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N$_2$O synthesis by microalgae: Pathways, significance and mitigations

A thesis presented in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

in Environmental Engineering

At Massey University, Palmerston North, New Zealand

Maxence Plouviez

2017
Abstract

Over the last decades, various studies have reported the occurrence of emissions of nitrous oxide (N\textsubscript{2}O) from aquatic ecosystems characterised by a high level of algal activity (e.g. eutrophic lakes) as well as from algal cultures representative of the processes used by the algae biotechnology industry. As N\textsubscript{2}O is a potent greenhouse gas (GHG) and ozone depleting pollutant, these findings suggest that large scale microalgal cultivation (and possibly, eutrophic ecosystems) could contribute to the global N\textsubscript{2}O budget. Considering the current rapid development of microalgal biotechnologies and the ubiquity of microalgae in the environment, this PhD research was undertaken to determine the biochemical pathway of microalgal N\textsubscript{2}O synthesis and evaluate the potential significance of microalgal N\textsubscript{2}O emissions with regard to climate change.

To determine the pathway of N\textsubscript{2}O synthesis in microalgae, Chlamydomonas reinhardtii and its associated mutants were incubated in short-term (24 h) laboratory \textit{in vitro} batch assays. For the first time, axenic \textit{C. reinhardtii} cultures (i.e. culture free of other microorganisms such as bacteria) fed nitrite (NO\textsubscript{2}\textsuperscript{-}) were shown to synthesise N\textsubscript{2}O under aerobic conditions. The results evidenced that N\textsubscript{2}O synthesis involves 1) NO\textsubscript{2}\textsuperscript{-} reduction into nitric oxide (NO), followed by 2) NO reduction into N\textsubscript{2}O by nitric oxide reductase (NOR). With regard to the first step, the results show that NO\textsubscript{2}\textsuperscript{-} reduction into NO could be catalysed by the dual system nitrate reductase-amidoxime reducing component (NR-ARC) and the mitochondrial cytochrome c oxidase (COX). Based on our experimental evidence and published literature, we hypothesise that N\textsubscript{2}O is
synthesised via NR-ARC-mediated NO$_2^-$ reduction under physiological conditions (i.e. low/moderate intracellular NO$_2^-$) but that under NO$_2^-$ stress (i.e. induced by high intracellular NO$_2^-$), N$_2$O synthesis involves both NR-ARC-mediated and COX-mediated NO$_2^-$ reductions. RNA sequencing analysis on *C. reinhardtii* samples confirmed that the genes encoding ARC, COX and NOR were expressed in NO$_2^-$-laden culture, although NO$_2^-$ addition did not trigger significant transcriptomic regulation of these genes. We therefore hypothesise that the microalgal N$_2$O pathway may be involved in NO regulation in microalgae where NOR acts as a security valve to get rid of excess NO (or NO$_2^-$).

To evaluate N$_2$O emissions during microalgal cultivation, N$_2$O emissions were quantified during the long term outdoor cultivation of commercially relevant microalgae species (*Chlorella vulgaris, Neochloris* sp. and *Arthrospira platensis*) in 50 L pilot scale tubular photobioreactors (92 days) and during secondary wastewater treatment in a 1000 L high rate algal pond (365 days). Highly variable N$_2$O emissions were recorded from both systems (0.0 – 38 μmol N$_2$O·m$^{-2}$·h$^{-1}$, n = 510 from the 50 L photobioreactors; 0.008 – 28 μmol N$_2$O·m$^{-2}$·h$^{-1}$, n = 50 from the high rate algal pond). Based on these data, we estimated that the large scale cultivation of microalgae for biofuel production in order to, for example, replace 30% of USA transport fuel with algal-derived biofuel (i.e. a commonly used sustainability target), could generate N$_2$O emissions representing up to 10% of the currently budgeted global anthropogenic N$_2$O emissions. In contrast, N$_2$O emissions from the microalgae-based pond systems commonly used for wastewater treatment would represent less than 2% of the currently budgeted global N$_2$O emissions from wastewater treatment. As emission factors to
predict N$_2$O emissions during microalgae cultivation and microalgae-based wastewater treatment are currently lacking in Intergovernmental Panel for Climate Change methodologies, we estimated these values to 0.1 – 0.4% (0.02 – 0.11 g N-N$_2$O·m$^{-3}$·d$^{-1}$) of the N load on synthetic media (NO$_3^-$) during commercial cultivation and 0.04 – 0.45% (0.002 – 0.02 g N-N$_2$O·m$^{-3}$·d$^{-1}$) of the N load during wastewater treatment. The accuracy of the emission factors estimated is still uncertain due to the variability in the N$_2$O emissions recorded and by consequence further research is needed. Nevertheless, further monitoring showed that the use of ammonium as N source and/or the cultivation of microalgae species lacking the ability to generate N$_2$O (e.g. $A$. platensis) could provide simple mitigation solutions.
Acknowledgments

“Who would become a Padawan without his Jedi master?”

I thought that starting my acknowledgements with this metaphor is a suitable way of describing the importance of my main supervisor, Prof Benoit Guieysse, whom I would like to acknowledge first and foremost and also thank for his wonderful guidance, motivation and amazing supervision throughout my journey as a PhD student. I would also like to acknowledge my co-supervisors: Prof Andy Shilton for his excellent advice and teaching methods, which have been challenging at times but always in the benefit of success and Dr Mike Packer for his expertise on microalgal cultivation and biology as well as for his advice on editing.

Many thanks to Massey University staff: Mrs Ann Marie Jackson who was always really understanding and helpful with experimental requirements; Mr. John Edwards and Mr. John Sykes who helped me solve technical issues; Glenda Rosoman and Michel Wagner for their efficient and friendly help with all of the administrative aspects of my PhD and Julia Good, Judy Farrand-Collins, Nereda Corbett, Kylie Evans as well as the whole school of engineering and advanced technology (SEAT) workshop team: Anthony Wade, Morio Fukuoka, Kerry Griffiths, Ian Thomas and Clive Bardell.

Particular thanks go out to Trish McLenachan for her assistance on genomics analysis that helped me to obtain key results throughout my PhD and to Nihal Jayamaha for his great expertise and advices on statistical analysis. I also wish to thank Dr Dave
Wheeler, Dr Emanuel Sanz-Luque and Prof Emilio Fernandez for their collaboration and contribution to this project which has led to valuable results.

A big thank you to Dr Quentin Béchet for his amazing help and who was always there for me as the “senior” PhD student. Many thanks to all of my colleagues with who I shared many laughs and interesting (sometimes surprising) scientific discussions/debates: Aidan Crimp; Matthew Sell, Paul Chambonnière, Roland Schaap and Zane Norvil. Thank you to the part-time team members, our interns, who have been of a great help. I am thinking of Quentin Frigeri, Helene Thuret-Benoist, Qiao Wang, Emilie Alaux, Romain Lebrun and Mathilde Lippi.

I also wish to thank the Royal Society of New Zealand (Marsden fund MAU1102) for financially supporting my doctoral scholarship.

Finally, I could never express enough gratitude to all of my friends and family for their support. I would especially like to thank my father Yoland Plouviez and my mother Marie-Pierre Capillon without whom I could not realise all of this and also for their trust and support during my studies. Another thank you goes to my uncle Serge Weyenbergh and my fiancée Carina Svensson who have continuously believed in me and supported me during my studies.

My last thank you goes to my beautiful daughter Maeli who illuminates my life and has given me the strength to finish up performing at my best.
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Structure of the thesis

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To link the chapters together and illustrate the logic to achieve the research objectives; a preface is included at the beginning of Chapter 1–3. The content of the chapters is the same as the manuscripts they are based on; however, in some cases supporting information is given to improve clarity. For example, in Chapter 2 supplementary figures have been added in the core of the chapter to make the reading easier by directly showing all the evidences supporting each conclusion.

The relevant publications for each chapter are presented in the next section. The structure of this thesis complies with Massey University guidelines for doctoral thesis by publication, 2015.
List of papers and contributions

Chapter 1


Chapter 2


Chapter 3

Plouviez, M.; Shilton, A.; Packer, M.; Thuret-Benoist H.; Alaux, E.; Guieysse, B. Nitrous oxide (N\textsubscript{2}O) emissions from microalgae cultures in 50 L photobioreactors. *(Accepted (with revisions) in Algal Research).*

Some of the key results discussed in Chapter 3 were also presented at the following conferences:


- International Water Association, Ecotechnologies for wastewater treatment, Cambridge, United Kingdom (June 2016): Plouviez, M.; Posadas, E.; Lebrun,
R.; Munoz, R.; Guieysse, B. Direct and indirect N₂O emissions during secondary domestic wastewater treatment in a pilot-scale high rate algal pond.

Maxence Plouviez was the main contributor and lead author on all the papers and also presented at the conference Biorefinery for Food & Fuels & Materials. While Maxence Plouviez designed and conducted all the experimental work and analysed the results, his supervisors offered advice and help editing papers (the statements of contribution to doctoral thesis containing publications can be found at the end of the appendices).
Thesis introduction

In recent years, billions of dollars have been invested in microalgal biotechnologies\(^1\) with the main belief that microalgae-based products (e.g. biofuels, animal feed) and services (e.g. pollution control) have intrinsic low carbon footprints. This is, however, without considering that microalgae can generate the potent greenhouse gas and ozone depleting pollutant, nitrous oxide (N\(_2\)O)\(^2\). Although carbon neutrality may be achieved via the recycling of atmospheric carbon dioxide (CO\(_2\)) during photosynthesis, N\(_2\)O emissions during microalgal cultivation have not yet been properly investigated.

The potential of microalgae to synthesise N\(_2\)O is of broad significance due to potential adverse effects on the environment. However, the mechanisms involved and the magnitude of microalgal N\(_2\)O emissions from microalgae-based engineered (and natural\(^3\)) systems are largely unknown, raising research questions such as: How and why microalgae synthesise N\(_2\)O? Could microalgal N\(_2\)O emissions impact the sustainability of the microalgae industry? How could these emissions be mitigated? In order to answer these critical questions, this PhD thesis seeks to achieve the following objectives:

1. Acquire knowledge on microalgal N\(_2\)O biochemistry and understand the metabolism behind N\(_2\)O synthesis.

2. Evaluate N\(_2\)O emissions from microalgal engineered systems.

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\(^2\) The ability of microalgae to synthesise N\(_2\)O was suggested more than 40 years ago and demonstrated in two mid-1980 studies.

\(^3\) As it will be discussed in Chapter 1, there is clear evidence that microalgal N\(_2\)O emissions may be significant during microalgal cultivation but also from natural ecosystems which was to our knowledge completely dismissed among expert committees.
3. Evaluate the potential environmental significance of microalgal N\textsubscript{2}O emissions, and propose mitigation strategies.

Chapter 1 defines the scope of the thesis and critically discusses the current knowledge about N\textsubscript{2}O synthesis in microalgae and N\textsubscript{2}O emissions from microalgae (eco)systems. Chapter 2 presents and discusses new findings about the biochemical pathway of N\textsubscript{2}O synthesis in microalgae. Chapter 3 presents the first long term investigations of N\textsubscript{2}O emissions from outdoor microalgal cultivation systems, followed by a discussion on significance, mitigation solutions, and future guidance. Chapter 4 then presents conclusions on all the findings obtained during this research and discusses future prospects.
List of abbreviations

AOA: Ammonia-oxidizing archaea
AOB: Ammonia-oxidizing bacteria
AOX: Alternative oxidase
ARC: Amidoxime reducing component
CN⁻: Cyanide ion
COX: Cytochrome c oxidase
DAF FM Diacetate: 4-amino-5-methylamino-2’7’-difluore-fluorescein diacetate
DEA NONOate: diethylamine NONOate
DCW: Dry cell weight
DO: Dissolved oxygen
E-flasks: Erlenmeyer flasks
EFs: Emissions factors
Fd: Ferredoxin
GC: Gas chromatography
GHG: Greenhouse gas
HNO: Nitroxylic
HRAP: High rate algae pond
IPCC: Intergovernmental Panel for Climate change
L-Arg: L-arginine
L-NNA: Nω-nitro-L-arginine
Log2FC: Log 2 fold change
NAD(P)H: Nicotinamide adenine dinucleotide phosphate
NH₃: Ammonia
NH₄⁺: Ammonium
NiR: Nitrite reductase
NO: Nitric oxide
NOFNiR: Nitric Oxide Forming Nitrite Reductase
NOR: Nitric oxide reductase
NO₂⁻: Nitrite
NO₃⁻: Nitrate