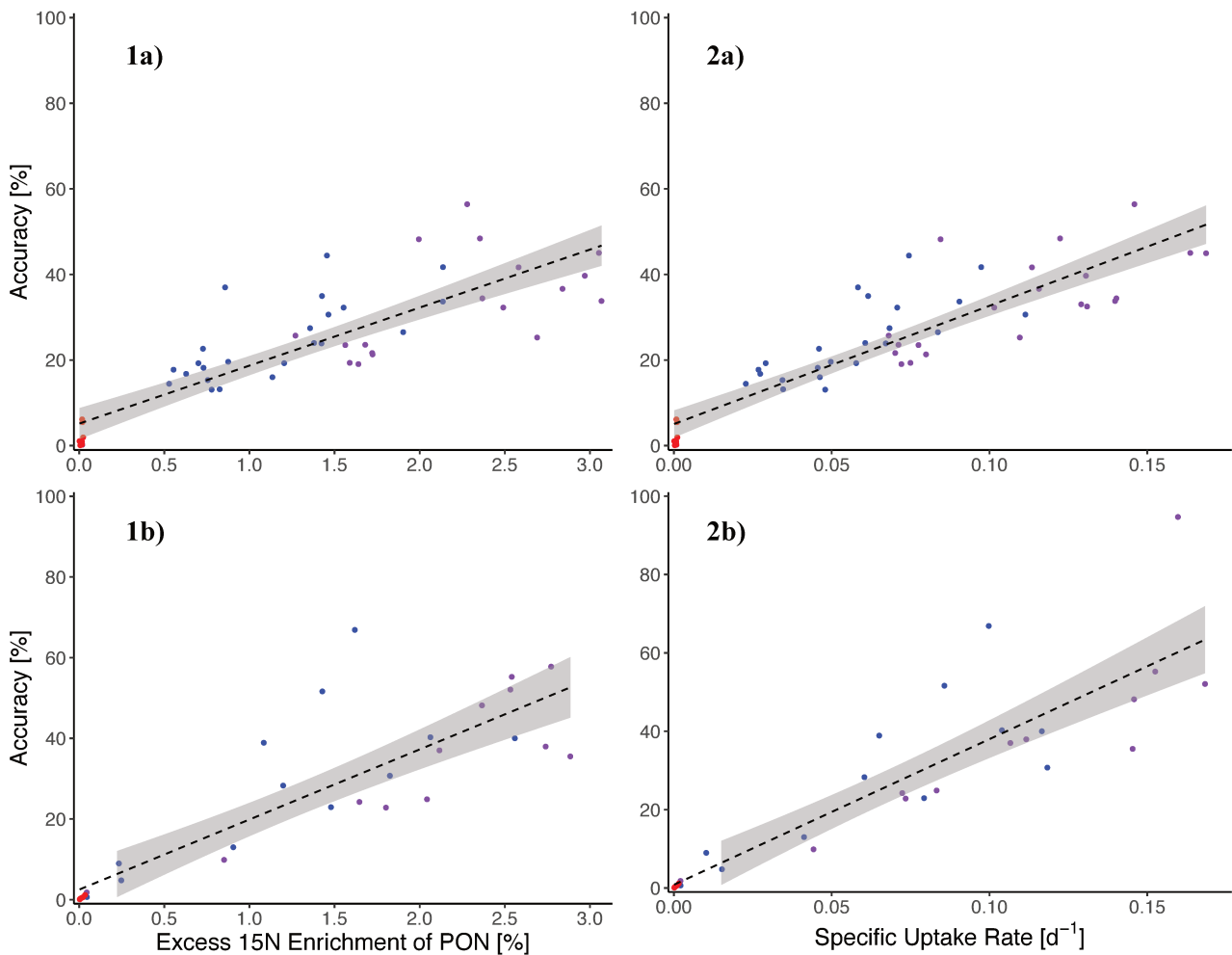
On average over all incubation types (dark, light, and 24h), N<sub>2</sub> fixation as determined by <sup>15</sup>N enrichment ( $\rho E_{N_2}$ ) estimated  $22.54 \pm 15.16\%$  (saturating light experiment) and  $24.74 \pm 21.01\%$  (sub-saturating light experiment) of the N<sub>2</sub> fixation as estimated by bulk methods ( $\rho B_{N_2}$ ). Sub-dividing these values into the different incubation types showed that dark phase incubations estimated  $24.43 \pm 9.28\%$  and  $28.92 \pm 19.95\%$  respectively, light phase incubations estimated  $1.36 \pm 2.18\%$  and  $0.14 \pm 0.53\%$  respectively, and 24h incubations estimated  $32.12 \pm 12.80\%$  and  $33.94 \pm 17.81\%$  respectively. Paired T-tests between the two N<sub>2</sub> fixation estimation methods, with associated DON and NH<sub>4</sub><sup>+</sup> fluxes factored in, confirm significant differences between the two methods for both experiments ( $p < 2.2e^{-16}$  and  $p = 0.003$  respectively). Mean of the differences was  $10.10 \pm 1.44$  and  $4.41 \pm 2.79$  femtomoles N cell<sup>-1</sup> d<sup>-1</sup> respectively. For context, these differences are roughly 75% and 55% the size of mean  $\rho B_{N_2}$  for each experiment respectively.

Accuracy of <sup>15</sup>N<sub>2</sub> measurements appeared to be correlated with excess <sup>15</sup>N enrichment ( $A_{PONf} - A_{PON0}$  (%); see Eq. 2.3) in the PON ( $R^2 = 0.71$  and  $0.54$  respectively), as well the specific uptake rate ( $V_E$ ; Eq. 2.3) of <sup>15</sup>N ( $R^2 = 0.79$  and  $0.53$  respectively). Accuracy increased at a higher rate in the sub-saturating light experiment, roughly 1.8 times in the excess <sup>15</sup>N enrichment of PON and 1.4 times in the specific uptake rate (Figs 1a-2b).

For the ARA, in the saturating light experiment, accuracy was between ~250-650%, whereas in the low sub-saturating light experiment, the accuracy was between ~100-130%, albeit with one seemingly erroneous high value of >1000%. Too few records using the ARA method were obtained to compare statistically with any reliability.

### *Method 2 - Measure of reasonability*

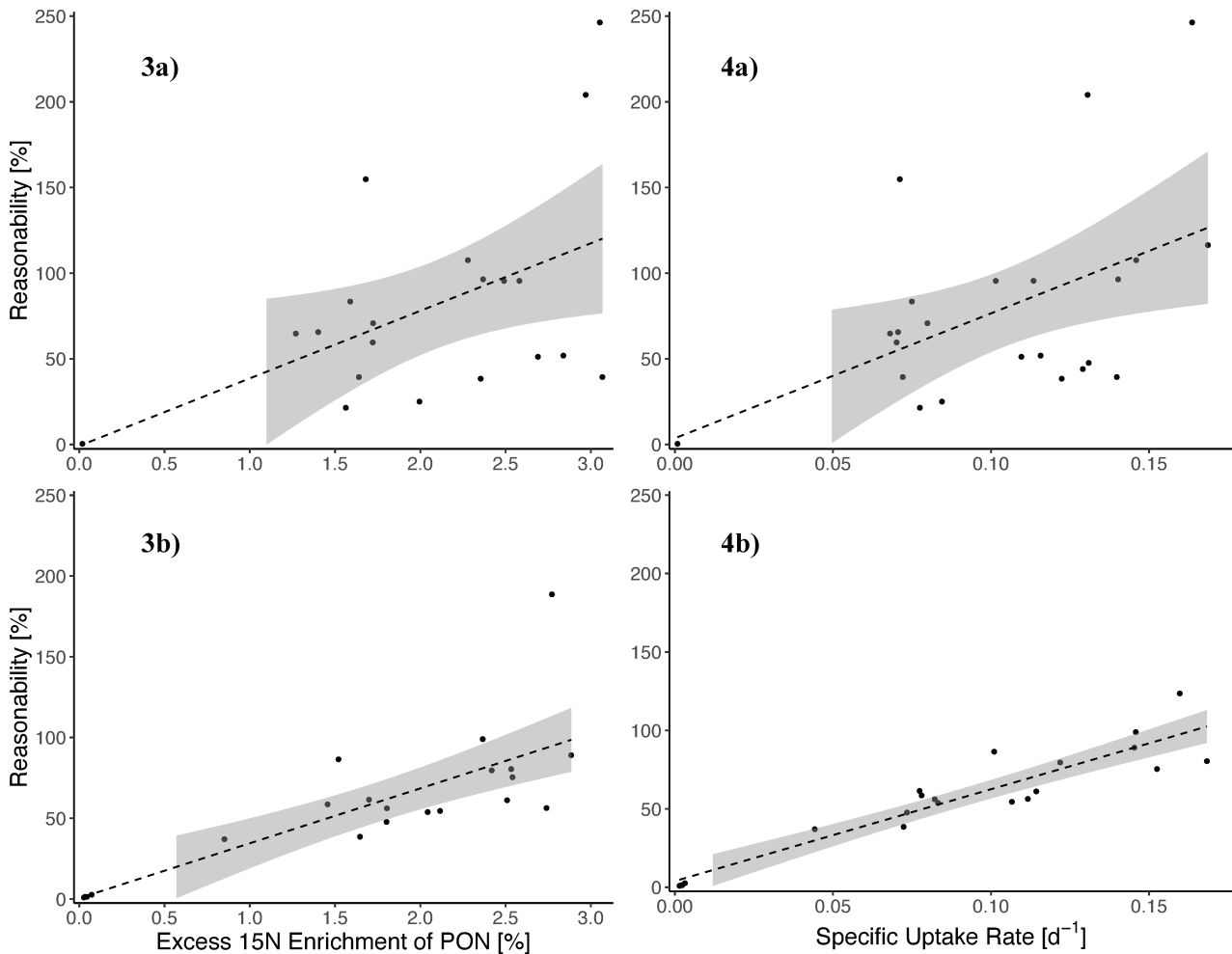
Mean percentage fulfilment of estimated N cost ('reasonability') by the cellular N<sub>2</sub> fixation rate (<sup>15</sup>N enrichment), using a fixed cellular N cost of 43 femtomoles N cell<sup>-1</sup> d<sup>-1</sup>, was  $65.20 \pm 36.44\%$  and  $55.48 \pm 33.62\%$  for the saturating light and sub-saturating light experiments respectively. Paired T-tests between the estimated N cost and the cellular N<sub>2</sub> fixation rate ( $\rho E_{N_2}$ ) confirm significant difference between the two values ( $p = 0.0020$  and  $p = 0.0001$  for the saturating and sub saturating light experiments respectively). There was a mean difference between estimated N cost and  $\rho E_{N_2}$  of



**Figures 4.1 & 4.2:** ‘Accuracy’ [%] of <sup>15</sup>N<sub>2</sub> incubation derived N<sub>2</sub> fixation measurements against excess <sup>15</sup>N enrichment [%] in the PON (Fig 4.1) and specific uptake rate [d<sup>-1</sup>] of <sup>15</sup>N (Fig 4.2) for the saturating light (1a and 2a) and sub saturating light experiments (1b and 2b). Dashed lines are regression lines and grey shading denotes the standard deviation of said regression lines. Red points are from light phase 12h incubations, blue points are from dark phase 12h incubations, and purple points are from 24h incubations.

3.98 ± 2.34 and 3.19 ± 1.41 femtomoles N cell<sup>-1</sup> d<sup>-1</sup> for the saturating and sub-saturating light experiments respectively. For reference, the mean values for the estimated N cost were 9.21 ± 5.26 and 8.10 ± 1.26 femtomoles N cell<sup>-1</sup> d<sup>-1</sup> for the saturating and sub-saturating light experiments respectively. ‘Reasonability’ appeared to correlate with both specific uptake rate (R<sup>2</sup> = 0.86) and excess <sup>15</sup>N enrichment in the PON (R<sup>2</sup> = 0.64), but only in the sub-saturating light experiment (Figs 3a-4b).

In the case of the ARA estimations, the average estimated N<sub>2</sub> fixation for the high light experiment produced ~600-900% of the required N. For the low light, this figure was closer to 200%, although again there was one potentially erroneous case where ARA predicted more than >1000% of the required N for observed population growth. Again, too few records using the ARA method were obtained to compare statistically with any reliability.



**Figures 4.3 & 4.4:** ‘Reasonability’ [%] of  $^{15}\text{N}_2$  incubation derived  $\text{N}_2$  fixation measurements against excess  $^{15}\text{N}$  enrichment [%] in the PON (Fig 4.3) and specific uptake rate [ $\text{d}^{-1}$ ] of  $^{15}\text{N}$  (Fig 4.4) for the saturating light (3a and 4a) and sub saturating light experiments (3b and 4b). Dashed lines are regression lines and grey shading denotes the standard deviation of said regression lines.

#### *Comparisons of incubations with the culture proper*

Cellular density was found to be statistically similar between incubations and the culture proper for only the dark phase incubations in the saturating light experiment, with non-similar incubation types under estimating cellular density by 13% and 30% in the light phase and 24h incubations respectively. In the sub-saturating light experiment, all incubation types were found to be statistically similar. POC concentration was statistically different in all incubation types for the saturating light experiments, slightly overestimating by 9% in the dark phase incubations, and underestimating by 30% and 34% in the light phase and 24h experiments respectively. In the, sub-saturating light experiments, POC concentration was statistically similar for all incubation types. PON concentration was significantly different in all incubation types for all experiments. For the

saturating light experiment, PON concentration was underestimated by 15% and 6% in the dark phase and 24h incubations, and very slightly overestimated in the light phase incubations. In the sub-saturating light experiments, all incubations overestimated PON concentrations by 12-22%. Values are listed exhaustively in table 4.1.

**Table 4.1:** Mean fulfilment (%) and statistical similarity (one sample T-test,  $\mu=100$ ) between incubations and the culture proper for the parameters of cellular density, POC concentration and PON concentration. These are separated into each incubation type and experimental condition. The  $\pm$  signified 95% confidence limits on the mean fulfilment. P-values in bold demonstrate statistical similarity ( $p > 0.05$ ) and p-values in italics demonstrate statistical difference ( $p < 0.05$ ).

	Saturating Light			Sub-Saturating Light		
	Dark Phase	Light Phase	24h	Dark Phase	Light Phase	24h
<b>Cellular density</b>						
Mean fulfilment (%)*	104 $\pm$ 4.4	87 $\pm$ 6.5	70 $\pm$ 5.7	94 $\pm$ 7.0	97 $\pm$ 11.0	97 $\pm$ 10.0
p-value	<b>0.0590</b>	<i>0.0004</i>	<i>1.714E-10</i>	<b>0.1149</b>	<b>0.5438</b>	<b>0.4850</b>
<b>POC concentration</b>						
Mean fulfilment (%)*	109 $\pm$ 6.7	69 $\pm$ 6.1	66 $\pm$ 4.9	99 $\pm$ 3.0	96 $\pm$ 6.3	100 $\pm$ 8.2
p-value	<i>0.0077</i>	<i>6.869E-09</i>	<i>9.296E-11</i>	<b>0.3184</b>	<b>0.1783</b>	<b>0.9904</b>
<b>PON concentration</b>						
Mean fulfilment (%)*	85 $\pm$ 4.4	108 $\pm$ 7.8	94 $\pm$ 6.0	112 $\pm$ 3.8	113 $\pm$ 6.5	122 $\pm$ 7.4
p-value	<i>6.043E-07</i>	<i>0.0469</i>	<i>0.0476</i>	<i>7.252E-07</i>	<i>0.0005</i>	<i>4.448E-06</i>

\* Mean fulfilment is a measure of closeness of a parameter measured in the incubation to the same parameter measured at the same time in the culture proper; with 100% meaning identical, below being under-estimated and above being over estimated

## Discussion

A frequently discussed issue in the field of marine biogeochemistry has been the resolution of the purported underestimation of global rates of N<sub>2</sub> fixation with relation to global rates of denitrification, which according to a number of lines of evidence, should be balanced over time. Among the major directions researchers have taken to alleviate said problem is to assess the efficacy of assays used to measure biological N<sub>2</sub> fixation in the lab or field. The two major biological assays used are the ARA and the <sup>15</sup>N<sub>2</sub> incubation methods. Both methods have been criticised of late, with the former suffering from inconsistent C<sub>2</sub>H<sub>2</sub>:N<sub>2</sub> conversion ratios used [Mague et al., 1974; Graham et al., 1980; Capone, 1993; Montoya et al., 1996; Wilson et al., 2012], and the latter coming under scrutiny with regards to poor estimation of the rate and efficiency of <sup>15</sup>N<sub>2</sub> equilibration with the medium [Mohr et al., 2010; Wannicke et al., 2018]. While a number of methods have been attempted to prove, or disprove each method's suitability, these assays are rarely verified by reporters using concurrent bulk N measurements and subsequent rates of N uptake using said measurements [Montoya et al., 1996]. Bulk N measurements require far fewer parameters to

calculate the end result, and do not suffer from issues such as inconsistent conversion ratios, nor slow uptake of  $^{15}\text{N}_2$ . As such, it can be easily assumed that rates calculated from bulk N measurements are more accurate than the other methods, and therefore can be used to assess the efficacy of said methods. The one issue is that the bulk N method does not discriminate between uptake of recently fixed N and flux of other sources of N, such as  $\text{NO}_3^-$ ,  $\text{NH}_4^+$  and DON. However, if fluxes of these materials are accounted for, then one can estimate the total  $\text{N}_2$  fixed over the incubation period using this method.

Here, we assessed the accuracy of a large number of  $\text{N}_2$  fixation estimations derived from  $^{15}\text{N}_2$  incubations and ARAs using *Crocospaera watsonii* cultures grown in two different light conditions.  $^{15}\text{N}_2$  incubations were subdivided into three types; 12h dark and light, which covered the dark and light phases respectively, and 24h incubations, which covered an entire diel cycle from the start of one dark phase to the next. These data were produced by the experiments described in the companion piece Randall et al. [in prep] or chapter 5 of this thesis. Accuracy was assessed using two methods. First, a measure of the difference between estimations of  $\text{N}_2$  fixation as derived from the former methods and the bulk N incorporation method. The second was a measure of the ‘reasonability’ of a measurement. For the second method, this involved comparing estimations of N incorporation using the first two methods against an estimated N requirement derived from the observed growth rate and a fixed N cost of producing a new cell ( $\sim 43$  femtomoles N cell $^{-1}$ ). A further consideration was the use of these data to assess how well (12-24h) incubations represent the cellular activity of diazotrophs within the culture proper, i.e. whether or not the incubation procedure induced a bias in the growth processes and to what extent if so.

#### *Accuracy of $^{15}\text{N}_2$ incubations and the ARA against bulk measurements*

A clear result of the use of this method to assess the accuracy of  $^{15}\text{N}_2$  incubations was that on average, for all incubation types and both experimental light conditions, there was clear and significant underestimation of  $\text{N}_2$  fixation. Initially, the most striking result was the complete underestimation of  $\text{N}_2$  fixation in the light phase. This result is almost certainly due to the lack of time for proper equilibration of  $^{15}\text{N}_2$  in order to catch the last small spillover of  $\text{N}_2$  fixation into the light phase [Wannicke et al., 2018]. Interestingly, while there is a large degree of variance for each measurement,  $\text{N}_2$  fixation results appeared to be less accurate on average in the dark phase incubations than in the 24h incubations. This result is again potentially due to slow equilibration of

$^{15}\text{N}_2$ ; 12h incubations dark phase incubations were stopped at the end of the dark phase, but  $\text{N}_2$  fixation appeared to continue on further.

There appeared to be a relatively strong correlation of accuracy with both the specific uptake rate of  $^{15}\text{N}_2$  ( $V_E$ ; equation 2.3) and excess enrichment of  $^{15}\text{N}$  in the PON ( $A_{\text{PONF}} - A_{\text{PON0}}$  (%); see Eq. 2.3) after incubations (Figs. 1a-2b). This points strongly towards variable and inconsistent equilibration of  $^{15}\text{N}_2$  gas within the medium, as if the equilibration was indeed consistent, we would observe a consistent ratio of  $\text{N}_2$  fixation as determined by bulk measurements and N fixation as determined by  $^{15}\text{N}_2$  uptake despite the uptake rate. Instead, a positive correlation of accuracy with specific uptake rate of  $^{15}\text{N}_2$  and excess enrichment of  $^{15}\text{N}$  in the PON suggests that there was incomplete enrichment of the medium. It must be stated, however, that while these results do confirm somewhat the work of Mohr et al. [2010], the required reduction in the isotopic enrichment value used to calculate the present results far exceeds the underestimation that said study suggests. In the current work, we estimate that  $\text{N}_2$  in the medium was subject to  $^{15}\text{N}_2$  enrichment of around 22% after  $^{15}\text{N}_2$  bubble addition (Eq. 2.1). For  $\text{N}_2$  fixation estimations calculated from the  $^{15}\text{N}$  enrichments ( $\rho E_{\text{N}_2}$ ) observed in this study to be made equal to values derived from bulk measurements ( $\rho B_{\text{N}_2}$ ), the  $^{15}\text{N}$ - $\text{N}_2$  enrichment used to calculate said value would have to be closer to ~6.6%. Our estimated enrichment is therefore overestimated by a factor of ~3.3. Mohr et al. [2010] suggests that for a 12-24h incubation, this overestimation should only be by a factor of ~1.25. As such, it may therefore indicate there is a general issue with the method ( $^{15}\text{N}_2$  incubations) itself, something that perhaps would not be alleviated with a change to the delivery method of  $^{15}\text{N}_2$  gas. The accuracy method we described here would have to be used with  $^{15}\text{N}$ -enriched seawater incubations to verify this, however.

ARA results were too few to perform reliable statistical tests, however, all successful assays predicted larger  $\text{N}_2$  fixation estimations as compared with equivalent estimations from the bulk N method. These were notably larger in the saturating light experiment, with ARA estimations being between 2.5 and 6.5 times the equivalent bulk N estimations. ARA estimations from the sub-saturating light experiment were instead closer to 100%, although with one potentially erroneous result being closer to 10 times the equivalent bulk estimation. It must be noted that exudation of nitrogenous compounds were accounted for in these results (Eq. 4.2), and as such the overestimation of  $\text{N}_2$  fixation by ARA cannot be rightly due to the classic explanation, that ARA

simply predicts 'gross' N<sub>2</sub> fixation. These results suggest an issue with this method overall, potentially due to inaccurate conversion ratios used.

#### *Reasonability of <sup>15</sup>N<sub>2</sub> incubations and the ARA against observed growth rate*

As with the previous measure of accuracy, 'reasonability' analysis demonstrated a clear and also suggest underestimation. This underestimation was roughly 40-50% on average, albeit with a large degree of variability ( $\pm 30\%$ ). However, the difference between the required N<sub>2</sub> fixation to fulfil cellular demand, and the actual measured N<sub>2</sub> fixation, were generally not large,  $\sim 3$  femtomoles N cell<sup>-1</sup> d<sup>-1</sup> on average. This suggests that while consistently underestimated, these results aren't particularly unreasonable. There was again an observed positive correlation with both specific uptake rate of <sup>15</sup>N<sub>2</sub> and excess <sup>15</sup>N in the PON, mirroring the above considerations (Figs. 3b and 4b). There was however no strong correlation of these parameters in the saturating light experiment (Figs. 3a and 4a), suggesting that there was less of an issue in said experiment with <sup>15</sup>N<sub>2</sub> gas enrichment equilibration.

ARA results suggest an overestimation of required N by 6 to 9 times in the saturating light experiment, and  $\sim 2$  times in the sub saturating light experiment. The former could therefore be considered a highly unreasonable and unrealistic results, especially considering the lack of DON or NH<sub>4</sub><sup>+</sup> exudation on the scale required to balance the N budget. The latter, in the sub-saturating light experiment, however is far more reasonable, potentially pointing towards a bias in accuracy when light is sub-saturating.

#### *Verification of the incubations vs the culture proper*

In the saturating light experiment, incubations were a relatively poor substitute for the culture proper. In particular POC content and cell density was below the equivalent level in the light and 24h incubations, suggesting that C fixation was particularly inhibited relative to the cultures (Table 4.1). PON content was also underestimated significantly in the dark phase and 24h incubations, albeit less unreasonably in the latter case. In addition, there was more POC in the incubations after the dark phase compared with the culture, suggesting less of the POC reserves necessary for N<sub>2</sub> fixation were appropriated for said purpose. These issues could be potentially an issue with gas exchange and gas limitation, as incubations had no ability to replenish N<sub>2</sub> or CO<sub>2</sub> once depleted. The cultures on the other hand had stirring, bubbling and direct CO<sub>2</sub> injection due to pH regulation. Alternatively, or perhaps concurrently, there may have been less homogeneity of light penetration in

the incubation bottles due to the relative lack of mixing. This is however somewhat unlikely, as incubation bottles were generally too thin to occlude significant amounts of light to certain parts of the bottle.

In the sub-saturating light experiment, incubations mirrored the cultures far more effectively, with cell content and POC content statistically similar on average for all incubation types (Table 4.1). There was however an issue of over-production of N for all types (by around 10-20%). It is uncertain why this occurred. Compared with the saturating light experiment, there was less N<sub>2</sub> fixation so gas limitation would probably not occur. It is however uncertain why N acquisition would be significantly greater. One potential explanation is that incubation bottles are sealed from the environment with little to no headspace, meaning less replenishment of O<sub>2</sub> due to gas exchange over the N<sub>2</sub> fixation period. Because cultures from the sub-saturating light experiment had relatively lower rates of C fixation compared with the saturating light experiment, there was likely a lower O<sub>2</sub> concentration, in their incubation bottles. This relative lack of O<sub>2</sub> have led to reduced the inhibition of nitrogenase and therefore higher relative rates of N<sub>2</sub> fixation.

Overall, while there were some issues with the use of incubations, there were few major deviations from the culture proper, most seemingly due to the lack of gas exchange in the incubation bottles. This, however, is a difficult issue to solve, and potentially impossible, as a closed bottle is required to maintain <sup>15</sup>N<sub>2</sub> equilibration with the medium.



## Conclusions

This study provides useful insight into the relative accuracy and reasonability of common methods used to estimate rates of N<sub>2</sub> fixation by living diazotrophs in culture. Our results reveal that <sup>15</sup>N<sub>2</sub> incubations tended to underestimate N<sub>2</sub> fixation when compared to estimates derived by bulk measurements of N performed on results from the exact same incubation bottles, suggesting that other N acquisition strategies such as DON uptake may well play a significant role in the overall N metabolism of cells. In addition they also tended to estimate less production than was required for observed cellular growth rates. For the second measure of accuracy, however, results were generally close in relative terms to the required N production for observed cellular growth, suggesting they are not particularly unreasonable estimates. The issue appeared to be due to slow, irregular dissolution of <sup>15</sup>N<sub>2</sub> gas, as demonstrated by the positive correlation of accuracy with specific uptake rate and excess <sup>15</sup>N in the PON, which should not occur if <sup>15</sup>N<sub>2</sub> gas is consistently and effectively equilibrated in the medium. While initially this would point towards the findings of Mohr et al. [2010], sensitivity analyses demonstrate that the enrichment overestimation was outside the range that said study suggested. This indicates that while poor enrichment due to the bubble method may be an issue, it may just be a general issue with <sup>15</sup>N<sub>2</sub> enrichment, or accessibility itself. While the enriched seawater method as suggested by Mohr et al. [2010] may alleviate the highlighted issues, generally reporters do not include verification of their results against the bulk N method of calculating N<sub>2</sub> fixation, including said study. Verification of the enriched seawater method would have to be performed against the bulk N method to determine if it is indeed more suitable.

The ARA method on the other hand, suffered very large deficiencies in its ability to accurately estimate N<sub>2</sub> fixation, with both methods of estimation overestimating by a large degree. This appeared to be more prominent in the saturating light experiment. Notably, as exudation of nitrogenous compounds were measured and controlled for, it could not be said the ARA provides a 'gross' estimation of N<sub>2</sub> fixation. Comparatively, while the <sup>15</sup>N<sub>2</sub> incubation method seems to underestimate N<sub>2</sub> fixation, it appeared to do so to a reasonable degree; ARA on the other hand provided results which could not be explained. Major research needs to be performed to regulate the issues observed with this method.

While initially it may be considered that using the bulk method may provide the solution to underestimation of N<sub>2</sub> fixation, this may not be the case for all. It was possible to use the bulk

method to quantify N<sub>2</sub> fixation in these experiments because many details were controlled for; no NO<sub>3</sub><sup>-</sup> was added to the medium, *Crocospaera watsoniii* cultures were isolated and no significant microbial growth was present, and clear signals of POC, PON, DON, and NH<sub>4</sub><sup>+</sup> could be detected. These issues may be simple to control in a laboratory setting, but in the field, specific and targeted methods such as the <sup>15</sup>N<sub>2</sub> incubation method are highly preferable because they can isolate specific fluxes of N with absolute certainty that said fluxes originate from biological N<sub>2</sub> fixation. However, it would be advantageous for all reviewers to assess their bulk N budgets when performing either method, as it adds a measure of reasonability to their <sup>15</sup>N<sub>2</sub> fixation estimates.

Our final consideration was to demonstrate the deficiencies in using bottle incubations to represent metabolic processes from a culture. Our results indicate that while most processes did not have major issues, there was notably a few issues that may be due to gas exchange and limitation. In particular, where there was a high degree of photosynthesis such as in the saturating light experiment, there appeared to be significant limitation of C fixation and therefore cell division. While these issues are not easily solved, they should always be taken into consideration analysing the results of any form of bottle incubation.

# Chapter Five: The effect of Irradiance on Particulate and Dissolved Carbon and Nitrogen Budgets of *Crocospaera watsonii*

## *ABSTRACT*

Here we describe the effect of saturating and sub-saturating irradiance on the carbon (C) and N budgets of continuous turbidostat cultures of *Crocospaera watsonii* WH8501, including the effects of irradiance on population growth, particulate organic C (POC) and N (PON) growth, cellular size and stoichiometry, N<sub>2</sub> and C uptake, and finally a particularly understudied area for UCYN diazotrophs, fluxes of NH<sub>4</sub><sup>+</sup> and dissolved organic N (DON). Both bulk measurements (PON, POC, DON, NH<sub>4</sub><sup>+</sup>) and isotopic incubations (<sup>13</sup>C and <sup>15</sup>N) were used to assess the above parameters. There was an observed increase in both population, C, and N growth rates in saturating light conditions versus sub-saturating, as well as an increase in cellular storage of C during the light phase and N over the dark phase. Per cellular rates of C and N uptake were, however, not significantly different between the two conditions. However, when accounting for fluxes of dissolved N (NH<sub>4</sub><sup>+</sup> and DON), there was a clear enhanced uptake of DON in the sub-saturating light condition compared to the saturating light experiment. This would suggest, despite similar total rates of total N uptake, there was enhanced relative N<sub>2</sub> fixation in the saturating light condition. There was a notable observed accumulation of NH<sub>4</sub><sup>+</sup> in both experiments, which was enhanced in the sub-saturating light condition (12% vs 4% of total N). Finally, results measuring <sup>15</sup>N-dissolved N exudation were reduced relative to bulk dissolved N exudation measurements; suggesting *Crocospaera watsonii* favours recently fixed N for somatic growth.

## Introduction

With the unique ability to utilise atmospheric dinitrogen ( $N_2$ ), a vastly abundant yet recalcitrant nitrogen (N) source,  $N_2$  fixation represents a key step in the marine N cycle. By linking  $N_2$  to the pool of bioavailable N, the rate of  $N_2$  fixation in the wider ocean ultimately decides the size of the marine N inventory. Consequently, this affects all other dependent processes, including anthropologically important processes such as the marine food web and the biological carbon pump [e.g. Capone et al., 2005, Falkowski, 1997]. It is therefore of utmost importance that a rigorous and complete understanding and quantification of  $N_2$  fixation, and what affects its rate, is ascertained and developed by marine biogeochemists. Current lines of debate surrounding the marine fixed N budget suggests that the the balance of processes inputting ( $N_2$  fixation) into and removing (denitrification and anammox) fixed N from the marine N inventory is significantly overbalanced in the direction of denitrification [Codispoti et al., 2001; Codispoti 2007], with a net loss of fixed N between 188 and 234 Tg N  $y^{-1}$ . This result is contentious for a number of reasons, as a number of lines of evidence suggest these processes should be balanced. These include Holocene carbon dioxide records [Gruber and Sarmiento, 1997], sedimentary  $\delta^{15}N$  records [e.g. Altabet et al., 2002; 2006], and the presence of a strong feedback mechanism pushing the marine N to phosphorous (P) ratio towards the canonical Redfield ratio of 16:1 [Tyrrell 1999]. Furthermore, if the total fixed N budget of  $6.6E+05$  Tg presented by Capone et al., [2008] is correct, the suggested rates of N removal reported above would deplete the world's oceans of N in less than 3500 years. As such, it is possible that our current estimates of  $N_2$  fixation require significant upward revision. Global models describing  $N_2$  fixation in the wider context of the marine N cycle often do not use direct descriptions of  $N_2$  fixation with regards to the cellular action of diazotrophs, instead preferring parameterisations designed to compensate for losses through denitrification and balance the budget over time. As such, working towards building a quantitative description of the cellular diazotrophic N budget with respect to different growth scenarios may reveal further insight into the question of whether or not  $N_2$  fixation is being underestimated.

Much of the work surrounding the field of marine  $N_2$  fixation has been performed on macroscopic diazotrophs such as the characteristic *Trichodesmium*. Recent advances in the field of environmental genomics have, however, expanded our understanding of the diversity of diazotrophs, especially in the direction of free living unicellular diazotrophs [e.g. Zehr, 2011]. As such, information regarding the N metabolism of these relatively novel organisms is sparse, but

necessary if we are to have a more complete understanding of N<sub>2</sub> fixation. One such organism which has gained popularity of study over the past few decades is *Crocospaera watsonii*, a culturable unicellular cyanobacterial diazotroph (UCYN-B) found throughout sub-tropical oceans worldwide [Zehr et al., 2001; Church et al. 2005; Zehr et al. 2007]. Recent efforts to describe the N metabolism of *Crocospaera watsonii* have pointed towards gross rates of N<sub>2</sub> fixation which outstrip the metabolic cost of growth [Dron et al., 2012], prompting discussion as to the fate of said surplus N production. Given the aforementioned issue with underestimation of global rates of N<sub>2</sub> fixation, the possibility of a large degree of N exudation by diazotrophs represents a potentially significant underestimation in rates derived using ‘net’ methods such as the <sup>15</sup>N<sub>2</sub> incubation method, and is therefore an important field of research.

The exudation of nitrogenous compounds has been previously observed in the filamentous diazotroph *Trichodesmium* spp. [Capone *et al.*, 1994; Glibert and Bronk, 1994; Mulholland and Capone, 2000; Mulholland et al., 2004], of which the majority of work on this topic has been conducted in the field. Previous studies which utilised techniques to assess the incorporation of <sup>15</sup>N<sub>2</sub> into both the particulate (biomass) phase and the dissolved (exudate) phase in the field show that exudate may account for from 10 to >50% of the total N fixed [Glibert and Bronk, 1994; Konno et al., 2010; Benavides et al., 2013b]. This has been suggested to be driven by processes such involving the destruction of the diazotrophic cell, such as ‘sloppy feeding’ [O’Neil and Roman, 1992], viral lysis [Fuhrman, 1999], and senescence [Berman-Frank et al., 2004]. While these processes are of course of vital importance to the cycling of N in the marine environment, it must be noted that they bear distinction from cellular metabolic processes which seek to passively or actively exude or excrete fixed N from the cell. This is difficult to study *in situ* as diazotrophs taken from the marine environment are not always able to be isolated from inter-microbial interactions. To garner a better idea of the magnitude in which diazotrophs exude or excrete N as a metabolic process, it is necessary to study them at the unicellular level, i.e. in a culture, in order to isolate them as much as possible from the above processes. This has notably been performed with canonically important, macroscopic colony forming species such as *Trichodesmium* spp., where significant overproduction and exudation has been noted [Mulholland et al., 2004; Mulholland and Bernhardt, 2005; Benavides et al., 2013a].

As of writing, information regarding the exudation of free living unicellular diazotrophs is seemingly limited to a few studies [Agawin et al., 2007, Benavides et al., 2013a, Berthelot et al.,

2015]. The study by Berthelot et al. [2015] is the only to our knowledge which uses *Crocospaera watsonii*. In said paper N release was compared in batch cultures of four species of diazotrophs: the colony forming *Trichodesmium* (IMS10101) with two strains of the unicellular *Crocospaera watsonii* (WH0003 and WH8501), and one strain of *Cyanothece* (ATCC51142). The findings of this study suggest that up to 1% of isotopically enriched fixed N can be found in the dissolved phase, but when comparing the total N accumulation (i.e. from both dissolved and particulate N pools) with the net N<sub>2</sub> fixation (from <sup>15</sup>N<sub>2</sub> incubations), 1-20% of the fixed N could be found in the dissolved phase. A more rigorously quantified rate of exudation is of utmost importance to defining the budgets.

In order to do define rigorous N budgets, including a quantification of N fluxes into and potentially out of the dissolved phase, turbidostat cultures of *Crocospaera watsonii* (WH8501) were established and used to study the various fluxes of N associated with diazotrophic cyanobacteria. Incorporation of N through N<sub>2</sub> fixation and potential interaction with dissolved organic N was studied using both bulk measurements by elemental analysis and incorporation of <sup>15</sup>N into biomass via <sup>15</sup>N<sub>2</sub> incubations. This was supplemented through use of the acetylene reduction assay (ARA), which was used to quantify gross nitrogenase activity. Interactions with dissolved organic N (DON) and NH<sub>4</sub><sup>+</sup> were assessed by both tracking bulk measurements of DON and NH<sub>4</sub><sup>+</sup> as well as analysing <sup>15</sup>N<sub>2</sub> incubation filtrates for incorporation of <sup>15</sup>N into the dissolved species of N. In order to develop a greater understanding of what drives these processes, this experiment was performed using two distinctly different light conditions. The light conditions chosen were in order to test the effect of saturating and sub-saturating light conditions [Rabouille et al., 2017] on the N budget. As phototrophic cyanobacteria, *Crocospaera watsonii* metabolic function relies significantly on light as an energy source; significantly varying the intensity of light which reaches the cells could therefore cause notable shifts in N metabolic function such as N<sub>2</sub> fixation and interaction with dissolved N pools. While it is reasonable to expect that increasing light intensity will result in larger reserves of energy for further N<sub>2</sub> fixation, it is unclear how this will affect N storage as well as potential exudation of dissolved N.

## Methods

### *Culture conditions and experimental design*

Cultures were grown in 5L cylindrical photobioreactors (PBRs) using YBCII medium [Chen et al., 1996]. This is is  $\text{NO}_3^-$  free in order to achieve obligate diazotrophy, and nutrient dense to ensure the cultures do not become limited by nutrients. Other forms of N were not included purposefully, aside from a small amount of DON in the form of vitamins ( $\sim 0.4 \mu\text{moles L}^{-1}$ ). DON and  $\text{NH}_4^+$  were however present from remnant cell lysis, cell exudation from previous generations, and DON remnants from the aged seawater used to make the medium. During the high frequency sampling period, an average of  $49.7 \pm 4.8$  and  $52.7 \pm 17.9 \mu\text{moles DON L}^{-1}$  could be found in the cultures, for the saturating and sub-saturating light experiments respectively. The PBRs used were designed to maintain constant temperature ( $27^\circ\text{C}$ ) throughout the experiment using a double wall with space to circulate temperature regulated water (using a cryostat) around the outside of the cultures. The PBRs were fitted with a pH probe and  $\text{CO}_2$  bubbling system to maintain pH at 8.2, and neon bulbs which kept light intensity at a constant. The light regime used was a standard 12:12 light-dark cycle. The light settings applied resulted in an average experienced irradiance of  $\sim 400 \mu\text{Einsteins m}^{-2} \text{s}^{-1}$  in the first experiment (saturating light) and  $60 \mu\text{Einsteins m}^{-2} \text{s}^{-1}$  in the second experiment (sub-saturating light). Finally, in order to maintain agitation of the cultures and eliminate the production of biofilms, bubbling of filtered atmospheric air was installed as well as constant stirring using a magnetic bar. Two PBRs were used per experiment as culture replicates.

Cultures were first grown in batch for a minimum of two weeks to acclimate cells to the intended light conditions of each experiment. After acclimation, light conditions were maintained throughout the experiment via turbidostat, which is the continuous dilution of cultures in order to offset self shading as a natural consequence of cellular growth. This was done manually by counting cells (Beckman Coulter Counter), calculating the correct dilution rate needed and then allowing pumps connected to medium to add medium slowly throughout the day, removing excess culture using an outlet tube. Cultures were maintained in turbidostat for at least two weeks each, aiming to observe relative stability in daily growth rate before executing the high frequency sampling phase of the experiment.

Once ready, high frequency measurements (summary: Fig. 3.1) of the various N dynamics targeted by the scope of the study were taken. Time points were fitted around extremes in the N and C

metabolism dynamics [Dron et al., 2012; 2013], i.e. at the transition from dark-light and vice-versa. This was done to capture the specific diel N and C cycles that are highly regulated in *Crocospaera watsonii* [Rabouille and Claquin, 2016].

### *Sampling and sample processing*

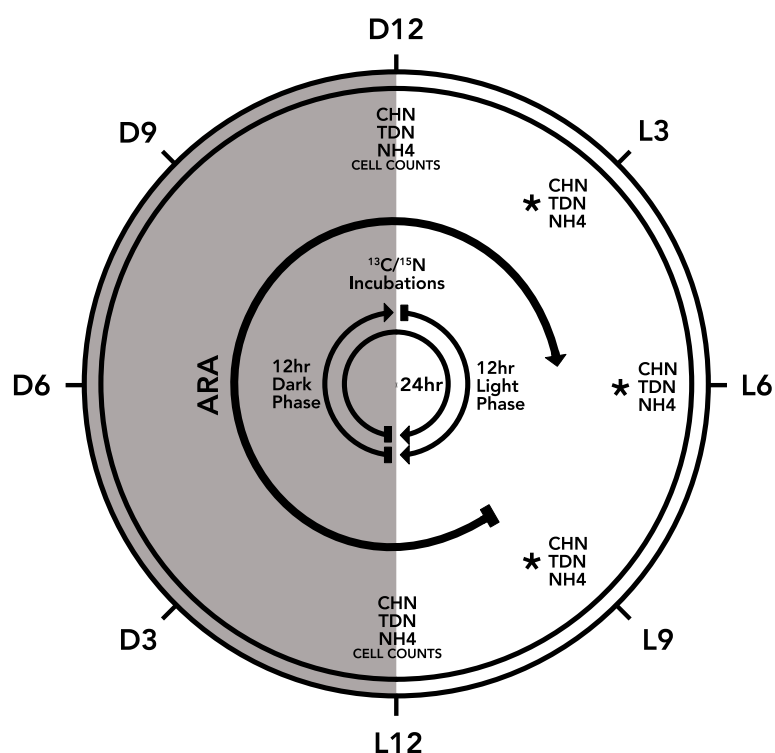
At the corresponding time points specified in Figure 3.1, the following samples were taken: cell counts, particulate C and N concentrations, total dissolved N and  $\text{NH}_4^+$  concentrations (and thus DON concentration),  $^{15}\text{N}_2$  and  $^{13}\text{C}$  incubations for determination of  $\text{N}_2$  fixation, DON and  $\text{NH}_4^+$  flux measurements, ARAs, and finally bacterial contamination verification. Corresponding methodologies for sampling and sample processing for each sample type may be found in the methodologies chapter of this thesis. Sampling for  $^{15}\text{N}_2$  and  $^{13}\text{C}$  incubations were taken with higher frequency for these experiments. Three types of incubation were performed: two 12h incubations covering both the dark phase and the light phase, and a 24h incubation, starting at the same time as the dark phase incubation, covering both the dark and light phases. These incubations were performed in triplicate (when possible) at each time point and for each culture, and this process was repeated over the entirety of the high frequency sampling phase of the experiment.

### *Analytical methodology*

Analytical methodology for the calculation of initial  $^{15}\text{N}_2$  enrichment in the medium ( $A_{\text{N}_2}$ ; Eq. 2.1), specific uptake rate of N and C into the PON and POC via isotopic enrichment ( $V_E$ ; Eq. 2.3) and bulk measurements ( $V_B$ ; Eq. 2.2), specific uptake rate of N into the dissolved fractions ( $\text{NH}_4^+$  and DON) of N via isotopic enrichment ( $V_{ED}$ ; Eq. 2.6) and bulk measurements ( $V_{BD}$ ; Eq. 2.5) and finally the volumetric rates for all 'V's above (Eqs. 2.4 and 2.7 for particulate and dissolved phases respectively), can all be found in the methods chapter of this thesis. Note, for the volumetric rates of N uptake into the  $\text{NH}_4^+$  and DON (exudation) some consideration was made to outlier treatment, specifically in instances where there was very low  $^{15}\text{N}$  enrichment in both the PON and the  $\text{NH}_4^+$  or DON; application of these values to the above equations result in impossibly large exudation rates. Upon further investigation, these values appear to be grouped below 0.2% in the enrichment of PON. To be cautious, data points in this group were set to 0, making the explicit assumption that exudation could only be negligible in these instances. The majority of these data points came from 12h light incubations.



Values for cellular (population) growth rate were calculated by fitting an exponential curve to a reconstructed population growth curve made by reconstituting the daily dilutions. These were calculated from initial inoculation of the PBRs to the end of the high frequency sampling phase. It must be noted however that the fairly low error from the curve fitting is due to the reconstruction of the growth curve. Daily cellular growth rates are subject to a degree of stochasticity and thus were somewhat more variable.



**Figure 5.1:** Diagram demonstrating sampling times for high frequency sampling phase of the nitrogen budget experiment. D refers to the dark phase (12h) and L refers to the light phase (12h). D<sub>n</sub> or L<sub>n</sub> refers to a discrete time point at the start of the nth hour of the respective phase. Where CHN, TDN, NH<sub>4</sub> or cell counts is marked, samples were taken for said parameters. Where this is coupled with an (\*), it refers to the fact that culture volume may have been too limiting at times to taken these. The centre two arrows demonstrate the three different timings for <sup>13</sup>C/<sup>15</sup>N<sub>2</sub> incubations (24h, 12h dark phase and 12h light phase). The third arrow demonstrates the timing for the acetylene reduction assays (ARAs).

## Results

### Cell size and Growth rates

Average cell diameter at the end of the light phase in the saturating light experiment was  $3.47 \pm 0.14 \mu\text{m}$  and  $3.23 \pm 0.09 \mu\text{m}$  in the sub-saturating light experiment. Average growth rate across the entire experiment was  $0.34 \pm 0.006 \text{ d}^{-1}$  in the saturating light and  $0.11 \pm 0.006 \text{ d}^{-1}$  in the sub-saturating light experiment. These growth rates were conserved in the incubations performed during the high frequency sampling period, albeit with some enhancement in the sub-saturating light ( $0.19 \pm 0.03$ ), and higher degrees of variance. Average growth rate calculated instead using growth in total POC content over the 24h incubations was  $0.29 \pm 0.13 \text{ d}^{-1}$  and  $0.14 \pm 0.13 \text{ d}^{-1}$ , This value for

the growth in total PON content over the 24h incubations was  $0.45 \pm 0.09 \text{ d}^{-1}$  and  $0.35 \pm 0.12 \text{ d}^{-1}$  for the saturating light and sub-saturating light experiments respectively.

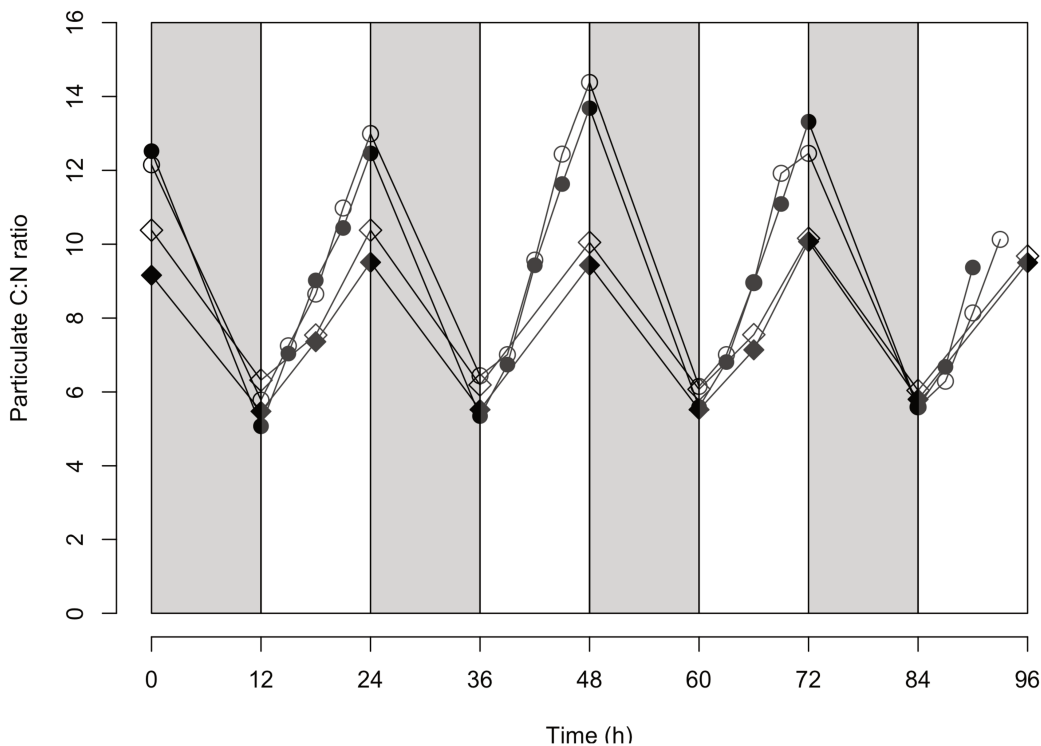
### PON and POC Cellular Content and Flux (cultures)

Cellular C was enriched at the end of the light phase and depleted at the end of the dark phase (Table 5.1). Cells from the saturating light experiment were on average significantly more enriched in C at both time points ( $p < 0.05$ ; Welch's two sample T-test) compared with the sub-saturating light condition, albeit with closer means and a larger degree of overlap at the end of the dark phase. Cellular N was enriched at the end of the dark phase, and relatively depleted at the end of the light. Cells from the saturating light experiment were again on average significantly more enriched at the end of the dark phase ( $p < 0.05$ ; Welch's two sample T-test) but not significantly different to the sub-saturating light condition at the end of the light phase ( $p = 0.053$ ; Welch's two sample T-test). Cellular C:N ratios were highly conserved between replicates and experiments, with the ratio mirroring the observed above trends in cellular C and N content. Between the two experimental enrichments, C:N was the same at the end of the dark phase, but higher at the end of the light phase in the saturating light condition by roughly 30% (Figure 5.2).

**Table 5.1:** Average POC and PON content per cell at the end of the dark and light phases as well as the average associated change over those phases. Data is divided into the values from the saturating light and sub-saturating light experiments. Data is derived from the main cultures and not the incubations. The '±' symbol denotes one standard deviation.

	Saturating Light		Sub-Saturating Light	
	Dark Phase	Light Phase	Dark Phase	Light Phase
<b>POC content (end of phase)</b> Per Cell (fmoles C cell <sup>-1</sup> )	348.35 ± 30.32	551.74 ± 53.91	319.43 ± 26.75	425.71 ± 25.62
<b>ΔPOC *</b> Per Cell (fmoles C cell <sup>-1</sup> )	203.39 ± 61.85		106.28 ± 39.26	
<b>PON content (end of phase)</b> Per Cell (fmoles N cell <sup>-1</sup> )	62.34 ± 5.82	42.57 ± 2.41	54.97 ± 1.97	41.34 ± 2.69
<b>ΔPON *</b> Per Cell (fmoles N cell <sup>-1</sup> )	19.77 ± 6.30		13.63 ± 3.33	

\* ΔPOC was negative during the dark phase and positive during the light phase. ΔPON was positive during the dark phase and negative during the light phase



**Figure 5.2:** Evolution of cellular C:N ratio over the course of both experiments. Red points and lines are from the high light experiments and blue points and lines are from the low light experiment. Circular points are from the first experimental replicate (PBR1) and diamond points are from the second experimental replicate (PBR2).

Particulate C and N uptake and loss measurements (Incubations)

*All results described here are listed summarily in Table 5.2, and come from measurements of 12h and 24h bottle incubations ( $^{13}\text{C}$ ,  $^{15}\text{N}_2$  and bulk POC and PON). To clarify, isotopic and bulk measurements were both performed within the same bottles. Where significance is mentioned, generally a Welch's two sample t-test was used.*

There was a variable observed flux of C out of the cells during the dark phase. Comparison of the two light conditions suggest a nominal but not significant ( $p > 0.05$ ) increase in the per cellular loss of POC in the saturating light condition vs. the sub-saturating light condition, which is reversed when normalising by total POC content instead (again not significant). Over the same period, we see a flux of N into the cells. Comparison of the two light conditions again shows no significant difference between the conditions per cell, for both  $^{15}\text{N}$  and bulk measurements. However, when normalising by total POC content we instead see a significant increase in the flux of N over the dark phase in the sub-saturating light experiments for the bulk measurements only.

For the light phase, there was again a variable flux of C but this time into the cells. Comparison of the two light conditions show statistically similar rates ( $p > 0.05$ ) in per cell activity for the  $^{13}\text{C}$  measurements, but a significantly increasing rate in the bulk measurements ( $p > 0.05$ ). Normalising instead by total POC shows similar rates in the bulk measurements but a significantly higher rate in

the <sup>13</sup>C measurements. For N, we see a continuation of N acquisition into the light phase for the saturating light experiment only. This constituted a large portion of N acquisition: 15-40% of acquisition depending on the normalisation used and method used. For the sub-saturating light experiment, there was no such continuation into the light phase, instead seeing only a small gain or loss when accounting for the variance. Total N acquisition was significantly greater in the saturating light experiment, but only by a small amount. When normalising by C content however, there appeared to be a statistically similar rate, if not slightly higher in the sub-saturating light experiment.

**Table 5.2:** Average per cell and per unit POC values for the flux of PON and POC into and out of the cells. Data is divided into the values from the high light and low light experiments and further by dark phase, light phase and 24h periods. Furthermore, values are calculated via either isotopic incubation (<sup>15</sup>N<sub>2</sub>) or changes in bulk N measured concurrently in the incubations. Values in bold were found to be significantly different to equivalent values in the opposing experimental condition (Welch Two Sample t-test; p < 0.05).

	Saturating Light			Sub-Saturating Light		
	Dark Phase	Light Phase	24h	Dark Phase	Light Phase	24h
<b>POC flux</b> Per Cell (fmoles C cell <sup>-1</sup> d <sup>-1</sup> )						
<sup>13</sup> C	-	118.64 ± 18.77	-	-	128.30 ± 9.51	-
Bulk Carbon*	-131.55 ± 48.92	<b>167.72 ± 27.54</b>	<b>36.17 ± 56.14</b>	-119.43 ± 26.99	<b>128.64 ± 29.92</b>	<b>9.49 ± 40.34</b>
<b>POC adjusted</b> (nmoles C per μmol C d <sup>-1</sup> )						
<sup>13</sup> C	-	<b>225.62 ± 44.4</b>	-	-	<b>332.98 ± 36.62</b>	-
Bulk Carbon*	-282.93 ± 115.02	305.59 ± 79.67	22.66 ± 139.92	-348.15 ± 90.88	338.13 ± 61.29	-10.02 ± 109.61
<b>PON flux</b> Per Cell (fmoles cell <sup>-1</sup> d <sup>-1</sup> )						
<sup>15</sup> N <sub>2</sub> †	2.93 ± 1.23	2.50 ± 1.91	5.43 ± 1.46	4.06 ± 1.50	0.57 ± 3.06	4.63 ± 2.67
Bulk Nitrogen †	13.19 ± 3.22	<b>5.77 ± 3.97</b>	<b>18.96 ± 2.33</b>	15.66 ± 2.2	<b>-1.26 ± 5.48</b>	<b>14.40 ± 5.01</b>
<b>POC adjusted</b> (nmoles N per μmol C d <sup>-1</sup> )						
<sup>15</sup> N <sub>2</sub> †	5.96 ± 2.07	3.23 ± 2.97	9.19 ± 2.12	11.08 ± 4.17	0.72 ± 7.61	11.80 ± 6.37
Bulk Nitrogen †	<b>27.70 ± 6.90</b>	<b>5.58 ± 7.29</b>	33.22 ± 2.35	<b>41.84 ± 5.57</b>	<b>-4.77 ± 13.77</b>	37.07 ± 12.59

\* Dark phase and Light phase are recorded values, '24h' is net mean values of both combined

† Total and Dark phase are recorded values, Light phase is derived by subtracting Dark phase from total, as recorded Light phase values were unreliable due to slow equilibration of <sup>15</sup>N<sub>2</sub> gas in this incubation type

### Nitrogenase activity (ARA)

For the saturating light experiment, the commencement of N<sub>2</sub> fixation was observed at ~D2, with the rate increasing rapidly from this point. The rate began to plateau at about D6, maintaining maximal rate for ~2hrs, and dropping slowly in rate after this. Fixation appeared to cross over into the light phase and stopped at around L3, with light phase fixation appearing to constitute roughly  $8.2 \pm 2.5\%$  of total fixation. For the sub-saturating light experiment, the start of the N<sub>2</sub> fixation period was instead at ~D3. Maximal rate was reached at ~D7 and in most cases began to reduce again in rate after <1-2h. Fixation barely crossed into the light phase, with the rate stopping no later than 1hr after the lights went on (D12 - L1). Only ~2% of total fixation occurred during the light.

### DON and NH<sub>4</sub><sup>+</sup> exudation measurements

*All results described here are listed summarily in Table 3x, and come from measurements of 12h and 24h bottle incubations (<sup>15</sup>N<sub>2</sub> and bulk NH<sub>4</sub><sup>+</sup> and DON). To clarify, isotopic and bulk measurements were both performed within the same bottles, which were incidentally the same incubations as used above in the POC and PON section. Where significance is mentioned, generally a Welch's two sample t-test was used.*

NH<sub>4</sub><sup>+</sup>: Bulk exudation of NH<sub>4</sub><sup>+</sup> was within the order of magnitude of ~250-2800 attomoles N cell<sup>-1</sup> per incubation. Between the two experiments, there appeared to be a significantly larger exudation on average in the sub-saturating light experiment ( $p < 0.05$ ). In general there was a large degree of variability in all measurements as evidenced by the standard deviations, although it would appear that there is generally net exudation of NH<sub>4</sub><sup>+</sup> in all samples. <sup>15</sup>N results show a total exudation 1-3 orders of magnitude lower than bulk. Between experiments, there appeared to be similar rates of exudation in total, albeit with higher variability in the sub-saturating light. Compared with the bulk results, there appeared to be clear temporal signals, with practically all exudation occurring in the light phase in the saturating light experiment, and the majority (80%) of exudation occurring in the light in the sub-saturating light experiment.

DON: From the bulk measurements, it would appear that for most incubations there was a net loss of DON from the medium, termed here as 'uptake' (into the cells) for simplicity. Exudation to the medium occurred only in some 24h incubations in the saturating light experiment, although incubations from all types had instances of very low DON uptake. On average, DON loss from the medium in the sub-saturating light experiment was 4-5 times that of the saturating light experiments

and 8-9 times when instead normalising by POC. Variability was very large for these measurements, but there was a far larger range of values in the sub-saturating light experiment, and as such DON uptake was found to be significantly greater in said experiment ( $p < 0.05$ ). When factored in to above bulk N uptake measurements, it would appear that on average, *Crocospaera watsonii* cells took up **7% and 42%** of their total N as DON over a 24h period (saturating and sub-saturating light experiments respectively).  $^{15}\text{N}$  results show instead exudation of similar orders of magnitude to  $^{15}\text{N}$ - $\text{NH}_4^+$  exudation, albeit significantly higher. Total DON exudation was significantly greater in the saturating light, with almost double the exudation. Furthermore, there was a larger exudation in the light phase for the saturating light experiment, with  $\sim 75\%$  of exudation occurring in the light as opposed to  $\sim 55\%$  in the sub-saturating light experiment.

**Table 5.3:** Average per cell and per unit POC values for the flux of DON and  $\text{NH}_4^+$  into and out of the cells. Data is divided into the values from the high light and low light experiments and further by dark phase, light phase and 24h periods. Furthermore, values are calculated via either isotopic incubation ( $^{15}\text{N}_2$ ) or changes in bulk N measured concurrently in the incubations. Values in bold were found to be significantly different to equivalent values in the opposing experimental condition (Welch Two Sample t-test;  $p < 0.05$ ).

	Saturating Light			Sub-Saturating Light		
	Dark Phase	Light Phase	24h	Dark Phase	Light Phase	24h
<b><math>\text{NH}_4^+</math> flux</b> Per Cell (attomoles cell <sup>-1</sup> d <sup>-1</sup> )						
$^{15}\text{N}_2^*$	<b>0.55 ± 0.61</b>	23.85 ± 8.58	24.40 ± 8.56	<b>4.158 ± 3.76</b>	16.442 ± 17.24	20.60 ± 16.82
Bulk Nitrogen †	<b>576.00 ± 371.54</b>	540.19 ± 223.87	<b>671.54 ± 377.44</b>	<b>1410.61 ± 790.62</b>	999.70 ± 795.75	<b>1723.46 ± 1114.89</b>
<i>POC adjusted</i> (fmoles N per μmoles C d <sup>-1</sup> )						
$^{15}\text{N}_2^*$	1.22 ± 1.35	42.88 ± 16.59	44.10 ± 16.54	11.76 ± 11.02	40.72 ± 43.86	52.48 ± 42.45
Bulk Nitrogen †	<b>1159.26 ± 747.91</b>	1271.22 ± 416.28	<b>1085.11 ± 724.48</b>	<b>3865.84 ± 2146.28</b>	2837.03 ± 2382.93	<b>4432.99 ± 2912.22</b>
<b>DON flux</b> Per Cell (attomoles cell <sup>-1</sup> d <sup>-1</sup> )						
$^{15}\text{N}_2^*$	42.57 ± 40.18	<b>140.71 ± 50.01</b>	<b>183.28 ± 29.77</b>	40.26 ± 19.08	<b>58.27 ± 54.07</b>	<b>98.54 ± 50.59</b>
Bulk Nitrogen †	<b>-1843.95 ± 1614.12</b>	-355.58 ± 1408.08	<b>-1377.25 ± 1494.62</b>	<b>-9955.22 ± 9370.73</b>	-7676.57 ± 7643.93	<b>-5899.41 ± 5564.90</b>
<i>POC adjusted</i> (fmoles N per μmoles C d <sup>-1</sup> )						
$^{15}\text{N}_2^*$	<b>3.44 ± 3.65</b>	17.55 ± 7.57	20.99 ± 6.63	<b>5.35 ± 3.68</b>	11.95 ± 9.19	17.30 ± 8.43
Bulk Nitrogen †	<b>-3849.58 ± 3221.74</b>	-2735.41 ± 554.55	<b>-1734.97 ± 2509.62</b>	<b>-26545.39 ± 25394.81</b>	-20610.59 ± 20328.30	<b>-15181.95 ± 14424.54</b>

\* Total and Dark phase are recorded values, Light phase is derived by subtracting Dark phase from total, as recorded Light phase values were unreliable due to slow equilibration of  $^{15}\text{N}_2$  gas in this incubation type

† All values are recorded values

## Synthesis and Discussion

The main aim of the experiments detailed here was to ascertain the effects of different light conditions on the daily budgets of C and N for cultures of *Crocospaera watsonii* (WH8501). As a diazotrophic cyanobacterium, *Crocospaera watsonii* directly links C and N acquisition with diel rhythmicity by fixing C in the day, and N at night. Higher intensity light fuels higher rates of C acquisition through photosynthesis, and this has been proven to be true in *Crocospaera watsonii* [Rabouille et al., 2017]. It is however unclear the effect this may have on N metabolism in this cyanobacteria. While it can be reasonably posited that a higher rate of C fixation and storage should theoretically stimulate larger rates of N<sub>2</sub> fixation and sustain higher growth rates, it is uncertain how this will affect cellular processes which interact with the dissolved phase of N, i.e. exudation and uptake.

With these factors in mind, we raise a number of questions regarding the effect that saturating and sub-saturating light intensities [Rabouille et al., 2017] may have on the budgets of C and N in *Crocospaera watsonii*. Firstly, we seek to identify the effect of said light conditions on the growth rate of cells, POC and PON content within the cultures to determine if greater light availability translates quantifiably into higher rates of growth for these parameters. Next, we consider the effect of these light conditions on cell size and average per cell C and N stoichiometry to determine the effect on cellular storage. The effect of light on the flux of C (i.e. respiration processes, photosynthesis, exudation; not individually parameterised) and N into and out of the cell was next assessed using the specific uptake rate equations [Montoya et al., 1996], and were quantified using both bulk changes in POC, PON, DON and NH<sub>4</sub><sup>+</sup>, well as uptake of <sup>15</sup>N into the biomass and dissolved phases. These rates were compared to determine the proportion of flux of dissolved N to the rates of flux of PON, again considering the effect that light has on these proportions. Our results show that when considered in the context of growth of the whole culture, C and N acquisition was indeed enhanced in saturating light conditions compared to sub-saturating light, but when normalised per cell, *Crocospaera watsonii* WH8501 does not drastically alter its C and N acquisition efficiency, with significant but minor uptake increase in the saturating light experiment. However, when flux of dissolved N is factored in, we instead see a drastic change in acquisition strategy in the sub-saturating light scenario, with cells relying on DON for ~42% of the N uptake, compared to ~7% in the saturating light. Finally, we see a notable disparity in dissolved N flux between recently fixed N (measured by <sup>15</sup>N uptake) compared with bulk exudation: in general bulk

N was exuded at a higher rate in the form of  $\text{NH}_4^+$ , with DON generally showing net uptake, whereas recently fixed N tended to be exuded at much lower rates, but with more DON exuded than  $\text{NH}_4^+$ . We discuss these findings in detail below.

### *Irradiance, culture growth and cellular size and elemental composition*

Whole culture population growth rate data as well as incubation specific population growth rate data showed a definitive increase in cellular production in the saturating light experiment compared with the sub-saturating light experiment. POC- and PON-related growth in the incubations also demonstrated an increase for the saturating light condition. This is further demonstrated by the larger relative size at the end of cell division ( $\sim 3.40 \mu\text{m}$  and  $3.20 \mu\text{m}$  respectively) and observed change in per cell C content over the light phase, where we observe increasing C content per cell, again despite cell division. This demonstrates a clear bulking up of C reserves, most likely in order to fuel energetically costly processes during the dark phase, i.e.  $\text{N}_2$  fixation. Between the two light conditions, C reserve production was greatly enhanced in the saturating light experiment: if we consider cellular C content at the end of the dark phase as a rough 'baseline' post- $\text{N}_2$  fixation C content per cell, we observe roughly double the increase in C content per cell for the saturating light experiment ( $\sim 200$  vs  $\sim 100$  femtomoles C cell $^{-1}$ ; Table 5.1). This mirrors what was previously seen for *Crocospaera watsonii* in Rabouille et al. [2017], where average per cellular carbohydrates and POC increased with irradiance. This increase plateaued at half the irradiance used in this study for the saturating light experiment ( $\sim 400$  vs  $\sim 200 \mu\text{Einsteins m}^{-2} \text{ s}^{-1}$ ).

N acquisition in the saturating light experiment had greater average rate ( $\sim 0.45$  vs  $\sim 0.33 \text{ d}^{-1}$ ), although a high degree of variance suggested that these values were not significantly different. Despite this, growth in N in the saturating light experiment had a higher upwards range, suggesting potential enhancement in N acquisition due to greater irradiance, albeit not consistently. In addition, we observed a greater upwards enrichment in per cell N at the end of the dark phase. The relatively small distance between these average values suggest that sub-saturating light may not greatly affect net N acquisition, and potentially that *Crocospaera watsonii* can operate at similar N efficiency despite less energy available. This is likely due to the observed change in N acquisition strategy in the sub-saturating light experiment; on average, roughly 42% of N acquisition could potentially attributed to DON, as significant flux of DON out of the culture medium was observed, which we attribute to DON uptake. This result is notable, as while dissolved N uptake replacing portions of N acquisition in the stead of  $\text{N}_2$  fixation is a common assumption in diazotrophs, DON is not often



considered as a significant N source for diazotrophs.  $\text{NH}_4^+$  and  $\text{NO}_3^-$  are more often considered, but despite this, our recent work [Randall et al., in prep; chapter 3] suggests that  $\text{NO}_3^-$  may not be important for the strain of *Crocospaera* used in both experiments. Said study was however not performed at sub-saturating light conditions as was the present experiment, and the results of both this study, and another which considered light as a factor for  $\text{NO}_3^-$  uptake in *Crocospaera watsonii* [Garcia and Hutchins, 2014] both reveal light as an important factor in  $\text{N}_2$  fixation replacement. This may suggest that the strain used in both the present study and in Randall et al. [in prep; chapter 3] may respond differently to  $\text{NO}_3^-$  if subjected to lower irradiance. Despite this, we observe a relatively greater use of DON in even the saturating light experiment than what was observed in the  $\text{NO}_3^-$  consumption experiment presented in Randall et al. [in prep; chapter 3]. Our result of enhanced DON uptake in sub-saturating light, along with the result of Garcia and Hutchins [2014] suggests quite an important shift in perspective of how diazotrophs may interact supplement their N needs with dissolved N: instead of preferentially uptake lower energetically costly N sources, it could indeed be that  $\text{N}_2$  fixation is regulated by light availability, with dissolved N used to supplement and regulate N uptake. A subtle, but important distinction.

#### *POC and PON cellular rates of uptake and loss*

In the above results section, within table 5.2, we outline a range of results using various methods of calculation for fluxes of N and C. For POC and PON we use measurements of  $^{13}\text{C}$  and  $^{15}\text{N}$  uptake and bulk changes in POC and PON content derived from the same incubations to give two measurements for N flux. Without accounting for fluxes in dissolved fractions of N and C, the latter can be considered net uptake of N and C, whereas the former can be considered net uptake of  $\text{N}_2$  [Montoya et al., 1996]. It must be however noted that by percentage, we determined underestimation in the  $^{15}\text{N}_2$  uptake measurements of ~40-80% when compared with bulk uptake of PON and DON, which is detailed in the companion paper Randall et al., [in prep; chapter 4]. Despite being relatively large by percentage, these results are generally not particularly unreasonable when considering the range of measured values, and in fact when accounting for DON uptake, they are not significantly different in the sub-saturating light experiment. However, these values were always underestimates, and must be considered as such. Still, they may at least be considered as a minimum rate of  $\text{N}_2$  fixation, as  $^{15}\text{N}$  uptake isolates the N uptake rate from other sources such as DON. For the assessment of POC and PON acquisition budgets, we decided to place greater consideration on values derived from the bulk uptake method; these measurements

involved require far fewer assumptions and conversion factors to calculate a final rate, and were thus deemed more trustworthy for the purpose of this study..

When viewed together, it would appear that there was on average a significantly greater (30%) net acquisition (bulk measurements) of carbon over the light phase in the saturating light experiment, confirming that higher irradiance led to greater uptake of carbon. Net loss over the dark phase was significantly similar between conditions, suggesting a similar scale of metabolic activity. N acquisition was, despite having overlapping ranges of rates, also significantly greater by 30% over the diel cycle (24h). N acquisition also saw that in the saturating light experiment, N uptake extends somewhat more prominently into the light phase (Table 5.2), which was barely seen in the sub-saturating light experiment. This extension into the light phase appears to drive the higher rate of N acquisition in the saturating light experiment. The same activity is seen when comparing this to ARA results for the same experiments: we indeed see a continuation of nitrogenase activity into the light for the saturating light experiment, but not for the sub saturating light. However, this fraction is not quite identical, with ARA suggesting that only ~8% of total fixation occurred in the light phase. This difference is quite significant, and likely due to the large differences in conditions between the two incubation methods. One particular issue may be the supply of gas. In the ARA, fresh gas, most notably oxygen, is pumped continuously into the incubation cell. However, during the isotopic incubations, the culture completely isolated from the atmosphere. Oxygen is therefore likely to be more scarce, especially due to the high rate of respiration over the dark phase. This could mean lower suppression of nitrogenase during the early hours of the light phase and thus a higher rate of N<sub>2</sub> fixation during this time.

The lack of difference in N acquisition budgets between the two experiments interestingly mirrors what we observed in the per cell N stoichiometry; that per cell, uptake rate of N is similar despite lower irradiance, and that this is done by altering N acquisition strategy. Furthermore, ratios between average saturating and sub-saturating light values for 24h PON growth rate, and both uptake measurements (<sup>15</sup>N and bulk) were roughly the same (~1.25, ~1.30 and 1.17 respectively), with no significant difference for any measurement type between the two conditions. It is however puzzling when viewed per cell, despite almost double POC growth rate in the saturating vs sub-saturating light experiment, and similarly greater C storage per cell, we observe identical C uptake rates in the light phase in the <sup>13</sup>C measurements and only ~1.3 times the C uptake rate in the bulk (not significantly different). This difference is however easily explained by the fact that cellular

division occurred during the light phase, meaning that because of the higher growth rate in the saturating light experiment, values were divided by a larger amount of cells thus reducing in appearance the amount of C fixed per cell. C and N uptake was however very similar to the average  $\Delta\text{POC}$  and  $\Delta\text{PON}$  per cell (Table 5.1) for both experiments, which was a good sign that C and N uptake measurements in the bottles is a decent proxy to represent culture proper cellular activity.

#### *Flux of $\text{NH}_4^+$ and DON in and out of the biomass*

As with before, two methods were used in the calculation of  $\text{NH}_4^+$  and DON fluxes into and out of the cell. Both results come from isotopic incubation filtrates, using both isotopic enrichment in the various dissolved N phases as well as assessing evolutions in bulk concentrations of dissolved N. These were the same incubations as used for the POC and PON results discussed above. As opposed to the budget assessment of the PON above, where methods of calculation were somewhat at odds, it is important here to consider both the bulk and  $^{15}\text{N}$  enrichments. That is because they communicate different things. The bulk method shows the overall net flux of DON or  $\text{NH}_4^+$  out of the cell whereas the  $^{15}\text{N}$  enrichment method shows net flux *specific* to freshly fixed N from the day. A note of warning for this section: many of the values listed here show a high degree of variance. *Crocospaera watsonii* is in general a slow growing cyanobacteria with a high degree of variability to its biological functions. In addition, as it would seem that  $\text{NH}_4^+$  and DON appear to flux into and out of the cell at an order of magnitude similar to the size of their external pools, and the timescale of these fluxes is not known, the variability may be due to temporal stochasticity in behaviour throughout the diel cycle. However, especially with the  $^{15}\text{N}_2$  incubations, definite signals were detected and as such, valuable information can be derived from these measurements.

We clearly observed net accumulation in the  $\text{NH}_4^+$  concentration within the filtrate over the incubations, potentially suggesting exudation by *Crocospaera* cells. Both bulk and  $^{15}\text{N}$  data support this, albeit to a much smaller degree in the  $^{15}\text{N}$ . Furthermore, we observed DON loss from the medium with some cases of exudation in the saturating light experiment, and to a smaller degree presence of  $^{15}\text{N}$ -DON in the medium. This can be interpreted as DON flux both into and out of the cell. For bulk results, we noted no clear temporal patterns in exudation of  $\text{NH}_4^+$  or uptake of DON. Per incubation (dark, light, and 24h) results were similar, and combined values from the light and dark phases did not result in similar exudation/uptake to the 24h incubations. This may suggest either altered metabolism in these two incubation types compared with the 24h, or potentially relatively constant cycling of uptake and exudation. Furthermore, the standard deviation of values

for each were often close to 100% of the average value. This suggests highly variable rates of uptake and exudation which may not be coupled to the diel cycle. Interestingly, over the 24h incubations we observed both greater average exudation of  $\text{NH}_4^+$  (~12% v.s. ~4%) and greater average DON uptake (~42% v.s. ~7%) relative to the bulk N production in the sub-saturating light experiment, despite roughly greater acquisition of N in the saturating light experiment. These results may suggest that DON utilisation may be less efficient than  $\text{N}_2$  fixation, resulting in more waste removal. Furthermore, in sub-saturating light environments, exudation may be utilised to 'feed' other cells in the culture. Both these ideas would have to be substantiated with more direct evidence, however. We must note that there is a possibility that the observed DON decrease and  $\text{NH}_4^+$  increase may be a result of photoammonification [Jeff et al., 2012]. We however note that while observed ammonium release may be the result of said process, ammonium build up and DON removal was detected in the dark phase as well as the light at similar rates. Furthermore, DON removal was generally at a much higher rate than  $\text{NH}_4^+$  release, although this may be being taken up continually, reducing the  $\text{NH}_4^+$  concentration as time passes. Finally, DON uptake and  $\text{NH}_4^+$  release were enhanced in the sub-saturating light experiment, despite photoammonification of DON generally being considered to be enhanced with higher irradiance. These factors suggest that a large fraction of DON uptake and  $\text{NH}_4^+$  release is biologically mediated.

Despite seeing a lack of temporal rhythmicity in the bulk measurements,  $^{15}\text{N}$  measurements of the dissolved phase showed much clearer results, albeit at a smaller order of magnitude. Smaller rates of exudation in the dissolved phase suggest a preference for recently fixed N for somatic growth. However both DON and  $\text{NH}_4^+$  appeared to be exuded out of cells, as there were small but clear signals of  $^{15}\text{N}$  in both phases. In general, DON was exuded more than  $\text{NH}_4^+$  (5-10 times), suggesting recently fixed N goes through an indeterminate number of steps before being released, with less being released immediately as  $\text{NH}_4^+$ . DON was exuded at a higher rate in the saturating light experiment (2 times) but  $\text{NH}_4^+$  exudation was roughly the same in both per cell, albeit with more variability in the sub-saturating light experiment. Higher rates of DON release in the saturating light experiment would appear to be a natural consequence of higher rates of  $\text{N}_2$  fixation, although it would seem that the proportion of recently fixed N which is released as DON is roughly similar (3% and 2% for the saturating and sub-saturating light experiments respectively).  $\text{NH}_4^+$  release was roughly 0.4% of total recently fixed N for both experiments. If this  $\text{NH}_4^+$  is released immediately after  $\text{N}_2$  fixation, it could be considered a background rate of inefficiency of incorporation. However, we must note again that high lability of  $\text{NH}_4^+$  may mean that the turnover time of  $\text{NH}_4^+$  in

the cultures is much faster than the observed incubation periods. Still, a positive accumulation of  $\text{NH}_4^+$  in both the bulk and isotopic measurements suggests a rate of exudation greater than the rate of uptake. It is however, odd, that DON is exuded at a higher rate than  $\text{NH}_4^+$  when considering  $^{15}\text{N}$  results, but fluxes in the opposite direction when considering the bulk data. While it is uncertain why this occurs, it may be that recently fixed DON exudate is of a less labile form than bulk DON, which is taken up preferentially, or exclusively.

While the reason for recently fixed DON and  $\text{NH}_4^+$  exudation is uncertain, for both, we observed greater exudation during the light phase, albeit with a higher proportion of net exudation in the light phase seeming to occur in the saturating light experiment for both  $\text{NH}_4^+$  (20% v.s. 3%) and DON (45% v.s. 25%). Higher rates of light phase exudation of DON indicate that exudation is likely related to a process which occurs during the light, such as photosynthesis or cell division. This could be waste release, RNA signalling, extracellular proteins, dissolved free amino acids, etc. In particular, this may also answer the question as to why there is a net release of isotopically labelled DON but a net uptake of DON in the bulk; as DON released here may be functional.

It must be noted that for both the dissolved  $^{15}\text{N}$  analyses here, the values listed are potentially underestimates. To explain, theoretically, all dissolved  $^{15}\text{N}$  found in the dissolved N pool, be it  $\text{NH}_4^+$  or DON, must first pass through the cellular PON pool. As such, the 'initial enrichment' used is an average of the enrichment of  $^{15}\text{N}$  in the cellular PON pool. This is accumulating during the same period as exudation is occurring, and it is not established at the beginning of the incubation. If exudation is occurring constantly, or is front-loaded at the beginning of the incubations, then a large portion is likely to be missed by these incubations. Furthermore, cycling of uptake and release would naturally act to dilute the  $^{15}\text{N}$  signals. As such, the values listed here could be considered minimums rather than the true value. More work needs to be done on the temporality of dissolved N flux, as well as the specificity of DON molecules are released.

## Conclusion

In this work, we performed a number of biogeochemical analyses on cultures of *Crocospaera watsonii* (WH8501) in order to better elucidate the C and N budget of the aforementioned strain of diazotroph. In particular, we focussed on the effect of vastly different light intensities (~60 & 400  $\mu\text{Einsteins m}^{-2} \text{ s}^{-1}$ ) on the various fluxes of N into and out of the cell. These specific light intensities were chosen to represent saturating light conditions and sub-saturating light conditions [Rabouille et al., 2017]. As well as C and  $\text{N}_2$  fixation, the flux of  $\text{N}_2$  into the cell, the flux of dissolved N ( $\text{NH}_4^+$  & DON) into or out of the cell were measured in cultures at different time points over multiple days.

As would be expected, the growth of both population and cell biomass (POC) were demonstrably greater in the saturating light experiment, and this translated to a larger rate of N acquisition through  $\text{N}_2$  fixation. The results of this study reveal however that, while highly variable, when normalised per cell the budgets of POC and PON are conserved despite disparate light saturation between the two experiments. The only difference was that there was observed continuation of  $\text{N}_2$  fixation into the light phase in the saturating light experiment only.

Factoring in the dissolved N budgets, however, augmented the story somewhat. Isotopic incubation results suggested that there may be an exudation of recently fixed N up to ~3.5%, with the majority of this being DON. However, using bulk measurements instead revealed that while extremely variable due to the lability of  $\text{NH}_4^+$ , 3-12% of recently acquired N may be exuded as  $\text{NH}_4^+$  over a full diel cycle, with potentially a greater percentage in low light conditions. Additionally, there was also a net removal of DON from the medium in most (but not all) incubations. If this is indeed up-taken by the cells, it would suggest that *Crocospaera watsonii* WH8501 is actively using DON to supplement its N costs. This was observed far more prevalently in the the sub-saturating light experiment, suggesting that in more limiting light conditions *Crocospaera watsonii* WH8501 may depend more upon less energetically costly N sources. This also presents a potential shift in perspective regarding how diazotrophs may supplement their N costs with dissolved N;  $\text{N}_2$  fixation is regulated by light availability, with less energetically costly N sources used to supplement and regulate N cost. This is an important distinction to the assumed paradigm that diazotrophs should prefer less energetically costly N sources to diazotrophy.

Finally, smaller proportional exudation of recently fixed dissolved N in comparison to bulk measurements suggests that recently fixed N is favoured for somatic growth and that the bulk of exudation comes from waste removal.

## General Conclusions of the Thesis:

The aims at the start of this PhD project was to improve our understanding of the N budgets of the unicellular cyanobacterial diazotroph *Crocospaera watsonii*, including the characterising and quantifying of its various interactions with dissolved N. This was done in effort to expand our knowledge of how global estimates of rates of marine N<sub>2</sub> fixation may be underestimated, a poignant source of debate in the field of marine biogeochemistry [e.g. Codispoti 2007; Eugster and Gruber, 2012]. We began by compiling an expansive literature review as to how our understanding of diazotrophy, and the inefficiencies of our methodology, may be affecting our global rate estimates. From this, we formulated a number of working questions which sought to further our initial aims through the framework established in the literature review. These questions were as follows:

- 1) How does *Crocospaera watsonii* interact with NO<sub>3</sub><sup>-</sup>? (Chapter 3)
- 2) How does irradiance affect *Crocospaera watsonii*'s C and N budget, including flux of DON and NH<sub>4</sub><sup>+</sup>? (Chapter 5)
- 3) What do our measurements say about the relative accuracy and reasonability of commonly used N<sub>2</sub> fixation assays? (Chapter 4)

Our empirical findings for the above research questions are reported in their respective chapters. We synthesise these findings here with respect to our initial thesis aims:

- 1) How does *Crocospaera watsonii* interact with NO<sub>3</sub><sup>-</sup>?
  - a) ***Crocospaera watsonii* were *not* found to uptake significant quantities of NO<sub>3</sub><sup>-</sup>:** Despite common understanding that marine diazotrophs tend to prefer less energetically costly N sources (e.g. NO<sub>3</sub><sup>-</sup>) to diazotrophy when available, there was only minor disappearance of NO<sub>3</sub><sup>-</sup> from cultures incubated long term with it. NO<sub>3</sub><sup>-</sup> lost from the medium could account for <2% of estimated N acquisition based on culture growth. Furthermore, isotopic uptake of <sup>15</sup>NO<sub>3</sub><sup>-</sup> into the biomass was found to be minor compared with uptake of <sup>15</sup>N<sub>2</sub>, and when biomass was rinsed with sterilised seawater, isotopic enrichment reduced, suggesting that adsorption onto the cell may be misrepresenting these rates of uptake.
  - b) **Rates of N<sub>2</sub> fixation by *Crocospaera watsonii* were *not* affected by incubation with NO<sub>3</sub><sup>-</sup>:** Again, despite common understanding that diazotrophy tends to be inhibited by NO<sub>3</sub><sup>-</sup>,



comparisons of N<sub>2</sub> fixation rates of cultures from otherwise identical conditions showed that there was no significant difference in N<sub>2</sub> fixation rate between NO<sub>3</sub><sup>-</sup> enriched and obligate diazotrophy cultures.

- c) **Cellular growth rate and physiology were not affected by NO<sub>3</sub><sup>-</sup>:** Population growth rate, cell size, cellular C:N ratio, rate of C fixation and C:N fixation rate were statistically similar between the two experimental conditions. NO<sub>3</sub><sup>-</sup> could not be said to enhance, inhibit or affect any of the measured parameters.

2) How does irradiance affect *Crocospaera watsonii*'s C and N budget, including flux of DON and NH<sub>4</sub><sup>+</sup>?

- a) ***Crocospaera watsonii* cell population growth rate and C acquisition rate over the diel cycle were enhanced (by 2-3) in saturating light conditions compared with sub-saturating, but N acquisition rate was statistically similar between conditions:** While N acquisition was slightly enhanced on average ( $0.45 \pm 0.09 \text{ d}^{-1}$  v.s.  $0.35 \pm 0.12 \text{ d}^{-1}$ ), the difference was not significant. This suggests that N acquisition is a priority for *Crocospaera watsonii*.

- b) **Per cellular C and N content was significantly greater in the saturating light experiment at almost all time points:** Cells tended to be more enriched in C in the saturating light experiment, and especially after the light phase, where there was almost double the accumulation of C storage in the cells compared with the sub-saturating light experiment. N was somewhat enhanced at the end of the dark phase in the saturating light experiment, but N at the end of the light phase after cellular division had occurred was identical between conditions. C:N ratio was also highly conserved between experiments, with the exception that there was a higher C:N ratio due to high C content at the end of the light phase in the saturating light experiment. This points towards tight regulation of N acquisition.

- c) **Per cellular C and N acquisition rates (from elemental analysis of incubations) suggest enhanced uptake in the saturating light experiment:** Higher light led to significantly greater net uptake of N and C over the diel cycle, but C loss (via respiration, DOC loss, etc.) over the dark phase was not significantly different between treatments. Higher N acquisition in the saturating light experiment seems to be driven by an extension of N acquisition into the light phase, which wasn't seen in the sub-saturating light experiment.

- d) **N acquisition appears to be supplemented by DON uptake, the rate of which was significantly greater in the sub-saturating light experiment:** DON loss from the medium (bulk measurements) could account for ~42% of observed N acquisition in the sub-saturating light experiment, whereas it could only account for ~7% in the saturating light experiment. This suggests that when in lower energy environments, *Crocospaera watsonii* depends more on non-diazotrophic sources of N.
- e) **NH<sub>4</sub><sup>+</sup> exudation (bulk measurements) was relatively enhanced sub-saturating light experiment:** NH<sub>4</sub><sup>+</sup> exudation was 12% of total N acquired in the sub-saturating light experiment compared with 4% in the saturating light experiment.
- f) **Measurements of <sup>15</sup>N enrichment of DON and NH<sub>4</sub><sup>+</sup> following <sup>15</sup>N<sub>2</sub> incubations showed greater enrichment in the DON compared with NH<sub>4</sub><sup>+</sup>:** This could suggest that recently fixed N is preferentially exuded as DON, for example in the form of proteins, RNA signalling, etc. However, this is not certain, as interactions with dissolved N are not rigorously defined in *Crocospaera watsonii*, and our analyses were not specific enough to be certain.
- g) **Overall, around 2-3% of recently fixed, <sup>15</sup>N enriched N could be found in the dissolved phase post incubation:** Proportions of recently fixed N were the same over both incubations for NH<sub>4</sub><sup>+</sup> (0.4%) and very similar for DON (3% v.s. 2% for the saturating light and sub-saturating light experiments respectively).
- 3) What do our measurements say about the relative accuracy and reasonability of commonly used N<sub>2</sub> fixation assays?
- a) **<sup>15</sup>N<sub>2</sub> incubations consistently underestimate N<sub>2</sub> fixation:** Comparison of N<sub>2</sub> fixation measurements derived from <sup>15</sup>N enrichments and bulk uptake of N measured by elemental analysis of the **same** incubations demonstrated consistent underestimation of N<sub>2</sub> fixation. This was true, even when accounting for dissolved N fluxes. Within the sub-saturating light experiment, DON uptake was high, and thus this underestimated values were statistically similar to 'bulk' N uptake rates from the same incubations, however this could not be said for the sub-saturating light experiment.
- b) **Underestimation of N<sub>2</sub> fixation was correlated with V<sub>E</sub>:** Specific uptake rate of <sup>15</sup>N (V<sub>E</sub>) was positively correlated with increasingly accurate estimations of N<sub>2</sub> fixation. This

suggests that inefficiency of equilibration of  $^{15}\text{N}_2$  gas may be affecting to some degree the accuracy of estimates.

- c) **ARA results consistently overestimate  $\text{N}_2$  fixation:** When using a  $\text{C}_2\text{H}_2:\text{N}$  conversion ratio of 4,  $\text{N}_2$  fixation estimates derived from nitrogenase activity measurements were almost always grossly overestimated when compared with bulk uptake of N.
- d)  **$^{15}\text{N}_2$  incubation results were more reasonable than ARA results:** Estimates were much closer to bulk uptake estimates than ARA results; suggesting that  $^{15}\text{N}_2$  incubations were better predictors of  $\text{N}_2$  fixation. Comparing results to an estimated required N amount for observed cell growth, again showed  $^{15}\text{N}_2$  incubation results were closer and therefore more reasonable estimates of  $\text{N}_2$  fixation than ARA.
- e) **Incubation appeared to diminish C fixation rate and cell division over longer incubations periods, while potentially enhancing N acquisition:** Comparison of end concentrations of the incubations with concentrations from the culture at the same time (reconstituted from dilutions) suggest cellular division and C fixation were reduced in the incubations. This may be the effect of the lack of gas exchange in the incubations.

With respect to our initial thesis aims, the results here are clear. With regards to quantification of the C and N budget of *Crocospaera watsonii* WH8501 over the diel cycle, we provide succinct budgets regarding C and N uptake, cellular stoichiometry and cell content. When factoring in irradiance, cultures behaved roughly as expected, with enhanced C and N uptake per cell, greater cellular storage of C and N, and higher growth overall in the culture. Interestingly, it appeared that N uptake was *less* affected by increased light than C uptake, suggesting that N uptake is a prioritised mechanism regardless of energetic availability. Interaction with dissolved phases of N (DON,  $\text{NO}_3^-$  and  $\text{NH}_4^+$ ) were however more unexpected. Despite the pervasive notion that  $\text{NO}_3^-$  *should* affect diazotrophy by way of altering N acquisition strategy and inhibiting  $\text{N}_2$  fixation, we instead found no significant interaction with  $\text{NO}_3^-$  for our strain of *Crocospaera watsonii*. The few studies that looked at *Crocospaera watsonii*'s interaction with  $\text{NO}_3^-$  before all document *some*, albeit disparate, interaction with  $\text{NO}_3^-$  [Dekazemacker and Bonnet, 2011; Knapp et al., 2012, Garcia and Hutchins, 2014]. However, our study appears to be the first focussed study which documents negligible interaction with  $\text{NO}_3^-$ . This result is especially noteworthy as our cultures were grown *long term* on  $\text{NO}_3^-$  when most studies expect that  $\text{NO}_3^-$  utilisation and inhibition of diazotrophy comes with longer term exposures [Knapp, 2012]. We found instead an important uptake of DON in place of  $\text{NO}_3^-$ . This interaction was apparently defined by light availability; in

lower irradiance cultures, there was a far superior ‘uptake’ of DON. This interaction is indeed interesting, as Garcia and Hutchins [2014] also noted enhanced uptake of dissolved N when light conditions were low, albeit with  $\text{NO}_3^-$  and not DON. From these results, we tentatively conclude two things. Firstly, it would appear that due to long term incubation with  $\text{NO}_3^-$  free medium, which was enriched with DON sourced from aged seawater and the exudation and lysate of older cells, the strain of *Crocospaera watsonii* we use has adapted to ignore  $\text{NO}_3^-$  and instead uptake DON. Secondly, it would appear that for *Crocospaera watsonii*, and potentially other diazotrophs, the supplementation of N acquisition with other sources of N may be predicated on the availability of light, with higher light availability favouring  $\text{N}_2$  fixation. This result is notable, as it delineates the concept of DIN (or DON) inhibition of diazotrophy away from N availability and towards light availability. This would suggest that observed inhibition of  $\text{N}_2$  fixation is more-so the result of reduced light availability, with uptake of non-diazotrophic N potentially a secondary mechanism designed to maintain sufficient amounts of N for cell growth. We note that this may not be true for  $\text{NH}_4^+$  however, as  $\text{NH}_4^+$  is far more labile than  $\text{NO}_3^-$ .

Investigation into the rate of exudation of DON and  $\text{NH}_4^+$ , or rather the net flux into the medium, reveal further interesting results. In our approach, we examined the net fluxes of N into the dissolved phase via both accumulation (or loss) of total ‘fixed N’, and accumulation of isotopically labelled fixed N in the medium. This approach was broadly similar to Berthelot et al. [2015]’s approach albeit with a broadly different culturing methodology (i.e. batch v.s. continuous). Accumulation of dissolved  $^{15}\text{N}$  in the medium after incubation was similar to the study by Berthelot et al. [2015], albeit with a nominally larger exudation in our study (0.5% v.s. 2-3% DON; 0.1% v.s. 0.4%  $\text{NH}_4^+$ ). Furthermore, in said study, there was an observed total flux of ‘fixed N’ into the medium after one diel cycle on the same order of magnitude as what was observed in the current study (0-20% v.s. 4-12% respectively). However there were notable differences. Firstly, DON fluxed *into* the medium in Berthelot et al. [2015]’s study whereas DON was fluxed *out of* the medium in our study. This is most likely due to different medium preparation; there was negligible DON in the aged seawater used to prepare medium in the former study, whereas in the current study DON was not negligible and present in fresh medium at concentrations likely approaching 20 - 50  $\mu\text{moles N L}^{-1}$ . Furthermore, in our study, medium was continuously fed to the cultures, meaning DON was continuously replenished and therefore established as a sustainable N source, which would be impossible in a batch culture. The next difference was that there was a much more important accumulation of  $\text{NH}_4^+$ , with net exudation of up to 12% of total fixed N accumulation

over the 24h period. It bears noting that our DON uptake and  $\text{NH}_4^+$  exudation may be linked: breakdown of DON to  $\text{NH}_4^+$  is potentially the mechanism used to access N from DON, meaning that  $\text{NH}_4^+$  accumulation may not be completely the result of exudation.

The results of our accuracy measurements are as what is generally expected: ARA tends to overestimate while  $^{15}\text{N}_2$  incubations tend to underestimate  $\text{N}_2$  fixation. We however present a novel approach to accuracy measurement: comparison of bulk N uptake (Eq. 4.2) to estimates by ARA and  $^{15}\text{N}_2$ . Normally bulk uptake of N wouldn't suffice as a measurement of  $\text{N}_2$  fixation, as it ignores specificity of N sources. However, by isolating *Crocospaera watsonii* as much as possible from other organisms and the environment, from  $\text{NO}_3^-$ , and by measuring fluxes of  $\text{NH}_4^+$  and DON, we can approximate  $\text{N}_2$  fixation using bulk uptake of N. Because the methods used to measure the parameters necessary for this are fewer, simpler, and more accurate than for the others, it can be expected that our 'bulk N' method is likely a more accurate representation of  $\text{N}_2$  fixation. From these results, we found an important correlation of measurement accuracy of the  $^{15}\text{N}_2$  incubation method with both V (specific uptake rate of  $^{15}\text{N}$  into the PON; Eq. 2.3) and  $^{15}\text{N}$  enrichment in the PON. This suggests a potential issue with the equilibration of  $^{15}\text{N}_2$  in the medium. While some may point towards the use of the bubble addition method, often decried since Mohr et al. [2010] suggested inefficient equilibration, it would appear that the issue may not be so simple. Firstly, Wannicke et al. [2018] has since updated the debate by suggesting that measurements using the bubble addition method are generally significantly similar to those using the purportedly superior pre-enriched seawater method first suggested by Mohr et al. [2010]. Furthermore, estimates of inaccuracy using our method suggest an unaccounted inaccuracy: necessary initial enrichment to push our values up to values matching the bulk N method suggests a larger inaccuracy than what was suggested by Mohr et al. [2010]. We therefore suggest that there are broader issues with the method in general.

#### Implications of our results and recommendations for future work

Amongst the largest implication of our results was the lack of interaction of *Crocospaera watsonii* with  $\text{NO}_3^-$ . Diazotrophy is often considered to be limited to regions where DN is depleted, such as oligotrophic gyres, partly due to the assumption that less energetically costly sources of N would be preferred to  $\text{N}_2$  fixation. Our work not only adds to a growing body of literature which challenges said assumption but goes a step further; not only did  $\text{NO}_3^-$  not replace diazotrophy nor inhibit it,  $\text{NO}_3^-$  was barely touched at all. The existence of a diazotroph which has seemingly adapted to not

significantly interact with  $\text{NO}_3^-$  bears important implications for diazotrophic ecology, as it allows diazotrophs to be placed in  $\text{NO}_3^-$  enriched waters while fixing  $\text{N}_2$ , which tend to be assumed to not support diazotrophy. This has further implications for the global  $\text{N}_2$  fixation budget, as it allows for the theoretical expansion of placement of diazotrophy into  $\text{NO}_3^-$  replete areas, thus pointing towards a potentially enhanced global budget for diazotrophy. Further work should be performed to characterise rates of  $\text{N}_2$  fixation in  $\text{NO}_3^-$  replete regions, and furthermore in other regions previously considered not suitable for diazotrophy, as outlined in the state of the art.

Our result regarding the uptake of DON, and the increased importance of DON supplementation with increasing light availability, also deserves further thought. Combining our result with the result of the study by Garcia and Hutchins [2014], we present an important potential paradigmatic change to our understanding of why and when diazotrophs supplement diazotrophy with dissolved N; diazotrophs may regulate their  $\text{N}_2$  fixation based on light availability, with DN supplementation as a secondary mechanism for regulation of N uptake. Further work should emphasise the relationship of diazotrophy of cyanobacterial phototrophs with light availability and its effect on DN supplementation; be it DON or  $\text{NO}_3^-$ . Furthermore, DON uptake and exudation should be characterised with more specificity in order to understand the mechanisms and likely species of DON which are taken up and exuded by diazotrophs such as *Crocospaera watsonii*.

Our accuracy results present a previously unused method to verify  $^{15}\text{N}_2$  incubation and ARA results; reporters seem to rarely report bulk N fluxes in the PON and DN. While this method may not be viable in the field, laboratory studies with sufficient biomass in unialgal cultures could easily adopt methodology to verify their results against bulk N fluxes, methods for which require far less handling, parameterisation and assumption. Furthermore, by verifying results from both bubble addition and pre-enriched seawater incubations against V and  $^{15}\text{N}$  enrichment in the PON, we may reveal more regarding this debate, and potentially settle it. Finally, it would appear that far more work is required to regulate the accuracy of the ARA method.

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