N_2O synthesis by microalgae: Pathways, significance and mitigations

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Abstract

Over the last decades, various studies have reported the occurrence of emissions of nitrous oxide (N_2O) 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_2O is a potent greenhouse gas (GHG) and ozone depleting pollutant, these findings suggest that large scale microalgae cultivation (and possibly, eutrophic ecosystems) could contribute to the global N_2O 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_2O synthesis and evaluate the potential significance of microalgal N_2O emissions with regard to climate change.

To determine the pathway of N_2O synthesis in microalgae, *Chlamydomonas reinhardtii* and its associated mutants were incubated in short-term (24 h) laboratory *in vitro* batch assays. For the first time, axenic *C. reinhardtii* cultures (i.e. culture free of other microorganisms such as bacteria) fed nitrite (NO_2^-) were shown to synthesise N_2O under aerobic conditions. The results evidenced that N_2O synthesis involves 1) NO_2^- reduction into nitric oxide (NO), followed by 2) NO reduction into N_2O by nitric oxide reductase (NOR). With regard to the first step, the results show that NO_2^- 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_2O 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_2O 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_2O 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_2O emissions during microalgal cultivation, N_2O 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_2O emissions were recorded from both systems (0.0 – 38 μmol $N_2O \cdot m^{-2} \cdot h^{-1}$, n = 510 from the 50 L photobioreactors; 0.008 – 28 μmol $N_2O \cdot m^{-2} \cdot h^{-1}$, 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_2O emissions representing up to 10% of the currently budgeted global anthropogenic N_2O emissions. In contrast, N_2O emissions from the microalgae-based pond systems commonly used for wastewater treatment would represent less than 2% of the currently budgeted global N_2O emissions from wastewater treatment. As emission factors to

predict N_2O 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 $N\text{-}N_2O \cdot m^{-3} \cdot d^{-1}$) of the N load on synthetic media (NO_3^-) during commercial cultivation and 0.04 – 0.45% (0.002 − 0.02 g $N\text{-}N_2O \cdot m^{-3} \cdot d^{-1}$) of the N load during wastewater treatment. The accuracy of the emission factors estimated is still uncertain due to the variability in the N_2O 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_2O (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.

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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

This thesis is based on manuscripts that have been published in, accepted in or ready to be submitted for publication in international peer-reviewed journals (Chapter 1 − 3). Some of the key results have also been peer-reviewed and accepted for presentation in international conferences (Chapter 3). The content of Chapter 1-3 therefore supports the thesis conclusions discussed in Chapter 4.

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

Plouviez, M.; Shilton, A.; Packer, M.; Guieysse, B. Nitrous oxide emissions from microalgae: Potential pathways and significance. (*Under preparation*)

Chapter 2

Plouviez, M.; Wheeler, D.; Shilton, A.; Packer, M., A.; McLenachan, P.A.; Sanz-Luque, E.; Francisco, O-C.; Fernández, E.; and Guieysse, B. The biosynthesis of nitrous oxide in the green algae *Chlamydomonas reinhardtii*. *(Published in the Plant Journal Plant J. doi:10.1111/tpj.13544).*

Chapter 3

Plouviez, M.; Shilton, A.; Packer, M.; Thuret-Benoist H.; Alaux, E.; Guieysse, B. Nitrous oxide (N_2O) 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:

- Biorefinery for Food & Fuels & Materials, Montpellier Supagro, France (June 2015): Plouviez, M.; Guieysse, B.; Shilton, A.; Packer, M.; Thuret-Benoist, H.; Alaux, E. N_2O (Nitrous oxide) emissions during full-scale microalgae cultivation outdoors.
- 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 N_2O 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_2O)[2]. Although carbon neutrality may be achieved via the recycling of atmospheric carbon dioxide (CO_2) during photosynthesis, N_2O emissions during microalgal cultivation have not yet been properly investigated.

The potential of microalgae to synthesise N_2O is of broad significance due to potential adverse effects on the environment. However, the mechanisms involved and the magnitude of microalgal N_2O emissions from microalgae-based engineered (and natural[3]) systems are largely unknown, raising research questions such as: How and why microalgae synthesise N_2O? Could microalgal N_2O 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_2O biochemistry and understand the metabolism behind N_2O synthesis.
2. Evaluate N_2O emissions from microalgal engineered systems.

[1] Mascarelli, A.L. (2009). Gold rush for algae. Nature 461: 460–461.
[2] The ability of microalgae to synthesise N_2O 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_2O 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_2O emissions, and propose mitigation strategies.

Chapter 1 defines the scope of the thesis and critically discusses the current knowledge about N_2O synthesis in microalgae and N_2O emissions from microalgae (eco)systems. Chapter 2 presents and discusses new findings about the biochemical pathway of N_2O synthesis in microalgae. Chapter 3 presents the first long term investigations of N_2O 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: Nitroxyl

HRAP: High rate algae pond

IPCC: Intergovernmental Panel for Climate change

L-Arg: L-arginine

L-NNA: *N*ω-nitro-L-arginine

Log2FC: Log 2 fold change

NAD(P)H: Nicotinamide adenine dinucleotide phosphate

NH_3: Ammonia

NH_4^+: Ammonium

NiR: Nitrite reductase

NO: Nitric oxide

NOFNiR: Nitric Oxide Forming Nitrite Reductase

NOR: Nitric oxide reductase

NO_2^-: Nitrite

NO_3^-: Nitrate