Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

N_2O 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

MASSEY
UNIVERSITY

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.

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.

Table of contents

List of illustrations

List of tables

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

NR: Nitrate reductase

NR-ARC: dual system nitrate reductase-amidoxime reducing companont

NOS : Nitric oxide synthase

N_2O: Nitrous oxide

OD: Optical density

OGBF: Otago Genome and Bioinformatics Facility

PAR: Photosynthetically active radiation

PBR: Photobioreactor

RNA: Ribonucleic acid

TAP: Tris-acetate-phosphate

THB: Truncated hemoglobin

TSS: Total suspended solids

WWT: Wastewater treatment

Chapter 1.

Literature review

Preface

This chapter reviewed the current knowledge about microalgal N_2O synthesis and defined research knowledge gaps. In addition, using simple and conservative estimations this chapter was evaluating if microalgal N_2O emissions could be a potential issue. The aim was to evaluate if we should consider microalgal N_2O emissions rather than to determine the current magnitude of these emissions (there is currently not enough data for the later).

This chapter is based on paper 1 (*under preparation*):

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

ABSTRACT

This chapter discusses the potential significance of nitrous oxide (N_2O) synthesis by microalgae in light of recent advances in microalgal biology and evidence from field studies. Based on the published literature, we conservatively estimate that the currently unaccounted N_2O emissions from eutrophic lakes alone could yield global N_2O emissions equating to 18% of the N_2O currently accounted from all rivers, estuaries and coastal zones. In addition, cultivating microalgae in order to meet the commonly used 'sustainability target' of displacing 30% of US transportation fuels with microalgal biofuels could generate emissions representing 15% of the amount of N_2O generated from all US national anthropogenic sources. While these estimates are highly uncertain given the lack of knowledge in the area, they support a case for improving our mechanistic understanding of the pathways and key triggers of microalgal N_2O synthesis considering that i) N_2O emissions have been repeatedly reported from a diverse range of aquatic ecosystems characterised by a high level of algal activity; ii) N_2O synthesis by pure microalgae cultures has been unambiguously demonstrated and current knowledge on N_2O synthesis from a broad range of organisms provides the foundation for six putative microalgal N_2O synthesis pathways; iii) microalgae are ubiquitously found in natural ecosystems and may be cultivated at a massive scale in the future; iv) the Intergovernmental Panel for Climate Change does not currently consider N_2O emissions from algae-based ecosystems such as eutrophic lakes.

1.1 Introduction

Microalgae are extensively used for the commercial production of animal/human feed and high value chemicals (Borowitzka, 2013) and considerable research is currently aiming at developing microalgae-based biotechnologies for wastewater treatment, biofuel generation and carbon dioxide (CO_2) bio-fixation (Wang et al., 2008; Rodolfi et al., 2009; Shilton et al., 2012). A key driver behind the popularity of microalgae biotechnologies is the perception that microalgae cultivation is intrinsically environmentally benign (Ahmad et al., 2011). However, the ability of microalgae to synthesise nitrous oxide (N_2O) has been suspected for decades and unambiguously demonstrated in pure cultures (Table 1.1). Guieysse et al, (2013) for example estimated the rate of N_2O emissions during microalgae cultivation with nitrate (NO_3^-) as a nitrogen (N)-source to 1.38 – 10.1 kg N-N_2O $ha^{-1} \cdot yr^{-1}$, which is higher than the rate of N_2O emissions from natural vegetation of 0.7 kg N-N_2O $ha^{-1} \cdot yr^{-1}$ (Smeets et al., 2009). Microalgal N_2O synthesis is concerning because N_2O is a potent greenhouse gas (GHG) and ozone-depleting atmospheric pollutant (Ravishankara et al., 2009) and because microalgae (a term henceforth use to broadly describe prokaryotic and eukaryotic phototrophs) are ubiquitous in the environment and their growth is often triggered by anthropogenic pollution.

In this broad context, this chapter analyses past studies acknowledging the potential for microalgal N_2O emissions (Table 1.1) in light of recent advances in microalgal biology and current knowledge of microbial pathways for N_2O synthesis. The potential environmental significance of microalgal N_2O emissions is then estimated and benchmarked using two simple case studies. The main objective of this chapter is not to

provide accurate estimates (there is insufficient data for the latter) but to highlight the potential current and future significance of a presently unaccounted N_2O source.

Table 1.1: N_2O emissions reported from studies acknowledging algal N_2O synthesis (chronological order of publication).

Ecosystem	N_2O fluxes[a]	Comments
Ocean	115 nmol·m^{-2}·h^{-1}	Cohen and Gordon, (1978) reported that, in surface water, N_2O emissions were correlated to the presence of nitrite (NO_2^-) and oxygen (O_2). These authors dismissed algae as key contributor.
Ocean	409 nmol·m^{-2}·h^{-1}	Pierotti and Rasmussen, (1980) recognised phytoplankton as a significant N_2O source in upper layer of upwelling regions.
Costal wetland	125 – 228 nmol·m^{-2}·h^{-1}	Smith et al, (1983) noted the potential of algal N_2O synthesis and attributed N_2O production to bacterial nitrification-denitrification.
Laboratory assays	933 – 1579 nmol·g-DW^{-1}·h^{-1}	Weathers, (1984) demonstrated N_2O synthesis in axenic microalgae culture supplied with NO_2^-.
Laboratory assays	1500 nmol.g-DW^{-1}·h^{-1}	Weathers and Niedzielski, (1986) demonstrated N_2O synthesis in axenic cyanobacteria cultures supplied with NO_2^-.
Ocean	123 – 132% saturation	Outdot et al, (1990) attributed N_2O emissions in upper (euphotic) zone to NO_3^- assimilation by phytoplankton.
Lakes	300 – 700 nmol·m^{-2}·h^{-1}	Mengis et al, (1997) attributed N_2O production in the upper layer of eutrophic lakes to "actively growing algae".
Ocean	88 nmol·m^{-2}·h^{-1}	Morell et al, (2001) acknowledged the existence of an algal N_2O pathway but did not discuss in further.
Lake	357 – 2450 nmol·m^{-2}·h^{-1}	Wang et al, (2006) concluded that "excessive N_2O emissions during algal bloom periods suggested that algae played a significant role in the emission of N_2O."
Open pond	580 nmol·m^{-2}·h^{-1}	Florez-leiva et al., (2010) reported N_2O emissions from a *Nannochloris* culture in an open pond
Laboratory assays	13 – 145 nmol·g-DW^{-1}·h^{-1}	Fagerstone et al, (2011) acknowledged the potential of microalgal synthesis but attributed N_2O synthesis in

		cultures of the microalga *Nannochloropsis salina* to bacteria.
Raceway pond	320 $nmol \cdot L^{-1}$	Ferrón et al, (2012) acknowledged the potential of algal synthesis but did not conclude on the sources of N_2O emissions recorded during the outdoor culture of marine diatom *Staurosira* sp.
Laboratory assays	109 – 1480 $nmol \cdot g\text{-}DW^{-1} \cdot h^{-1}$	Guieysse et al, (2013) demonstrated N_2O synthesis by axenic *Chlorella vulgaris* and linked this activity to NO_2^- reduction.
Photobioreactor	240 – 1250 $nmol \cdot m^{-2} \cdot h^{-1}$	Guieysse et al, (2013) reported that N_2O synthesis during *Chlorella vulgaris* culture was correlated to light intensity.
Photobioreactor	0 – 36 $nmol \cdot g\text{-}DW^{-1} \cdot h^{-1}$	Mezzari et al, (2013) attributed N_2O emissions to microalgae.
Photobioreactor	9698 – 10761 $nmol \cdot g\text{-}DW^{-1} \cdot h^{-1}$	Harter et al, (2013) acknowledged the potential of microalgal synthesis but attributed N_2O emissions in *Dunaliella salina* cultures to anaerobic bacteria.
Laboratory assay	0.087 – 0.3 $nmol \cdot L^{-1} \cdot h^{-1}$	Kamp et al, (2013) attributed N_2O emissions from *Thalassiorira weissflogii* cultures to NO_3^- dissimilatory reduction by this diatom.
Raceway pond	2 – 5685 $nmol \cdot g\text{-}DW^{-1} \cdot h^{-1}$	Alcántara et al, (2015) linked N_2O emissions to *Chlorella vulgaris* activity during synthetic wastewater in a laboratory scale raceway pond.

[a] The fluxes herein listed correspond to the flux reported in the zone when phototrophic activity is evidenced or suspected (e.g. surface water; upper layer etc); when possible, these fluxes were re-calculated in nmol $N_2O \cdot m^{-2} \cdot h^{-1}$ (natural aquatic and engineered systems) or nmol $N_2O \cdot g\text{-}DW^{-1} \cdot h^{-1}$ (laboratory studies).

1.2 N_2O emissions from microalgae-based (eco)systems

As illustrated in Table 1.1, very few studies have hitherto acknowledged the potential of algal N_2O synthesis, regardless of whether or not the emissions reported in these studies were attributed to algae (Pierotti and Rasmussen, 1980; Weathers, 1984; Weathers and Niedzielski, 1986; Outdot et al., 1990; Mengis et al., 1997; Wang et al., 2006; Guieysse et al., 2013; Mezzari et al., 2013; Alcántara et al., 2015) or other organisms (Cohen and Gordon, 1978; Smith et al., 1983; Morell et al., 2001; Fagerstone et al., 2011; Harter et al., 2013). Of course it cannot be inferred that N_2O production from an ecosystem harbouring strong photosynthetic activity (Charpentier et al., 2010; Arévalo-Martínez et al., 2015) necessarily involves microalgae, directly or indirectly. There is also no evidence that algal and bacterial sources are mutually exclusive and two studies providing evidence of bacterial nitrification/denitrification also highlighted the occurrence of additional unknown N_2O sources: Pierotti and Rasmussen, (1980) thus reported evidence of high productivity of primary producers correlated to NO_3^- uptake in oceanic upwelling regions suggesting N_2O was partly produced by phytoplankton, while Lamontagne et al, (2003) established that experimental N_2O concentrations exceeded N_2O estimations based on known biological sources and sinks of N_2O in river, and hypothesised the possibility of missing N_2O sources.

Despite the uncertainty around the origin of N_2O emissions, the observations summarised in Table 1.1, and other studies (Charpentier et al., 2010; Arévalo-Martínez et al., 2015), evidence that correlation between phototrophic activity and N_2O emissions have been repeatedly reported for decades. N_2O emissions were for example correlated to O_2 concentration maxima in the surface layer of tropical oceans (Outdot et

al., 1990) and eutrophic lakes (Mengis et al., 1997) in two studies suggesting microalgal N_2O synthesis during NO_3^- assimilation. A possible pathway was even proposed as early as 1977 (Hahn and Junge, 1977; Cohen and Gordon, 1978) and later laboratory studies unambiguously demonstrated the ability of axenic microalgae and cyanobacterial cultures to synthesise N_2O in the presence of NO_2^- (Weathers, 1984; Weathers and Niedzielski, 1986). Interestingly, while several authors demonstrated or hypothesised N_2O emissions from eutrophic environments (Mengis et al., 1997; Wang et al., 2006) and microalgae cultures (Guieysse et al., 2013; Kamp et al., 2013; Mezzari et al., 2013; Alcántara et al., 2015) were caused by microalgae, others concluded N_2O emissions from oxic microalgae cultures were caused by bacteria (Fagerstone et al., 2011; Harter et al., 2013). This divergence is not surprising if we consider that for years bacterial nitrification and denitrification were the only known major biological sources of N_2O in the environment (Wrage et al., 2001), an assumption still at the core of national greenhouse gas (GHG) inventories and sustainability analysis. The biological mechanisms of N_2O synthesis are however far more diverse than initially believed (Hayatsu et al., 2010) as, for example, this compound can be emitted during fungal heterotrophic denitrification in soil (Laughlin and Stevens, 2002) and via an archaeal ammonium oxidation in the ocean (Löscher et al., 2012; Hatzenpichler, 2012).

The difficulty of tracking the biological sources of N_2O in diverse ecosystems is understandable considering that bacteria and microalgae often exist in symbiotic/synergic relationships (Croft et al., 2005) and may have similar N_2O metabolic pathways (Alcántara et al., 2015): as illustrated in Figure 1.1 and Box 1.1, our current knowledge of the 'conventional pathways' of N_2O biosynthesis (i.e. enzymes and substrates involved) and of microalgal and plant biology, provides the basis for six possible routes for N_2O synthesis in microalgae (Box 1.1).

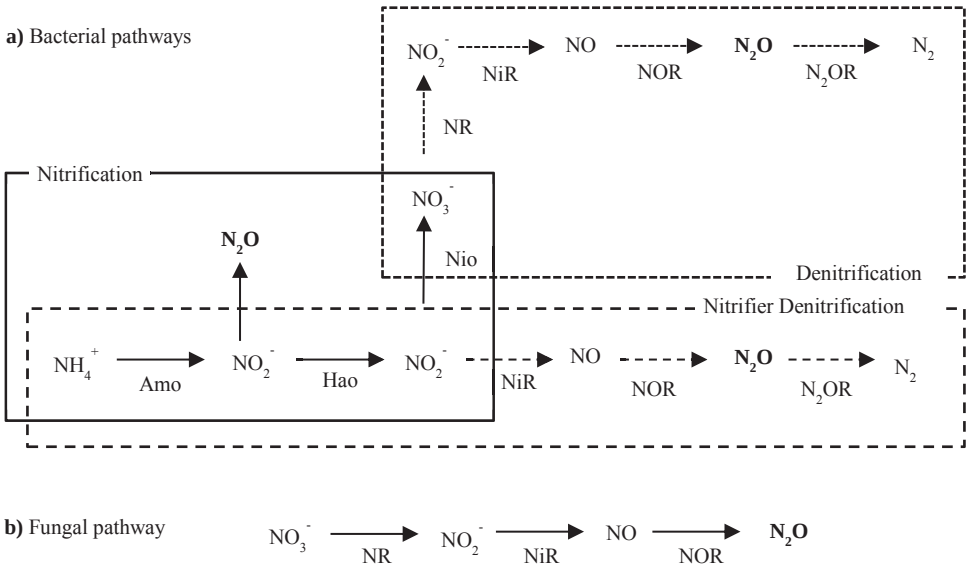


Figure 1.1: Common N_2O pathways **a)** N_2O synthesis by bacteria adapted from Wrage et al, (2001) N_2O synthesis during nitrification (solid line), denitrification (dot dash line) and nitrifier-denitrification (dashed line). The overlaps between the processes show the possible link occurring in particular environments such as coupled nitrification-denitrification at the aerobic/anaerobic interface; **b)** Fungal denitrification pathway. (Amo = Ammonia monooxygenase; Hao = Hydroxylamine oxidoreductase; NO_3^- = nitrate; NO_2^- = nitrite; NiR = Nitrite Reductase; NO = Nitric oxide; NOR = Nitric oxide reductase; N_2O = Nitrous oxide; Nio = Nitrite oxidoreductase; N_2OR = Nitrous oxide reductase).

Box 1.1 Putative pathways of microalgal N_2O synthesis

N_2O synthesis from nitrate assimilation: This pathway was first suggested by Hahn and Junge, (1977) who proposed that N_2O was a degradation product from hyponitrous acid ($H_2N_2O_2$) or nitroxyl (HNO), two potential intermediates in the pathway of nitrate (NO_3^-) assimilatory reduction into cellular material. N_2O synthesis was later linked to nitrite (NO_2^-) extracellular supply at high concentration (Weathers, 1984; Guieysse et al., 2013) and Guieysse et al. (2013) refined the putative 'assimilatory pathway` in *C. vulgaris* by linking the reduction of intracellular NO_2^- by nitrate reductase (NR) into NO or HNO and the subsequent reduction of these precursors into N_2O (Figure 1.2), see Guieysse et al, (2013) for further discussion.

Recent findings by Chamizo-Ampudia et al, (2016) showed that in the microalgae *Chlamydomonas reinhardtii* NO_2^- reduction into NO is actually catalysed by the dual NR-ARC (amidoxime reducing component) system. This system allows NO generation in the presence of both NO_2^- and NO_3^- (Chamizo-Ampudia et al., 2016). Further research is required to understand if and how NR itself or the dual system NR-ARC influence microalgal N_2O synthesis.

N_2O emissions during microalgal nitrification: When pre-cultivated using ammonium (NH_4^+) as sole exogenous N-source and subsequently subjected to N-deprivation, various microalgae have the ability to release NO_3^- and NO_2^- concomitantly to an increase in NR activity (Morris and Syrett, 1963, 1965; Kessler and Oesterheld, 1970). The generation of NO_3^- or NO_2^- via the oxidation of cellular nitrogen compounds was suspected to have caused NR-expression following N-deprivation (Kessler and Oesterheld, 1970). Given the potential functions of NR and

NO_2^- during N_2O synthesis (as discussed above), the possibility of a 'microalgal N_2O-nitrification pathway' involving NR-mediated reduction of endogenous NO_2^- into NO or HNO deserves consideration.

N_2O synthesis during nitrate dissimilation: Diatoms can use NO_3^- as electron acceptor for energy generation via `dissimilatory pathway`(Kamp et al., 2011). Kamp et al. (2013) further concluded that N_2O synthesis could occur as a by-product of NO_3^- dissimilatory reduction to NH_4^+ under anoxic conditions and darkness. Weathers and Niedzielski, (1986) also hypothesised a similar pathway could cause N_2O synthesis in cyanobacteria.

Other potential pathways leading to N_2O synthesis in microalgae: As discussed above, NO is a potential precursor of N_2O in microalgae. Three alternative N_2O pathways involving NO formation in microalgae can be theorised based on known pathway of NO synthesis in these microorganisms (experimental evidence is still lacking):

NO_2^- reduction into NO by mitochondria: Plant leaves mitochondria can generate NO under conditions favouring NO_2^- accumulation and O_2 deprivation (Gupta et al., 2011). Two enzyme complexes from the mitochondrial electron transport chain, the cytochrome c oxidase (COX) and the alternative oxidase (AOX), are indeed able to reduce NO_2^- to NO (Tischner et al., 2004; Gupta and Igamberdiev, 2011). The reduction of NO_2^- via the mitochondrial electron transport chain was first demonstrated in the microalgae *Chlorella sorokiniana* (Tischner et al., 2004).

L-arginine oxidation into NO by nitric oxide synthase: NO generation during nitric oxide synthase (NOS) mediated oxidation of the amino acid L-arginine into L-citrulline

(using nicotinamide adenine dinucleotide phosphate (NADPH) as electron donor) is well established in plant cells (Gupta et al., 2011). Although this mechanism cannot be ruled out in microalgae, prior studies using NOS inhibitor N_{ω}-nitro-L-arginine demonstrated that NO production by NOS is unlikely in *C. vulgaris* (Guieysse et al., 2013) and *C. reinhardtii* (Sakihama et al., 2002). These species can indeed synthesise both, NO and N_2O when N_{ω}-nitro-L-arginine is supplied to the culture medium.

NO_2^- *reduction into NO by hemoglobin:* Hemoglobins are widely spread in plant cells where, among numerous functions, they can scavenge NO (Perazzolli et al., 2004; Hoy and Hargrove, 2008; Gupta et al., 2011). Interestingly, plant and cyanobacterial hemoglobins are also known to reduce NO_2^- to NO (Sturms et al., 2011; Tiso et al., 2012; Ciaccio et al., 2015) under hypoxic/anoxic conditions, a mechanisms potentially related to NO_2^- detoxification (Sturms et al., 2011), or nitrogen metabolism (Ciaccio et al., 2015).

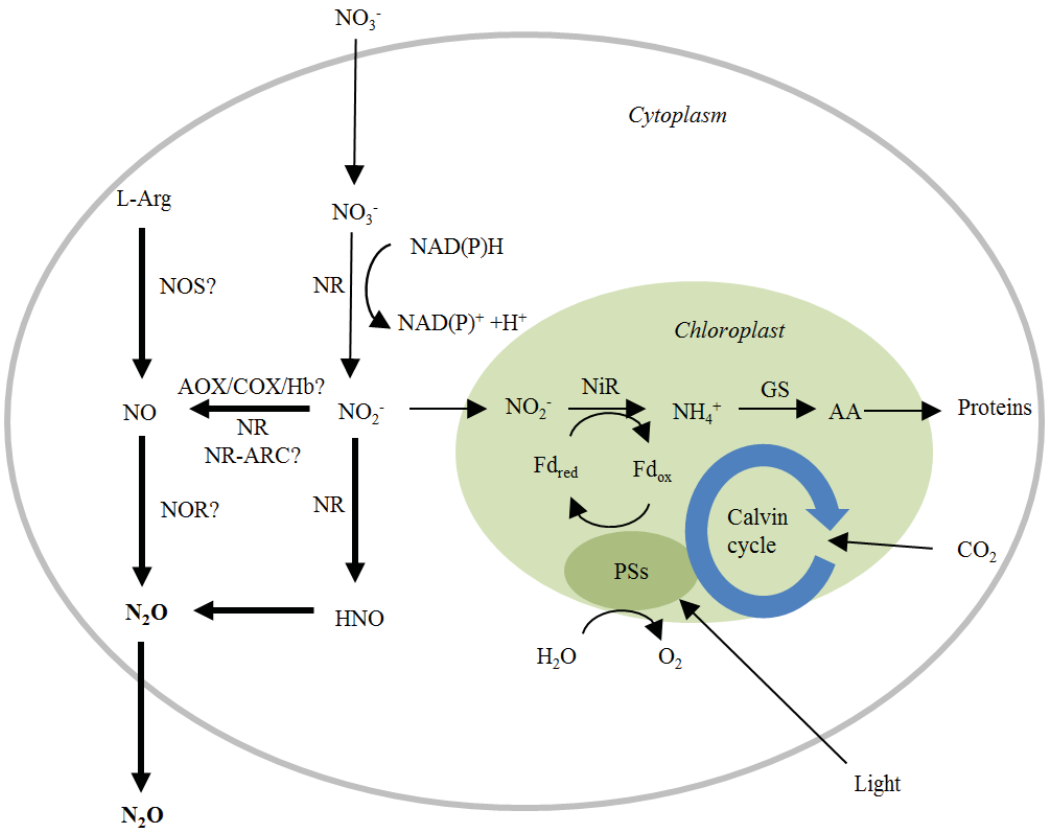


Figure 1.2: Nitrate assimilation in microalgae and N_2O putative pathways (bold); nitrate assimilation by microalgae involves the reduction of nitrate (NO_3^-) into nitrite (NO_2^-) by nitrate reductase (NR) in the cytoplasm using nicotinamide adenine dinucleotide phosphate (NAD(P)H) as an electron donor, followed by NO_2^- reduction into ammonium (NH_4^+) in the chloroplast by nitrite reductase (NiR) using reduced ferredoxin (Fd) as an electron donor, and the subsequent incorporation of NH_4^+ into glutamine by glutamine synthase (GS) (Sanz-Luque et al., 2015). Nitrate assimilation is rapid in the presence of light because reduced ferredoxin is regenerated during photosynthesis and is therefore available for NO_2^- reduction by NiR, together with excess NAD(P)H available for NR activity. NO_2^- formation is thought to be the limiting step of the NO_3^- assimilation (Huppe and Turpin, 1994), explaining why NO_2^- does not normally accumulate in photosynthesising cells (Abrol et al., 1983). Microalgae assimilate NO_3^- at much lower rates in the dark than under illumination (Grant and Turner, 1969) and under darkness, adenosine triphosphate (ATP) and NAD(P)H are produced via respiration by using carbohydrate reserves or reduced organic compounds from the environment and used directly by NR, or indirectly by NiR, by the way of Fd-NAD(P)H oxidoreductase, to reduce Fd.

(PSs = photosystems; AA = amino acid; L-Arg = L-Arginine; NOR = nitric oxide reductase; NOS = nitric oxide synthase; AOX/COX/Hb = Alternative oxidase/Cytochrome oxidase/Hemoglobin; NR-ARC = Nitrate reductase-amidoxime reducing component; ? = putative enzymes).

1.3 Potential significance of N_2O emissions from microalgae-based (eco)systems

As shown in Section 1.2, there is significant evidence that microalgae produce N_2O in various natural and engineered ecosystems and various pathways can be proposed to explain these emissions. This ability has a number of environmental ramifications given that N_2O is a globally significant ozone-depleting pollutant and a GHG. Of particular relevance, the Intergovernmental Panel for Climate Change (IPCC) currently quantifies global N_2O emissions from anthropogenic and natural sources without consideration that microalgae can produce N_2O. This section focuses on the impact of N_2O as a GHG.

1.3.1 Eutrophic lakes

In its gas inventory methodology, the IPCC (Ciais et al., 2013), documents that N_2O emissions from rivers, estuaries and coastal zones have increased because of intensive anthropogenic activities such as agriculture. However, the US Environmental Protection Agency (US EPA, 2010) notes that lakes (which can be affected by anthropogenic activities) are "poorly studied" in this regard and are "not tabulated as a source of N_2O in the IPCC assessments". Global N_2O emissions from eutrophic lakes and reservoirs were estimated based on the yearly average production of 0.9 kg $N\text{-}N_2O \cdot ha^{-1} \cdot yr^{-1}$ (357 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$) reported in the lowest emitting zone (pelagic zone) of the eutrophic Lake Taihu (China) assuming that i) 30 – 40% of the world's lakes are affected by eutrophication (Yang et al., 2008), and ii) lakes occupy a total

surface of 4.2 million km^2 (Downing et al., 2006). Using the lower end of the range for eutrophic lake area, we conservatively estimate that the lakes and reservoirs currently considered as eutrophic (Yang et al., 2008) could emit 110 kt $N\text{-}N_2O \cdot yr^{-1}$, which is equivalent to 18% of the current IPCC estimate of 600 kt $N\text{-}N_2O \cdot yr^{-1}$ for N_2O emitted from all rivers, estuaries and coastal zones globally (Ciais et al., 2013). As can be seen in Table 1.1, the global estimate of 110 kt $N\text{-}N_2O \cdot yr^{-1}$ is based on experimental emissions from eutrophic lakes in agreement with fluxes reported from other ecosystems including estuaries (Lamontagne et al., 2003); oceans (Pierotti and Rasmussen, 1980); coastal wetlands (Smith et al., 1983), and other eutrophic lakes (Mengis et al., 1997). However, Wang et al, (2006) also reported that the infralittoral zone rich in microalgae yielded annual fluxes of 2450 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$. The role microalgae are playing in these highly variable but poorly understood emissions becomes clear as Wang et al, (2006) quantified N_2O emissions temporally and were thus able to link maximum emissions to periods of algae blooms. The dominant phytoplankton groups in Lake Taihu are cyanobacteria, diatoms and green algae (Chen et al., 2003; Ma et al., 2014) and members of these groups are ubiquitous and have the ability to synthesise N_2O (Table 1.2). Hence, while the fact that lakes may generate significant emissions that are currently unaccounted is an issue *per se*, concerns grow when we consider the variability of the fluxes reported.

1.3.2 Microalgae cultivation for biofuel production

While relatively minor today, the cultivation of microalgae as a biofuel feedstock is being proposed on a massive scale (Quinn et al., 2012; Pate, 2013). Moody et al, (2014) thus calculated that United States would require 35 million ha of microalgal cultivation

systems to switch 30% of conventional transport fuels to algae-derived biodiesel (based on a biomass yield average of 9.4 $g \cdot m^{-2} \cdot d^{-1}$ and a lipid content of 30%, Moody et al., 2014). Assuming microalgae are cultivated in 0.25 m deep raceway ponds operated at 7 days hydraulic retention time emitting 4.4 – 32.4 nmol $N_2O \cdot h^{-1} \cdot g\text{-}DW^{-1}$ (Guieysse et al., 2013), microalgae cultivation over 35 million ha would release 24 – 183 kt N-$N_2O \cdot yr^{-1}$ (0.71 – 5.2 kg N-$N_2O \cdot ha^{-1} \cdot yr^{-1}$), which represents up to 15% of the 1200 kt N-$N_2O.yr^{-1}$ generated from every anthropogenic source for the entire United States (US EPA, 2015). Given that US and European environmental standards and directives demand GHG reductions over the biofuels life cycle (Sorda et al., 2010), potentially massive GHG emissions undermine the dogma of the environmentally friendly algal biofuel alternative (Chisti, 2008; Khan et al., 2009; Ahmad et al., 2011; Gouveia, 2011). The accuracy of the estimate provided above is unknown and cannot be assessed because microalgae are currently not cultivated as such large scale. This simple calculation nevertheless illustrates why it is important to consider the potential issue now rather than later.

1.3.3 Unknown 'unknowns'

The significance of N_2O production by microalgae has numerous implications because it represents a substantially different mechanism of production that potentially involves multiple species and pathways. Based on the information summarised in Table 1.2, there is evidence that microalgal N_2O emissions are influenced by factors such as cell biology (Weathers, 1984; Weathers and Niedzielski, 1986), N-source and concentration (Weathers, 1984; Weathers and Niedzielski, 1986; Guieysse et al., 2013; Kamp et al., 2013; Alcántara et al., 2015), light supply (Weathers, 1984; Weathers and Niedzielski,

1986; Guieysse et al., 2013; Kamp et al., 2013), and cell age and concentration (Guieysse et al., 2013). As shown in a recent study into microalgae-based wastewater treatment (Alcántara et al., 2015), simply changing the nitrogen source from ammonia to nitrite caused the resulting N_2O flux to rocket from 2 to 5685 nmol·g-total suspended solids^{-1}·h^{-1}. This massive change in yield must be considered in a context where algae are ubiquitously found in multitudes of engineered and aquatic ecosystems, and raises a spectrum of research questions such as why are there such massive variations and how could these emissions be mitigated?

Table 1.2: Biological, environmental, operational and design parameters potentially triggering microalgal N_2O emissions

Parameter	Comments	References
Microalgae species	The green microalgae *Chlorella vulgaris, Chlorella rubescens, Homosphaericas* sp., *Scenedesmus obliquus, Coelastrum* sp., *Chlorococcum vacuolarum* and *Nannochloris*, the diatom *Thalassiosira weissflogii* and the cyanobacteria *Aphanocapsa 6308, Aphanocapsa 6714,* and *Nostoc* sp have been shown to synthesise N_2O at species-dependent rates.	(Weathers, 1984; Weathers and Niedzielski, 1986; Florez-Leiva et al., 2010; Guieysse et al., 2013; Alcántara et al., 2015; Kamp et al., 2013)
Microalgal concentration	*C. vulgaris* N_2O production rates were linearly correlated to microalgal concentration.	(Guieysse et al., 2013)
Cellular stage	4-days old *C. vulgaris* cells generated more N_2O than 7-days old cells (over 4 hours of incubation with nitrite under darkness).	(Guieysse et al., 2013)
Light intensity	N_2O synthesis increased 10 – 15 times when photosynthesis was inactivated in the presence of NO_2^- in batch laboratory assays, although daytime N_2O emissions were recorded during outdoor *C. vulgaris* cultures in column photobioreactors.	(Weathers, 1984; Weathers and Niedzielski, 1986; Guieysse et al., 2013; Kamp et al., 2013; Alcántara et al., 2015)
Nitrogen supply	Several studies have shown a clear correlation between NO_3^- assimilation and/or the presence/supply of NO_2^- and N_2O synthesis by microalgae.	(Weathers, 1984; Weathers and Niedzielski, 1986; Guieysse et al., 2013; Kamp et al., 2013; Alcántara et al., 2015)

1.4 Conclusions

N_2O emissions have been repeatedly recorded from a diverse range of microalgae-based ecosystems and N_2O synthesis by pure microalgae has been demonstrated in the laboratory. Current knowledge provides the foundation for inferring at least six pathways for N_2O synthesis in algae. Using simple (albeit conservative) estimates based on the data currently available, we demonstrate algal emissions from currently unaccounted (e.g. eutrophic lakes) and future (algal cultivation) sources could be potentially globally significant. That the understanding in this area is in its infancy is a concern when we consider that N_2O has a severe impact on the ozone layer and as a greenhouse gas. The ubiquitous nature of algae throughout engineered and natural ecosystems, in conjunction with variations in N_2O flux that are measured in multiple orders of magnitude, provides strong motivation for further developing and understanding this phenomenon. Acknowledging and understanding N_2O emissions from microalgae is also of paramount importance to improve strategic investments in the fields of microalgal biotechnology.

References

Abrol, Y., Sawhney, S., and Naik, M. (1983). Light and dark assimilation of nitrate in plants. Plant. Cell Environ. **6**: 595–599.

Ahmad, A.L., Yasin, N.H.M., Derek, C.J.C., and Lim, J.K. (2011). Microalgae as a sustainable energy source for biodiesel production : A review. Renew. Sustain. Energy Rev. **15**: 584–593.

Alcántara, C., Muñoz, R., Norvill, Z., Plouviez, M., and Guieysse, B. (2015). Nitrous oxide emissions from high rate algal ponds treating domestic wastewater. Bioresour. Technol. **177**: 110–117.

Arévalo-Martínez, D.L., Kock, A., Löscher, C.R., Schmitz, R.A., and Bange, H.W. (2015). Massive nitrous oxide emissions from the tropical South Pacific Ocean. Nat. Geosci. **8**: 530–533.

Borowitzka, M.A. (2013). High-value products from microalgae - their development and commercialisation. J. Appl. Phycol. **25**: 743–756.

Chamizo-Ampudia, A., Sanz-Luque, E., Llamas, Á., Ocaña-Calahorro, F., Mariscal, V., Carreras, A., Barroso, J.B., Galván, A., and Fernández, E. (2016). A dual system formed by the ARC and NR molybdoenzymes mediates nitrite-dependent NO production in *Chlamydomonas*. Plant. Cell Environ. **10**: 2097-2107.

Charpentier, J., Farías, L., and Pizarro, O. (2010). Nitrous oxide fluxes in the Central and Eastern South Pacific. Global Biogeochem. Cycles **24**: 1–14.

Chen, Y., Qin, B., Teubner, K., and Dokulil, M.T. (2003). Long-term dynamics of phytoplankton assemblages : Microcystis-domination in Lake Taihu, a large shallow lake in China. J. Plankton Res. **25**: 445–453.

Chisti, Y. (2008). Biodiesel from microalgae beats bioethanol. Trends Biotechnol. **26**: 121–131.

Ciaccio, C., Ocaña-Calahorro, F., Droghetti, E., Tundo, G.R., Sanz-Luque, E., Polticelli, F., Visca, P., Smulevich, G., Ascenzi, P., and Coletta, M. (2015). Functional and spectroscopic characterization of *Chlamydomonas reinhardtii* truncated hemoglobins. PLoS One **10**: 1–24.

Ciais, P. et al. (2013). Carbon and other biogeochemical cycles. In Climate Change 2013: The physical science basis. Contribution of working group I to the fifth assessment report of the intergovernmental panel on climate change, V.B. and P.M.M. Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, ed (Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA), pp. 465–570.

Cohen, Y. and Gordon, L.I. (1978). Nitrous oxide in the oxygen minimum of the eastern tropical North Pacific : evidence for its consumption during denitrification and possible mechanisms for its production. Deep. Res. **25**: 509–524.

Croft, M.T., Lawrence, A.D., Raux-deery, E., Warren, M.J., and Smith, A.G. (2005). Algae acquire vitamin B_{12} through a symbiotic relationship with bacteria. Nature **438**: 90–93.

Downing, J. a., Prairie, Y.T., Cole, J.J., Duarte, C.M., Tranvik, L.J., Striegl, R.G., McDowell, W.H., Kortelainen, P., Caraco, N.F., and Melack, J.M. (2006). The global

abundance and size distribution of lakes, ponds, and impoundments. Limnol. Oceanogr. **51**: 2388–2397.

US Environmental Protection Agency (2010) Methane and nitrous oxide emissions from natural sources. http://dx.doi.org/EPA 430-R-10-001.

Fagerstone, K.D., Quinn, J.C., Bradley, T.H., De Long, S.K., and Marchese, A.J. (2011). Quantitative measurement of direct nitrous oxide emissions from microalgae cultivation. Enviro. Sci. Technol. **45**: 9449–9456.

Ferrón, S., Ho, D.T., Johnson, Z., I., and Huntley, M., E. (2012). Air − Water fluxes of N_2O and CH_4 during microalgae (*Staurosira sp.*) cultivation in an open raceway pond. Environ. Sci. Technol. **46**: 10842–10848.

Florez-Leiva, L. Tarifeño, E. Cornejo, M. Kiene, R. Farías, L. (2010) High production of nitrous oxide (N2O), methane (CH4) and dimethylsulphoniopropionate (DMSP) in a massive marine phytoplankton culture, Biogeosciences Discuss. 7 6705–6723.**Gouveia, L.** (2011). Microalgae as a feedstock for biodiesel (SpringerBriefs in Microbiology). pp 1-68.

Grant, B.. R.. and Turner, I. ldym (1969). Light-stimulated nitrate and nitrite assimilation in several species of algae. Comp. Biochem. Physiol. **29**: 995–1004.

Guieysse, B., Plouviez, M., Coilhac, M., and Cazali, L. (2013). Nitrous Oxide (N_2O) production in axenic Chlorella vulgaris microalgae cultures: Evidence, putative pathways, and potential environmental impacts. Biogeosciences **10**: 6737–6746.

Gupta, K.J., Fernie, A.R., Kaiser, W.M., and Van Dongen, J.T. (2011). On the origins of nitric oxide. Trends Plant Sci. **16**: 160–168.

Gupta, K.J. and Igamberdiev, A.U. (2011). The anoxic plant mitochondrion as a nitrite: NO reductase. Mitochondrion **11**: 537–543.

Hahn, J. and Junge, C. (1977). Atmospheric nitrous oxide: a critical review. Zeitschrift fur Naturforsch. **32**: 190–214.

Harter, T., Bossier, P., Verreth, J., Bodé, S., Van der Ha, D., Debeer, A.-E., Boon, N., Boeckx, P., Vyverman, W., and Nevejan, N. (2013). Carbon and nitrogen mass balance during flue gas treatment with *Dunaliella salina* cultures. J. Appl Phycol **25**: 359–368.

Hatzenpichler, R. (2012). Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. Appl. Environ. Microbiol. **78**: 7501–7510.

Hayatsu, M., Kanako, T., and Masanori, S. (2010). Various players in the nitrogen cycle : Diversity and functions of the microorganisms involved in nitrification and denitrification. Soil Sci. Plant Nutr. **54**: 37–41.

Hoy, J.A. and Hargrove, M.S. (2008). The structure and function of plant hemoglobins. Plant Physiol. Biochem. **46**: 371–379.

Huppe, H.C. and Turpin, D.H. (1994). Integration of carbon and nitrogen metabolism in plant and algal cells. Annu. Rev. Plant Physiol. **45**: 577–607.

Kamp, A., de Beer, D., Nitsch, J.L., Lavik, G., and Stief, P. (2011). Diatoms respire nitrate to survive dark and anoxic conditions. Proc. Natl. Acad. Sci. U. S. A. **108**: 5649–5654.

Kamp, A., Stief, P., Knappe, J., and De Beer, D. (2013). Response of the ubiquitous pelagic diatom *Thalassiosira weissflogii* to darkness and anoxia. PLoS One **8**: 1–11.

Kessler, E. and Oesterheld, H. (1970). Nitrification and induction of nitrate reductase in nitrogen-deficient algae. Nature **228**: 287–288.

Khan, S. a., Rashmi, Hussain, M.Z., Prasad, S., and Banerjee, U.C. (2009). Prospects of biodiesel production from microalgae in India. Renew. Sustain. Energy Rev. **13**: 2361–2372.

Lamontagne, M.G., Duran, R., and Valiela, I. (2003). Nitrous oxide sources and sinks in coastal aquifers and coupled estuarine receiving waters. Sci. Total Environ. **309**: 139–149.

Laughlin, R.J. and Stevens, R.J. (2002). Evidence for fungal dominance of denitrification and codenitrification in a grassland soil. Soil Sci. Soc. Am. J. **66**: 1540–1548.

Löscher, C.R., Kock, A., Könneke, M., LaRoche, J., Bange, H.W., and Schmitz, R. A. (2012). Production of oceanic nitrous oxide by ammonia-oxidizing archaea. Biogeosciences **9**: 2419–2429.

Ma, J., Boqiang, Q., Paerl, H.W., Brookes, J.D., Wu, P., Zhou, J., Deng, J., Guo, J., and Li, Z. (2014). Green algal over cyanobacterial dominance promoted with nitrogen and phosphorus additions in a mesocosm study at Lake Taihu, China. Environ. Sci. Pollut. Res. **22**: 5041–5049.

Mengis, M., Gachter, R., and Wehrli, B. (1997). Sources and sinks of nitrous oxide (N_2O) in deep lakes. Biogeochemistry **38**: 281–301.

Mezzari, M.P., Márcio, L.B., Nicoloso, R.S., Ibelli, A.M.G., Bortoli, M., Viancelli, A., and Soares, H.M. (2013). Assessment of N_2O emission from a photobioreactor treating ammonia-rich swine wastewater digestate. Bioresour. Technol. **149**: 327–332.

Moody, J.W., McGinty, C.M., and Quinn, J.C. (2014). Global evaluation of biofuel potential from microalgae. Proc. Natl. Acad. Sci. U.S.A. **111**: 8691–8696.

Morell, J.M., Capella, J., Mercado, A., Bauzá, J., and Corredor, J.E. (2001). Nitrous oxide fluxes in Caribbean and tropical Atlantic waters: Evidence for near surface production. Mar. Chem. **74**: 131–143.

Morris, I. and Syrett, P. (1963). The development of nitrate reductase in *Chlorella* and its repression by ammonium. Arch. Mikrobiol. **41**: 32–41.

Morris, I. and Syrett, P.J. (1965). The effect of nitrogen starvation on the activity of nitrate reductase and other enzymes in *Chlorella*. J. Gen. Microbiol. **38**: 21–28.

Outdot, C., Andrie, C., and Montel, Y. (1990). Nitrous oxide production in the tropical Atlantic Ocean. Deep. Res. **37**: 183–202.

Pate, R.C. (2013). Resource requirements for the large-scale production of algal biofuels. Biofuels **4**: 409–435.

Perazzolli, M., Dominici, P., C Romero-Puertas, M., Zago, E., Zeier, J., Sonoda, M., Lamb, C., and Delledonne, M. (2004). *Arabidopsis* non-symbiotic hemoglobin AHb1 modulates nitric oxide bioactivity. Plant Cell **16**: 2785–2794.

Pierotti, D. and Rasmussen, R.A. (1980). Nitrous oxide measurements in the eastern tropical Pacific Ocean. Tellus **32**: 56–70.

Quinn, J.C., Catton, K., Wagner, N., and Bradley, T.H. (2012). Current large-scale US biofuel potential from microalgae cultivated in photobioreactors. Bioenergy Res. **5**: 49–60.

Ravishankara, A.R., Daniel, J.S., and Portmann, R.W. (2009). Nitrous oxide (N_2O): The dominant Ozone-depleting substance emitted in the 21st Century. Science. **326**: 123–125.

Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., and Tredici, M.R. (2009). Microalgae for oil : strain selection , induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng. **102**: 100–112.

Sakihama, Y., Nakamura, S., and Yamasaki, H. (2002). Nitric oxide production mediated by nitrate reductase in the green alga *Chlamydomonas reinhardtii*: an alternative NO production pathway in photosynthetic organisms. Plant Cell Physiol. **43**: 290–297.

Sanz-Luque, E., Chamizo-Ampudia, A., Llamas, A., Galvan, A., and Fernandez, E. (2015). Understanding nitrate assimilation and its regulation in microalgae. Front. Plant Sci. **6**: 1-17.

Shilton, A.N., Powell, N., and Guieysse, B. (2012). Plant based phosphorus recovery from wastewater via algae and macrophytes. Curr. Opin. Biotechnol. **23**: 884–889.

Smeets, E.M.W., Bouwman, L.F., Stehfest, E., van Vuuren, D.P., and Posthuma, A. (2009). Contribution of N_2O to the greenhouse gas balance of first-generation biofuels. Glob. Chang. Biol. **15**: 1–23.

Smith, C.J., Delaune, R.D., and Patrick, Jr, W.H. (1983). Nitrous oxide emission from Gulf Coast Wetlands. Geochemica Cosmochim. Acta **47**: 1805–1814.

Sorda, G., Banse, M., and Kemfert, C. (2010). An overview of biofuel policies across the world. Energy Policy **38**: 6977–6988.

Sturms, R., Dispirito, A.A., and Hargrove, M.S. (2011). Plant and cyanobacterial hemoglobins reduce nitrite to nitric oxide under anoxic conditions. Biochemistry **50**: 3873–3878.

Tischner, R., Planchet, E., and Kaiser, W.M. (2004). Mitochondrial electron transport as a source for nitric oxide in the unicellular green alga Chlorella sorokiniana. FEBS Lett. **576**: 151–155.

Tiso, M., Tejero, J., Kenney, C., Frizzell, S., and Gladwin, M.T. (2012). Nitrite reductase activity of Non-symbiotic Hemoglobins from *Arabidopsis thaliana*. Biochemistry **51**: 5285–5292.

US Environmental Protection Agency (2015). Inventory of U.S. greenhouse gas emissions and sinks : 1990-2013. https://www.epa.gov/climatechange/emissions/usinventoryreport.html

Wang, B., Li, Y., Wu, N., and Lan, C.Q. (2008). CO_2 bio-mitigation using microalgae. Appl. Microbiol. Biotechnol. **79**: 707–718.

Wang, H., Wang, W., Yin, C., Wang, Y., and Lu, J. (2006). Littoral zones as the "hotspots" of nitrous oxide (N_2O) emission in a hyper-eutrophic lake in China. Atmos. Environ. **40**: 5522–5527.

Weathers, P.J. (1984). N_2O Evolution by Green Algae. Appl. Envionmental Microbiol. **48**: 1251–1253.

Weathers, P.J. and Niedzielski, J.J. (1986). Nitrous oxide production by cyanobacteria. Arch Microbiol **146**: 204–206.

Wrage, N., Velthof, G.L., van Beusichem, M.L., and Oenema, O. (2001). Role of nitrifier denitrification in the production of nitrous oxide. Soil Biol. Biochem. **33**: 1723–1732.

Yang, X., Wu, X., Hao, H., and He, Z. (2008). Mechanisms and assessment of water eutrophication. J. Zhejiang Univ. Sci. B **9**: 197–209.

Chapter 2.

Microalgal N_2O pathway

Preface

From the critical literature review presented in Chapter 1 we determined six putative microalgal N_2O pathways. Consequently, laboratory experiments were performed to study the biochemistry of microalgal N_2O synthesis. This study aimed to identify the key enzymes and intermediates involved during microalgal N_2O synthesis, and understand why microalgae synthesise N_2O. The findings discussed in this chapter are critical to the prediction of environmental or operational conditions that may favour or repress microalgal N_2O emissions.

For clarity, the key results obtained during the research undertaken in this chapter are summarised in diagrams at the end of each key section. The chapter then finishes with one figure combining the findings and illustrating the contribution of this research to new knowledge.

This chapter is based on paper 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).*

Parts of this work were performed with the collaboration of Prof. Emilio Fernández Reyes[4], Dr. Emanuel Sanz-Luque, Dr. Francisco Ocaña-Calahorro, and Dr. Dave Wheeler[5]. While Prof. Emilio Fernández Reyes shared his expertise on *C. reinhardtii* biology, transferred *C. reinhardtii* strains to our laboratory (i.e. *Chlamydomonas* NiR and NOR mutants) and helped editing the manuscript, two of his team members Dr. Emanuel Sanz-Luque and Dr. Francisco Ocaña-Calahorro created the NOR mutant used in this study. Dr. Dave Wheeler, with his expertise on RNA sequencing, helped in designing the 'RNA-sequencing experiment', performed the bio-informatic analysis, and helped editing the manuscript.

[4] Department of Biochemistry and Molecular Biology, University of Cordoba, Spain. Prof. Fernández Reyes is a world expert in *Chlamydomonas* genetics and N uptake.
[5] Institute of fundamental science (Bioinformatics/genomics), Massey University, New Zealand.

ABSTRACT

This chapter reports, for the first time, nitrous oxide (N_2O) synthesis by aerobic *Chlamydomonas reinhardtii* cultures supplied with nitrite (NO_2^-). In darkness, this biosynthesis was characterised by an immediate and short period (0 – 15 min) of N_2O production followed by a 20 h plateau of low production and a late phase (>20 h) of high production. Bioassays using enzyme inhibitors and *C. reinhardtii* mutants showed that NO_2^- was reduced into nitric oxide (NO) by either a dual enzymatic system involving nitrate reductase (NR), in the initial phase, or mitochondrial cytochrome c oxidase (COX), in the late phase, and that NO was subsequently reduced into N_2O by the enzyme NO reductase (NOR). High throughput RNA sequencing of *C. reinhardtii* 6145c samples confirmed that genes encoding putative COXs and NOR were expressed in NO_2^--laden oxic cultures, although these enzymes did not show significant transcriptional regulation. Based on experimental evidence and published literature, we hypothesise that under physiological conditions where nitrate, NO_3^-, is the main N source, microalgal N_2O synthesis involves 1) NO_3^- reduction into NO_2^- by nitrate reductase; followed by 2) NO_2^- reduction into NO by a dual system of NR and amidoxime-reducing NO-forming nitrite reductase; and 3) NO reduction into N_2O by NOR. NO_2^- reduction by COX appears to act as a means to detoxify NO_2^- when the intracellular concentration of this compound is high.

2.1 Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas and a major ozone depleting pollutant (Ravishankara et al., 2009) that can be emitted from algal cultures (Weathers, 1984; Weathers and Niedzielski, 1986), including verified axenic cultures (Guieysse et al., 2013), and eutrophic ecosystems characterised by a high level of primary activity (Mengis et al., 1997; Wang et al., 2006). Although little is known about N_2O synthesis in microalgae, nitrite (NO_2^-) has long been suspected as substrate (Weathers, 1984; Weathers and Niedzielski, 1986). Guieysse et al, (2013) later proposed that *Chlorella vulgaris* synthesises N_2O via the reduction of NO_2^- into nitric oxide (NO) or nitroxyl (HNO) by nitrate reductase (NR), followed by the reduction of NO into N_2O by a NO reductase (NOR) or the spontaneous dimerization of HNO into N_2O. However, if NO can indeed be reduced into N_2O under aerobic conditions, several alternative NO-generation scenarios could also lead to N_2O synthesis such as:

1. NO_2^- reduction into NO by other molybdo-enzymes than NR, such as the amidoxime reducing component in the microalgae *Chlamydomonas* (Chamizo-Ampudia et al., 2016) or the xanthine oxidase/dehydrogenases in higher plants (Gupta et al., 2011).
2. NO_2^- reduction into NO by mitochondrial cytochrome c oxidase (COX) and alternative oxidase (AOX), as demonstrated in plant cells (Planchet et al., 2005; Gupta et al., 2016) and the microalgae *Chlorella sorokiniana* (Tischner et al., 2004).
3. L-arginine oxidation into NO by nitric oxide synthase (NOS), activity described in plants despite the protein involved has not been identified yet (Gupta et al., 2011).

4. NO_2^- reduction into NO by hemoglobin (Sturms et al., 2011; Tiso et al., 2012; Ciaccio et al., 2015) which is known to occur in plants, microalgae and cyanobacteria under hypoxic/anoxic conditions (Sturms et al., 2011; Ciaccio et al., 2015).

This study was conducted to determine the biochemical pathway of N_2O synthesis in microalgae with the view that this knowledge is critical to the understanding of the nitrogen cycle in aquatic ecosystems (Weathers, 1984; Hayatsu et al., 2010; Kamp et al., 2013) and the assessment of the environmental impacts of algal biotechnologies and anthropogenic eutrophication (see Chapter 3). In this study, we have thus taken advantage of the many biological and genomic resources available for the extensively studied unicellular green microalgae *Chlamydomonas reinhardtii* (Navarro et al., 2000; Harris, 2001; Merchant et al., 2007; Chlamydomonas centre: http://www.chlamycollection.org/). The model organism *C. reinhardtii* has been especially suitable for investigating N_2O synthesis because 1) nitrogen (N) assimilation and regulation have been extensively studied in this species (Navarro et al., 2000; Sanz-Luque et al., 2015a), and 2) the mechanism for generation of NO via NO_2^- reduction has already been shown (Sakihama et al., 2002; Wei et al., 2014, Chamizo-Ampudia et al., 2016). As the ability of this strain to synthesise N_2O was unknown prior to this study, its potential was first demonstrated in axenic cultures. A series of *in vitro* biochemical assays using mutants and/or specific inhibitors were subsequently conducted to determine the main pathway, and these findings were substantiated using RNA sequencing analysis.

2.2 Materials and methods

2.2.1 Strains selection and maintenance

Table 2.1 lists the wildtype and mutants strains of *C. reinhardtii* used during this study. All strains were maintained on a sterilised solid medium containing 15 $g \cdot L^{-1}$ of agar agar in a buffered Tris-Acetate-Phosphate (TAP) medium (Appendix A). Unless otherwise stated, liquid cultures were conducted in TAP media. Importantly, NR- and nitrite reductase (NiR)-deficient mutants were cultivated using ammonium (NH_4^+) as the nitrogen source (as these strains were unable to grow on nitrate NO_3^- and NO_2^-). Each week, 250 mL Erlenmeyer-flasks (E-flasks) were filled with 125 mL of working media and inoculated with a single colony of *C. reinhardtii*. The microalgae were cultivated in a Minitron incubator (Infors HT, Switzerland) at 25 ± 1°C under continuous agitation (180 rpm), constant illumination (21 W of PAR m^{-2} at the culture surface, using five 18 W Polylux coolwhite tubes), and in an atmosphere of 2% (vol.) CO_2 in air. The E-flasks were closed with cotton tops and autoclaved with the media before inoculation. To provide algae cultures free of extracellular N, 25-50 mL of 5.5 days old (i.e. exponential phase) axenic *C. reinhardtii* cultures (described above) were centrifuged at 2900 g for 3.5 minutes, and the cells pellet was re-suspended in a N-free and acetate-free TAP medium to reach a concentration of 0.25 g- dry cell weight$\cdot L^{-1}$ (g-DCW$\cdot L^{-1}$) DCW was measured in triplicates by calculating the difference in weight of membrane filters (0.45 µm, 47 mm, Merck Millipore, Billerica, MA) before and after filtration of 5 mL of microalgal suspension. Before being weighed the filters were initially pre-dried at 105°C for 24 h and 1 h at 105°C following filtration (Béchet et al., 2015).

Table 2.1: Reference strain, wildtype, and mutant strains used during this study (strain numbers refer to the number given by *Chlamydomonas* centre http://chlamycollection.org).

Strain	Phenotype	Reference
6145c	Reference strain used during this study (wild-type); grows on nitrate and nitrite	Navarro et al., (2000)
M3	NiR deficient; cannot grow on nitrate nor nitrite	Navarro et al., (2000)
2929	Lack NR; cannot grow on nitrate nor nitrite	Sakihama et al., (2002)
704	Wild type strain used as parental strain for mutagenesis on NOR (CYP55 gene); grows on nitrate and nitrite	This study
amiCYP55	Lower NOR expression (46%) than the parent strain 704; grows on nitrate and nitrite	This study
409	Wild-type; grows on nitrate and nitrite	(Pröschold et al., 2005)
217	Wild-type; grows on nitrate and nitrite	Sakihama et al., (2002)
112	Parent strain of the mutant 2929	*Chlamydomonas* centre
530	Acetate requiring mutant, parent strain of the mutant 2929	*Chlamydomonas* centre
124	NR, NiR and HANiT deficient, cannot grow on nitrate nor nitrite	(Pröschold et al., 2005)
125	NR, NiR and HANiT deficient, cannot grow on nitrate nor nitrite	(Pröschold et al., 2005)

2.2.2 General protocol used during *in vitro* batch assays

Following the protocol described by Guieysse et al, (2013), 122.5 ± 2.5 mL serum flasks were filled with 50 mL of the N-free algal suspension described in the section above and supplemented with a N source and/or other chemicals as needed (see below). The flasks were then sealed with a rubber top and an aluminium cap (to prevent gas exchange and contamination) and incubated at 25°C for 24 - 48 hr under continuous agitation (180 rpm) and either continuous illumination (21 W of photosynthetically active radiation (PAR)·m^{-2} at the culture surface) or darkness. The following experiments were thus conducted:

1. *C. reinhardtii* 6145c was supplied with different N sources (10 mM NO_2^-, NO_3^-, or NH_4^+) or NO_2^- at various concentrations (3, 6, 12 and 24 mM).
2. *C. reinhardtii* 409, 217, 112, 530, 124, 125 were supplied 10 mM NO_2^- in darkness.
3. *C. reinhardtii* NR mutant 2929 was supplied with 10 mM NO_2^- in darkness.
4. *C. reinhardtii* NiR mutant M3 was supplied with 10 mM NO_2^- or NO_3^- both in darkness and under illumination.
5. *C. reinhardtii* wild-type 704 and amiCYP55 (NOR knock down mutant) were supplied with 10 mM NO_2^- in darkness (The NOR knock-down strain was isolated from wild-type strain 704 upon transformation with the vector pChlamiRNA3-CYP55, see Appendix B for the full description of the NOR amiRNA-strain construction).

The NO_2^- concentrations chosen were useful to study the N_2O pathway and aimed to easily detect N_2O synthesis. It is however important to note that the concentration of 10 mM NO_2^- used in most of our assays is irrelevantly high in comparison to typical microalgal cultivation (NO_3^- is at 7 mM in TAP medium). Nitrite toxicity was assessed

and although the occurrence of intracellular stress potentially involving various responses (e.g. NO) could not be dismissed, high NO_2^- concentrations (3 – 12 mM) did not affect *C. reinhardtii* growth kinetics (Appendix C).

2.2.3 Inhibition assays

2.2.3.1 Nitrate reductase

Based on the protocol described by Guieysse et al., (2013), *C. reinhardtii* 6145c (125 mL) was cultivated in 250 mL E-flasks for 10 days in TAP medium before aliquots (100 mL) were withdrawn, centrifuged, and re-suspended in either "normal" TAP or "modified" TAP where molybdate (MoO_2^{4-}) was replaced by orthotungstate (WO_2^{4-}) at the concentration of 0.253 $mg \cdot L^{-1}$ in order to inhibit NR (Deng et al., 1989). These cultures were incubated 4 days before aliquots (25-50 mL) were withdrawn, centrifuged, and re-suspended in N-free medium. These algal suspensions were then transferred to flasks, supplemented with 10 mM NO_2^-, and incubated in darkness as described above.

2.2.3.2 Nitric Oxide Synthase (NOS)

N_2O synthesis by *C. reinhardtii* was tested in TAP medium supplemented with 10 mM of the NOS substrate L-arginine (L-arg, ≥98% Sigma-Aldrich, USA) and/or 10 mM of the NOS-inhibitor *N*ω-nitro-L-arginine (L-NNA, ≥98% Sigma-Aldrich, USA). These tests were conducted in darkness with and without 10 mM NO_2^- supplementation (Guieysse et al., 2013).

2.2.3.3 Cytochrome c oxidase (COX)

Cyanide (KCN ≥97%, Sigma-Aldrich, USA) was added at 2 mM to cultures of *C. reinhardtii* 6145c and *C. reinhardtii* NR mutant 2929 supplied with 10 mM NO_2^- and incubated in darkness. KCN is an inhibitor of heme-containing enzymes (Sakihama et al., 2002; Tischner et al., 2004) known to affect mitochondrial cytochrome c oxidase (COX) (Way, 1984) at concentrations lower than 5 mM (Gans and Wollman, 1995).

2.2.4 Polymerase Chain Reaction (PCR)

Culture purity was assessed by extracting (Isolate II genomic DNA kit, Bioline, UK) and amplifying DNA using the primers and methods described by Guieysse et al., (2013) (Appendix D).

2.2.5 RNA-sequencing

2.2.5.1 Batch assays

All glassware was cleaned with Ultrapure diethylpyrocarbonate-treated water (RNAase-free) and all equipment that came in contact with the microalgal cultures was treated with RNAase Zap (Ambion, ThermoFischer Scientific USA) on the day of the experiment. *C. reinhardtii* 6145c was cultivated as described in Section 2.2.1 and, on the day of the experiment, aliquots of microalgal suspension were withdrawn, directly poured into 122.5 ± 2.5 mL serum flasks and incubated in darkness (microalgal cultures were directly poured from E-flasks to serum flasks to prevent any potential genetic drift

due to centrifugation and/or re-suspension of the microalgal pellet in N-free medium). Six serum flasks were thus incubated at constant temperature (25°C) and agitation (180 rpm) for 1 h before the addition of 10 mM NO_2^- to three of the flasks henceforth referred to as the 'treatments', with the remaining flasks being labelled as 'controls'. Based on the kinetics recorded over 24 hr of incubation in darkness (see Section 2.3.1), gas and liquid samples were withdrawn from each flask after 1.25 (t1: 15 min after NO_2^- supply), 4 (t2: 3 h after NO_2^- supply) and 25 hours (t3: 24 h after NO_2^- supply) to measure N_2O and extract RNA, respectively. Initial and final pH was measured by using a pH 510 pH/°C (Eutech instruments). Initial and final biomass concentrations were measured by dry cell weight (DCW) following DW protocol from Béchet et al, (2015). NO_3^-/NO_2^- concentrations were analysed by using a Dionex ICS-2000 Ion Chromatograph (Dionex Corporation, Sunnyvale, USA).

The same protocol was repeated three times before performing the RNA-sequencing. The amount of N_2O recorded for each time point between triplicates over the three repeated experiments never exceeded 14% standard error of the mean (for each time point n = 9, p = 0.05). See Appendix E.1 for more detail.

2.2.5.2 RNA sample preparations and bioinformatics

RNA was extracted with a plant NucleoSpin RNA kit (Macherey-Nagel). The presence of high quality RNA was confirmed in each sample by using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Waldbronn, Germany). Standard paired-end Nextera sequencing libraries were generated from poly-A purified RNA by NZGL Ltd at the Otago Genome and Bioinformatics Facility (OGBF). The resulting libraries were sequenced on one lane of a HiSeq2500 platform by the OGBF. Adaptors were

removed from the raw data with the software *fastqMCF* (Aronesty, 2013) before quality trimming using the software SolexaQA++ v.3.3 (Cox et al., 2010) with the default parameters. The short reads were aligned to a *C. reinhardtii* reference genome (*C. reinhardtii* v.5.5, Merchant et al., 2007) using TopHat v2.1.0 (Kim et al., 2013) with the "-library-type" switch set to "fr-firststrand". Read counts were generated from the alignments using htseq-count (Anders et al., 2015) based on *C. reinhardtii* v.5.5 gene models. Finally, differential expression analysis was performed based on the control and treatment read counts using the statistical R-package DESeq2 (Love et al., 2014). For each time point, if statistically significant (*p-value* < 0.05), the differential expression is given by a log2-fold change (log2FC). If positive, the log2FC indicates the up expression (i.e. increase) of particular genes between treatment and control. By contrast, if the log2FC is negative it indicates the down expression (i.e. decrease) of particular genes between treatment and control.

2.2.6 N_2O measurement

The gaseous concentration of N_2O was measured in flask headspace by sampling 3-5 mL with a gas-tight syringe and needle. The results herein presented show the total amount of N_2O produced in the flasks assuming the dissolved N_2O concentration in the aqueous phase was in equilibrium with the gas phase. N_2O losses and pressure changes caused by sampling were accounted for (Appendix F.1). As described by Guieysse et al, (2013), the gas sample was analysed by ECD-gas-chromatography (Shimadzu GC-2010, Shimadzu, Japan) using a 250 µL sample loop (380°C), a Alltech Porapak QS 80/100 column (70°C, sigma-Aldrich, USA) and an electron conductivity detector

(315°C). Calibration was achieved using a 100 ppm N_2O standard in N_2 (Appendix F.2).

2.2.7 NO detection

To detect NO, 10 µM of DAF-FM diacetate (≥98%, Sigma-Aldrich, USA) was added to 5.5 days-old *C. reinhardtii* cultures subsequently incubated for 0.5 − 1 h in serum flasks (25°C, constant agitation at 180 rpm) under illumination (5 $W \cdot m^{-2}$ at the culture surface). These suspensions were then centrifuged at 2900 g for 3.5 minutes, supernatants were discarded, and the biomass pellets were mixed and re-suspended in N-free medium. Because DAF-FM rapidly reacts with NO, NO_2^- (10 mM) was added one minute before microscope observation. The fluorescence in the cells was observed under fluorescence microscopy (Olympus BX51 microscope, Japan) with excitation at 520-550 nm for chlorophyll auto-fluorescence (characterised by a red colour) and at 460 – 490 nm for DAF FM triazol fluorescence (characterised by a green colour) using U-MWIG2 and U-MWIBA2 filters (Olympus, Japan), respectively. The specificity of DAF-FM for NO or HNO were tested using diethylamine NONOate (DEA NONOate ≥98% Sigma-Aldrich, USA) and Angeli`s salt (≥98% Sigma-Aldrich, USA), respectively.

2.2.8 Quality controls and data analysis

Positive controls (the wild-type *C. reinhardtii* 6145c in darkness supplied with 10 mM NO_2^-) were performed for each batch assays experiments. Over the duration of the study positive controls were thus repeated 40 times. In order to dismiss possible abiotic

N_2O production, negative controls (N-free medium supplied with 10 mM NO_2^-; or N-free medium supplied with chemical inhibitors and 10 mM NO_2^-; or autoclaved microalgal suspension supplied with 10 mM NO_2^-) were also performed. While positive significant N_2O production was always recorded from the positive controls, no significant N_2O production was recorded in the negative controls over 24 h of incubation. Details about data distribution and standard deviations (SD) can be found in Appendix G.1-2.

The amount of 'background' N_2O level initially found in the flasks was experimentally estimated to 1.45 ± 0.18 nmole (average ± SD; n = 56). Based on this value and the standard error of 57% recorded at the gas sampling time of 0.25 h (Appendix G.1), the limit of quantification in the flasks was estimated to 2.9 nmole (i.e. 200% of the background value). When N_2O production was higher than this threshold, N_2O production was deemed significant and specific production values (nmol $g \cdot DCW^{-1}$) were calculated as the amount of N_2O produced a time t (nmole) minus the background level (1.45 nmole), divided by the initial cell mass (g-$DCW \cdot L^{-1}$). Standard deviations (SD) represent combined uncertainties of N_2O and dry cell weight measurements (experimentally estimated to 5%). Summary of the all data analysis can be found in Appendix H (Tables H.1 to H.4).

2.3 Results and discussion

2.3.1 N_2O emissions from axenic *C. reinhardtii* cultures

Prior to this study, the ability of *C. reinhardtii* to synthesise N_2O had never been investigated. Preliminary batch assays were conducted and N_2O synthesis was only recorded in *C. reinhardtii* 6145c cultures supplied with NO_2^- (Figure 2.1). In contrast no significant N_2O synthesis was recorded in flasks not supplied with NO_2^- or in negative controls (i.e. flasks containing autoclaved cultures or sterile medium in the presence of NO_2^-).

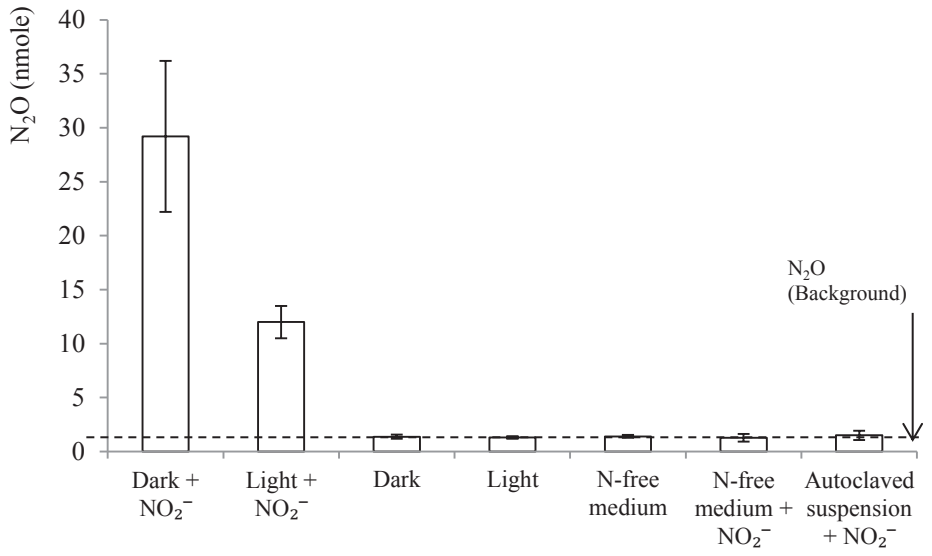


Figure 2.1: N_2O produced (average nmole ± SD) in triplicates of sealed batch cultures of *C. reinhardtii* 6145c (initial DCW of 0.22 g-DCW·L^{-1}) with or without 10 mM NO_2^- supplementation and incubated for 24 h in darkness or illumination; sterile N-free medium with or without NO_2^- supplementation; and autoclaved culture of *C. reinhardtii* 6145c supplied with 10 mM NO_2^- and incubated in darkness.

The production of N_2O could be confirmed for all wild-type *C. reinhardtii* strains tested, although the rates of emission were strain-dependent (Appendix I). Diagnostic PCR confirmed the absence of bacterial or archaeal genes in these cultures (Appendix D), demonstrating for the first time the ability of axenic *C. reinhardtii* to generate N_2O under aerobic conditions. N_2O production was linearly correlated with initial microalgae biomass concentration (Figure 2.2), providing further evidence that biological processes in the algae were the source of N_2O.

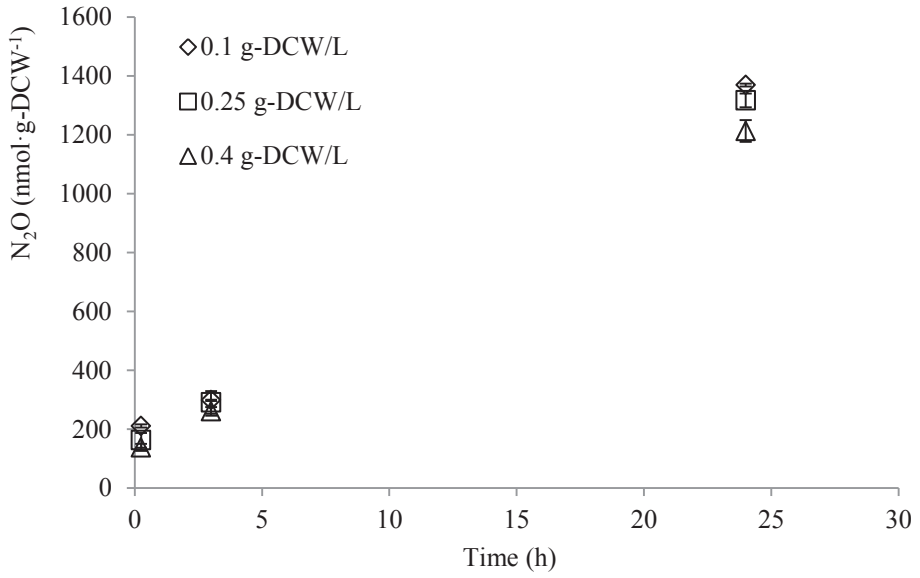


Figure 2.2: N_2O production (average nmol·g-DCW^{-1} ± SD) by triplicate cultures of *C. reinhardtii* 6145c at 3 different initial cellular concentrations (0.1, 0.25, 0.4 g-DCW·L^{-1}) incubated for 24 h with 10 mM NO_2^- in darkness.

Interestingly, there are clear differences in the kinetics of N_2O emission from NO_2^--laden *C. vulgaris* and *C. reinhardtii* cultures. For example, *C. vulgaris* has previously been shown to produce N_2O at a constant rate (zero-order) over 48 hours of incubation in darkness (Guieysse et al. 2013). In contrast, N_2O emission from *C. reinhardtii* incubated under nearly identical conditions was characterised by a '3-phases' kinetics of N_2O production (Figure 2.3a and b)[6]. As seen in Figure 2.3a, a short period of high N_2O production started immediately following NO_2^- addition (5.1 nmole after 10 – 15 min, or 1300 nmol·g-DCW^{-1}·h^{-1}). As can be seen in Figure 2.3b this initial response was followed by a phase of low production (22 – 32 nmole over 4 – 20 hours, or 57 nmol·g-DCW^{-1}·h^{-1}), and a final phase of vigorous production over the remaining duration of the experiment (32 – 126 nmole over 20 – 52 hours, or 300 nmol·g-DCW^{-1}·h^{-1}). The 20 h "lag" phase recorded during *C. reinhardtii* N_2O synthesis suggests a long-term response triggering N_2O emissions, whereas the shorter response suggests that N_2O synthesis is mediated by enzymes which are already active.

[6] While the general trends herein reported were reproducible, the exact duration and magnitude of the immediate response were variable (i.e. compare N_2O production after 1 and 4 h shown in Figure 2.3a and 2.3b, respectively), difficult to determine (as N_2O production could not be continuously monitored), and impacted by quantification uncertainty in the low gaseous N_2O levels recorded at the start of the experiments (Figure G.1 in Appendix G). Nevertheless, because the immediate response lasted at least 0.25 h, the '0 – 0.25 h' total production levels rates disclosed in Figure 2.5, 2.10 and Appendix H can be considered as representative.

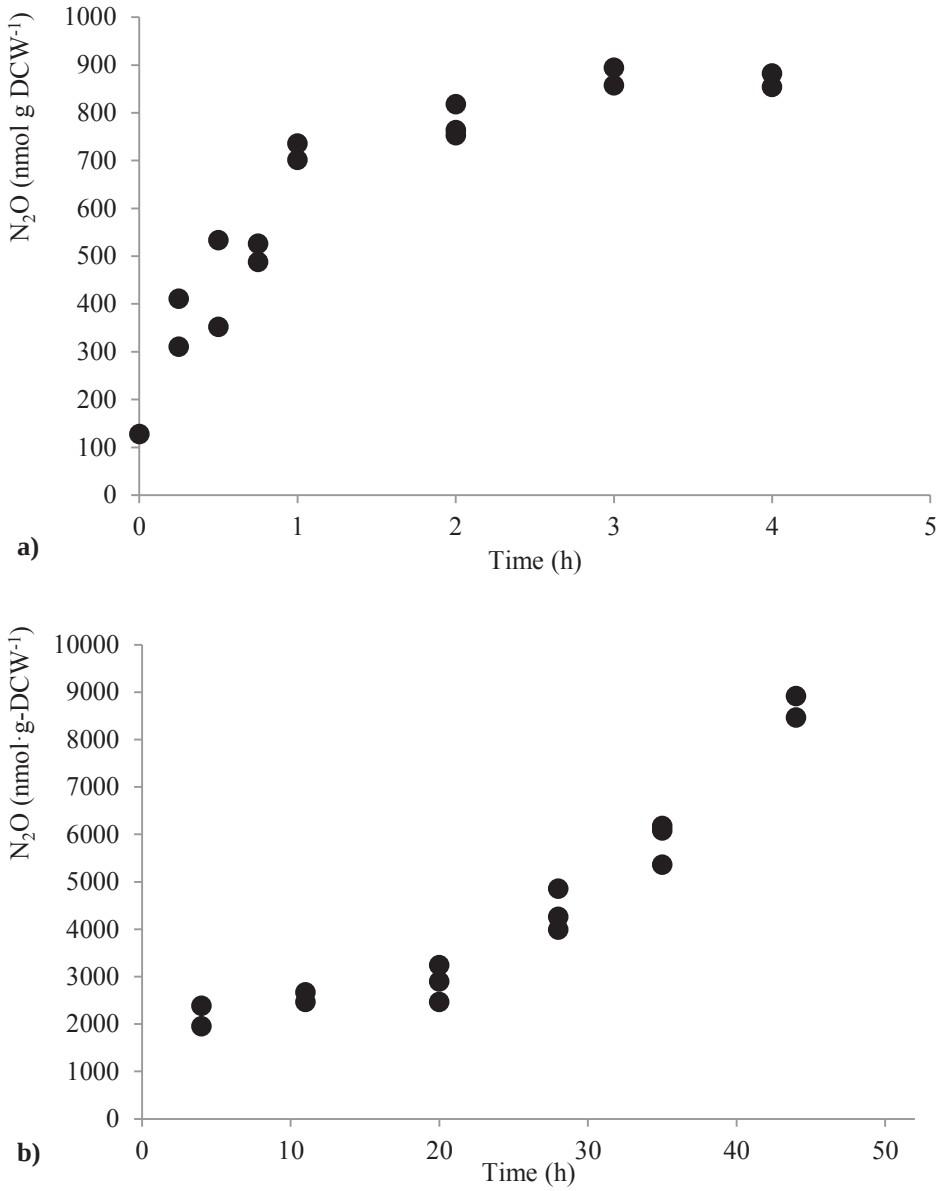


Figure 2.3: N_2O production (nmol·g-DCW^{-1}) between **a)** 0 – 5 h and **b)** 4 – 52 h by *C. reinhardtii* 6145c (initial DCW of 0.22 g-DCW·L^{-1}) supplied with 10 mM NO_2^- incubated in darkness.

After 24 h incubation higher levels of N_2O were generated in cultures incubated in darkness than cultures incubated under illumination (Figure 2.1), which is in agreement with past studies on axenic *C. vulgaris* when photosynthesis was inactive (darkness) or chemically inhibited (Weathers, 1984; Guieysse et al., 2013).

The 4 – 28 h N_2O production rates achieved by *C. reinhardtii* 6145c (100 nmol $N_2O \cdot h^{-1} \cdot g\text{-}DCW^{-1}$) were, however, significantly lower than rates previously recorded with *C. vulgaris* (1000 nmol $N_2O \cdot h^{-1} \cdot g\text{-}DCW^{-1}$). This divergence may be explained by differences in pathways and regulatory mechanisms.

For the first time, the ability of *C. reinhardtii* to synthesise N_2O was demonstrated (Figure 2.4).

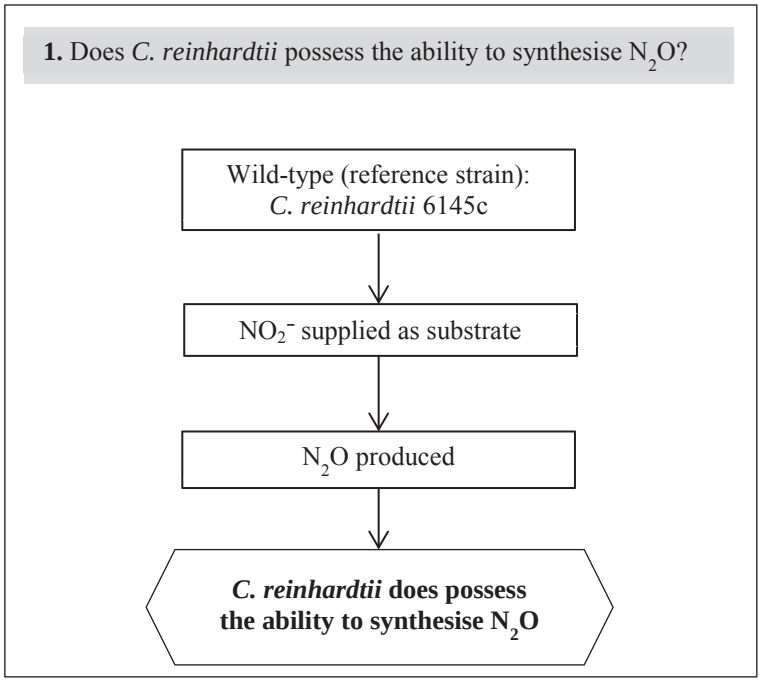


Figure 2.4: Diagram summarising the findings from Section 2.3.1: N_2O emissions from axenic *C. reinhardtii* cultures.

2.3.2 NO_2^- acts as substrate during microalgal N_2O synthesis

As shown in Chapter 1 (Box 1.1: Other potential pathways leading to N_2O synthesis in microalgae), several routes and substrates (i.e. nitrogen in different forms) could lead to

N_2O synthesis in microalgae. Knowing the potential link between N_2O synthesis and nitrate assimilation, batch assays were conducted with NO_2^-, NO_3^-, and NH_4^+. As seen in Figure 2.5, while the supply of NO_2^- triggers significant N_2O emissions (1200 nmol·g-DCW^{-1} after 24 h), the supply of NO_3^- or NH_4^+ did not trigger any significant emissions from axenic *C. reinhardtii* cultures (details are given in Appendix H, Table H1).

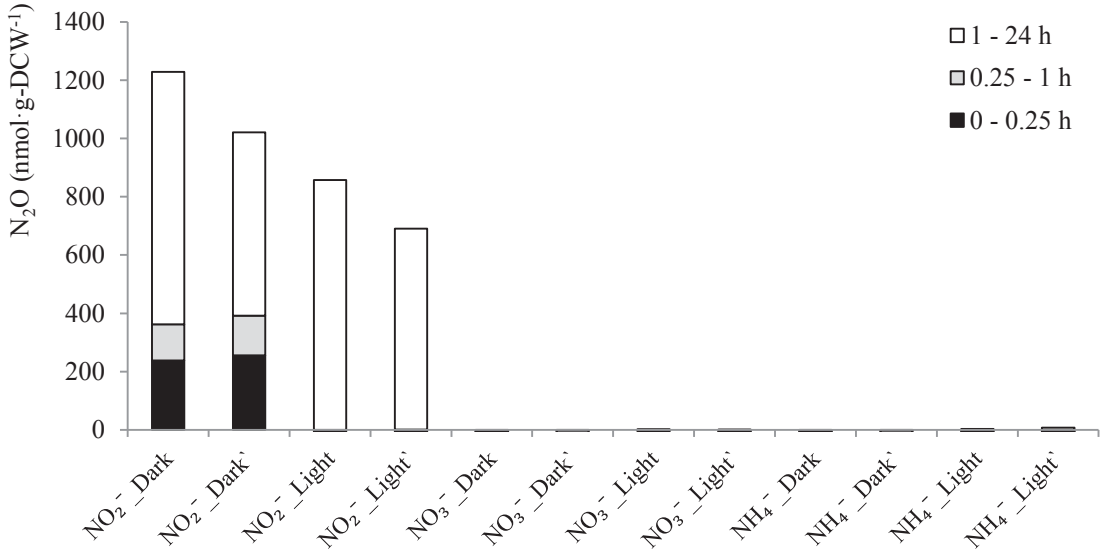


Figure 2.5: N_2O production (nmol·g-DCW^{-1}) by cultures of *C. reinhardtii* 6145c (initial DCW of 0.22 g-DCW·L^{-1}) supplied with either 10 mM NO_2^- or NO_3^- or NH_4^+ and incubated in darkness or under illumination for 24 h.

Further results showed that N_2O emissions were linearly correlated to extracellular NO_2^- concentration (Figure 2.6). The results therefore revealed an association between NO_2^- reduction and N_2O synthesis in agreement with past studies (Weathers, 1984; Weathers and Niedzielski, 1986; Guieysse et al., 2013; Kamp et al., 2013; Alcántara et al., 2015). This association between NO_2^- and N_2O was also confirmed in a different

setting (2 L photobioreactor) when the supply of NO_2^- triggered rapid N_2O synthesis by actively growing *C. reinhardtii* 6145c cultures (Appendix J).

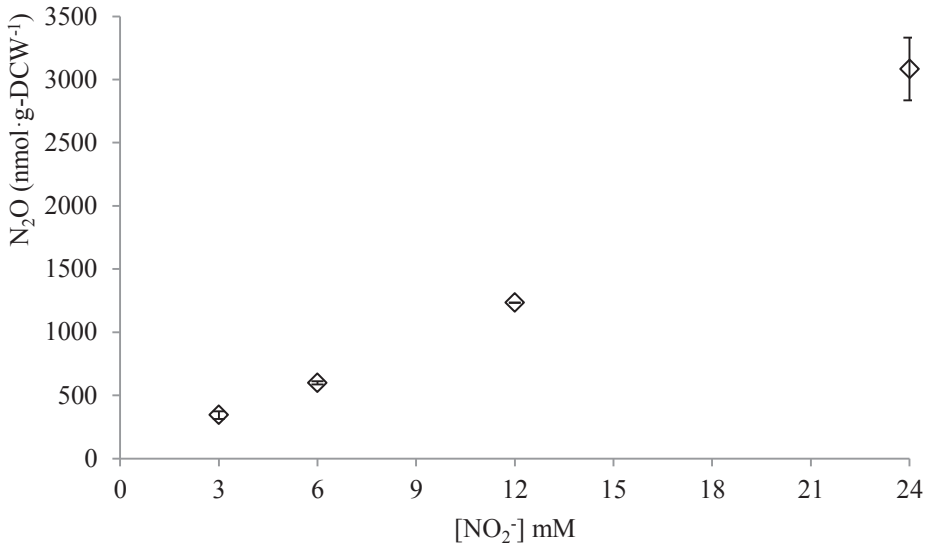


Figure 2.6: N_2O production (average nmol·g-DCW^{-1} ± SD) by triplicate cultures of *C. reinhardtii* 6145c (initial DCW of 0.25 g-DCW·L^{-1}) incubated for 24 h in darkness supplied with 3, 6, 12, or 24 mM NO_2^-.

This finding did not however dismiss the possibility of N_2O synthesis from nitric oxide synthase activity (NOS) as hypothesised in Chapter 1 (Box 1.1: Other potential pathways leading to N_2O synthesis in microalgae). NOS normally catalyses the oxidation of the amino acid L-arginine (L-arg) to L-citrulline and NO. Although a putative NOS-like activity was suggested in *C. reinhardtii* (de Montaigu et al., 2010) two observations provide compelling evidence that the NO_2^--independent pathway involving L-arg conversion by NOS is unlikely to be involved in the N_2O synthesis observed in our cultures. Firstly, N_2O synthesis was not recorded in NO_2^--free cultures supplied with L-arg. Secondly, N_2O production in nitrite-laden cultures actually increased slightly in the presence of the NOS inhibitor L-NNA (Figure 2.7). These

results agree with previous findings achieved over shorter durations (4 hr) using *C. reinhardtii* (Sakihama et al., 2002) and *C. vulgaris* (Guieysse et al., 2013).

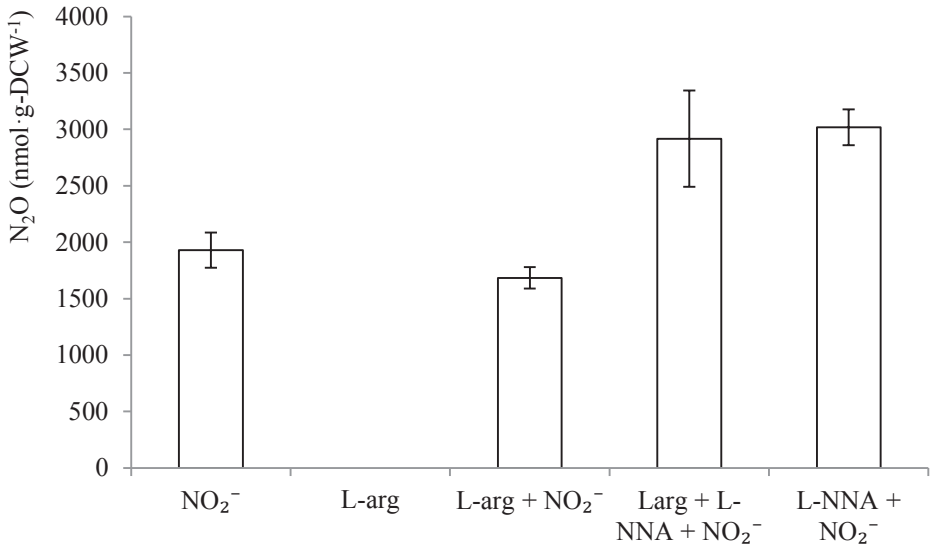


Figure 2.7: N_2O production (average nmol·g-DCW^{-1} ± SD) by triplicate cultures of *C. reinhardtii* 6145c (initial DCW of 0.25 g-DCW·L^{-1}) supplied with either NO_2^- 10 mM, or L-arg 10 mM, or L-Arg and NO_2^- at 10 mM, or L-arg, NNA and NO_2^- at 10 mM or NNA and NO_2^- at 10 mM and incubated for 24 h in darkness.

The results confirmed that only NO_2^- acted as a substrate during N_2O synthesis in *C. reinhardtii* (Figure 2.8).

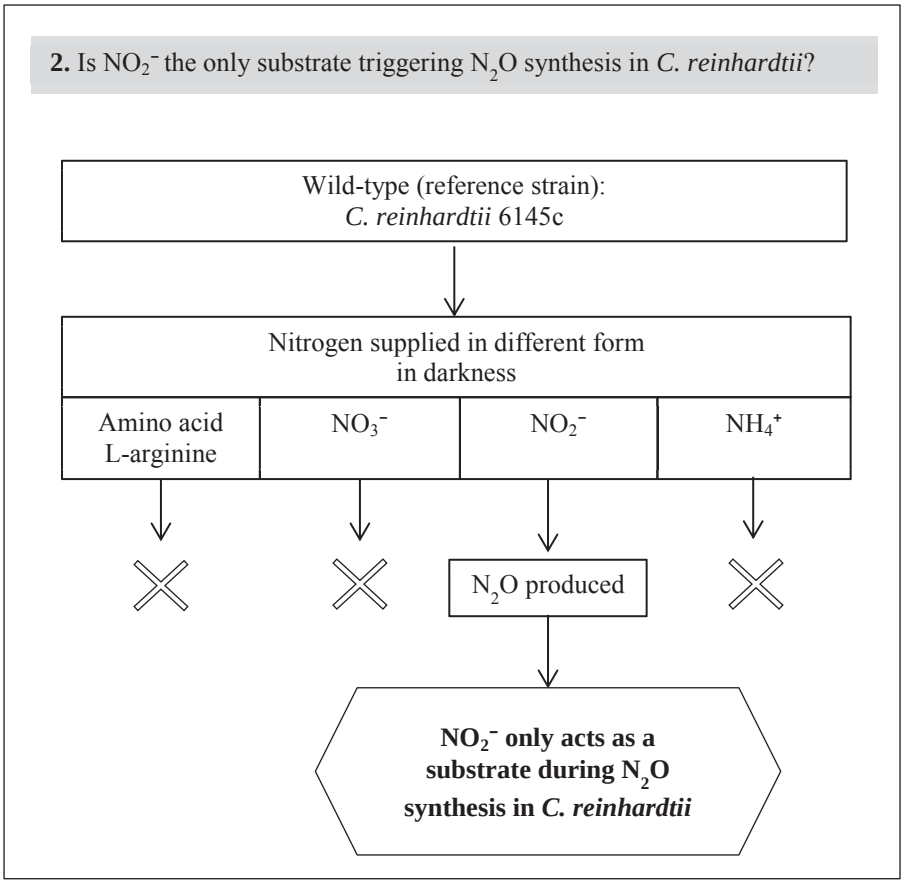


Figure 2.8: Diagram summarising the findings from Section 2.3.2: NO_2^- acts as substrate during microalgal N_2O synthesis.

2.3.3 NO is a key intermediate during N_2O synthesis

NO has been suggested as the potential intermediate during N_2O synthesis in *C. vulgaris* (Guieysse et al., 2013). NO is synthesised by many marine microalgae (Kim et al., 2008; Kumar et al., 2015), and fresh water microalgae (Mallick et al., 1999, 2000; Tischner et al., 2004; Estevez and Puntarulo, 2005) such as *C. reinhardtii* (Sakihama et al., 2002; Chang et al., 2013; Wei et al., 2014; Sanz-Luque et al., 2015b, Chamizo-Ampudia et al., 2016). In *C. reinhardtii*, NO synthesis has been associated with

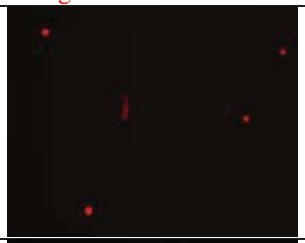
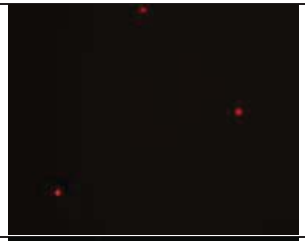
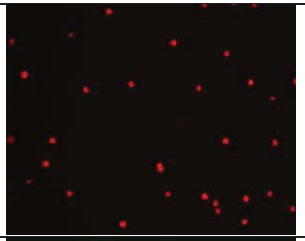
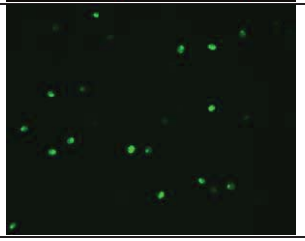
nitrogen assimilation (Sanz-Luque et al., 2013, 2015a) and both the hypoxic and nitrogen stress response (Zhang and Mehta, 2008; Hemschemeier et al., 2013; Wei et al., 2014). As a clear link between NO generation and N_2O synthesis was still missing in microalgae, the following section specifically addresses this knowledge gap.

Table 2.2 shows the microscopic observations for chlorophyll auto-fluorescence (characterised by a red colour) and DAF FM triazol fluorescence (characterised by a green colour) of *C. reinhardtii* 6145c cells pre-incubated with DAF-FM diacetate; cells pre-incubated with DAF-FM diacetate but not supplied with NO_2^-; cells supplied with NO_2^- but not pre-incubated with DAF-FM diacetate; cells pre-incubated with DAF-FM diacetate and subsequently exposed to NO_2^-. While red fluorescence was observed in all samples, green fluorescence was only observed in *C. reinhardtii* 6145c cells pre-incubated with DAF-FM diacetate and subsequently exposed to NO_2^- (Table 2.2).

Microscopic observations showed that strong green fluorescence was always detected in *C. reinhardtii* cells incubated with the NO fluorophore DAF-FM diacetate and subsequently supplied with an NO donor (Appendix K1). Based on this positive control, the presence of green fluorescence in *C. reinhardtii* 6145c cells pre-incubated with DAF-FM diacetate and exposed to NO_2^- suggests NO_2^- reduction caused NO synthesis, in agreement with the results of Sakihama et al, (2002) and Chamizo-Ampudia et al, (2016). In addition, fluorescence was not observed when the NO scavenger bovine hemoglobin was added to the microalgal suspension before incubation with DAF-FM diacetate and a supply of NO_2^- (Table K.1, Appendix K), confirming NO synthesis from NO_2^-. Taken together these results demonstrate that the

conditions triggering N_2O synthesis (Figure 2.1, 2.5 and 2.6) also trigger NO production in *C. reinhardtii*.

Table 2.2: Fluorescence of *C. reinhardtii* 6145c cells incubated with DAF FM Diacetate and supplied NO_2^-. Under the hypothesis that NO_2^- biological reduction yielded NO; red text shows negative control (when DAF FM diacetate and/or NO_2^- were not present), whereas the green text show the treatment (DAF FM diacetate and NO_2^- were both present). Microscopic photographs were taken with a Micropublisher 5 colour CCD camera (QImaging, Canada).

Conditions:	Control cells (no pre-incubation with DAF-FM Diacetate, no NO_2^- supply): these tests were conducted to characterize the green and red fluorescence backgrounds.	Cells pre-incubated with DAF-FM Diacetate (no NO_2^- supply): These tests were conducted to identify potential interfering effects of the pre-incubation on fluorescence detection.	Cells supplied NO_2^- (10 mM) but no pre-incubated with DAF-FM Diacetate: These tests were conducted to identify potential interfering effects of NO_2^- on fluorescence detection.	Cells pre-incubated with DAF-FM Diacetate and supplied NO_2^- (10 mM)
Microscopic photographs (×40) (excitation wavelengths 520 – 550 nm; emission wavelengths: 580 – 797 nm)				
Microscopic photographs (×40) (excitation wavelengths 460 – 480 nm; emission wavelengths: 510-580 nm)				

Note: the observed red colour is due to auto-fluorescence of the chlorophyll. The observed green colour indicates NO formation (more particularly the formation of DAF FM triazol produced when DAF FM reacts with NO).

As HNO has also been proposed as a possible intermediate in microalgal N_2O synthesis via NO_2^- reduction (Guieysse et al, 2013), an additional control was performed where *C. reinhardtii* 6145c cells pre-incubated with DAF-FM diacetate were supplied with Angeli's salt as an HNO donor (Table K.1, Appendix K). A weak fluorescence was found, which is surprising considering that DAF-FM is known to react with the NO oxidation products dinitrogen trioxide (N_2O_3) and nitrosonium anion (NO^+). However, the aqueous degradation of Angeli's salt releases both NO_2^- and HNO under physiological condition (Dutton et al., 2004; Miranda et al., 2005), meaning that 'indirect' NO_2^- supply may have caused NO synthesis in these tests. While our results strongly suggest that NO was generated during N_2O synthesis (Figure 2.9), further work is needed to fully verify this hypothesis, especially given that knowledge of HNO biology is still very limited (Fukuto et al., 2005).

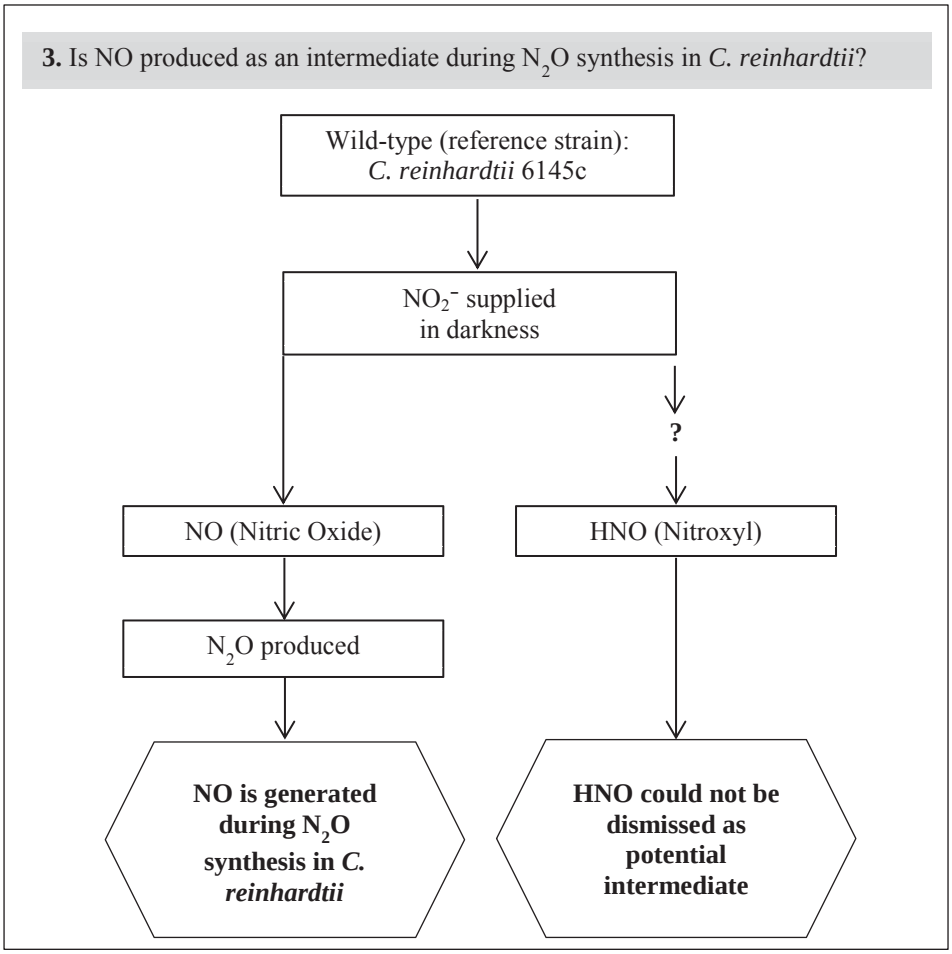


Figure 2.9: Diagram summarising the findings from Section 2.3.3: NO is a key intermediate during N_2O synthesis.

2.3.4 Short-term N_2O synthesis involves NR but late synthesis involves other enzymes

In microalgae NR normally reduces NO_3^- into NO_2^- via a 2 electron reduction during the first step of nitrate assimilation. NR has long been suspected to catalyse NO_2^- reduction into NO in both plants and *C. reinhardtii* (Yamasaki, 2000; Rockel et al., 2002; Sakihama et al., 2002; Meyer et al., 2005). This 'NR-NiR activity' (Yamasaki, 2000; Sakihama et al., 2002; Rockel et al., 2002; Meyer et al., 2005) was shown to be

dependent on the amount of NO_2^- accumulated in cell cytoplasm and to be often occurring at low levels (0.1–1% of NR total activity) due to competition between NO_3^- and NO_2^- for the same reduction sites (Meyer et al., 2005). More recently, NR was shown to be providing electrons to a NO forming nitrite reductase, thereby enabling NO_2^- reduction into NO by the dual NR-ARC (amidoxime reducing component) system of two molybdoenzymes (Chamizo-Ampudia et al., 2016). Renamed as NOFNiR (Nitric Oxide Forming Nitrite Reductase), the dual system was shown to mediate NO production both *in vitro* and *in vivo* in *C. reinhardtii*, when the intracellular concentration of NO_2^- increases in the presence of NO_3^- (Chamizo-Ampudia et al., 2016). In *C. vulgaris*, the involvement of NR during N_2O synthesis was suggested by evidence that N_2O synthesis was partially repressed in cells pre-cultivated with tungstate, a specific inhibitor of molybdoenzymes such as NR (Deng et al., 1989). The hypothesis of NR-mediated N_2O synthesis was therefore tested in *C. reinhardtii*.

C. reinhardtii 2929 is a mutant which is lacking NR. As shown in Figure 2.10, 'immediate' (0 – 15 min) release of N_2O was not recorded in NO_2^--laden cultures of the NR-deficient strain, although similar 0 – 24 h N_2O emissions were recorded in NO_2^--laden cultures of the NR-lacking mutant 2929 (2700 nmol·g-DCW^{-1}) and the reference strain 6145c (2500 nmol·g-DCW^{-1}) after 24 h of incubation. Similar observations (Appendix L) were reported in *C. reinhardtii* 6145c pre-cultivated with the NR-inhibitor tungstate which partially inhibits NR in *Chlamydomonas* (Llamas et al., 2000). Interestingly, Sakihama et al., (2002) reported short term (10 minutes) NO production in response to NO_2^- supply in *C. reinhardtii* wild-type 217 but not in NR mutant 2929. As further evidence in Figure K.1 (Appendix K), fluorescence was not observed in NO_2^--laden NR mutant 2929 cultures pre-incubated with DAF-FM

diacetate (this protocol could only be used for short-term exposure to nitrite due to the reactivity of DAF-FM). Altogether, these results indicate that N_2O synthesis involved NO_2^- reduction into NO by the dual system NR-ARC immediately following NO_2^- addition (0 – 15 min), whereas another enzymatic system was involved in the late N_2O response. The possible involvement of other molybdoenzymes during the late N_2O production phase (e.g. xanthine oxidase) was also dismissed based on the tungstate inhibition assays.

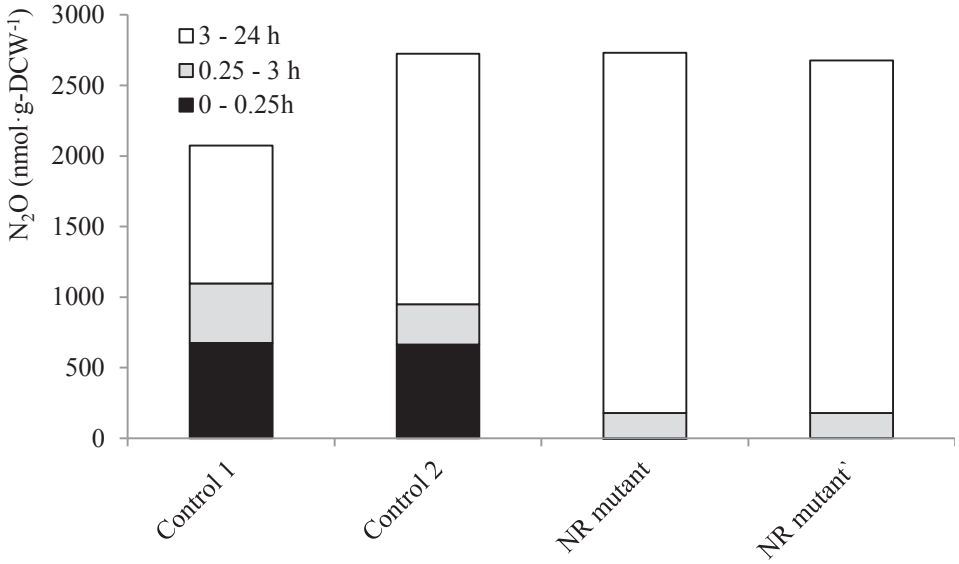


Figure 2.10: N_2O production (nmol·g-DCW^{-1}) between 0 – 15 min; 15 min – 3 h; 3 – 24 h *C. reinhardtii* 6145c (initial DCW of 0.25 g-DCW·L^{-1}) and NR deficient mutant 2929 (initial DCW of 0.25 g-DCW·L^{-1}) supplied with 10 mM NO_2^- and incubated in darkness. See Appendix H, Table H.1 and Table H.2, for the results from all replicates.

2.3.5 NR and NiR activities impacts N_2O synthesis under NO_3^- supply

NO_3^- is the main nitrogen source available to microalgae in many 'natural' environments (Raven and Giordano, 2013) and it is commonly used during commercial microalgae cultivation (Borowitzka, 2005). NR and NiR sequentially catalyse the assimilatory reduction of NO_3^- into NO_2^- and NH_4^+, thereby contributing to regulation of the intracellular concentration of NO_2^-. Consequently, both NR and NiR have key functions during N_2O synthesis. As NO_2^- 'fuels' N_2O synthesis via NO, the activities of NR and NiR are likely to impact N_2O emissions under physiological conditions (when NO_3^- is the main N source and the intracellular concentration of NO_2^- is low), as indeed shown by the fact NiR-deficient mutants supplied NO_3^- synthesised large amounts of N_2O under illumination (Figure 2.11).

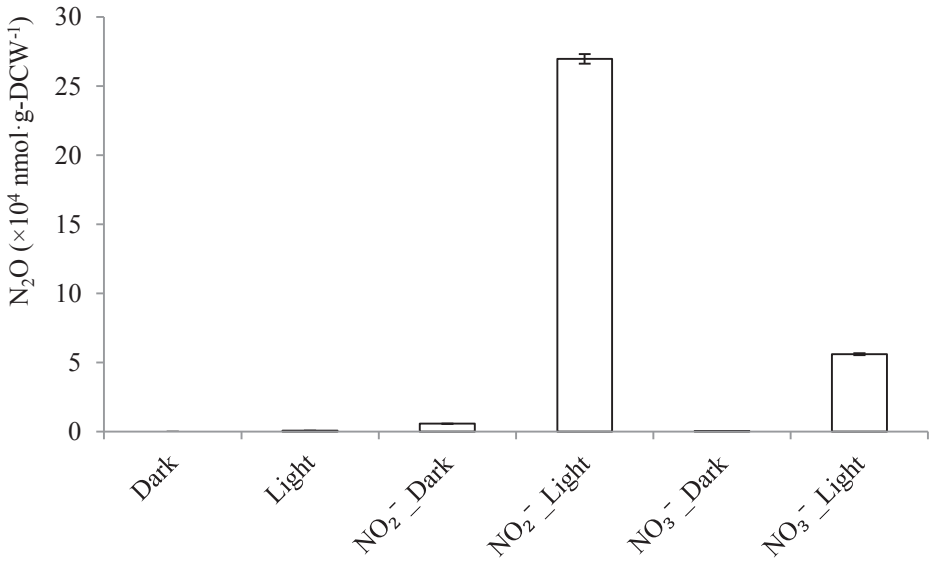


Figure 2.11: N_2O production (average nmol·g-DCW^{-1} ± SD) by triplicate cultures of *C. reinhardtii* NiR mutant M3 (initial DCW of 0.25 g-DCW·L^{-1}) supplied with either NO_2^- or NO_3^- at 10 mM and incubated for 24 h in darkness or under illumination.

Because the NiR mutant M3 possessed an active NR with activity similar to the 6145c NR (Navarro et al., 2000) and NR is activated by light and NO_3^- (Kaiser and Huber, 2001), this NiR mutant was able to carry out the stoichiometric reduction of NO_3^- into NO_2^- (Navarro et al., 2000) but could not reduce NO_2^- into NH_4^+. Nitrite therefore over-accumulated in illuminated NiR-deficient cells (Navarro et al., 2000), which probably resulted in increased NO turnover boosting N_2O emissions. Nitrite-laden cultures of NiR-deficient mutants were also shown to release N_2O both in darkness and under illumination (Figure 2.11).

The indirect impact of NR and NiR on intracellular NO_2^- accumulation suggests that microalgal N_2O emissions should mainly occur under illumination when NO_3^- is the dominant N source, as reported by Guieysse et al. (2013) during the outdoor cultivation of *C. vulgaris* supplied NO_3^- as sole N source (See further evidence in Chapter 3 Section 3.1.3.1). The results presented in Sections 2.3.4 and 2.3.5 confirmed the direct relation between nitrate assimilation and N_2O synthesis: In the presence of NO_3^-, NR supplies NO_2^- as the substrate for N_2O synthesis and if NiR is lacking or inactivated (i.e. during a period of low photosynthetic activity) it favours NO_2^- accumulation, potentially leading to N_2O synthesis (Figure 2.12).

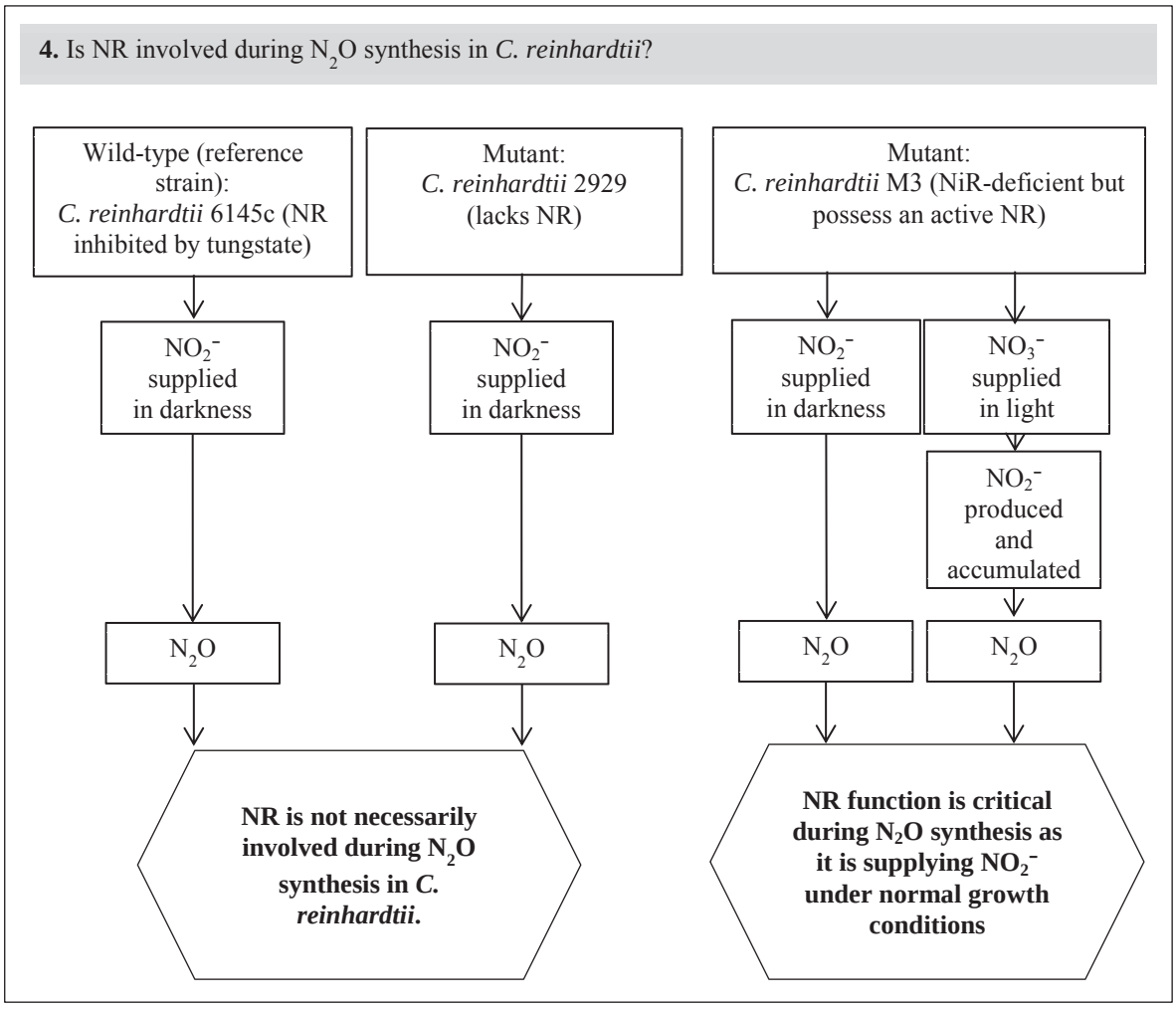


Figure 2.12: Diagram summarising the findings from section 2.3.4: Short-term N_2O synthesis involves NR but late synthesis involves other enzymes in *C. reinhardtii*, and 2.3.5: NR and NiR activities impacts N_2O synthesis under NO_3^- supply.

2.3.6 Late N_2O synthesis involves NO_2^- reduction to NO by mitochondrial COX

NO_2^- reduction via the mitochondrial electron transport chain was first demonstrated in the green microalgae *C. sorokiniana* (Tischner et al., 2004) and mitochondria are a known source of NO in higher plants (Planchet et al., 2005; Igamberdiev et al., 2014; Gupta et al., 2016). Mitochondrial COX or AOX can reduce NO_2^- into NO under anaerobic conditions (Tischner et al., 2004; Planchet et al., 2005; Gupta and Igamberdiev, 2011) and, at a low rate, under aerobic conditions (Tischner et al., 2004; Planchet et al., 2005). As experimental evidence linking these mitochondrial enzymes to N_2O synthesis was lacking, *C. reinhardtii* 6145c and the NR deficient mutant *C. reinhardtii* 2929 were supplied with NO_2^- in the presence of the COX inhibitor KCN (2 mM). This treatment with 2 mM CN^- resulted in immediate inhibition of both N_2O (Appendix H, Table H.1) and NO (Appendix K), which could be explained by the fact that CN^- can also inhibit NR (Tischner et al., 2004; Planchet et al., 2005). However, and in contrast to NR chemical inhibition or repression, N_2O synthesis was inhibited by 90% and 95% in the wild-type 6145c and NR mutant 2929 cultures, respectively, when 2 mM KCN was added in the cultures (Figure 2.13). Together, these results suggest that mitochondrial COX catalyses NO_2^- reduction into NO during the late period of N_2O production.

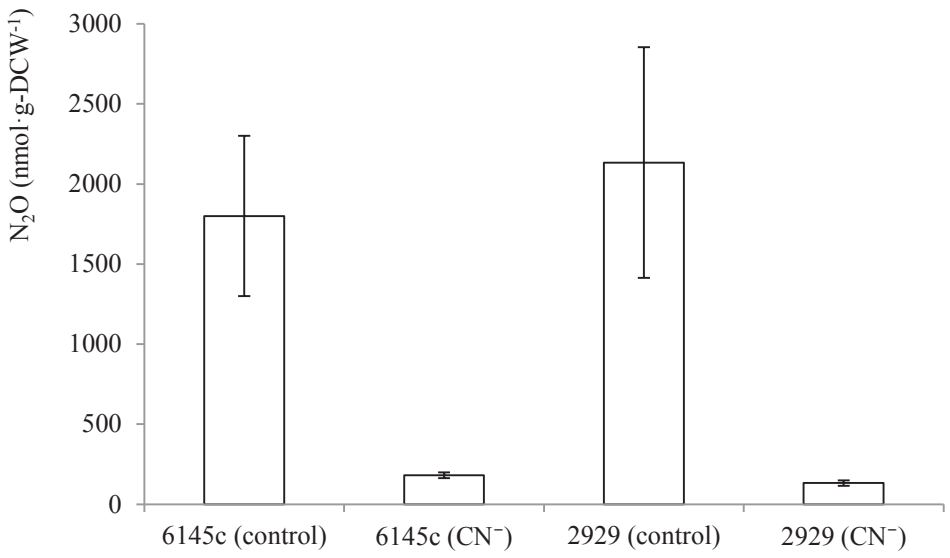


Figure 2.13: N_2O production (average nmol·g-DCW^{-1} ± SD) by triplicate cultures of *C. reinhardtii* 6145c (0.25 g·L^{-1}) and cultures of *C. reinhardtii* 2929 (0.25 g·L^{-1}) supplied 10 mM NO_2^- and 2 mM KCN ("CN^-") against CN^-- free cultures ("control"); all cultures were incubated for 24 h in darkness.

Given that N_2O synthesis by both *C. reinhardtii* strains was not completely abolished following KCN addition, AOX may also be partially responsible for NO_2^- reduction into NO. Tischner et al, (2004) demonstrated that the *C. sorokiniana* NiR mutant continuously generated a high amount of NO under anaerobic and aerobic conditions, and that this production could be catalysed by both COX and AOX. AOX-mediated NO_2^- reduction to NO was also demonstrated in the model plant *Arabidopsis thaliana* (Gupta et al., 2016). Unfortunately the potential involvement of AOX could not be experimentally dismissed as the chemical reaction of the known AOX inhibitor salicylhydroxamic acid (SHAM) was found to generate N_2O from NO_2^- (44 ± 8 nmole N_2O in abiotic tests after 3 h). It should also be noted that NO_2^- is a known antidote to cyanide, and that NO may also impact the inhibitory effect of CN^- (Leavesley et al., 2008).

2.3.7 Possible role of hemoglobins during N_2O synthesis in *C. reinhardtii*

Plant hemoglobins are known to reduce NO_2^- into NO (Hoy and Hargrove, 2008; Gupta et al., 2011) and microalgal hemoglobins can catalyse this reaction under hypoxic/anoxic conditions (Sturms et al., 2011), a mechanism thought to be related to either NO_2^- detoxification (Sturms et al., 2011) or survival under anoxia (Hemschemeier et al., 2013). *C. reinhardtii* possesses 12 truncated hemoglobins (THB 1-12) and some of these proteins could be able to reduce NO_2^- (Huwald et al., 2015; Ciaccio et al., 2015). *C. reinhardtii* THB1 is in turn involved in NO signalling, NO_3^- assimilation, NR activity regulation, and, of particular relevance, NO scavenging by its dioxygenase activity under aerobic conditions (Sanz-Luque et al., 2015b). This latter activity of THB1, by promoting transformation of NO into NO_3^-, may help in the regulation of NO intracellular accumulation during nitrate assimilation. Further research on the role of hemoglobins (e.g. as NO synthases) during N_2O synthesis is needed.

The results discussed in Section 2.3.6 showed that mitochondrial COX was probably the enzyme catalysing the reduction of NO_2^- into NO during the late phase of N_2O synthesis. However, as mentioned in Section 2.3.6 and the paragraph above, the potential involvement of AOXs and hemoglobins (THBs) as NO synthases could not be entirely dismissed (Figure 2.14). In consequence, further research is needed.

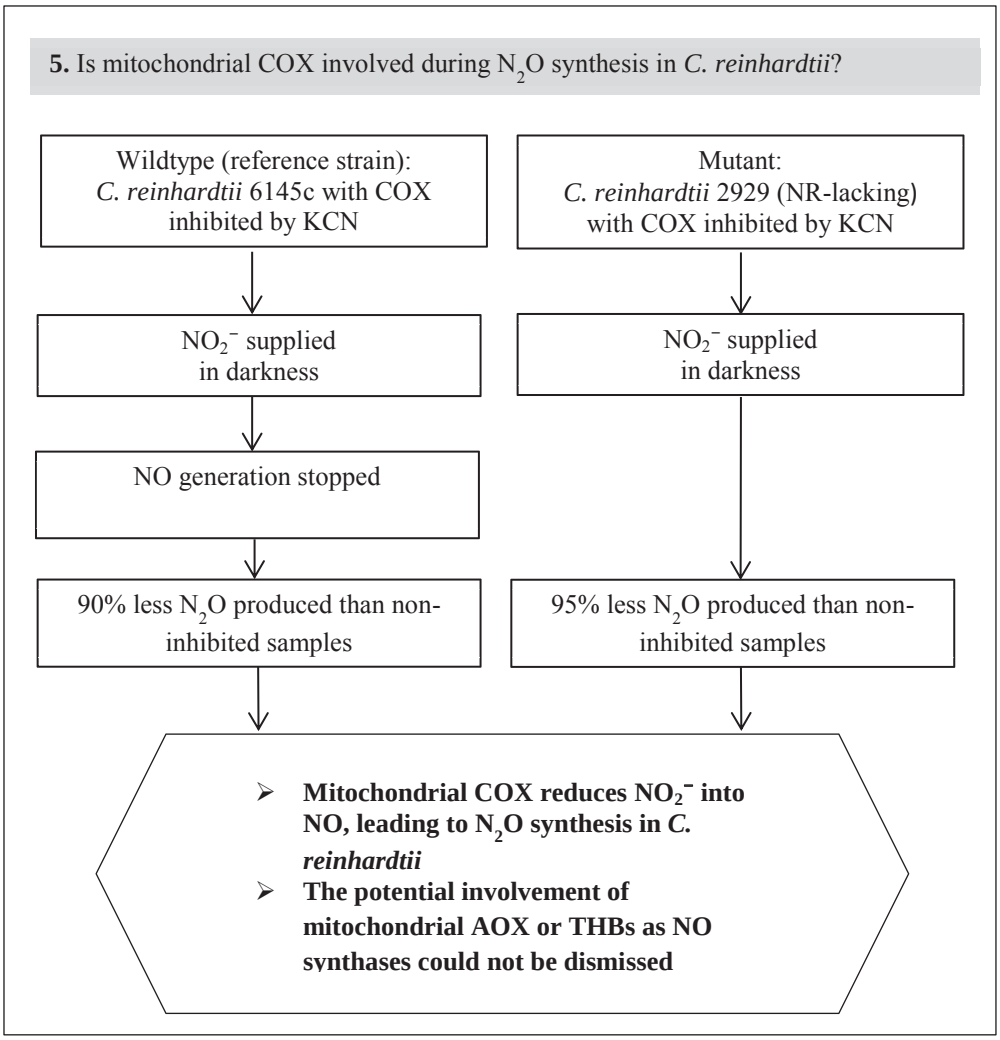


Figure 2.14: Diagram summarising the findings from Section 2.3.6: Late N_2O synthesis involves NO_2^- reduction to NO by mitochondrial COX and Section 2.3.7: Possible role of hemoglobins during N_2O synthesis in *C. reinhardtii*.

2.3.8 N_2O synthesis by *C. reinhardtii* involves NO reduction into N_2O by NOR

Although concurrent NO and N_2O syntheses in response to NO_2^- supply were repeatedly observed in *C. reinhardtii* cultures (Figure 2.1; 2.5; 2.6; Table 2.2), NO

reduction into N_2O is difficult to explain because NO is typically oxidized into nitrogen dioxide (NO_2) and other products (*e.g.* $ONOO^-$) under aerobic conditions (Murphy et al., 1998; Lamattina et al., 2003). *C. reinhardtii* however, harbours a gene (CYP55) similar to a fungal gene encoding a nitric oxide reductase (NOR) (Chang et al., 2011; Guieysse et al., 2013; see further evidence below), an enzyme capable of reducing NO into N_2O under hypoxia (Morozkina and Kurakov, 2007; Shoun et al., 2012). When supplied with NO_2^-, a *C. reinhardtii* NOR-knock down mutant (amiCYP55 strain with gene CYP55 silenced using artificial micro iRNA) synthesised 70 – 90% less N_2O[7] than its respective parent (Figure 2.15).

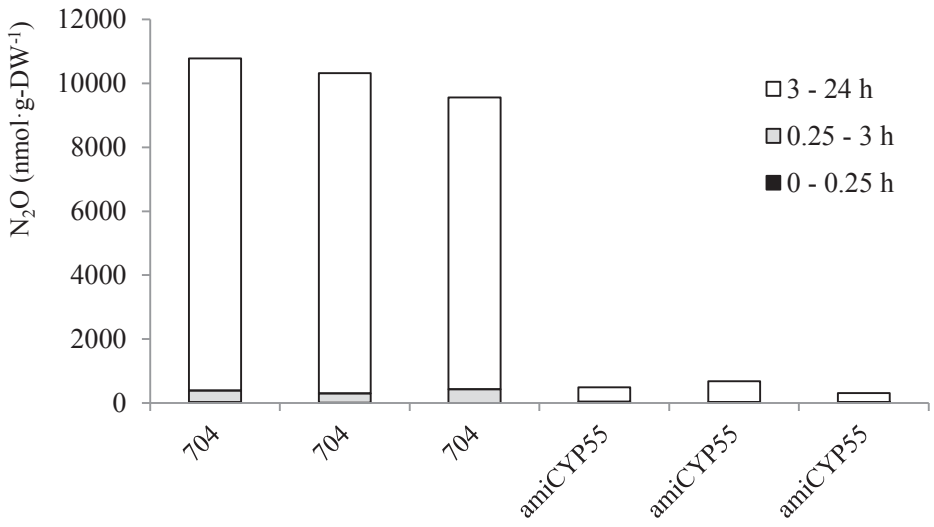


Figure 2.15: N_2O production (nmol·g-DCW^{-1}) by triplicate cultures of *C. reinhardtii* 704 (initial DCW of 0.25 g-DCW·L^{-1}) and amiCYP55 (CYP55-silenced) mutant (initial DCW of 0.25 g-DCW·L^{-1}) supplied with 10 mM NO_2^- and incubated for 24 h in darkness.

[7] N_2O synthesis was not fully inhibited likely due to the fact NOR expression was not fully repressed in the amiCYP55 strain (i.e. 54% of the wildtype strain 704).

The results discussed above suggest that wild-type *C. reinhardtii* synthesises N_2O via NOR-mediated NO reduction under aerobic conditions (Figure 2.16). Interestingly, N_2O synthesis increased when NO_2^--laden *C. reinhardtii* 6145c was incubated under anoxic conditions (Appendix M). This finding may be explained by the absence of competition between NO_2^- and O_2 for the COX reaction sites (Gupta et al., 2005), as well as reduced rates of NO oxidation (Gupta et al., 2016) favoring NO reduction into N_2O by NOR.

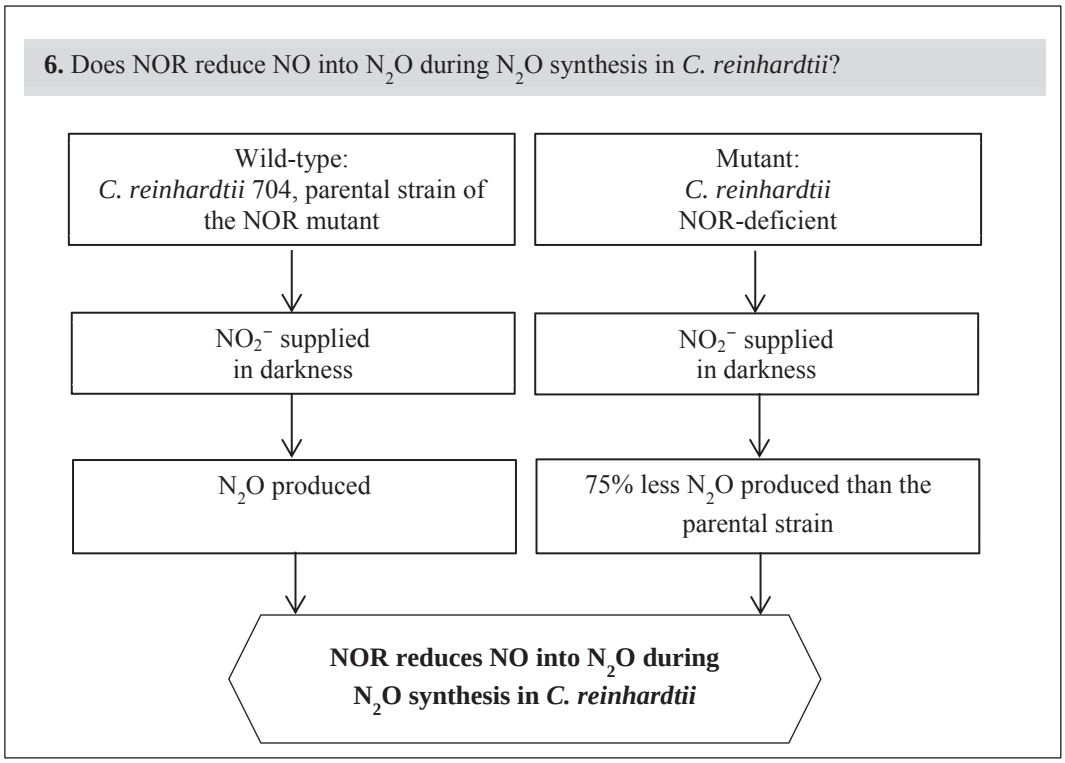


Figure 2.16: Diagram summarising the findings from Section 2.3.8: N_2O synthesis by *C. reinhardtii* involves NO reduction into N_2O by NOR.

2.3.9 RNA-seq based transcriptomics suggest that microalgal N_2O synthesis is a side reaction of NO metabolism

RNA-seq uses high throughput sequencing data to measure gene expression levels (Marguerat and Bähler, 2010). Unlike older microarray based transcriptomic methods, RNA-seq has high reproducibility and sensitivity, and a large dynamic range of expression level detection (Wang et al., 2009). In order to better understand the transcriptional response underlying N_2O production in *C. reinhardtii*, RNA-seq was carried out on triplicate samples collected at 0.25, 3, and 24 hours after the addition of NO_2^- in the "treatment" cultures. These particular time points were chosen based on the three phases N_2O kinetic in *C. reinhardtii* (Section 2.3.1).

Illumina RNA sequencing of the resulting libraries generated 241,151,809 paired-end 125bp reads with 95.78% of total base pair having a Phred quality score of > Q30. Mapping these reads to the published *C. reinhardtii* genome (*C. reinhardtii* v.5.5, Merchant et al., 2007) resulted in an average mapping rate of 73.9%. Following RNA-seq analysis, it became clear that the supply of NO_2^- in darkness had an effect on *C. reinhardtii* 6145c cells (Figure E.2 in Appendix E). The RNA-seq data was used to explore the expression of key candidate genes thought to be involved in N_2O production in *C. reinhardtii*. Table 2.3 presents for each time point, the differential expression and the normalised read counts in treatment samples for the genes thought to be involved during N_2O synthesis in *C. reinhardtii*. The differential expression is then given by a log2 fold change (log2FC) which indicate the up (*i.e.* increase) or down (*i.e.* decrease) expression of genes between controls and treatments at each time point. The log2FC was only trusted if the differential expression was statistically significant (adjusted *p-value* threshold of 0.05).

Table 2.3: Log2FC between control and treatment groups of candidate genes potentially involved in microalgal N_2O synthesis. Numbers in parenthesis represent mean normalised counts.

Genes	annotation	Time (h) after exposure to NO_2^-		
		0.25	**3**	**24**
NIT1	Nitrate reductase (NR)	0.0 (1)	0.0 (0)	0.0 (0)
NII 1	Nitrite reductase (NiR)	-0.2 (2)	-0.4 (4)	-0.7 (1)
CYP55	Nitric oxide reductase (NOR)	0.2 (5150)	0.3 (2974)	-0.1 (1487)
ARC	Amidoxime reducing component	0.0 (10)	-0.2 (7)	-0.8 (15)
THB1	Truncated Hemoglobin	**0.7* (1142)**	0 (1142)	**0.5* (1052)**
THB2	Truncated Hemoglobin	**-0.6* (133)**	-0.3 (342)	**-1.4* (57)**
COX2a	Cytochrome c oxidase subunit II	-0.1 (945)	-0.2 (534)	-0.4 (420)
COX3	Cytochrome c oxidase subunit III	-0.1 (1583)	-0.1 (920)	**-0.5* (921)**
COX5c	Cytochrome c oxidase subunit	-0.1 (1445)	-0.2 (824)	-0.3 (518)
MT-CO1	Mitochondrial cytochrome c oxidase subunit 1	0.0 (2)	-0.3 (2)	0.4 (0)
COX2b	Cytochrome c oxidase subunit	-0.1 (3801)	-0.2 (2409)	0.0 (1482)
COX11	Involved in the insertion of Copper into the CuB center of subunit Cox1p	-0.1 (2)	0.0 (1)	0.4 (3)
COX13	Cytochrome c oxidase subunit 10	-0.1 (2219)	-0.1 (1462)	-0.2 (742)
COX15	Cytochrome c oxidase assembly factor	0.2 (2)	0.0 (0)	0.5 (7)
COX16	Cytochrome c oxidase assembly factor	0.0 (2)	0.0 (2)	-0.1 (5)
COX17	Involved in the delivery of Copper to mitochondria	0.2 (8)	-0.1 (5)	1.2 (7)
COX18	Cytochrome c oxidase assembly factor	0.2 (4)	0.1 (4)	0.0 (4)
COX19	Cytochrome c oxidase assembly factor	0.2 (21)	0.2 (8)	-0.1 (6)
COX191	Involved in cytochrome c oxidase expression	0.1 (3)	0.0 (9)	0.4 (19)
COX23	Cytochrome c oxidase assembly factor involved in Copper homeostasis	-0.2 (49)	-0.4 (37)	0.0 (51)
COX90	Cytochrome c oxidase subunit	-0.1 (910)	-0.2 (465)	0.0 (473)
AOX1	Alternative oxidase	**0.5* (1050)**	-0.5 (575)	**0.5* (736)**
AOX2	Alternative oxidase	-0.2 (17)	0.4 (6)	**1.4* (14)**

* Log2FC values shown in bold are significant based on a Benjamin-Hochberg adjusted *p-value* < 0.05 (Benjamini and Hochberg, 1995). Log2 fold changes of associated genes (e.g. nitrate assimilation regulation) are given in Table E.3 (Appendix E).

Surprisingly, genes encoding NR, ARC, NiR, and NOR did not show significant differential expression between the treatments and the controls at any of the time points tested in this study. Overall, the transcriptomics data was consistent with the biochemical evidence that NO_2^- reduction into NO leading to 'late' N_2O synthesis is not NR-mediated (it does not dismiss the eventuality of active enzyme e.g. NR-ARC dual system). This may be due to the NO generated, as the production of high amounts of NO is known to cause transcriptional repression of nitrate assimilation genes (de Montaigu et al, 2010). Nitrite response via transcriptional regulation of NOR may not have been triggered/required under the experimental conditions tested, but the data clearly show that NOR was indeed being synthesised (the average normalised read counts across all samples for NOR was 3156, n = 9).

In contrast to the genes discussed above, THB1 and THB2 genes were significantly differentially expressed after 24 h of NO_2^- exposure (Table 2.3). *C. reinhardtii* THB1, which was significantly upregulated in the treatment cells after 0.25 and 24 h of NO_2^- exposure (Log2FC of 0.7 and 0.5, respectively), is also involved in NO signaling, NO_3^- assimilation, NR activity regulation and, of particular relevance, NO scavenging under normoxia via its dioxygenase activity (Sanz-Luque et al., 2015b). Consequently, during NO_3^- assimilation, THB1 may help to regulate NO intracellular accumulation by promoting the transformation of NO into NO_3^- in parallel to NO reduction to N_2O. The upregulation of THB1 and downregulation of THB2 agree with the transcriptional NO response described by Sanz-Luque et al. (2015b), and therefore provide additional evidence of both the involvement of NO as N_2O intermediate, and the involvement of THBs in the response to NO synthesis.

Transcriptomic data also showed that while several COX genes were strongly expressed, only one COX gene was differentially expressed and down-regulated after 24 h of NO_2^- exposure (Log2FC of -0.5; Table 2.3). As in the case of NOR, transcriptional regulation of COXs might not have been required under the experimental conditions tested. AOX1 and AOX 2 were also up-regulated with Log2FC of 0.5 and 1.4, respectively. AOX are regulatory enzymes balancing electrons transport in mitochondria (Vanlerberghe and McIntosh, 1997). Considering that NO has been shown to induce AOX in the well-studied terrestrial plant *Arabidospis* (Huang et al., 2002), AOX synthesis could have been activated in response to NO generation by COX in *C. reinhardtii*.

The RNA-seq data are consistent with the biochemical evidence that NO is generated during N_2O synthesis. The data also showed that the transcriptional regulation of NR, ARC, NiR, and NOR genes was potentially not required under NO_2^- loading. However, the up-regulation of AOXs and THB1 genes was necessary, most likely as a response to NO generation. It is worth noticing that the observation of genes not significantly differentially expressed does not rule out the possibility that the encoded proteins are present and active. It is also possible that the expressions of the genes of interest are 'hidden' behind the molecular 'noise' existing in samples. For instance, the transcriptomic data showed significant down regulation of several eukaryotic initiation factors (Table E.2 in Appendix E) specifically recorded in NO_2^--replete cultures, indicative of cells that have activated stress response pathways (Langland et al., 1996; Hinnebusch, 1997; Roy et al., 2010; Pakos-zebrucka et al., 2016). Activation of these pathways possibly explains the large amount of differential expression we observed in our transcriptomic comparisons and this complicates the identification of other genes potentially involved in this process.

We are currently in the process of carrying out a detailed RNA-seq study to fully characterise the molecular responses to NO_2^- loading. Indeed, further analysis is needed to evaluate the full metabolic effect of high NO_2^- in *C. reinhardtii* as 180, 740 and 3914 genes were significantly differentially expressed between control and treatment groups after 0.25, 3, and 24 hr of NO_2^- exposure, respectively.

2.4 Implications and integration of findings to new knowledge

2.4.1 Biological implications

This study showed that axenic *C. reinhardtii* supplied with NO_2^- can synthesise N_2O under aerobic conditions via NO_2^- reduction into NO followed by NO reduction into N_2O. NO_2^- reduction into NO was initially catalysed by the dual NR-ARC system, but this activity rapidly ceased and was later replaced by NO_2^- reduction into NO by COX (Figure 2.17). Under physiological conditions, NO_3^- is the main N source and the intracellular concentration of NO_2^- is low. The reduction of NO_3^- into NO_2^- by NR therefore provides both the nitrogen source for growth and, under specific circumstances the substrate for NO biosynthesis by the dual system NR-ARC (Chamizo et al., 2016). Moderate NO production would be expected to take place at moderate cytosolic NO_2^- concentrations and, under such condition, the dioxygenase activity of THB1 could efficiently modulate the intracellular concentrations of NO, NO_2^-, and NO_3^- (Sanz-Luque et al., 2015b). In contrast, significant exposure to high intracellular NO_2^- concentrations (as applied in this work) appears to cause COX to reduce significant amounts of NO_2^- into NO (Figure 2.17).

Both NO_2^- and NO are known to generate harmful products (Faure et al., 1991; Beckman and Koppenol, 1996) and cells have developed mechanisms to prevent the intracellular accumulation of these molecules, such as NO oxidation into NO_3^- by THBs (Sanz-Luque et al., 2015b, Figure 2.17) and NO_2^- excretion (Faure et al., 1991; Navarro et al., 2000). NO_2^- reduction into N_2O via NO could therefore provide algae cells periodically experiencing exposure to hypoxia or anoxia (e.g. hypoxic oceans,

maturation ponds) with a means to detoxify NO_2^- and NO. This activity may be the fortuitous consequence of COX activity under aerobic conditions, or it may provide additional detoxification capacity in microalgal cells (i.e. NOR would be acting as a security valve eliminating NO).

Interestingly, the yield of algal N_2O synthesis was lower under aerobic conditions (0.03 % g-N-N_2O synthesised - g N-input^{-1} after 24 h of incubation in darkness for strain 6145c) than under anoxia (0.8 % g-N-N_2O synthesised - g N-input^{-1} after 24 h of incubation in darkness for strain 6145c, See Table G1 in Appendix G), suggesting the existence of a competitive NO metabolism under aerobic conditions (e.g. NO_x generation, Lamattina et al., 2003). Clearly, our understanding of the function and regulation of the enzymes and substrates involved in N_2O synthesis in microalgae remains limited. Nevertheless, the evidence herein presented (such as the ability of *C. reinhardtii* to reduce NO into N_2O under aerobic conditions) provides new advances in algal biology in light of the importance of nitrate assimilation and NO metabolism in these organisms.

2.4.2 Environmental implications

As discussed above, N_2O synthesis under physiological conditions is linked to NO_3^- assimilation in *C. reinhardtii* (Figure 2.17) in a mechanism similar to the fungal N_2O denitrification pathway (Shoun et al., 2012), with the significant difference that the microalgae carry out N_2O synthesis under aerobic conditions. This finding has broad implications because the pathway of NO_3^- assimilation is conserved among microalgae species from the Chlorophyta division (Sanz-Luque et al., 2015a) and its regulation

involves NO (de Montaigu et al., 2010; Sanz-Luque et al., 2013, 2015a). Moreover, several microalgae species from at least 3 divisions (Chlorophyta, Bacillariophyta, Cyanobacteria) have the ability to synthesise NO (Kim et al., 2008; Kumar et al., 2015; Mallick et al., 1999; Tischner et al., 2004) and/or N_2O (Weathers, 1984; Weathers and Niedzielski, 1986; Guieysse et al., 2013; Kamp et al., 2013; Alcántara et al., 2015), and *Chlorella variabilis* harbors a close homolog gene to the *Chlamydomonas* NOR gene (Guieysse et al., 2013). All these observations suggest that the ability to synthesise N_2O could be found in numerous microalgae species. This ability may also explain why correlations between primary productivity and N_2O emission rate have been reported for decades (Pierotti and Rasmussen, 1980; Outdot et al., 1990; Mengis et al., 1997; Wang et al., 2006), even under very low exogenous NO_2^- concentration (probably due to intracellular NO_2^- generation during NO_3^- assimilation; Pierotti and Rasmussen, 1980). Because microalgae are ubiquitous in the environment and often associated with anthropogenic pollution (e.g. algal blooms), the potential significance of microalgal N_2O biosynthesis should be recognised in greenhouse gas inventories such as the methodology proposed by the Intergovernmental Panel for Climate change. Fortunately, the evidence that mandates a broader recognition of the potential issue also provides guidance for mitigation strategies: distinct differences in the abilities (Chapter 1, Section 1.3.3) and kinetics of microalgal N_2O emissions have been reported. For example, nitrite-laden *C. vulgaris* was previously shown to constantly produce N_2O over 48 hours of incubation in darkness (Guieysse et al., 2013). These divergences indicate differences in pathways and regulatory mechanisms that, in turn, could be exploited to reduce N_2O emissions during algal cultivation.

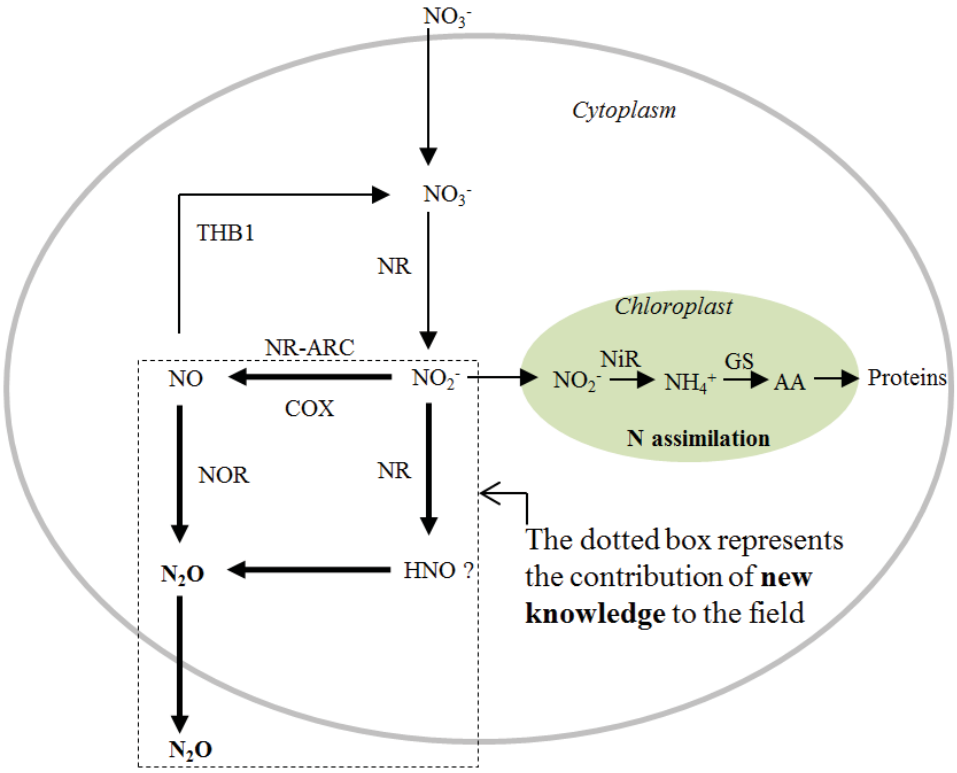


Figure 2.17: N_2O synthesis in *C. reinhardtii*. The dashed box represents the new knowledge introduced to the field of microalgal biochemistry. NR = nitrate reductase, NR-ARC = dual system of NR and ARC, also called NR-NOFNiR, NiR = nitrite reductase, GS = Glutamine synthase, AA = amino acid; NOR = nitric oxide reductase; COX = Cytochrome c oxidase, THB1 = hemoglobin 1, ? = putative molecule).

2.5 Conclusions

This research demonstrated, for the first time, that *C. reinhardtii* has the ability to synthesise N_2O. Microalgal N_2O synthesis is related to nitrate assimilation and is a consequence of the intracellular accumulation of NO_2^-. Under physiological conditions NO_2^- is reduced to NO by the dual system NR-ARC. When NO_2^- is present in high concentrations, its reduction into NO is also mediated by mitochondrial COX. The NO generated is then reduced into N_2O by NOR. We therefore hypothesise that N_2O synthesis may be a biochemical route to regulate NO (or NO_2^-) levels in microalgae.

References

Alcántara, C., Muñoz, R., Norvill, Z., Plouviez, M., and Guieysse, B. (2015). Nitrous oxide emissions from high rate algal ponds treating domestic wastewater. Bioresour. Technol. **177**: 110–117.

Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq-A Python framework to work with high-throughput sequencing data. Bioinformatics **31**: 166–169.

Aronesty, E. (2013). Comparison of Sequencing Utility Programs. Open Bioinforma. J. **7**: 1–8.

Béchet, Q., Chambonnière, P., Shilton, A., Guizard, G., and Guieysse, B. (2015). Algal productivity modeling: A step toward accurate assessments of full-scale algal cultivation. Biotechnol. Bioeng. **112**: 987–996.

Beckman, J.S. and Koppenol, W.H. (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am.J.Physiol **271**: 1424–1437.

Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate : A practical and powerful approach to multiple testing. J. R. Stat. Soc. **57**: 289–300.

Borowitzka, M.A. (2005) *Algal culturing techniques*, ed Andersen AR, (Elsevier Academic Press, Amsterdam) pp 205-218.

Chamizo-Ampudia, A., Sanz-Luque, E., Llamas, Á., Ocaña-Calahorro, F., Mariscal, V., Carreras, A., Barroso, J.B., Galván, A., and Fernández, E. (2016). A dual system formed by the ARC and NR molybdoenzymes mediates nitrite-dependent NO production in *Chlamydomonas*. Plant. Cell Environ. **39**:2097-2107.

Chang, H., Hsu, Y., Kang, C., Lee, T. (2013) Nitric oxide down-regulation of carotenoid synthesis and PSII activity in relation to very high light-induced singlet oxygen production and oxidative stress in *Chlamydomonas reinhardtii*. Plant Cell Physiol. **54**: 1296–1315.

Chang, R.L., Ghamsari, L., Manichaikul, A., Hom, E.F.Y., Balaji, S., Fu, W., Shen, Y., Hao, T., Palsson, B.Ø., Salehi-ashtiani, K., and Papin, J.A. (2011). Metabolic network reconstruction of *Chlamydomonas* offers insight into light-driven algal metabolism. Mol. Syst. Biol. **7**: 1–13.

Ciaccio, C., Ocaña-Calahorro, F., Droghetti, E., Tundo, G.R., Sanz-Luque, E., Polticelli, F., Visca, P., Smulevich, G., Ascenzi, P., and Coletta, M. (2015). Functional and spectroscopic characterization of *Chlamydomonas reinhardtii* truncated hemoglobins. PLoS One **10**: 1–24.

Cox, M.P., Peterson, D.A., and Biggs, P.J. (2010). SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. BMC Bioinformatics **11**: 485.

Deng, M., Moureaux, T., and Caboche, M. (1989). Tungstate, a molybdate analog inactivating nitrate reductase, deregulates the expression of the nitrate reductase structural gene. Plant Physiol. **91**: 304–309.

Dutton, A.S., Fukuto, J.M. and Houk, K.N. (2004) Mechanisms of HNO and NO production from Angeli's Salt: Density functional and CBS-QB3 theory predictions. J. Am. Chem. Soc., **126**, 3795–3800.

Estevez, M.S. and Puntarulo, S. (2005). Nitric oxide generation upon growth of Antarctic *Chlorella* sp. cells. Physiol. Plant. **125**: 192–201.

Faure, J.D., Vincentz, M., Kronenberger, J., and Caboche, M. (1991). Co-regulated expression of nitrate and nitrite reductases. Plant J. **1**: 107–113.

Fukuto, J.M., Switzer, C.H., Miranda, K.M., and Wink, D.A (2005). Nitroxyl (HNO): chemistry, biochemistry, and pharmacology. Annu. Rev. Pharmacol. Toxicol. **45**: 335–355.

Gans, P. and Wollman, F.-A. (1995). The effect of cyanide on state transitions in *Chlamydomonas reinhardtii*. Biochim. Biophys. Acta **1228**: 51–57.

Guieysse, B., Plouviez, M., Coilhac, M., and Cazali, L. (2013). Nitrous Oxide (N_2O) production in axenic *Chlorella vulgaris* microalgae cultures: Evidence, putative pathways, and potential environmental impacts. Biogeosciences **10**: 6737–6746.

Gupta, A.K., Kumari, A., Mishra, S., Wany, A., and Gupta, K.J. (2016). The functional role of nitric oxide in plant mitochondrial metabolism. **77**. http://dx.doi.org/10.1016/bs.abr.2015.10.007.

Gupta, K.J., Fernie, A.R., Kaiser, W.M., and Van Dongen, J.T. (2011). On the origins of nitric oxide. Trends Plant Sci. **16**: 160–168.

Gupta, K.J. and Igamberdiev, A.U. (2011). The anoxic plant mitochondrion as a nitrite: NO reductase. Mitochondrion **11**: 537–543.

Gupta, K.J., Stoimenova, M., and Kaiser, W.M. (2005). In higher plants, only root mitochondria, but not leaf mitochondria reduce nitrite to NO, in vitro and in situ. J. Exp. Bot. **56**: 2601–2609.

Harris, E.H. (2001). *Chlamydomonas* as a model organism. Annu. Rev. Plant Physiol. Plant Mol. Biol. **52**: 363–406.

Hayatsu, M., Kanako, T., and Masanori, S. (2010). Various players in the nitrogen cycle: Diversity and functions of the microorganisms involved in nitrification and denitrification. Soil Sci. Plant Nutr. **54**: 37–41.

Hemschemeier, A., Düner, M., Casero, D., Merchant, S.S., Winkler, M., and Happe, T. (2013). Hypoxic survival requires a 2-on-2 hemoglobin in a process involving nitric oxide. Proc. Natl. Acad. Sci. **110**: 10854–10859.

Hinnebusch, A. (1997). Translational regulation of yeast GCN4. J. Biol. Chem. **272**: 21661-21664.

Hoy, J.A. and Hargrove, M.S. (2008). The structure and function of plant hemoglobins. Plant Physiol. Biochem. **46**: 371–379.

Huang, X., Von Rad, U., and Durner, J. (2002). Nitric oxide induces transcriptional activation of the nitric oxide-tolerant alternative oxidase in Arabidopsis suspension cells. Planta **215**: 914–923.

Huwald, D., Schrapers, P., Kositzki, R., Haumann, M., and Hemschemeier, A. (2015). Characterization of unusual truncated hemoglobins of *Chlamydomonas reinhardtii* suggests specialized functions. Planta **242**: 167–185.

Igamberdiev, A.U., Ratcliffe, R.G., and Gupta, K.J. (2014). Plant mitochondria: Source and target for nitric oxide. Mitochondrion **19**: 329–333.

Kaiser, W.M. and Huber, S.C. (2001). Post-translational regulation of nitrate reductase: mechanism, physiological relevance and environmental triggers. J. Exp. Bot. **52**: 1981–

1989.

Kamp, A., Stief, P., Knappe, J., and De Beer, D. (2013). Response of the Ubiquitous Pelagic Diatom Thalassiosira weissflogii to Darkness and Anoxia. PLoS One **8**: 1–11.

Kim, D., Kang, Y.S., Lee, Y., Yamaguchi, K., Matsuoka, K., Lee, K.-W., Choi, K.-S., and Oda, T. (2008). Detection of nitric oxide (NO) in marine phytoplankters. J. Biosci. Bioeng. **105**: 414–417.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S.L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. **14**: http://dx.doi:10.1186/gb-2013-14-4-r36

Kumar, A., Castellano, I., Patti, F.P., Palumbo, A., and Buia, M.C. (2015). Nitric oxide in marine photosynthetic organisms. Nitric Oxide **47**: 34–39.

Lamattina, L., García-Mata, C., Graziano, M., and Pagnussat, G. (2003). Nitric oxide: the versatility of an extensive signal molecule. Annu. Rev. Plant Biol. **54**: 109–136.

Langland, J.O., Langland, L.A, Browning, K.S., and Roth, D.A (1996). Phosphorylation of plant Eukaryotic Initiation Factor-2 by the pPKR , and inhibition of protein synthesis *in vitro*. J Biol Chem. **271**: 4539–4544.

Leavesley, H.B., Li, L., Prabhakaran, K., Borowitz, J.L., and Isom, G.E (2008) Interaction of cyanide and nitric oxide with cytochrome c oxidase: Implications for acute cyanide toxicity. Toxicol Sci **101**:101–111.

Llamas, A., Kalakoutskii, K.L., and Fernández, E. (2000). Molybdenum cofactor amounts in *Chlamydomonas reinhardtii* depend on the Nit5 gene function related to molybdate transport. Plant, Cell Environ. **23**: 1247–1255.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. **15**: 1–34.

Mallick, N., Mohn, F.H., and Soeder, C.J. (2000). Evidence supporting nitrite-dependent NO release by the green microalga *Scenedesmus obliquus*. J. Plant Physiol. **157**: 40–46.

Mallick, N., Mohn, F.H., Soeder, C.J., and Gmbh, F.J. (1999). Studies on nitric oxide (NO) formation by the green alga *Scenedesmus obliquus* and the diazotrophic cyanobacterium *Anabaena Doliolum*. Chemosphere **39**: 1601–1610.

Marguerat, S. and Bähler, J. (2010). RNA-seq: From technology to biology. Cell. Mol. Life Sci. **67**: 569–579.

Mengis, M., Gachter, R., and Wehrli, B. (1997). Sources and sinks of nitrous oxide (N_2O) in deep lakes. Biogeochemistry **38**: 281–301.

Merchant, S.S. et al. (2007). The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. Science. **318**: 245–250.

Meyer, C., Lea, U.S., Provan, F., Kaiser, W.M., and Lillo, C. (2005). Is nitrate reductase a major player in the plant NO (nitric oxide) game? Photosynth. Res. **83**: 181–189.

de Montaigu, A., Sanz-Luque, E., Galván, A., and Fernández, E. (2010). A soluble guanylate cyclase mediates negative signaling by ammonium on expression of nitrate reductase in *Chlamydomonas*. Plant Cell **22**: 1532–1548.

Morozkina, E.V., and Kurakov, A.V. (2007). Dissimilatory nitrate reduction in fungi under

conditions of hypoxia and anoxia: a review. Appl. Biochem. Microbiol. **43**: 607–613.

Murphy, M.P., Packer, M. a., Scarlett, J.L., and Martin, S.W. (1998). Peroxynitrite: A biologically significant oxidant. Gen. Pharmacol. **31**: 179–186.

Navarro, T., Guerra, E., Fernández, E., and Galván, A. (2000). Nitrite reductase mutants as an approach to understanding nitrate assimilation in *Chlamydomonas reinhardtii*. Plant Physiol. **122**: 283–289.

Outdot, C., Andrie, C., and Montel, Y. (1990). Nitrous oxide production in the tropical Atlantic Ocean. Deep. Res. **37**: 183–202.

Pakos-zebrucka, K., Koryga, I., Mnich, K., Ljujic, M., Samali, A., and Gorman, A.M. (2016). The integrated stress response. EMBO. reports. **17**: 1–22.

Pierotti, D. and Rasmussen, R.A. (1980). Nitrous oxide measurements in the eastern tropical Pacific Ocean. Tellus **32**: 56–70.

Planchet, E., Jagadis Gupta, K., Sonoda, M., and Kaiser, W.M. (2005). Nitric oxide emission from tobacco leaves and cell suspensions: rate limiting factors and evidence for the involvement of mitochondrial electron transport. Plant J. **41**: 732–743.

Proschold, T., Harris, E.H., and Coleman, A.W. (2005). Portrait of a species: *Chlamydonomas reinhardtii*. Genetics **170**: 1601–1610.

Raven, J.A. and Giordano, M. (2013). Combined nitrogen. In *the physiology of microalgae*, eds Borowitzka M.A., John, B., Raven J.A., pp 143–154.

Ravishankara, A.R., Daniel, J.S., and Portmann, R.W. (2009). Nitrous oxide (N_2O): The dominant Ozone-depleting substance emitted in the 21st Century. Science. **326**: 123–125.

Rockel, P., Strube, F., Rockel, A., Wildt, J., and Kaiser, W.M. (2002). Regulation of nitric oxide (NO) production by plant nitrate reductase *in vivo* and *in vitro*. J. Exp. Bot. **53**: 103–110.

Roy, B., Vaughn, J.N., Kim, B.H., Zhou, F., Gilchrist, M.A., and Von Arnim, A.G. (2010). The h subunit of eIF3 promotes reinitiation competence during translation of mRNAs harboring upstream open reading frames. RNA **16**: 748–761.

Sakihama, Y., Nakamura, S., and Yamasaki, H. (2002). Nitric oxide production mediated by nitrate reductase in the green alga *Chlamydomonas reinhardtii*: an alternative NO production pathway in photosynthetic organisms. Plant Cell Physiol. **43**: 290–297.

Sanz-Luque, E., Chamizo-Ampudia, A., Llamas, A., Galván, A., and Fernández, E. (2015a). Understanding nitrate assimilation and its regulation in microalgae. Front. Plant Sci. **6**. http://dx.doi:10.3389/fpls.2015.00899.

Sanz-Luque, E., Ocaña-Calahorro, F., de Montaigu, A., Chamizo-Ampudia, A., Llamas, A., Galván, A., Fernández, E. (2015b). THB1, a truncated hemoglobin, modulates nitric oxide levels and nitrate reductase activity. Plant J. **81**: 467–479.

Sanz-Luque, E., Ocaña-Calahorro, F., Llamas, A., Galván, A., Fernández, E. (2013). Nitric oxide controls nitrate and ammonium assimilation in *Chlamydomonas reinhardtii*. J. Exp. Bot. **64**: 3373–3383.

Shoman, M.E. and Aly, O.M. (2016). Nitroxyl (HNO): A reduced form of nitric oxide with distinct chemical, pharmacological, and therapeutic properties. Oxid. Med. Cell. Longev. **2016**. http://dx .doi:10.1155/2016/4867124.

Shoun, H., Fushinobu, S., Jiang, L., Kim, S.-W., and Wakagi, T. (2012). Fungal denitrification and nitric oxide reductase cytochrome P450nor. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **367**: 1186–1194.

Sturms, R., Dispirito, A.A., and Hargrove, M.S. (2011). Plant and cyanobacterial hemoglobins reduce nitrite to nitric oxide under anoxic conditions. Biochemistry **50**: 3873–3878.

Tischner, R., Planchet, E., and Kaiser, W.M. (2004). Mitochondrial electron transport as a source for nitric oxide in the unicellular green alga *Chlorella sorokiniana*. FEBS Lett. **576**: 151–155.

Tiso, M., Tejero, J., Kenney, C., Frizzell, S., and Gladwin, M.T. (2012). Nitrite Reductase activity of nonsymbiotic hemoglobins from *Arabidopsis thaliana*. Biochemistry **51**: 5285–5292.

Vanlerberghe, G.C. and McIntosh, L. (1997). Alternative oxidase: from gene to function. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48**: 703–734.

Wang, H., Wang, W., Yin, C., Wang, Y., and Lu, J. (2006). Littoral zones as the "hotspots" of nitrous oxide (N_2O) emission in a hyper-eutrophic lake in China. Atmos. Environ. **40**: 5522–5527.

Wang, Z., Gerstein, M., and Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. Nat. Rev. Genet. **10**: 57–63.

Way, J.L. (1984). Cyanide intoxication and its mechanism of antagonism. Annu. Rev. Pharmacol. Toxicol. **24**: 451–481.

Weathers, P.J. (1984). N_2O evolution by green algae. Appl. Envionmental Microbiol. **48**: 1251–1253.

Weathers, P.J. and Niedzielski, J.J. (1986). Nitrous oxide production by cyanobacteria. Arch Microbiol **146**: 204–206.

Wei, L., Derrien, B., Gautier, A., Houille-Vernes, L., Boulouis, A., Saint-Marcoux, D., Malnoë, A., Rappaport, F., de Vitry, C., Vallon, O., Choquet, Y., and Wollman, F.-A. (2014). Nitric oxide-triggered remodeling of chloroplast bioenergetics and thylakoid proteins upon nitrogen starvation in *Chlamydomonas reinhardtii*. Plant Cell **26**: 353–372.

Yamasaki, H. (2000). Nitrite-dependent nitric oxide production pathway: implications for involvement of active nitrogen species in photoinhibition *in vivo*. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **355**: 1477–1488.

Zhang, L. and Mehta, S. (2008). Copper-induced proline synthesis is associated with nitric oxide generation in Chlamydomonas reinhardtii. Plant Cell Physiol. **49**: 411–419.

Chapter 3.

Evaluation of potential microalgal N_2O emissions in engineered systems

---

Preface

As discussed in Chapter 1, microalgal N_2O emissions are currently not recognised in international greenhouse gas inventories. In Chapter 2 we suggested that the ability of microalgae to synthesise N_2O is wide-spread among microalgae species, which implies that microalgal N_2O synthesis could be a global significant N_2O source.

Following the pathway study (Chapter 2), we initially planned to culture microalgae in 2 L bench scale reactors operated indoor to investigate the potential impact of cell age, cell concentration, nutrient concentrations, pH, temperature, mixing intensity, CO_2 supply and light supply on N_2O emissions. These particular variables were chosen due to their relevance to microalgal activity and N_2O emissions (Chapter 1 and 2). The data obtained would have been useful to construct a mechanistic model to predict N_2O emissions from microalgae culture as a function of relevant operational parameters.

Unfortunately, considering the complexity of the pathway (and technical issues with the use of probes needed to continuously record N_2O), we had to allocate more time on elucidating the pathway and reduce the modelling scope to only measuring data previously unavailable.

Given the lack of data available from representative systems (e.g. non-sterile long-term full-scale cultivation outdoors) it remained difficult to assess the potential environmental significance of N_2O emissions during microalgal cultivation. In order to address this important knowledge gap, the studies described in this chapter investigate the potential environmental significance of N_2O emissions from large scale outdoor microalgae culture. For this purpose, two well studied engineered systems were chosen and monitored for N_2O emissions (See Box 3.1 for the selection of cultivation systems).

In the light of the findings presented in Chapter 2 (i.e. N_2O emissions rates can be species-dependent) three microalgae species (*Chlorella vulgaris*, *Neochloris* sp., and *Arthrospira platensis)* with commercial potential were cultivated in pilot 50 L photobioreactors (PBRs) and N_2O was monitored daily from the PBRs gas headspace. Because from the three microalgae species chosen, *C. vulgaris* was the most studied for its ability to synthesise N_2O, further monitoring (e.g. using different N-sources) was performed with this species. In addition, simple microalgae-based systems (e.g. ponds) are widely used for wastewater treatment (WWT) and N_2O was also monitored from a 1000 L high rate algal pond (HRAP) operated under field conditions and fed primary domestic wastewater. The significance of N_2O emissions from the 50 L PBRs and the HRAP was then assessed for microalgal biomass cultivation to produce a biofuel feedstock, and microalgae-based domestic WWT, respectively. It must be noted that these estimates were generated to answer the question "Should we be concerned about potential microalgal N_2O emissions" rather than "What is the magnitude of microalgal

N_2O emissions"? Finally, tools for estimating N_2O emissions from microalgal cultivation were lacking, therefore, following current IPCC methodologies (i.e. greenhouse gas inventories) we estimated preliminary and simple tool (i.e. emissions factors) to predict microalgal N_2O emissions.

Box 3.1: Choice of cultivation systems

Microalgae can be cultivated in a variety of systems including PBRs and raceway ponds[8]. While the advantages and limitations of closed versus open cultivation systems are well discussed in the literature (Ugwu et al., 2008; Brennan and Owende, 2010), it is generally accepted that raceways are more cost-effective to build and operate but more prone to environmental interference (e.g. biological contamination). Regardless of the actual configuration, all algae cultivation systems are typically designed to optimise light supply while providing sufficient gas transfer for CO_2 supply and O_2 removal (Béchet et al., 2013). However, N_2O emissions had never been investigated long-term in any outdoor systems at pilot scale, so N_2O monitoring was performed in two well developed engineered systems:

Bubble column cylindrical photobioreactor

Various closed reactor geometries have been described in the literature. A column cylindrical photobioreactor was used for this research because Béchet et al, (2013) described this type of reactor as simple to build and operate with low energy requirement for efficient mixing[9]. Preliminary N_2O monitoring performed by Guieysse

[8] also called high rate algae pond in the context of wastewater treatment
[9] Béchet et al., 2013 verified that the microalgal suspension was well-mixed with no sedimentation occurring.

et al, (2013) showed that N_2O was easily detected in the headspace of this particular reactor geometry.

High rate algal pond fed with wastewater

Open systems, such as raceway ponds (also named high rate algal pond) are shallow (0.1 – 0.5 m), in order to favour light supply, and are generally mixed with a paddle wheel (Craggs et al., 2013). High rate algal pond for secondary wastewater treatment (WWT) are considered cost-effective in comparison to other WWT options (Craggs et al., 2013; Alcántara et al., 2015).

Considering that parts of this chapter (Chapter 3) are based on two different papers, the chapter has been divided in three distinct sub-sections to improve clarity. Sub-sections 1 and 2 are based on a journal paper and conference papers, respectively. Sub-section 3 discusses the relevance and implications of the findings obtained from the studies discussed in Sub-sections 1 and 2.

Sub-section 1 is based on paper 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*)

In addition, part of the results discussed in Sub-section 1 (i.e. N_2O monitoring performed during *C. vulgaris* cultivation) and Sub-section 2 (i.e. Part (6 months) of the N_2O monitoring performed during domestic wastewater treatment in high rate algal

pond) have been peer-reviewed, accepted, and presented in two international conferences (Abstract in Appendix N).

- Biorefinery for Food & Fuels & Materials, Montpellier Supagro, France (June 2015): **Plouviez, M**.; Guieysse, B.; Shilton, A.; Packer, M.; Thuret-Benoist.; 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 primary domestic wastewater treatment in a pilot-scale high rate algal pond.

3.1 N_2O emissions from commercial microalgae species cultivated in 50 L photobioreactors

ABSTRACT

This study investigated the potential environmental significance of N_2O emissions from outdoor microalgal cultivation. *Chlorella vulgaris*, *Neochloris* sp., and *Arthrospira platensis* were cultivated in 50 L pilot scale photobioreactors (PBRs) operated in fed batch mode and fed with either nitrate (NO_3^-) or ammonium (NH_4^+) as the nitrogen source. Whereas N_2O emissions were not detected from *A. plantensis* cultures over 32 days of cultivation, *Neochloris* sp., and *C. vulgaris* cultures emitted 50.5 – 14200 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$ (36 days of cultivation, n = 136, median = 2000 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$) and 9.60 – 38000 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$ (90 days of cultivation, n = 332, median = 4100 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$), respectively, when NO_3^- was supplied as N-source. Based on the emissions of 1500 – 8000 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$ (25 – 75% of the data were used instead of the full data range to remove outliers) recorded from *C. vulgaris* fed NO_3^-, emissions factors were estimated to be 0.1 – 0.4% of the N load of 25 g $N.d^{-1}$. Further monitoring of *C. vulgaris* cultures showed that N_2O emissions were positively correlated to biomass concentration ($R^2 = 0.77$) and light intensity ($R^2 = 0.57$). No N_2O emissions were detected when *C. vulgaris* was cultivated using NH_4^+ as N-source (31 days of cultivation, n = 84), or when *A. platensis* cultures were cultivated with NO_3^- as N-source (36 days of cultivation, n = 90). Therefore it would appear that the selection of appropriate N-source and algae species management could provide simple solutions for N_2O mitigation strategies.

3.1.1 Introduction

While N_2O synthesis by microalgae has been suspected for decades (Hahn and Junge, 1977; Pierotti and Rasmussen, 1980; Weathers, 1984; Weathers and Niedzielski, 1986), the potential environmental implications of this ability have only been recently acknowledged (Chapter 1). N_2O emissions have thus been reported during the cultivation of at least 10 microalgae species (Table 1.2 Chapter 1; Chapter 2). Of particular relevance to environmental assessment, N_2O emissions have been reported at pilot or full scale during the cultivations of *Nannochloris* in a 48 m^3 open pond (up to 580 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$; Florez-Leiva et al., 2010); *Staurosira* sp. in a 64 m^3 raceway pond (up to 320 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$ over 15 h; Ferrón et al., 2012); *Chorella vulgaris* in a 50 L column photobioreactor (240 – 1250 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$ over 24 h; Guieysse et al., 2013). As can be seen, there is a lack of data available from representative systems (*e.g.* non-sterile long-term full-scale outdoor cultivation on a meaningful scale) and the emission rates hitherto reported are highly variable. This variability can be explained by the complexity of mechanisms potentially influencing N_2O production and N cycling in algae cultures, especially in non-axenic cultures where N_2O may be produced by associated microorganisms. N_2O can indeed be generated during nitrite (NO_2^-) reduction to N_2O by algae (Chapter 2), which can occur during the assimilatory denitrification of nitrate (NO_3^-) into ammonium (NH_4^+) in algae, and/or during NO_2^- reduction into N_2O or NH_4^+ oxidation into NO_2^- by associated microorganisms (Alcántara et al., 2015). These mechanisms thus involves common substrates, products, and intermediates (e.g. NO_2^- is reduced in nitric oxide, NO, which is then reduced into N_2O) which extracellular and intracellular concentrations depends on numerous factors

(e.g. pH-dependant NH_4^+/NH_3 equilibrium, NH_3 volatilization, NH_4^+, NO_3^- and NO_2^- transport and uptake in microbial cells).

In order to provide a significant data set for impact assessment (i.e. non-sterile long-term full-scale outdoor cultivation) and better understand the impact of process parameters associated with N_2O synthesis during microalgae cultivation, this study investigates the potential environmental significance of N_2O emissions during pilot scale outdoor microalgae culture for biomass production. For this purpose, two microalgae (*C. vulgaris*, *Neochloris* sp.) and one cyanobacterium (*Arthrospira platensis*) were cultivated in pilot 50 L column photobioreactors (PBRs) and N_2O concentrations in the inlet and outlet gas streams were monitored. These strains were selected for their commercial potential (Spolaore et al., 2006; Gouveia, 2011) and the reactor geometry was selected for its simplicity and suitability for N_2O monitoring (Guieysse et al., 2013). As *C. vulgaris* ability to synthesise N_2O is well documented (Weathers, 1984; Guieysse et al., 2013; Alcántara et al., 2015), the influence of N supply on N_2O emissions during the outdoor cultivation of this species was also tested. Finally, the potential significance of microalgal N_2O emissions during mass-scale algae cultivation for biodiesel production was theoretically assessed using long-term monitoring data from *C. vulgaris* cultivation.

3.1.2 Materials and methods

3.1.2.1. Microalgae species and inoculum preparation

C. vulgaris, *Neochloris* sp., and *A. platensis* were selected based on their commercial potential (Spolaore et al., 2006; Gouveia, 2011). *C. vulgaris* and *Neochloris* sp. were obtained from Novis et al, (2009) and cultivated in buffered BG-11[10] medium (initial N concentration of 0.25 g $N \cdot L^{-1}$) whereas *A. platensis* was obtained from UTEX (reference number: 1926) and cultivated in Zarrouk[10] medium (initial N concentration of 0.41 g $N \cdot L^{-1}$). Axenic cultures were first inoculated from colonies maintained on solid medium. Liquid cultures were then prepared under sterile conditions and incubated in a Minitron incubator (Infors HT, Switzerland) at 25 ± 1°C under continuous agitation (180 rpm), constant illumination (21 W of PAR m^{-2} at the culture surface, using five 18 W Polylux coolwhite tubes), and in an atmosphere of 2% (vol.) CO_2 in air during 7 days. The resulting cultures were then used to inoculate 2 L bench scale PBRs.

Each 2 L PBR was made of two acrylic tubes (inner tube: height of 22 cm and a diameter of 14 cm; outer tube: height of 22 cm and a diameter of 19 cm) hermetically sealed with a metal lid. The outer tube was filled with water allowing temperature control (± 1ºC) using a temperature controller and the PBRs were placed in the centre of 7 "cool white" bulbs (Osram Duluxstar Mini Twist Lumilux Daylight, Power of 7 W). The 2 L PBRs were operated under continuous illumination (16 $W \cdot m^{-2}$) at 18 ± 1°C and CO_2-enriched air bubbling (2% vol., 1 $L \cdot min^{-1}$) to mix the cultures, supply excess inorganic carbon and remove oxygen. At the beginning of each inoculation, 1.5

[10] Appendix A

L of fresh medium were added into the 2 L PBRs and aeration was started at least 30 min before inoculation to allow the system to reach equilibrium. Prior to inoculation, 0.5 L of inoculum liquid cultures were re-suspended in freshly prepared medium after centrifugation and this re-suspended algal suspension was added in the 2 L PBR. The cultures were operated in batch mode until the biomass concentration reached a level of 1 $g \cdot L^{-1}$, and then the cultures were operated on a on a semi-continuous mode until needed.

3.1.2.2 PBR design and operation

Four 50 L acrylic column PBRs (Figure 3.1) were used for outdoor algae cultivation (Béchet et al., 2010). Before starting new cultures the PBRs were filled with 50 L of filtered tap water (1 μm pore size) with 5% chlorine to disinfect the inner surface of the PBRs. PBRs were then rinsed with filtered tap water once and filled with approximately 45 L filtered tap water and immediately aerated for at least 2 h to remove any residual chlorine. Stock solutions of dissolved chemicals were then added followed by 0.5 L of freshly re-suspended algal inoculums from the 2 L indoor PBRs (Section 3.1.2.1). Tap water was used to top up and reach a final working volume of 50 L. Microalgae were cultivated in batch regime until biomass concentration reached approximatively 1 $g \cdot L^{-1}$ and then after semi-continuously by replacing 25 L of their respective media every 5, 4 and 3.5 days according to the period of operation (Appendix O). The four reactors were located on the laboratory roof (Latitude: 40° 23′ 13.88′ S; Longitude: 175° 37′ 6.06′ E) and were aligned along an East/West axis to prevent shading. Media mixing and carbon supply was provided by a bubbling CO_2 enriched (2% v/v) air at a flow rate of approximatively 1 $L \cdot min^{-1}$. Gas flow rates were

controlled using rotameters (air: Ki Air Instruments: 0 − 20 $L \cdot min^{-1}$; CO_2: Aalborg PMR 1-013799: 0 − 500 $mL \cdot min^{-1}$) and adjusted when necessary. Each PBR was covered at the top and equipped with a gas exhaust pipe for gaseous sampling. Hourly weather data (solar irradiance and air temperature) for New Zealand were obtained from the New Zealand Institute of Water and Atmospheric research (NIWA, http://cliflo.niwa.co.nz/) for June 2012 and March – December 2014 (Station: Palmerston North Ews located in Palmerston north).

Figure 3.1: *C. vulgaris* cultivated in 50 L bubble column photobioreactors (2 m × 0.19 m inner diameter, 1 m^2 of illuminated area as described by Béchet et al. (2010)).

3.1.2.3 Liquid sampling

Following microalgal suspension re-circulation for 2 min using a pump (Ebara pump, Type CDXM\A, flow rate: approximately 25 $L \cdot min^{-1}$) liquid samples were withdrawn

at least once daily. Microalgae concentration as dry cell weight (DCW) and optical density (OD) were by consequent measured once daily. The value of the pH and the concentrations of nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and dissolved oxygen (DO) were monitored when needed. Direct pH and DO measurements were performed by submerging the pH and DO probes in the microalgal suspension. The other analyses (i.e. DCW, OD, and nitrogen compounds) were performed on the liquid samples withdrawn.

3.1.2.4 Gaseous sampling and N_2O measurements

Gas samples (10 mL) were withdrawn from the PBR headspace at least twice daily (9:00 am and 5:00 pm) using a gas-tight syringe. Following the method described in section 2.2.6, the concentration of N_2O in these samples was quantified using a Shimadzu GC-2010 gas-chromatography system (Shimadzu, Japan). The concentrations of N_2O in the surrounding air (controls) and in the gas mixture bubbled in the reactors were quantified at each sampling event and discounted from the PBR N_2O exhaust gas concentration when computing net N_2O productivities. To test whether the difference of concentrations calculated was statistically significant, two sample t-tests ($\alpha = 0.05$, $n > 13$) were performed on the data set for each PBR at each monitoring time (Appendix P). Net productivities were calculated based on the gas law formulae (Equation 3.1).

$$\Phi_{N_2O} = \frac{1}{A} \times \left(\frac{(fN_2O_{reactor} - fN_2O_{air}) \times P_{atm} \times \varphi_{gas}}{R \times T}\right)$$, Equation 3.1

Where:

Φ_{N_2O} = Net N_2O flux (nmol·m^{-2}·h^{-1})

P_{atm} = Atmospheric pressure (Pa)

φ_{gas} = Bubbled gas flux (m^3·h^{-1})

$fN_2O_{reactor}$ = N_2O fraction from the reactor estimated by the GC (nmol·mol^{-1})

fN_2O_{air} = N_2O fraction from the surrounding air estimated by the GC (nmol·mol^{-1})

R = Gas constant (8.31 J·mol^{-1}·K^{-1})

T = Air temperature (K)

A = Illuminated area (m^2)

Assuming:

$T_{in} = T_{out}$

$\Phi_{gas_{in}} = \Phi_{gas_{out}}$

$P_{in} = P_{out} = P_{atm}$

3.1.2.5 Analytical procedures

OD was measured at 683 nm using a Helios-Alpha spectrophotometer (Thermo Scientific, USA). DCW concentration was determined as followed: 1) Membrane filters (0.45 µm, 47 mm, Merck Millipore, Billerica, MA) were pre-dried at 105°C for 24 h before being weighed, 2) A known volume of microalgal suspension was filtered and to remove any dissolved salt the filters were rinsed with the same volume of distilled water, 3) the filters were placed at 105°C for 1 h before being weighed again, and 4) the

DCW was determined by subtracting the initial weight (i.e. filter) to the final weight (i.e. filter + biomass) measured (Béchet et al., 2015a). The dissolved oxygen (DO) concentration and pH were monitored using a multimeter (Orion Star A326, Thermo Scientific) equipped with the relevant probes (pH 510 pH/°C, Eutech instruments). Aqueous concentrations of NO_3^- and NO_2^- in filtered samples were quantified using a Dionex ICS-2000 Ion Chromatograph (Dionex Corporation, USA) equipped with a Dionex IonPac AS11-HC column (250 mm × 4 mm) eluted at 1 $mL \cdot min^{-1}$ with a 13 mM KOH aqueous solution. Ammonium (NH_4^+) was measured using AQUAfast ammonia thermal digestion kit (Thermo Scientific, USA).

3.1.2.6 Microsensors for dissolved N_2O measurement

Dissolved N_2O was measured by amperic detection with N_2O specific microsensors (Unisense A/S, Denmark) connected to a 4 channel high-sensitivity meter (picoameter) multi-meter (Unisense, Denmark). The sensor was directly submerged in the microalgal suspension of the PBR in order to detect dissolved N_2O in the aqueous phase. The reduction of N_2O at the metal cathode surface is detected as an electric signal and the reduction rate of N_2O is directly proportional to N_2O concentration. Although the use of these sensors seemed promising for continuous N_2O monitoring in the microalgal suspension (Appendix Q), it appeared the sensor suffered from high cost versus time of use, irreproducibility, membrane fragility, high various interferences (e.g. NO interferes with the N_2O signal) and high temperature dependence (Jenni et al., 2012). Thus, although this probe confirmed N_2O synthesis in microalgal suspension, their use was discontinued.

3.1.3 Results and discussion

3.1.3.1 N_2O emissions from microalgae cultivated in 50 L PBRs

In order to evaluate N_2O emissions from commercial microalgae cultures, N_2O was measured from *C. vulgaris* cultures fed either NO_3^- or NH_4^+, *Neochloris* cultures fed NO_3^-, and *A. platensis* cultures fed NO_3^- (Table 3.1).

Table 3.1: N_2O emissions recorded during microalgae cultivation in 50 L column photobioreactors (n = sampling size for N_2O measurements). Summary of operational parameters can be found in Appendix O.

Date (days of monitoring)	Season	Microalgae species	Nitrogen Source	n	Number of PBR operated	N_2O emissions ($nmol \cdot m^{-2} \cdot h^{-1}$)	Median N_2O emissions ($nmol \cdot m^{-2} \cdot h^{-1}$)[a]	N_2O emissions ($nmol\ N_2O \cdot g\text{-}DCW^{-1} \cdot h^{-1}$)
12/06/12 to 18/06/12 (6 days)	Winter	*C. vulgaris*	NO_3^-	76	1	400.0 – 10800	3400	10.0 – 270
					2	9.55 – 2940	1400	0.25 – 73.0
					3	3700 – 14900	6100	92.0 – 370
					4	290.0 – 5150	2500	7.30 – 130
28/03/14, 31/03/14 and 11/04/14 (3 days)	Autumn	*C. vulgaris*	NO_3^-	26	1	840.0 – 12150	2600	16.0 – 270
					2	270.0 – 14250	2300	7.70 – 400
04/06/14 to 04/07/14 (32 days)	Winter	*S. platensis*	NO_3^-	90	1	Not significant*	Not significant*	Not significant*
					2	Not significant*	Not significant*	Not significant*
04/06/14 to 10/07/14 (36 days)	Winter	*Neochloris*	NO_3^-	136	1	50.5 – 11600	2100	2.30 – 280
					2	130.0 – 14200	1900	3.50 – 340
28/07/14 to 28/08/14 (32 days)	Winter	*C. vulgaris*	NO_3^-	84	1	85.0 – 11490	2600	13.0 – 510
					2	63.0 – 11100	2300	9.60 – 540
28/07/14 to 28/08/14 (32 days)	Winter	*C. vulgaris*	NH_4^+	84	1	Not significant*	Not significant*	Not significant*
					2	Not significant*	Not significant*	Not significant*
13/10/14 to 28/10/14 (15 days)	Spring	*C. vulgaris*	NO_3^-	44	1	910.0 – 29300	6100	32.0 – 800
					2	330.0 – 15600	4000	14.0 – 350
04/11/14 to 28/11/14 (24 days)	Spring	*C. vulgaris*	NO_3^-	64	1	14.2 – 38000	12800	0.50 – 980
					2	32.0 – 27900	7000	2.60 – 800
01/12/14 to 10/12/14 (12 days)	Summer	*C. vulgaris*	NO_3^-	32	1	930.0 – 35600	11200	33.0 – 1180
					2	580.0 – 11400	8700	33.0 – 350

*Significance was statistically validated using two sample t-test, $\alpha = 0.05$ (Appendix P).
a N_2O emissions distributions were positively skewed justifying the choice of median instead of mean.

As shown by the monitoring data summarised in Table 3.1, statistically significant daily N_2O emissions (up to 38000 and 14200 nmole·m^{-2}·h^{-1}, respectively) were recorded from *C. vulgaris* and *Neochloris* cultures fed NO_3^-. In contrast, N_2O was never detected from *C. vulgaris* cultures fed NH_4^+ or *A. platensis* cultures fed NO_3^-. While 10 microalgae species have been indicated to generate N_2O emissions in the literature (Chapter 1), this ability had not previously been investigated in *Neochloris* and *A. platensis*. While the lack of N_2O emissions from *A. platensis* cultures evidences N_2O generation by associated microorganisms was insignificant in this system, this particular finding cannot be extrapolated to other systems because *A. platensis* was cultivated in Zarrouk medium at high pH (Morais and Costa 2007; Borowitzka, 2013). Therefore, N_2O emissions are henceforth associated with "algal cultures" rather than "algae species" in order to acknowledge the potential contribution from associated microorganisms (there is no evidence that emissions from algae and associated organisms are mutually exclusive). Such distinction is also often irrelevant to impact assessment (e.g. greenhouse gas inventories) as N_2O emissions should be quantified based on anthropogenic activities, whether direct (e.g. commercial cultivation) or indirect (pollution-based eutrophication), rather than biological origins. In algae cultures, N_2O emissions could thus potentially be associated with NO_2^- reduction by algae (see further discussion below), NH_4^+ oxidation by bacteria or archaea, or NO_2^- reduction by bacteria and fungi. N_2O production was however never detected in cultures fed NH_4^+, suggesting N_2O synthesis by ammonium oxidizing bacteria (AOA) and archaea (AOB) was not significant in this study. In comparison, the ability of *C. vulgaris* to synthesise N_2O is relatively well documented (Weathers, 1984; Guieysse et al., 2013; Alcántara et al., 2015) and has been linked to NO_2^- reduction under aerobic conditions (Guieysse et al., 2013). Finally, N_2O emission during NO_2^- reduction by

bacteria and fungi typically occurs in low-oxygen environments, although this pathway cannot be entirely dismissed under aerobic conditions (Wrage et al., 2001; Kampschreur et al., 2009). Here, we report N_2O emissions measured from *C. vulgaris* culture fed NO_3^- (320 data points over 92 days of cultivation) covering a broad range of algae concentrations, dissolved NO_2^- concentration, solar irradiance, and air and broth temperatures (Figure 3.2). The emission rates thus recorded were highly variable (Table 3.1) with positive values ranging from 9.60 – 38000 $nmol \cdot m^{-2} \cdot h^{-1}$.

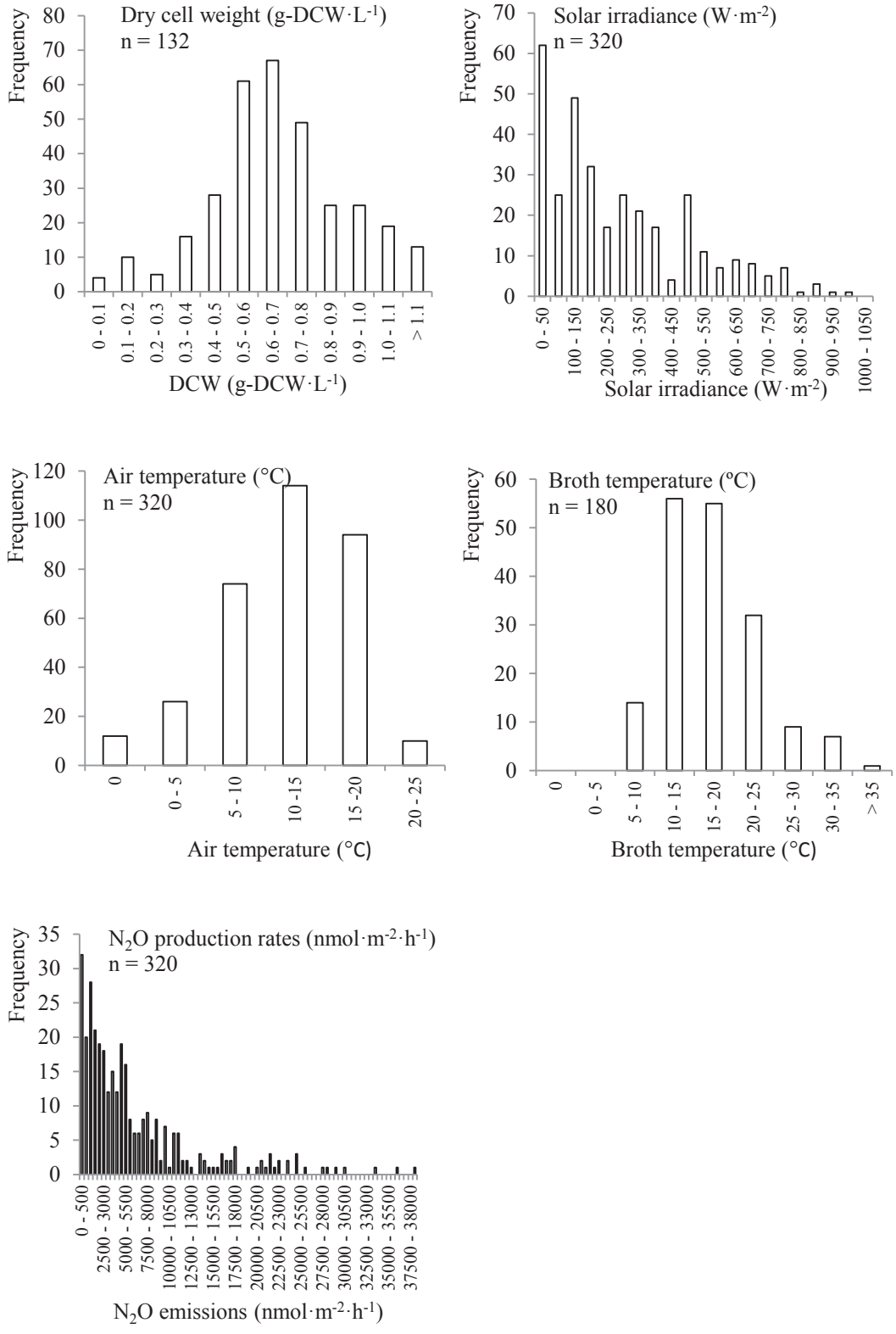


Figure 3.2: Histograms of dry cell weight (g-DCW·L^{-1}), solar irradiance (W·m^{-2}), air temperature (°C), broth temperature (°C), and N_2O production (nmol·m^{-2}·h^{-1}) measured during *C. vulgaris* cultivation in 50 L photobioreactors.

Numerous parameters influencing algal activity could potentially impact N_2O emissions during algae cultivation. In particular, Guieysse et al. (2013) reported that N_2O emissions by axenic *C. vulgaris* in laboratory NO_2^--laden batch cultures were positively correlated to extracellular NO_2^- concentration (albeit not linearly) and cell mass, and negatively correlated to light intensity. In the present study, *C. vulgaris* was cultivated outdoors in 50 L pilot scale PBRs where mixing, carbon supply and influent composition were kept constant, so these parameters should not have had a direct influence on N_2O emission variability. In addition, pH (7.00 ± 0.04, n = 65, p = 0.05) and DO concentration (100% at all times) varied little, suggesting these parameters were unlikely to be responsible for the variability in N_2O emissions. In analysing raw data sets (Figures R1-6, Appendix R), no clear statistical correlation could be found between N_2O emissions and daily DCW (R^2 = 0.070), solar irradiance (R^2 = 0.080), nitrite concentration (R^2 = 0.030), and air or broth temperature (R^2 = 0.050 and 0.0030, respectively). However, a stepwise linear regression analysis with backward elimination (personal communication[11]) showed that DCW and solar irradiance were significant variables at 95% confidence (*p-value* < 0.05) with increasing biomass concentration and solar irradiance having a positive effect on N_2O emissions (see Appendix S for full description of the regression analysis). However, even though both parameters were significant, the regression equation obtained from the analysis was inadequate because of a low R^2 (R^2 = 0.12). These results may be due to a combination of high data noise and data distribution bias (i.e. most samples were taken at 9 am and 5 pm within narrow ranges of light intensities, see Figure 3.2). The following section further examines, and specifically discusses, the possible influence of each variable recorded on N_2O emissions. In order to circumvent the effect of noise, data were

[11] Advised by Dr. Nihal Jayamaha, Massey University.

clustered in deciles for the following parameters: dry cell weight, solar irradiance, and air and broth temperature.

Biomass concentration: In agreement with the findings from Guieysse et al., (2013) and the findings discussed in Chapter 2 (Section 2.3.1) for axenic *C. vulgaris* cultures and *C. reinhardtii* cultures, respectively, Figure 3.3, shows averaged N_2O emissions were positively correlated ($R^2 = 0.77$) with averaged daily biomass concentrations (g-DCW·L^{-1}) calculated within each daily biomass concentration decile (these concentrations were normally distributed, Figure 3.2). Specific N_2O productions (nmol N_2O·g-DCW^{-1}·h^{-1}) are also given in Table 3.1 to compare with other monitoring studies.

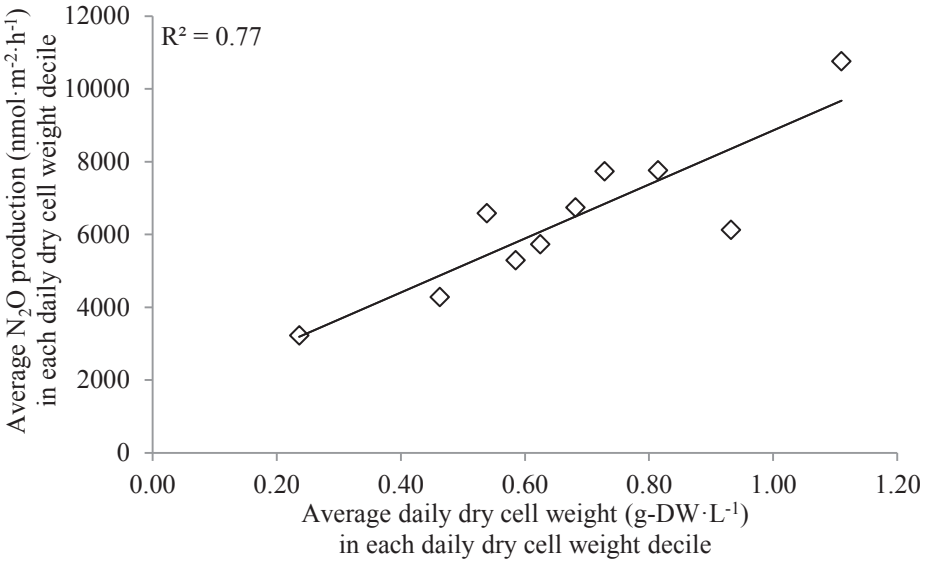


Figure 3.3: N_2O production (nmol·m^{-2}·h^{-1}) as a function of daily biomass concentration (g-DCW·L^{-1}) for each daily DCW population decile (in each decile n = 32).

Additional laboratory experiments showed that *C. vulgaris* cell number was linearly correlated to both OD and DCW (Appendix T.1). During outdoors cultivation, DCW and OD were also linearly correlated (Appendix T.2) as well as cell number and DCW (R^2 = 0.95; Figure T.3, Appendix T), therefore DCW measurement was likely representative of cell numbers in the pilot reactors. Cell viability testing[12] (Béchet et al., 2015b) was also quantified in June 2012 and during the entire period of monitoring, *C. vulgaris* viability always remained high (96 ± 1.0; 98 ± 0.8; 97 ± 0.9; and 98 ± 1.0 % in PBRs 1, 2, 3, and 4, respectively; for each reactor n = 13, confidence at 95%). These results suggest that variability in N_2O emissions were unlikely caused by microalgae viability or uncertainty in biomass quantification.

Cell growth kinetics: Laboratory experiments on *C. vulgaris* showed that cellular age did not impact N_2O specific emissions over 24 hours of incubation in darkness supplied with NO_2^-, however the initial production rate (0 - 4 h) of 3-days old *C. vulgaris* cells was 10-fold higher than for 7-days old cells (Supplementary information S5 of Guieysse et al., 2013). In our study, daily monitoring (e.g. August) showed no significant variation of N_2O emissions overtime despite the fact we can expect the average cell age to increase (Appendix U).

In addition, a thorough analysis of the monitoring data acquired in June 2012 using instantaneous biomass productivity predicted[13] based on the local conditions actually experienced by the microalgae (e.g. light intensities) did not evidence any statistical correlation between instantaneous productivities predicted at the time of sampling and

[12] Testing performed by another operator, Dr Quentin Béchet.
[13] predicted instantaneous productivities were obtained from Dr Quentin Béchet and were calculated following Béchet et al., 2014: Full-Scale validation of a model of algal productivity (Supplementary information S4 and S7).

N_2O emissions (Figure 3.4). As can be seen, N_2O was even produced during 'negative growth' (when cellular respiration offsets production). This lack of 'instantaneous' correlation may simply be due to different time scales (e.g. N_2O may accumulate in the solution so a change in cellular output may not be immediately recorded as change in reactor output). We therefore cannot conclude on the impact of cell growth kinetics on N_2O emission variability during pilot scale cultivation and further research is needed to evaluate the influence of growth kinetics on N_2O emissions.

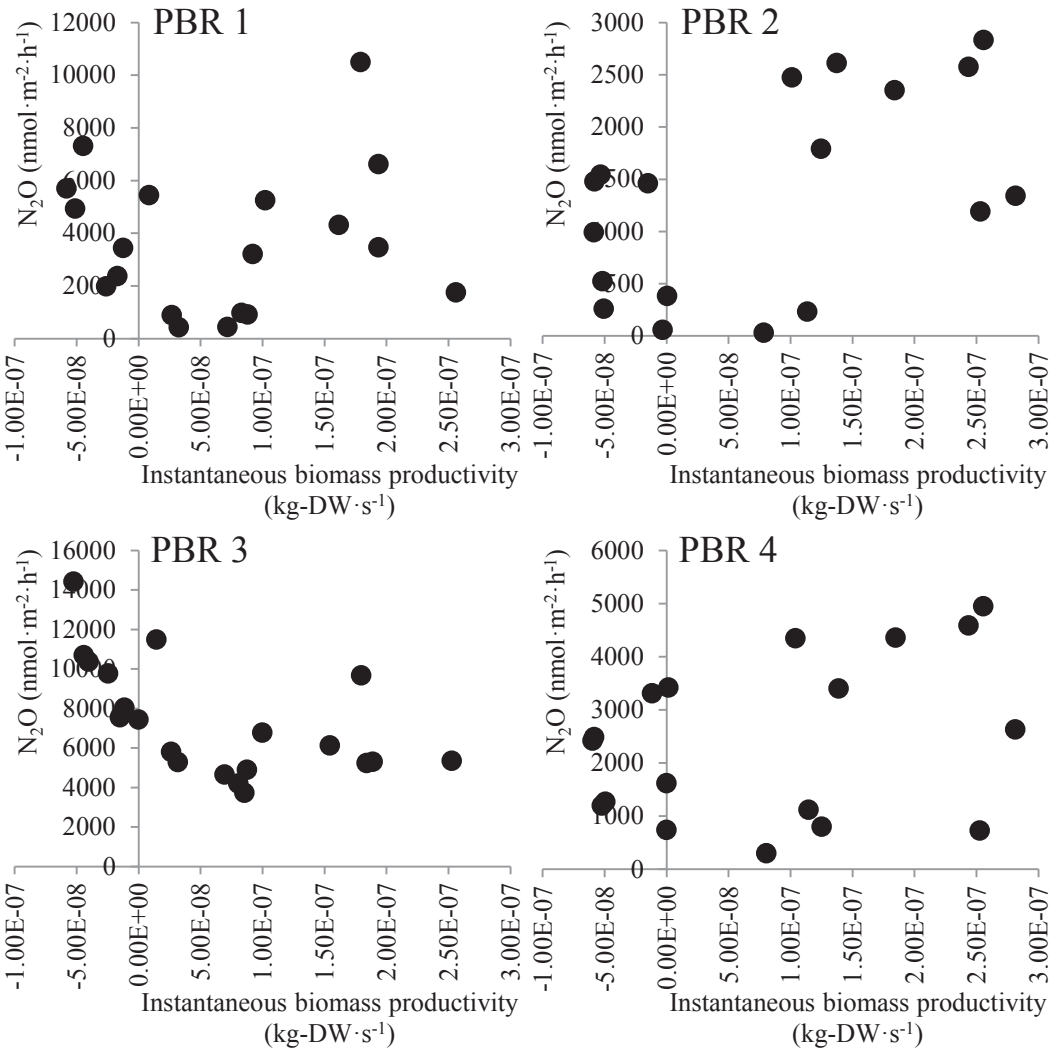


Figure 3.4: N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) as a function of predicted instantaneous biomass productivities ($kg\text{-}DW \cdot s^{-1}$) for each *C. vulgaris* cultures (PBRs 1 – 4) from the monitoring performed in June 2012.

Light intensity: A positive correlation between light intensity and N_2O emissions was evidenced by decile analysis (Figure 3.5a, $R^2 = 0.57$) as well as comparison of weekly data averages (Figure 3.5b, $R^2 = 0.56$).

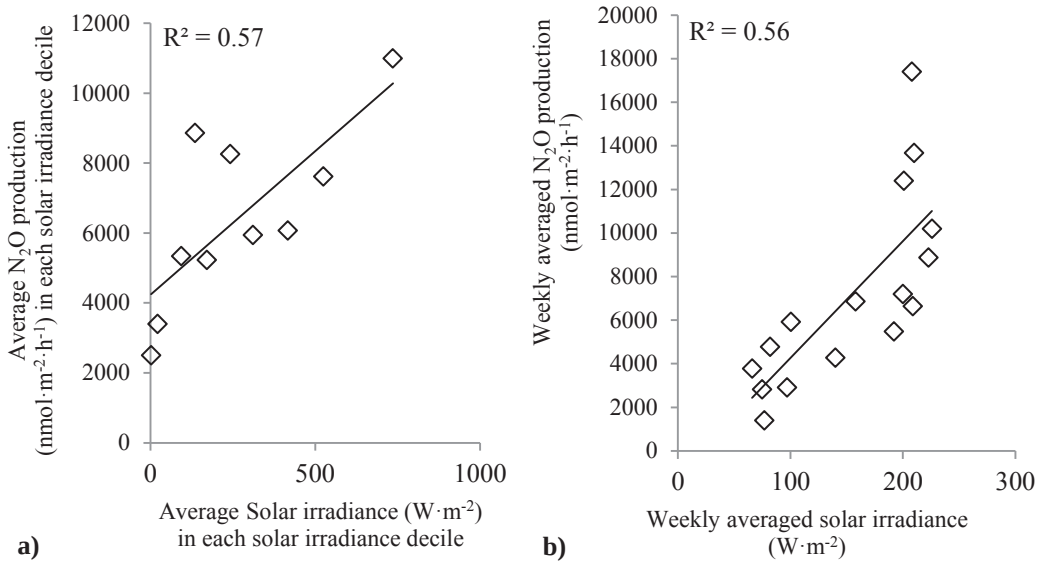


Figure 3.5: a) N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) as a function of solar irradiance ($W \cdot m^{-2}$) for each solar irradiance population decile (in each decile n = 32). **b)** Weekly averaged N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) as a function of weekly averaged solar irradiance ($W \cdot m^{-2}$).

A causality between light supply and N_2O synthesis was also indicated during the outdoor cultivation of *Neochloris* (Appendix V) and *C. vulgaris* fed NO_3^- (Figure 3.6). As can be seen N_2O emissions increased as solar irradiance increased from 9.00 am to 11.00 am (equivalent to 0 to 75 min in Figure 3.6), rapidly ceased when the reactor was completely shaded, and quickly resumed when shading was removed. These findings agrees with data collected during *C. vulgaris* cultivation in the same PBR (NO_3^- as N-source) in May 2011 (Guieysse et al., 2013), but contrast the results from the same authors (and from Chapter 2) who showed N_2O synthesis by axenic *C. vulgaris* supplied with NO_2^- was boosted under darkness during *in vitro* laboratory assays.

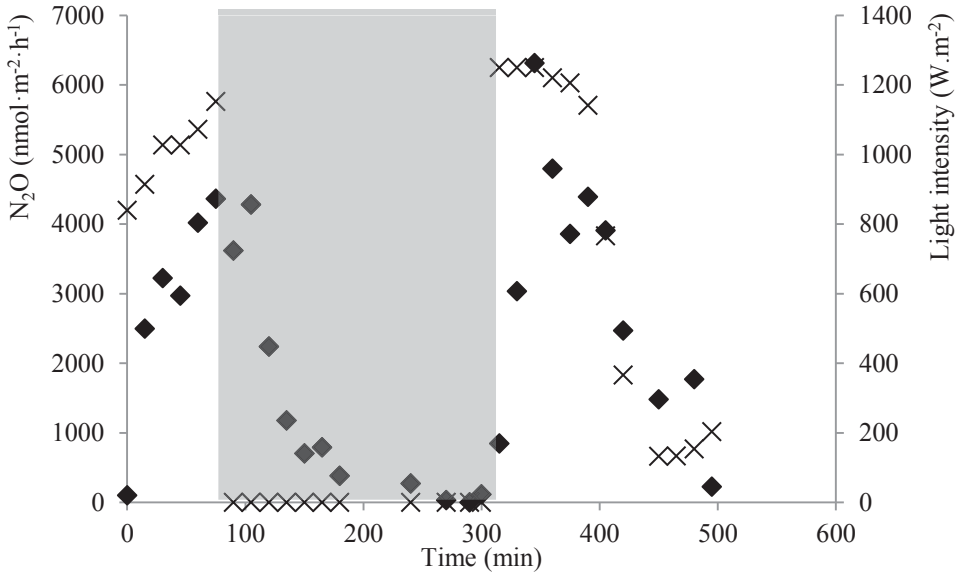


Figure 3.6: Changes in N_2O production (black ◊, nmol·m^{-2}·h^{-1}) and solar irradiance (×, W m^{-2}) during *C. vulgaris* cultivation in 50 L PBR: the shaded area (75 – 300 min) represents the time when the reactor was shaded from the sun.

The opposite impacts of light supply on N_2O emissions under NO_3^- outdoors (this study) and NO_2^- indoors supply (Guieysse et al., 2013) may be explained by light-dependent mechanisms influencing NO_3^- assimilation and therefore impacting intracellular NO_2^- accumulation (e.g. imbalances between the rate of NO_3^- reduction into NO_2^- and the rates of NO_2^- reduction into NH_4^+ and N_2O, Guieysse et al., 2013) and/or NR activity (e.g. regeneration of the cofactor Nicotinamide adenine dinucleotide phosphate via photosynthesis or inhibition in response to NO_2^- and NO generation). It is therefore possible that the rate of N_2O synthesis in light-limited (e.g. outdoors) *C. vulgaris* cultures fed NO_3^- is limited by the light-dependent reduction of NO_3^- into NO_2^-, while the rate of N_2O synthesis in *C. vulgaris* fed NO_2^- increases when photosynthesis-repression prevents NO_2^- reduction into NH_4^+ by nitrite reductase. Consequently, if N_2O production from outdoor *C. vulgaris* cultures fed NO_3^- is indeed

limited by light supply, we would expect a detrimental impact of cell concentration at constant light irradiance (as the algae will experience longer periods of darkness when cycling between light and dark PBR areas). This was suggested by the good correlation seen between monthly averaged N_2O emissions and monthly averaged light irradiance 'normalized' for cell density ($R^2 = 0.92$, Appendix W). While this correlation provides indication for future research and modeling, it must however be taken with caution given that no correlation was seen when data were not clustered, and the monthly data distribution was uneven with large variability in the N_2O emissions (Figure W.2 Appendix W).

N-supply: As confirmed in Chapter 2 (Section 2.3.2), NO_2^- was the substrate of microalgal N_2O synthesis and, indeed, as showed in Figure 3.6 NO_2^- addition significantly boosted N_2O emissions from outdoors *C. vulgaris* cultures fed NO_3^- (this was confirmed during a parallel indoor experiment, Appendix X).

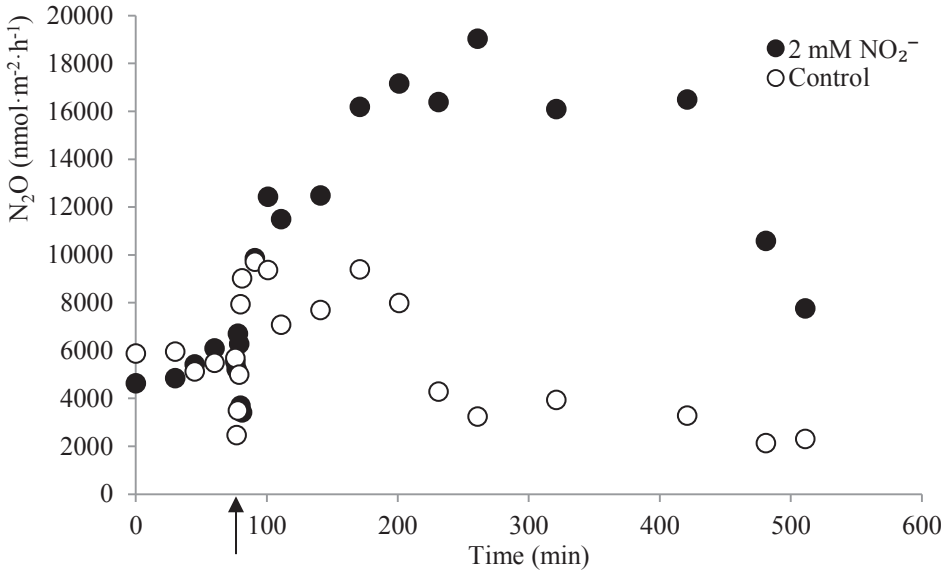


Figure 3.7: N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) recorded during *C. vulgaris* cultivation in 50 L PBR and supplemented with 2 mM NO_2^- (the arrow indicates when NO_2^- was added) or kept free of external NO_2^- as control.

Surprisingly, no statistical correlation between extracellular NO_2^- concentration and N_2O emission rates in cultures fed NO_3^- was found (Appendix R), probably due to the fact that extracellular NO_2^- concentration remained low and differed significantly from intracellular NO_2^- concentration when this compound was not added in the culture. Algae cells produce NO_2^- intracellularly via NR-mediated NO_3^- reduction (Navarro et al., 2000) and subsequently excrete this compound into the culture, as shown in algal cultures fed with NO_3^- where both NO_2^- excretion (0.04 – 0.36 mM NO_2^-) and N_2O emissions (63.0 – 11100 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$, n = 84) were reported. Interestingly, NO_2^- excretion (0 – 0.09 mM NO_2^-) was also observed in the culture fed with NH_4^+ but NO_2^- synthesis was not associated with detectable N_2O emissions. Nitrite excretion may be explained if NO_2^- formation involved organic nitrogen (Kessler and Oesterheld, 1970) as NH_4^+ is known to repress NR activity (Fernandez and Galvan, 2008), although N_2O

generation was still detected when *C. vulgaris* fed NH_4^+ was also supplied NO_2^- these emissions were still lower than emissions from *C. vulgaris* culture fed NO_3^- and then supplied NO_2^- (Appendix X). NO_2^- may also be produced from bacterial or archaeal NH_4^+ oxidation (de Godos et al., 2010; Limpiyakorn et al., 2011) but the presence of ammonium oxidizing microorganisms was unlikely under the environmental conditions (e.g. high light) occurring in the PBRs (Alcántara et al., 2015) and not evidenced by N_2O production in NH_4^+ laden cultures.

Temperature: Large daily and seasonal changes in culture temperature can be experienced during outdoor algae cultivation, especially in PBRs (Béchet et al., 2010). During this study *C. vulgaris* was able to synthesise N_2O under a broad range of broth temperatures (10 – 30ºC). Even when clustering the data, no correlation could be established between N_2O emissions and ambient air or broth temperature (Figure 3.8a and b). From the data it is therefore difficult to determine whether temperature influenced N_2O emissions.

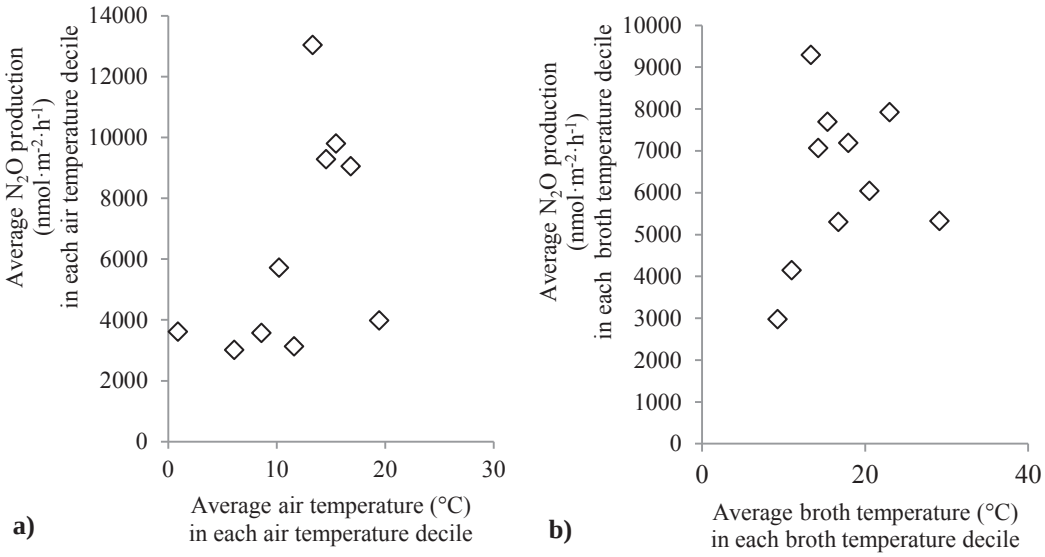


Figure 3.8: a) N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) as a function of air temperature (°C) for each air temperature population decile (in each decile n = 32). **b)** N_2O emissions ($nmol \cdot m^{-2} \cdot h^{-1}$) as a function of broth temperature (°C) for each broth temperature population decile (in each decile n = 18)[14].

3.1.3.2 Potential significance of N_2O emissions during algal biofuel production

In Chapter 1 (Section 1.3.2), we showed how the production of algal biomass as biofuel feedstock in the USA could generate significant N_2O emissions (up to 15% of the N_2O generated from all anthropogenic activities for the entire USA). The calculation performed was based on the only data available at the time: one day monitoring of *C. vulgaris* cultivated in a 50 L PBR (Guieysse et al., 2013). In order to provide a better estimate, a new calculation was performed considering the N_2O emissions rates

[14] Exponential fit is generally used to fit data sets involving temperature. Considering the weak correlation between N_2O emissions and ambient air temperature ($R^2 = 0.27$) or broth temperature ($R^2 = 0.10$) the trendlines were removed from the figures.

recorded from *C. vulgaris* fed NO_3^- over 90 days cultivation covering a broad range of environmental conditions.

The distribution of the N_2O emissions recorded was positively skewed (i.e. the distribution had a long right tail most likely due to the presence of outliers, Figure 3.2), so to remove outliers an emission range representing the 1st and 3rd quartile of the monitoring data (1500 – 8000 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$, n = 165, see Appendix Y for data distribution over this range) was used instead of the full data range (9.60 – 38000 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$). Based on these rates, the cultivation of algae over 35 million ha of raceway ponds[15] would release 132 – 685 kt $N\text{-}N_2O \cdot yr^{-1}$, which is equivalent to 2 – 10 % of the amount of N_2O globally emitted by all anthropogenic sources each year (6.9 Tg $N\text{-}N_2O \cdot yr^{-1}$, Ciais et al., 2013). While caution is needed as these estimates are still uncertain due to the variability in the emissions reported, they confirm the need for considering microalgal N_2O emissions during microalgal cultivation.

3.1.3.3 N_2O emission factors

N_2O emissions from managed and natural soils, wetlands, and wastewater treatment plants are currently estimated as a fraction of nitrogen load (kg N) transformed into N_2O (kg $N\text{-}N_2O$) by using specific N_2O emission factors (EFs, kg $N\text{-}N_2O \cdot kg\ N^{-1}$, De Klein et al., 2006; Doorn et al., 2006). Based on this approach, the emissions of 1500 – 8000 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$ (equivalent to 0.02 – 0.11 g $N\text{-}N_2O \cdot m^{-3} \cdot d^{-1}$) recorded during *C. vulgaris* cultivation on synthetic media with NO_3^- as the main N-source would be equivalent to 0.1 – 0.4% of the N load of 25 g $N.d^{-1}$. These EFs are 3 – 11 times higher

[15] As described in Chapter 1 (Section 1.3.2), in the USA 35 million ha of cultivation systems would be required to replace 30% of conventional transport fuels with algae-derived biodiesel.

than the EF given by the IPCC for centralised wastewater treatment (0.035%, Doorn et al., 2006) but 3 – 10 times lower than the EF given by the IPCC for agricultural soils (1%, De Klein et al., 2006). While the EFs determined during this study provide indication for future research, they however are specific to *C. vulgaris* cultivation and are uncertain due to the variability in the N_2O emissions recorded. Therefore, further data would be required to: 1) improve the accuracy of the EF estimated during this study; and 2) to estimate specific EFs (e.g. according to microalgae species).

3.1.3.4 Mitigation strategies

As illustrated by the case of the cyanobacteria *A. platensis*, species control may provide a simple means to mitigate N_2O emissions during algae cultivation. Similarly, Weathers and Niedzielski, (1986) reported that the cyanobacteria *Anacystis nidulans* did not produce N_2O when supplied NO_3^- (or NO_2^-) while other cyanobacteria could. It is worth considering that from the literature and the results presented in this thesis (Chapter 2), the kinetic and yields of N_2O synthesised can be different according to microalgae species. Consequently, further monitoring would be required to evaluate the potential ability and the magnitude of N_2O emissions from commercial microalgae species cultivated on a large scale (e.g. culture of the cyanobacteria *Aphazinomenon* and the eukaryotic algae *Dunaliella salina*).

If species control is not feasible, the use of NH_4^+ as N-source may provide a simple and economical solution to prevent N_2O emissions from microalgal cultivation, as shown here when *C. vulgaris* was fed NH_4^+. In addition, N_2O emission from *C. vulgaris* cultures fed with NH_4^+ was triggered by artificially raising the NO_2^- concentration by 0.5 mM and the N_2O production rates were 25 times lower than the rates recorded

when the same test was performed in cultures fed NO_3^- (Appendix X). Further consideration is still needed when using NH_4^+ as the nutrient source. NH_4^+ is converted into ammonia (NH_3) at high pH (when photosynthesis is carbon-limited and pH not actively controlled), which can inhibit growth, causing costly N losses (Andersen, 2005; García et al., 2006), and generating indirect N_2O emissions (Nevison, 1998; De Klein et al., 2006) via NH_3 volatilisation and deposition (Cai, 1997).

3.1.4 Conclusions

This study provides the first long-term monitoring data of N_2O emissions reported during commercially relevant algae cultivation outdoors. Significant and variable N_2O emissions were detected during the outdoor cultivation of *C. vulgaris* (9.60 – 38000 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$) and *Neochloris* sp. (50.5 – 14200 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$) in 50 L PBRs fed NO_3^-. No emissions were however recorded from *C. vulgaris* in 50 L PBRs fed NH_4^+ or *A.platensis* in 50 L PBRs fed NO_3^-. Further monitoring of *C. vulgaris* cultivation showed that N_2O emission rates were positively correlated with biomass concentration and light intensity. Nevertheless, it was still difficult to explain the variability in the emissions reported. Future research should account for variables that could explain uncertainty in the N_2O emissions such as growth kinetics which were lacking in our study. Based on the rates reported of 1500 – 8000 nmol $N_2O \cdot h^{-1} \cdot m^{-2}$ (representing 25 – 75% of the data to remove outliers), cultivating algae to produce globally meaningful amounts of algal biodiesel has the potential to generate significant global N_2O emissions. Based on these rates, N_2O emissions factors were estimated at 0.1 – 0.4% of the N load for *C. vulgaris* fed synthetic media. While these estimates are uncertain and further research is needed to improve accuracy, they are the first documented in the literature. Finally, the use of NH_4^+ as an N-source and/or cultivating microalgae species that do not appear to synthesise N_2O (e.g. *A. platensis*) offer possible mitigation solutions. Nevertheless, N_2O emissions from microalgal production should be carefully monitored and accounted for in impact assessment such as life cycle analysis.

3.2 N_2O emissions from HRAP fed real domestic wastewater

ABSTRACT

While the intergovernmental panel for climate change (IPCC) currently estimate that 200 kt of N_2O are indirectly generated from wastewater effluent discharge, direct N_2O emissions during centralised wastewater treatment are considered negligible. In order to determine if direct N_2O emissions from microalgae-based pond systems could also be considered negligible, N_2O emissions were recorded from an outdoor 1000 L pilot high rate algal pond fed primary wastewater over 1 year. The HRAP was operated at a HRT of 7 – 10 days and the microalgal/bacterial suspension was mixed with a paddle wheel. The HRAP effluent was monitored weekly over 1 year for total suspended solid (TSS), pH, dissolved oxygen (DO), total nitrogen (TN), nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), and N_2O. N_2O emissions were found to range from 5.10 to 19000 μg $N\text{-}N_2O \cdot m^{-2} \cdot d^{-1}$ (median of 2300 μg $N\text{-}N_2O \cdot m^{-2} \cdot d^{-1}$, n = 50). Based on the emissions of 450 – 4600 μg $N\text{-}N_2O \cdot m^{-2} \cdot d^{-1}$ (25 – 75% of the data were used to remove outliers), emissions factors were estimated to be 0.04 – 0.45% of the N load of 4.5 ± 0.75 g $N \cdot d^{-1}$ (n = 50). Using these emissions we estimated global direct N_2O emissions during secondary microalgae-based wastewater treatment at 0.36 – 3.8 kt $N\text{-}N_2O \cdot yr^{-1}$. This range represents less than 2% of global N_2O emissions budgeted by the IPCC for wastewater treatment. Therefore, N_2O emissions from microalgae-based system for wastewater treatment could be considered as minor.

3.2.1 Introduction

Simple microalgae-based pond systems (e.g. stabilisation/maturation ponds) are widely used for wastewater treatment (WWT, Shilton and Walmsley, 2005) because they are considered affordable and sustainable due to their low-energy requirements and the potential for bio-energy generation (Shilton et al., 2008).

In the last decade, increasing attention has been given to N_2O emissions from biological nutrient removal processes (e.g. activated sludge) during advanced centralised wastewater treatment (Kampschreur et al., 2009; Foley et al., 2010; Ahn et al., 2010; Law et al., 2012; Ye et al., 2014). However, and surprisingly, relatively little focus has been given to potential N_2O emissions from microalgae-based systems for wastewater treatment. To date, two studies have focused on N_2O emissions from laboratory scale photobioreactors and high rate algal ponds (HRAPs) fed wastewater (Mezzari et al., 2013; Alcántara et al., 2015); and two other studies investigated GHG emissions (including N_2O), from stabilisation ponds (Hernandez-Paniagua et al., 2014; Glaz et al., 2016). While Hernandez-Paniagua et al, (2014) reported emissions of 7 – 600 μg $N\text{-}N_2O \cdot m^{-2} \cdot d^{-1}$ in a stabilisation pond located in Mexico, Glaz et al, (2016) measured N_2O emissions up to 0.001 μg $N\text{-}N_2O \cdot m^{-2} \cdot d^{-1}$ and 0.5 μg $N\text{-}N_2O \cdot m^{-2} \cdot d^{-1}$ from stabilisation ponds in Western Australia and Quebec, respectively.

Due to the large variation in the N_2O emissions recorded and no clear understanding of why there is such large variation (Hernandez-Paniagua et al., 2014; Glaz et al., 2016), there is a need for further monitoring N_2O from microalgae-based pond systems. In addition, the global significance of N_2O emissions from microalgae-based pond systems for WWT is currently unknown. Because these systems require large land

areas in comparison with centralised WWT (Craggs et al., 2013) the magnitude of N_2O emissions from microalgae-based pond systems could be massive.

In order to evaluate the potential significance of N_2O emissions from microalgae-based systems for WWT, N_2O emissions were carefully monitored from a 1000 L HRAP fed domestic wastewater for a period of one year. Using the data obtained, the potential global significance of N_2O emissions from microalgae-based pond systems treating wastewater was then estimated.

3.2.2 Materials and methods

3.2.2.1 1000 L high rate algal pond (HRAP) design and operation

The 0.25 m deep pilot 1000 L HRAP (Figure 3.9) was made of concrete and was located at Palmerston North wastewater treatment plant, New Zealand (Latitude: 40° 23′ 7.486′ S; Longitude: 175° 34′ 47.417′ E). The system was inoculated on January 2015 with an algal/bacterial suspension obtained from Rongotea maturation pond, New Zealand (Latitude: 40° 17′ 42.319′ S; Longitude: 175° 24′ 48.466′ E) and was fed continuously (100 $L \cdot d^{-1}$) with primary treated domestic wastewater to reach a hydraulic retention time (HRT) of 10 days, later decreased to 7 days. Mixing was maintained with a paddle wheel (11 rpm, central average fluid velocity calculated at 0.166 $m \cdot s^{-1}$).

Figure 3.9: 1000 L HRAP fed primary wastewater

3.2.2.2 Sampling and N_2O measurements

Liquid samples (125 mL) were withdrawn once weekly. The value of the pH and dissolved oxygen (DO) were monitored directly by submerging the pH and DO probes in the HRAP microalgal/bacterial suspension. The other analyses (i.e. DCW, nitrogen compounds) were performed on the liquid samples withdrawn.

To measure dissolved N_2O concentration, 6 mL of HRAP microalgal/bacterial suspension was withdrawn from the pond (using a syringe to avoid any gas transfer) and, by piercing the septum, was transferred into 12.5 mL sealed vials previously flushed with N_2 (neutral and carrier gas for the GC). During each sampling, the liquid suspension was taken with care to prevent air entrainment. Following 30 min incubation at ambient temperature, an equilibrium between gaseous and aqueous phase N_2O concentration was reached in the vial. Because the equilibrium was rapid and not affected by significant N_2O production (or consumption) it was assumed that the N_2O measured at t = 30 min was representative of the N_2O in the HRAP at the time of sampling (Appendix Z). Gas samples were withdrawn from the vial headspace and analysed by ECD-gas-chromatography as described in Section 3.1.2.3. Dissolved N_2O concentration was then back calculated from the gaseous N_2O concentration using Henry`s law. The amount of atmospheric N_2O emitted was calculated based on Equation 3.2:

$$\Phi_{N_2O} = Kla_{N_2O} \times ([N_2O]^* - [N_2O]), \text{ Equation 3.2}$$

Φ_{N_2O} = N_2O flux ($mol \cdot L^{-1} \cdot s^{-1}$)

Kla_{N_2O} = N_2O mass transfer coefficient (s^{-1})

$[N_2O]^*$ = Dissolved N_2O concentration at equilibrium ($mol \cdot L^{-1}$)

$[N_2O]$ = Dissolved N_2O concentration ($mol \cdot L^{-1}$)

$Kl_a(O_2)$, was experimentally determined by filling the HRAP with 1000 L of tap water, under continuous mixing with the paddle wheel and bubbling with N_2 to decrease O_2 concentration below 1 $mg \cdot L^{-1}$. Bubbling was then stopped and O_2 concentration was measured every minute for 4 hours. The logged data were then used to estimate $Kl_a(O_2)$ = 0.18 h^{-1} in tap water by integrating O_2 concentration as a function of time (Akita and Yoshida, 1973). The mass transfer coefficient for oxygen, $Kl_a(O_2)$ was used to compute the mass transfer coefficients of N_2O, $Kl_a(N_2O)$ of 0.17 h^{-1} at 20ºC (Equation 3.3), based on the diffusivities of these gases (Ye et al., 2014). A standard temperature correction factor of 1.024 was used for temperature adjustment (Tchobanoglous et al., 2003).

$$Kla_{N_2O} = Kla_{O_2} \times \sqrt{\frac{D_{F_{N_2O}}}{D_{F_{O_2}}}}, \text{ Equation 3.3}$$

Kla_{N_2O} = N_2O mass transfer coefficient (s^{-1})

Kla_{O_2} = O_2 mass transfer coefficient (s^{-1})

$D_{F_{O_2}}$ = Molecular diffusivity of O_2 in water ($1.98 \times 10^{-9} \cdot m^{-2} \cdot s^{-1}$ at 20°C)

$D_{F_{N_2O}}$ = Molecular diffusivity of N_2O in water ($1.84 \times 10^{-9} \cdot m^{-2} \cdot s^{-1}$ at 20°C)

3.2.2.3 Analytical procedures

See Section 3.1.2.4

3.2.3 Results and discussion

3.2.3.1 N_2O emissions from a HRAP

This study presents the first long-term investigation of direct N_2O emissions during real domestic wastewater treatment in an outdoor HRAP. In agreement with biomass productivity documented during the operation of a ha-scale HRAP in New Zealand (Craggs et al., 2012), the seasonal microalgal/bacterial productivity measured as total suspended solids (TSS) ranged from 2.75− 10.5 g-TSS·m^{-2}·d^{-1} and 3.90 − 15.2 g-TSS·m^{-2}·d^{-1} during operation at 10 and 7 days HRT, respectively.

Over one year of monitoring (i.e. broad range of environmental conditions), the HRAP was a source of N_2O with direct emissions fluctuating from 7.60 – 28000 nmol N_2O·m^{-2}·h^{-1} (Figure 3.10). These significant and highly variable N_2O emissions represented 5.10 – 19000 µg $N\text{-}N_2O$·m^{-2}·d^{-1} with a median value of 2300 µg $N\text{-}N_2O$·m^{-2}·d^{-1}. N_2O emissions rates recorded from the HRAP were positively skewed, justifying the choice in presenting median instead of mean, see Appendix AA.

As seen in Figure 3.10, N_2O emissions recorded under 7 days HRT operation (70 – 19000 µg $N\text{-}N_2O$·m^{-2}·d^{-1} with a median value of 4500 µg $N\text{-}N_2O$· m^{-2}·d^{-1}) were significantly higher (two sample t-test, $\alpha = 0.05$, *p-value* < 0.05; See Appendix AA) than N_2O emissions recorded under 10 days HRT operation (5.1 – 6000 µg $N\text{-}N_2O$·m^{-2}·d^{-1} with a median value of 700 µg $N\text{-}N_2O$·m^{-2}·d^{-1}).

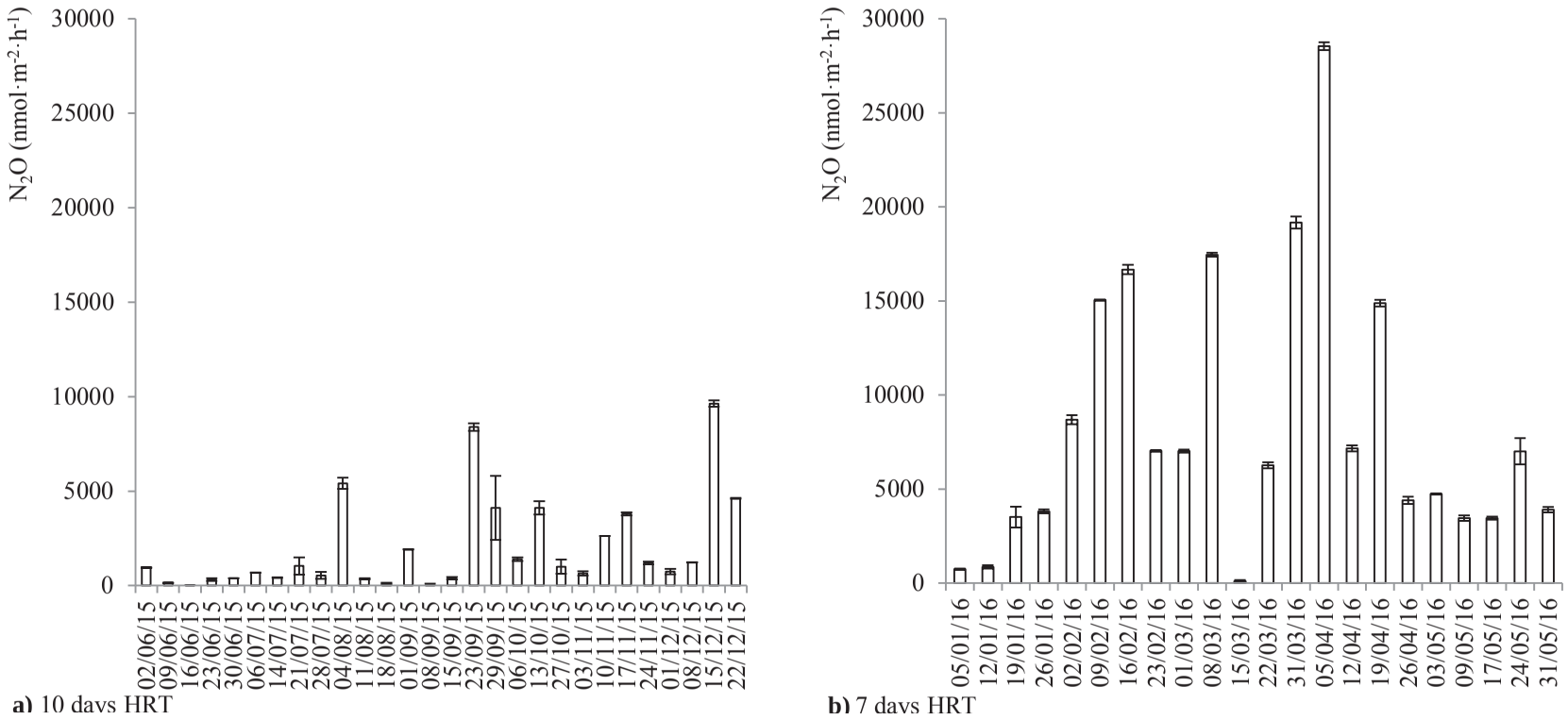


Figure 3.10: a) N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) from weekly samples when the HRAP was operated at 10 days HRT. **b)** N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) from weekly samples when the HRAP was operated at 7 days HRT (the error bars represent the (Max – Min)/2 value between duplicates).

Further analysis showed that the daily averaged pond temperature and daily averaged solar irradiance were also significantly higher (two sample t-test, $\alpha = 0.05$, *p-value* < 0.05) during operation at 7 days HRT than during operation at 10 days HRT (Appendix AA). It is therefore difficult to forecast if the increase in N_2O emissions was related to the change in HRT or the change in temperature and solar irradiance.

Figure 3.11 showed N_2O emissions were not correlated to total suspended solids (R^2 = 0.11), NO_2^- concentration ($R^2 = 0.05$), pH ($R^2 = 0.00$), or solar irradiance ($R^2 = 0.04$). On the other hand, N_2O emissions were found to be weakly positively correlated to broth temperature ($R^2 = 0.25$) and negatively correlated to DO concentration (R^2 = -0.54). Microbial activity is known to increase at temperatures > 4°C (Braker et al., 2010; Béchet et al., 2015a). N_2O synthesis by microorganisms such as denitrifiers and/or microalgae is also known to considerably increase at low oxygen concentrations (Wrage et al., 2001; Chapter 2, Section 2.3.8). Thus, the variability in the N_2O emissions recorded may be explained by changes in microorganisms' activity. Because operation and temperature influence microbial dynamics (Braker et al., 2010; Ferrero et al., 2012), the variability of the N_2O emissions recorded may also be explained by changes in microbial population in the HRAP.

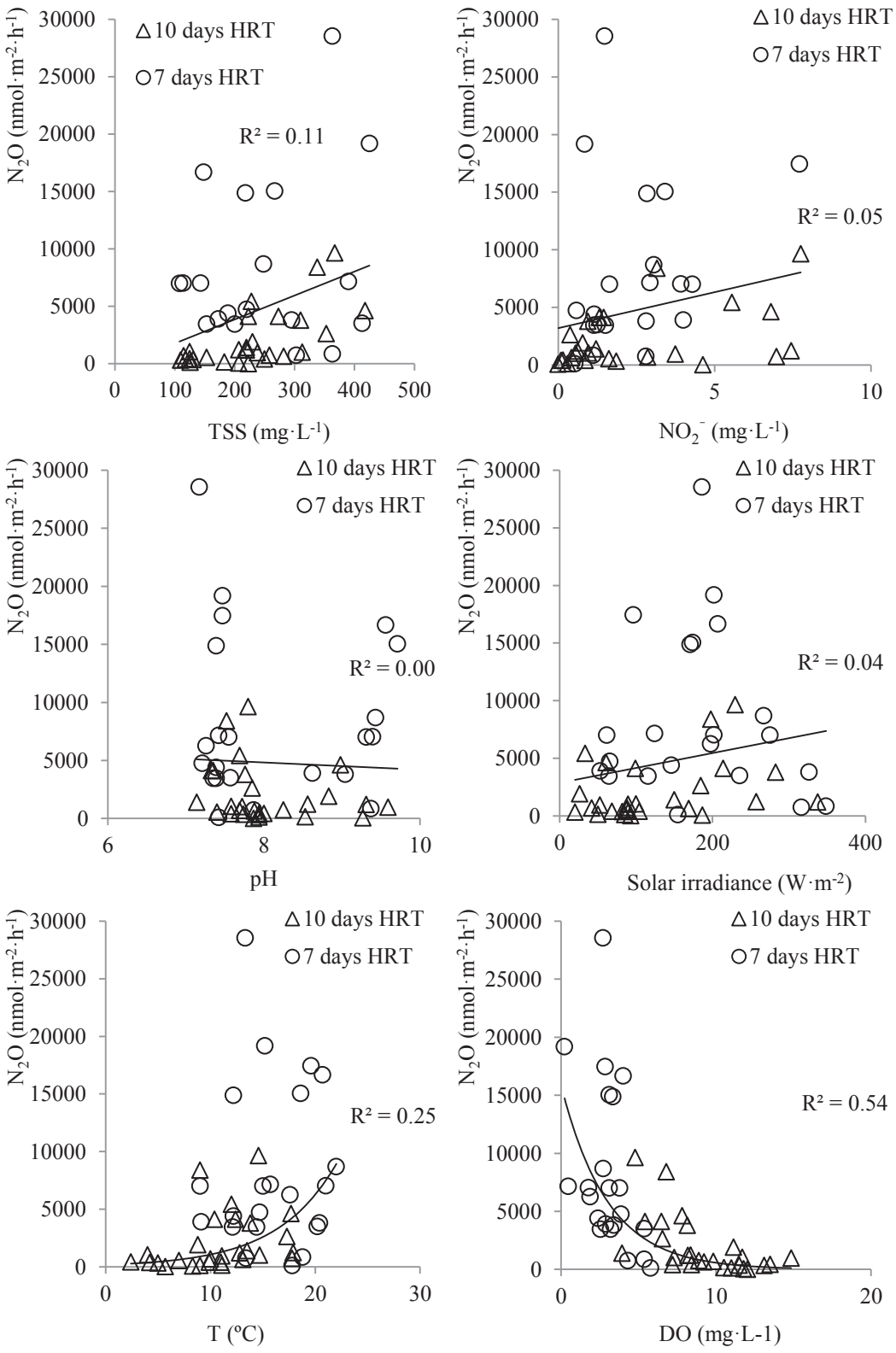


Figure 3.11: N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) against TSS ($mg \cdot L^{-1}$), NO_2^- ($mg \cdot L^{-1}$), pH, solar irradiance ($W \cdot m^{-2}$), T (°C), and DO ($mg \cdot L^{-1}$) during wastewater treatment in a HRAP operated at 10 and 7 days HRT.

While identifying the actual source of N_2O in the HRAP was beyond the scope of only measuring N_2O emissions for impact assessment (e.g. greenhouse gas inventories), various mechanisms can be proposed to explain the N_2O emissions. In wastewater N_2O can be biologically generated via bacterial nitrification, bacterial denitrification, bacterial nitrification-denitrification, archaeal ammonium oxidation (Limpiyakorn et al., 2011), and microalgal N_2O synthesis (Chapter 1 and 2). In the present study, denitrification was unlikely to be significant during operation at 10 days HRT because the culture was well-mixed and remained oxic at all times (DO concentration typically fluctuated between 2 $mg \cdot L^{-1}$ at night to super saturated values at day time). On the other hand, denitrification could explain some of the N_2O emissions recorded when the HRAP was operated at 7 days HRT because the culture was experiencing anoxia (< 1 $mg \cdot L^{-1}$ during night time). In addition, bacterial and archaeal nitrifying activity may explain the production of NO_3^- (48.5 ± 7.50 $mg \cdot L^{-1}$, 95% confidence, n = 49) and NO_2^- (2.42 ± 0.63 $mg \cdot L^{-1}$, 95% confidence, n = 46), even during the relatively 'cold' periods of the year. As seen in Figure 3.12, at most times (at least 75% of period of operation) NO_3^- and NO_2^- were the most prevalent N compounds in the HRAP. Consequently, bacterial and archaeal nitrification could also be a source of N_2O in the HRAP.

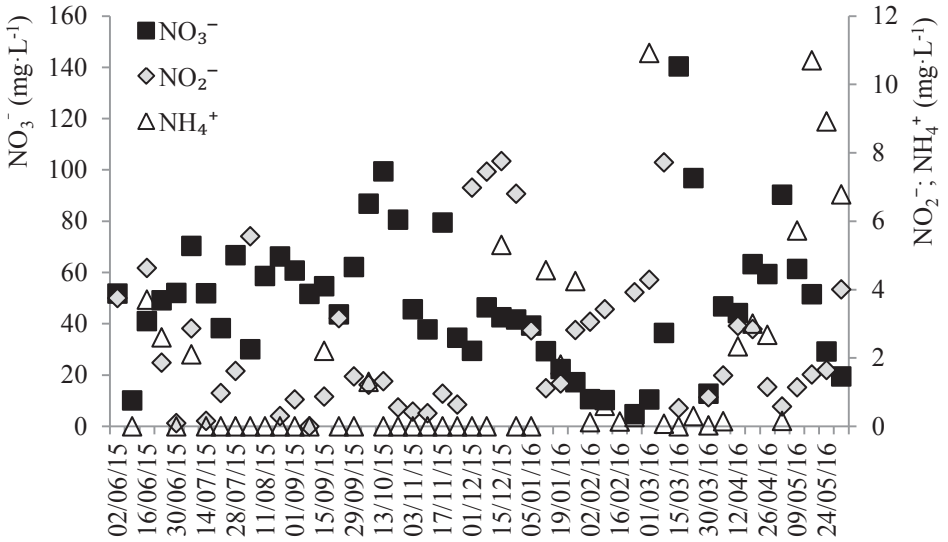


Figure 3.12: NO_3^- ($mg \cdot L^{-1}$), NO_2^- ($mg \cdot L^{-1}$) and NH_4^+ ($mg \cdot L^{-1}$) concentrations in the filtered HRAP microalgal/bacterial suspension.

Nitrite is known to fuel microalgal N_2O synthesis. Consequently, the NO_2^- produced in the HRAP could be utilised by the microalgae (but also potentially nitrifying bacteria), leading to N_2O production. Overall the co-occurrence of mechanisms leading to N_2O emissions could not be totally disregarded. Further work is required (i.e. metagenomics) to determine the source(s) of N_2O in the HRAP and to improve our understanding of N_2O emissions in HRAPs fed wastewater[16].

[16] In addition, out of the scope of this thesis, full wastewater treatment analysis (e.g. pollutant removal efficiencies) will be performed and used in another thesis.

3.2.3.2 N_2O emission factors

The direct N_2O emissions measured during this study ranged from 5.1 to 19000 µg N-$N_2O \cdot m^{-2} \cdot d^{-1}$. As the distribution of the rates recorded was positively skewed (Appendix AA), an emission range representing the 1st and 3rd quartile of the monitoring data (450 – 4600 µg N-$N_2O \cdot m^{-2} \cdot d^{-1}$, n = 25) was used instead of the full data range. Following the approach described in Section 3.1.3.3, the emissions of 450 – 4600 µg N-$N_2O \cdot m^{-2} \cdot d^{-1}$ (equivalent to 0.0018 – 0.018 g N-$N_2O \cdot m^{-3} \cdot d^{-1}$) represented 0.04 – 0.45% of the TN influent load of 4.5 g $TN \cdot d^{-1}$ fed to the HRAP. As seen in Figure 3.13a, this range is significantly higher than the 0.0047% g N-$N_2O \cdot$g N-$input^{-1}$ reported by Alcántara et al., (2015) for a HRAP fed synthetic wastewater under laboratory conditions (operated at 7 days HRT with a 12:12 h light-dark cycle). Surprisingly, even the lowest value estimated from our study is higher than the guideline value of 0.035% given by the IPCC for advanced centralised wastewater treatment, but is within the range of emission factors (0 – 14.6%) reported during full scale biological wastewater treatment (e.g. 1000 – 60000 person equivalent activated sludge) around the globe (Kampschreur et al., 2009).

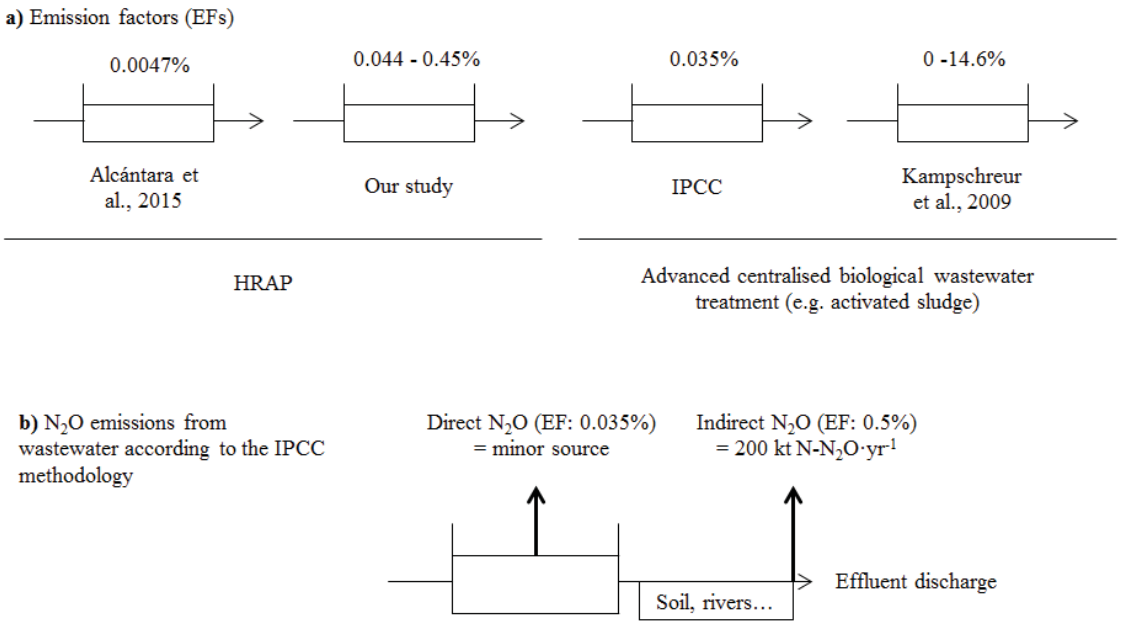


Figure 3.13: a) Comparison of N_2O emission factors documented in the literature and estimated from the N_2O measured from a 1000 L HRAP. **b)** IPCC methodology to estimate N_2O emissions from wastewater.

3.2.3.3 Significance of N_2O emissions from a pilot HRAP fed with domestic wastewater

As illustrated in Figure 3.13b, the IPCC currently does not account for direct N_2O emissions from advanced centralised wastewater treatment (e.g. biological wastewater treatment such as activated sludge) because these emissions are considered negligible in comparison with indirect emissions from effluent discharge (Doorn et al., 2006). The IPCC thus estimates that 200 kt $N\text{-}N_2O \cdot yr^{-1}$ are globally generated from wastewater effluent (Ciais et al., 2013).

In order to evaluate whether direct N_2O emissions from microalgae based pond systems are significant on a global scale, we conservatively estimated that 225000 ha of pond

systems are used by 685 million individuals worldwide (Box 3.2). Based on the N_2O emissions measured (450 – 4600 µg $N\text{-}N_2O \cdot m^{-2} \cdot d^{-1}$), global N_2O emissions from microalgae-based ponds were estimated at 0.36 – 3.8 kt $N\text{-}N_2O \cdot yr^{-1}$. These emissions represent 0.18 – 1.9% of global N_2O emissions budgeted for wastewater treatment discharge by the IPCC (200 kt $N\text{-}N_2O \cdot yr^{-1}$). Consequently, global direct N_2O emissions from microalgae-based ponds systems for wastewater treatment should have a relatively minor environmental impact.

This outcome is insensitive to uncertainty in the data used and the assumptions made. Indeed, N_2O emissions from pond systems are likely to be lower than the emissions of 0.36 – 3.8 kt $N\text{-}N_2O \cdot yr^{-1}$ for the following reasons:

1. N_2O emissions documented from actual stabilisation ponds are significantly lower than the rates recorded in the HRAP (See Section 3.2.1).
2. The microalgal/bacterial biomass, which is potentially generating N_2O, has generally a lower productivity in stabilisation ponds than in HRAP (Craggs et al., 2014).
3. Potential seasonal and temporal effects on N_2O emissions are unknown and were not considered (e.g. low photosynthetic activity in winter).

Box 3.2: In 2015, the World Health Organisation (WHO) documented that 46% of the world population of 7.3 billion individuals live in rural areas and that 51% of the rural population had access to improved sanitation facilities (WHO/UNICEF, 2015) which represent 1.7 billion individuals. The WHO also estimated that in rural areas, 20% of the classified improved sanitation facilities used were septic tanks (WHO/UNICEF, 2015); 1.37 billion individuals would be using other systems which should include ponds. Because the WHO consider that a sanitation facility is “improved” if it

hygienically separates human excreta from human contact without necessarily the subsequent management of faecal waste (WHO/UNICEF, 2015), and pond systems are widely used among small communities in rural areas (Shilton and Walmsley, 2005); we assumed that 50% of the individuals using improved sanitation systems other than septic tank were dependent on ponds (i.e. 685 million individuals). Based on the world biological oxygen demand (BOD) load of 60 g BOD.inhabitant^{-1}.d^{-1} (Henze and Comeau, 2008), the total amount of BOD generated by rural communities was estimated to 41100 t BOD.d^{-1}. Ponds are usually in a two stage process designed on a single BOD surface loading rate which is dependent on temperature and can be calculated using the widely used design equation (Equation 3.4) established by Mara (Shilton and Mara, 2005).

$$\lambda s = 350(1.107 - 0.002T)^{T-25}, \text{ Equation 3.4}$$

$\lambda s =$ Surface BOD loading rate (kg·ha^{-1}·d^{-1})

$T =$ Mean air temperature (°C)

Thus, based on the world average temperature reaching 16°C in 2016 (global analysis, August 2016 from National Centres for Environmental information: http://www.ncdc.noaa.gov/sotc/), a surface loading rate of 183 kg BOD.ha^{-1}.d^{-1} was calculated. Dividing the estimated BOD generated by rural communities of 41100 t BOD.d^{-1} by the loading rate of 183 kg BOD.ha^{-1}.d^{-1}, 225000 ha of ponds would be required to treat rural communities' wastewater.

3.2.4 Conclusions

Positive N_2O emissions of 5.1 – 19000 µg $N\text{-}N_2O \cdot m^{-2} \cdot d^{-1}$ (n = 50) were recorded from a 1000 L HRAP fed primary wastewater over one year of monitoring. Based on the conservative rates reported (450 – 4600 µg $N\text{-}N_2O \cdot m^{-2} \cdot d^{-1}$, representing 25 – 75% of the data) N_2O emissions factors were estimated at 0.04 – 0.45% of the N load for wastewater treatment. Using this data, we estimated that N_2O emissions from microalgae-based wastewater treatment ponds would have a relatively minor environmental impact, representing 0.18 – 1.9% of the global N_2O emissions currently budgeted by the IPCC for wastewater treatment. Nevertheless further research is needed (e.g. metagenomics) to improve our understanding of the dynamics in microorganisms in HRAP and their role in N_2O emissions.

3.3 Relevance of the monitoring studies

Section 3.1 and 3.2 presented the first long-term monitoring of direct N_2O emissions from microalgae cultivated in photobioreactors fed synthetic media and a high rate algal pond fed real domestic wastewater.

Tools for estimating N_2O emissions from microalgal cultivation were lacking. Based on the methodology described by the IPCC for GHG inventory[17], emissions factors (EFs) were calculated in order to estimate N_2O emissions from microalgal cultivation. EFs of 0.1 – 0.4% of the N load were estimated for microalgae culture fed NO_3^--laden synthetic media and EFs of 0.04 – 0.45% of the N load were estimated for microalgae culture fed wastewater. To date, these preliminary emission factors are the first documented for microalgae-based cultivation on synthetic media and wastewater. Although the accuracy of the EFs needs to be improved, emphasis should be given on the methodology used to compute those EFs.

Microalgae biotechnology may still be at its infancy the results presented in this chapter confirmed that N_2O emissions can occur during microalgae cultivation and that consequently, N_2O emissions should be considered for accurate environmental assessments of microalgal cultivation. Importantly, as discussed in Chapter 1, algal-mediated N_2O emissions may occur from a range of microalgae-dominated engineered and natural ecosystems and the global magnitude of N_2O emissions from these systems is currently unknown.

[17] As described in Section 3.1.3.4, N_2O emissions from managed and natural soils, wetlands and wastewater treatment plants are currently estimated as a fraction of nitrogen load (kg N) being transformed to N_2O, by using specific N_2O emission factors (kg $N_2O \cdot kg\ N^{-1}$) which are based on field N_2O emissions (See De Klein et al., 2006; Doorn et al., 2006).

Due to anthropogenic activities such as agriculture, the NO_3^- concentration in natural aquatic ecosystems is increasing (Vitousek, 1994). Since it is now clear that microalgal N_2O emissions are related to nitrate assimilation (Chapter 2) and that these emissions can potentially be significant when NO_3^- is the main N-source (Section 3.1.3.2) N_2O emissions from microalgae-based ecosystems should not be neglected. As microalgae are ubiquitous, it is necessary to investigate the potential of microalgae-based ecosystems as a source of N_2O. Particular focus should be given to eutrophic lakes which are known to be affected by periods of algal blooms and as a result could be a major source of N_2O (especially if eutrophication is of anthropogenic origin).

References

Ahn, J., Kim, S., Park, H., and Rahm, B. (2010). N_2O emissions from activated sludge processes, 2008− 2009: results of a national monitoring survey in the United States. Environ. Sci. Technol. **44**: 4505–4511.

Akita, K. and Yoshida, F. (1973). Gas holdup and volumetric mass transfer coefficient in bubble columns. Effects of Liquid Properties. Ind. Eng. Chem. Process Des. Dev. **12**: 76–80.

Alcántara, C., Muñoz, R., Norvill, Z., Plouviez, M., and Guieysse, B. (2015). Nitrous oxide emissions from high rate algal ponds treating domestic wastewater. Bioresour. Technol. **177**: 110–117.

Andersen, R.A. Berges, J.A, Harrison, P.J. Watanabe, M.M. Appendix A: Recipes for freshwater and seawater media, in: R.A. Andersen (Ed) Algal culturing techniques, Elsevier Academic Press (San Diego), 2005 pp. 429–538.

Béchet, Q., Chambonnière, P., Shilton, A., Guizard, G., and Guieysse, B. (2015). Algal productivity modeling: A step toward accurate assessments of full-scale algal cultivation. Biotechnol. Bioeng. **112**: 987–996.

Béchet, Q., Feurgard, I., Guieysse, B., and Lopes, F. (2015b). The colorimetric assay of viability for algae (CAVA): a fast and accurate technique. J. Appl. Phycol. 27: 2289–2297.

Béchet, Q., Shilton, A., Fringer, O.B., Muñoz, R., and Guieysse, B. (2010). Mechanistic modeling of broth temperature in outdoor photobioreactors. Environ. Sci. Technol. **47**: 2197-2203.

Béchet, Q., Shilton, A., and Guieysse, B. (2013). Modeling the effects of light and temperature on algae growth : State of the art and critical assessment for productivity prediction during outdoor cultivation. Biotechnol. Adv. **31**: 1648–1663.

Borowitzka, M.A. (2005) Culturing microalgae in outdoor ponds in: R.A. Andersen (Ed) Algal culturing techniques, Elsevier Academic Press (San Diego) pp. 205 –218.

Braker, G., Schwarz, J., and Conrad, R. (2010). Influence of temperature on the composition and activity of denitrifying soil communities. FEMS Microbiol. Ecol. **73**: 134–148.

Brennan, L. and Owende, P. (2010). Biofuels from microalgae — A review of technologies for production , processing , and extractions of biofuels and co-products. Renew. Sustain. Energy Rev. **14**: 557–577.

Cai, G. (1997). Ammonia volatilization. In Nitrogen in soils of China. Vol 74 of the series Developments in Plant and Soil sciences. pp 193-213.

Ciais, P. et al. (2013). Carbon and other biogeochemical cycles. In Climate Change 2013: The physical science basis. Contribution of working group I to the fifth assessment report of the intergovernmental panel on climate change, V.B. and P.M.M. Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, ed (Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA), pp. 465–570.

Craggs, R., Sutherland, D., and Campbell, H. (2012). Hectare-scale demonstration of high rate algal ponds for enhanced wastewater treatment and biofuel production. J. Appl. Phycol. **24**: 329–337.

Craggs, R.J., Lundquist, T.J., and Benemann, J.R. (2013). Wastewater treatment and algal biofuel production in Algae for biofuels and energy. M.A. Borowitzka and N.R. Moheimani, eds (Springer), pp. 99–113.

Doorn, M., Towprayoon, S., Viera, S.N.M., Irving, W., Palmer, C., Pipatti, R., Wang, C.(2006). Chapter 6 in 2006 IPCC guidelines for national greenhouse gas inventories. **5**:1-28.

Fernández, E. and Galván, A. (2008). Nitrate assimilation in Chlamydomonas. Eukaryot. Cell **7**: 555–559.

Ferrero, E.M., de Godos, I., Rodríguez, E.M., García-Encina, P.A., Muñoz, R., and Bécares, E. (2012). Molecular characterization of bacterial communities in algal-bacterial photobioreactors treating piggery wastewaters. Ecol. Eng. **40**: 121–130.

Ferrón, S., Ho, D.T., Johnson, Z., I., and Huntley, M., E. (2012). Air − Water fluxes of N_2O and CH_4 during microalgae (*Staurosira sp.*) cultivation in an open raceway pond. Environ. Sci. Technol. **46**: 10842–10848.

Florez-Leiva, L. Tarifeño, E. Cornejo, M. Kiene, R. Farías, L. (2010) High production of nitrous oxide (N2O), methane (CH4) and dimethylsulphoniopropionate (DMSP) in a massive marine phytoplankton culture, Biogeosciences Discuss. 7 6705–6723.

Foley, J., de Haas, D., Yuan, Z., and Lant, P. (2010). Nitrous oxide generation in full-scale biological nutrient removal wastewater treatment plants. Water Res. **44**: 831–844.

García, J., Green, B.F., Lundquist, T., Mujeriego, R., Hernández-Mariné, M., and Oswald, W.J. (2006). Long term diurnal variations in contaminant removal in high rate ponds treating urban wastewater. Bioresour. Technol. **97**: 1709–1715.

Glaz, P., Bartosiewicz, M., Laurion, I., Reichwaldt, E.S., Maranger, R., and Ghadouani, A. (2016). Greenhouse gas emissions from waste stabilization ponds in Western Australia and Quebec (Canada). Water Res. **101**: 64–74.

de Godos, I., Blanco, S., García -Encina, P.A., Becares, E., and Muñoz, R. (2010). Influence of flue gas sparging on the performance of high rate algae ponds treating agro-industrial wastewaters. J. Hazard. Mater. **179**: 1049–1054.

Gouveia, L. (2011). Microalgae as a feedstock for biodiesel (SpringerBriefs in Microbiology). pp 1-68.

Guieysse, B., Plouviez, M., Coilhac, M., and Cazali, L. (2013). Nitrous Oxide (N_2O) production in axenic *Chlorella vulgaris* microalgae cultures: Evidence, putative pathways, and potential environmental impacts. Biogeosciences **10**: 6737–6746.

Hahn, J. and Junge, C. (1977). Atmospheric nitrous oxide: a critical review. Zeitschrift fur Naturforsch. **32**: 190–214.

Henze, M. and Comeau, Y. (2008). Wastewater characterization. Biol. Wastewater Treat. Princ. Model. Des.pp 33–52.

Hernandez-Paniagua, I.Y., Ramirez-Vargas, R., Ramos-Gomez, M.S., Dendooven, L., Avelar-Gonzalez, F.J., and Thalasso, F. (2014). Greenhouse gas emissions from stabilization ponds in subtropical climate. Environ. Technol. **35**: 727–734.

Jenni, S., Mohn, J., Emmenegger, L., and Udert, K.M. (2012). Temperature dependence and interferences of NO and N_2O microelectrodes used in wastewater treatment. Environ. Sci. Technol. **46**: 2257-2266.

Kampschreur, M.J., Temmink, H., Kleerebezem, R., Jetten, M.S.M., and van Loosdrecht, M.C.M. (2009). Nitrous oxide emission during wastewater treatment. Water Res. **43**: 4093–4103.

Kessler, E. and Oesterheld, H. (1970). Nitrification and induction of nitrate reductase in nitrogen-deficient algae. Nature **228**: 287–288.

de Klein, C., Novoa, S.A., Ogle, S., Smith, K.A., Rochette, P., Wirth, T.C., McConkey, B.G., Mosier, A., Rypdal, K., Wlash, M., and Williams, S.. (2006). N_2O emissions from managed soils, and CO_2 emissions from lime and urea application. Chapter 11 in 2006 IPCC guidelines for national greenhouse gas inventories. **4**: 1–54.

Law, Y., Ye, L., Pan, Y., and Yuan, Z. (2012). Nitrous oxide emissions from wastewater treatment processes. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **367**: 1265–77.

Limpiyakorn, T., Sonthiphand, P., Rongsayamanont, C., and Polprasert, C. (2011). Abundance of amoA genes of ammonia-oxidizing archaea and bacteria in activated sludge of full-scale wastewater treatment plants. Bioresour. Technol. **102**: 3694–3701.

Mezzari, M.P., Márcio, L.B., Nicoloso, R.S., Ibelli, A.M.G., Bortoli, M., Viancelli, A., and Soares, H.M. (2013). Assessment of N_2O emission from a photobioreactor treating ammonia-rich swine wastewater digestate. Bioresour. Technol. **149**: 327–332.

de Morais, M.G. and Costa, J.A. V (2007). Biofixation of carbon dioxide by Spirulina sp. and Scenedesmus obliquus cultivated in a three-stage serial tubular photobioreactor. J. Biotechnol. 129: 439–445.

Navarro, T., Guerra, E., Fernández, E., and Galván, A. (2000). Nitrite reductase mutants as an approach to understanding nitrate assimilation in *Chlamydomonas reinhardtii*. Plant Physiol. **122**: 283–289.

Nevison, C. (1998). Indirect N_2O emissions from agriculture. In good practice guidance and uncertainty management for national greenhouse gas inventories. pp. 381–397.

Novis, P.M., Halle, C., Wilson, B., and Tremblay, L.A. (2009). Identification and characterization of freshwater algae from a pollution gradient using rbcl sequencing and toxicity testing. Arch. Environ. Contam. Toxicol. **57**: 504–514.

Pierotti, D. and Rasmussen, R.A. (1980). Nitrous oxide measurements in the eastern tropical Pacific Ocean. Tellus **32**: 56–70.

Shilton, A.N. and Mara, D. (2005). Pond process design - an historical review. In pond treatment technology, A. Shilton, ed (IWA Publishing, London-Seattle), pp. 145–167.

Shilton, A.N., Mara, D.D., Craggs, R., and Powell, N. (2008). Solar-powered aeration and disinfection, anaerobic co-digestion, biological CO_2 scrubbing and biofuel production: the energy and carbon management opportunities of waste stabilisation ponds. Water Sci. Technol. **58**: 253-258.

Shilton, A.N. and Walmsley, N. (2005). Introduction to pond treatment technology. In Pond treatment technology, A.N. Shilton, ed (IWA Publishing, London-Seattle), pp. 1–13.

Spolaore, P., Joannis-Cassan, C., Duran, E., and Isambert, A. (2006). Commercial applications of microalgae. J. Biosci. Bioeng. **101**: 87–96.

Tchobanoglous, G., Burton, F.L., Stensel, H., (2003). [Metcalf & Eddy, I.]. Wastewater Engineering – Treatment and Reuse, 4th Edition (NY: McGraw-Hill).

Ugwu, C.U., Aoyagi, H., and Uchiyama, H. (2008). Photobioreactors for mass cultivation of algae. Bioresour. Technol. **99**: 4021–4028.

Vitousek, P.M. (1994). Beyond global warming: Ecology and global change. Ecology **75**: 1861–1876.

Weathers, P.J. (1984). N_2O Evolution by green algae. Appl. Envionmental Microbiol. **48**: 1251–1253.

Weathers, P.J. and Niedzielski, J.J. (1986). Nitrous oxide production by cyanobacteria. Arch Microbiol **146**: 204–206.

WHO/UNICEF (2015). Progress on sanitation and drinking water 2015 Update and MDG Assessment. World Health Organization. http://www.who.int/water_sanitation_health/monitoring/jmp-2015-update/en/ pp 1-90.

Wrage, N., Velthof, G.L., van Beusichem, M.L., and Oenema, O. (2001). Role of nitrifier denitrification in the production of nitrous oxide. Soil Biol. Biochem. **33**: 1723–1732.

Ye, L., Ni, B.J., Law, Y., Byers, C., and Yuan, Z. (2014). A novel methodology to quantify nitrous oxide emissions from full-scale wastewater treatment systems with surface aerators. Water Res. **48**: 257–268.

Chapter 4.

Conclusions and future prospects

4.1 Conclusions

While the synthesis of N_2O by microalgae was suggested and documented for 40 years, the 'microalgal N_2O bio-origin' was rarely acknowledged in studies evaluating N_2O emissions from microalgae-based (eco)systems. From the critical literature review, it became clear that N_2O synthesis by microalgae needed further investigation. Firstly, basic calculations using available data showed that N_2O emissions from microalgae-based engineered (e.g. microalgal biomass as biofuel feedstock) and aquatic ecosystems (e.g. eutrophic lakes) could be significant. Secondly, the biochemical pathway and metabolic functions of microalgal N_2O synthesis were poorly understood. Only two putative microalgal N_2O pathways were described in the literature, but based on the knowledge of the 'conventional mechanisms' leading to N_2O emissions in biological systems we were able to suggest six putative microalgal N_2O pathways. Using this knowledge as a basis, the synthesis of N_2O in microalgae was then investigated using the model microalga *C. reinhardtii*.

The laboratory *in vitro* batch assays performed within this thesis demonstrated for the first time that *C. reinhardtii* cultures had the ability to synthesise N_2O under aerobic conditions when supplied with NO_2^-. Combining biochemical assays involving *C. reinhardtii* wildtype 6145c and its associated NR, NiR, and NOR mutant supplied with NO_2^-, we demonstrated that microalgal N_2O synthesis was linked to nitrate assimilation and was occurring in 2 steps respectively involving 1) intracellular NO_2^- reduction to NO and 2) NO subsequently reduction into N_2O by NOR. In regard to the first step, NO_2^- reduction into NO in darkness was found to be initially catalysed by the dual NR-ARC enzyme system, but this activity rapidly ceased and was later substituted by NO_2^-

reduction into NO by mitochondrial COX. Given the impacts of NR and NiR on intracellular NO_2^- accumulation (i.e. low/moderate intracellular NO_2^-), we hypothesised that N_2O synthesis should mainly occur under illumination and via NR-ARC-mediated NO_2^- reduction under physiological conditions (i.e. when NO_3^- is the dominant N source). By contrast, N_2O should be synthesised via both NR-ARC and COX-mediated NO_2^- reduction under NO_2^- stress (i.e. induced by high intracellular NO_2^-). High throughput RNA sequencing of *C. reinhardtii* 6145c samples showed that the genes encoding NR, ARC, NiR, NOR, COXs, AOXs, and THBs (proteins potentially involved during microalgal N_2O synthesis) were expressed in *C. reinhardtii* 6145c samples supplied with NO_2^- and incubated in darkness. While NO_2^- response via transcriptional regulations of NR, ARC, NiR, COX and NOR may not have been required in the conditions tested, AOX1-2 and THB1-2 were significantly differentially expressed between control and NO_2^--supplied tests. Overall, the RNA sequencing data were consistent with the biochemical evidence that under high NO_2^- loading:

1. NO is generated during N_2O synthesis because the gene THB1, which is known to be up-regulated in the presence of NO, was up-regulated in the tests supplied with NO_2^-.
2. NO_2^- reduction into NO leading to 'late' N_2O synthesis is not mediated by NR, as NO_2^- supply did not trigger any significant transcriptional regulation of NR gene in the tests.
3. NO_2^- stress was evidenced by the significantly different up-regulation of initiation factors genes in the tests (with NO_2^-) versus controls (no NO_2^-).

In light of the findings obtained from the laboratory *in vitro* batch assays performed, we concluded that N_2O synthesis may represent a means of regulating NO (and NO_2^-)

level in microalgal cells where NOR acts as a security valve to get rid of excess NO (or NO_2^-). The evidence of the microalgal N_2O pathway presented in this thesis has major biological and environmental implications.

- Biological implications: given the importance of nitrate assimilation and NO metabolism in microalgae, the microalgal N_2O pathway provides new advances in microalgal biology.
- Environmental implications: given that nitrate assimilation is highly conserved in some microalgae, the ability to synthesise N_2O could be widely spread among algal species. Knowing the ubiquity of microalgae in the environment, the aerobic microalgal N_2O pathway could be a globally significant N_2O source.

To the best of our knowledge, the potential environmental significance of N_2O emissions from microalgal cultivation was unknown and N_2O emissions during microalgal cultivation had never been thoroughly assessed. We thus presented the first long-term N_2O monitoring in outdoors microalgal engineered systems. N_2O emissions were quantified during the outdoors cultivation of commercially-relevant microalgae species in 50 L pilot scale column photobioreactors and during secondary wastewater treatment in a 1000 L high rate algal pond.

- From the N_2O monitoring performed during commercially-relevant microalgae species cultivation, we showed that: significant and variable N_2O emissions were recorded from *C. vulgaris* and *Neochloris sp.* cultures fed NO_3^- (9.60 – 38000 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$ n = 332, and 50 – 14200 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$ n = 136, respectively). While further monitoring of *C. vulgaris* cultures indicated that N_2O emissions were correlated with biomass concentration ($R^2 = 0.77$) and with

light intensity (R^2 = 0.57), it was still difficult to explain the variability recorded. Nevertheless, our findings appeared to confirm the hypothesis that when NO_3^- is the main N source, N_2O emissions occur during illumination. Also, a NO_2^- supply in *C. vulgaris* cultures fed NO_3^- triggered N_2O emissions, supporting the relation between nitrate assimilation, NO_2^-, and N_2O synthesis. Based on the conservative N_2O emissions recorded (1500 – 8000 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$, representing 25 – 75% of the data), we estimated that *C. vulgaris* as feedstock for replacing 30% of all US transport fuels could generate N_2O emissions representing up to 10% of the global anthropogenic N_2O emissions currently budgeted by the IPCC. Because no significant emissions were recorded from *A. platensis* cultures fed NO_3^- or *C. vulgaris* cultures fed NH_4^+ we were able to propose the use of NH_4^+ or species management as potential mitigation solutions.

- From the N_2O monitoring performed during wastewater treatment in a HRAP, we showed that significant and variable N_2O emissions of 5.1 to 19000 µg $N\text{-}N_2O \cdot m^{-2} \cdot d^{-1}$ (equivalent to 8 – 28000 nmol $N_2O \cdot m^{-2} \cdot h^{-1}$ n= 50) were recorded. Because N_2O can be produced by various microorganisms in HRAP fed wastewater, it was difficult to explain the actual source and the variability in the N_2O emissions recorded. Nevertheless, based on the conservative N_2O emissions rates measured (450 – 4600 µg $N\text{-}N_2O \cdot m^{-2} \cdot d^{-1}$, representing 25 – 75% of the data), we estimated that microalgae-based wastewater treatment ponds would generate minor global N_2O emissions representing less than 2% of the global N_2O emissions from wastewater treatment currently budgeted by the IPCC.

Based on the N_2O productions recorded we also estimated the first emission factors of 0.1 – 0.4% of the N load for microalgal cultivation fed NO_3^--laden synthetic media and 0.04 – 0.45% of the N load for microalgal cultivation fed wastewater. It is important to note that while the accuracy of the EFs estimated is still uncertain due to the variability in the N_2O emissions recorded, emphasis should be given on the methodology used as it provides good insight for future assessments. Similarly, while the other estimates generated within this thesis (i.e. case scenarios) must be interpreted with caution due to the variability observed, they support a strong case for improving our understanding of microalgal N_2O synthesis and considering microalgal N_2O emissions.

Overall, from the work performed within this thesis we clearly showed that 1) microalgae have the ability to synthesise N_2O and 2) N_2O can be generated during microalgal cultivation. The variability of N_2O emissions and the poor correlations between operational parameters generated uncertainty in our estimations. Nevertheless, we advised that microalgal N_2O emissions should be monitored and accounted for in greenhouse gas inventories and life cycle analysis of microalgal cultivation. Microalgae biotechnology is still at its infancy so there is an opportunity to understand and anticipate the 'N_2O emissions issue' before it becomes globally significant. Further monitoring from full scale microalgal cultivation should be performed and the influence of variables such as cellular growth kinetic should be investigated as it could explain some of the N_2O emissions variability. In addition, because our research did not include the evaluation of potential N_2O emissions from aquatic ecosystems, emphasis should be given to the study of these ecosystems with particular focus on eutrophic lakes, known to be affected by periods of algae blooms.

4.2 Future prospects

Concerning the N_2O pathway, quantitative PCR (qPCR) and proteomics analyses would be required to confirm the RNA-seq data. New series of laboratory batch experiments with *C. reinhardtii* and its associated NR, NiR, NOR (and COX) mutants combined with qPCR and proteomics analyses could also be performed to fully understand the influence of growth conditions on the N_2O biochemical pathway. For instance, *C. reinhardtii* could be incubated in darkness or under illumination, and with or without NO_2^- at various concentrations (10, 50, 100, 500 µM and 1, 2, 5, and 10 mM). Then, qPCR and proteomics analyses targeting the key genes and the related enzymes NR, ARC, NiR, NOR, COXs, AOXs, and THBs, would help to determine the differing N_2O synthesis according to the growth conditions. The results would be valuable for understanding how microalgal cells modulate NO_2^-, NO, and N_2O under physiological or stress conditions.

The work performed during this thesis mainly focused on 2 microalgae species. *C. reinhardtii* was chosen because it is a model in phycology and *C. vulgaris* because it is a species widely used for its commercial potential. Interestingly, both species had the ability to synthesise N_2O and are member of the Chlorophyta Division (green algae). Thus, it would be interesting to perform transcriptomic analyses (i.e. RNA-seq and qPCR on targeted genes) on samples of *C. vulgaris* but also on microalgae species from another family (e.g. The diatom *T. weissflogii*), and on another phototrophs (e.g. The cyanobacteria *Aphanocapsa*), to determine if the N_2O pathway (i.e. key enzymes, intermediates, functions, and regulations) is similar among phototrophs. There may be a strong case to focus on *Bacillariophyceae* (i.e. Diatoms) and cyanobacteria because

species from both families have already been shown to synthesise N_2O (Chapter 1). Also, these organisms are unique and have the reputation to spread through massive blooms which are known to trigger major environmental issues (e.g. hypoxic ocean; harmful algae bloom).

Understanding the N cycle has been the aim of many scientists in the past century. Considering that microalgae were 'an un-recognized N_2O source[18]' and that the N_2O pathway is probably widely spread among microalgae species, a search for homologs of the genes involved in microalgal N_2O synthesis in current databases such as National Center for Biotechnology Information (NCBI) and then selection of commercial microalgae species and environmental samples (e.g. eutrophic ecosystems) could be performed. The data would aid in evaluating how common is the microalgal N_2O pathway in the environment, and if this pathway is conserved in microalgae. Additionally, in order to investigate the microbial N_2O dynamics in microalgae-based systems metagenomics analysis complemented with metatranscriptomics could be implemented on similar samples.

From the work performed during this thesis, it is difficult to explain the variability in N_2O emissions recorded during pilot scale cultivation. To better evaluate the impact of operational parameters on N_2O emissions, controlled experiments with the commercially relevant microalgae *C. vulgaris* could be performed. Considering the complexity of the N_2O pathway and the high number of variables that can potentially

[18] Based on the current knowledge of the N cycle, various studies (and personal communication) that focused on the N cycle and N_2O synthesis, have noted some counter-intuitive pattern (e.g. unexplained N losses; N_2O synthesis from oxic waters).

influence N_2O synthesis, factorial analysis in bench scale 2 L reactors (operated indoor under fully controlled conditions) could help identifying environmental and cultivation (operation and design) parameters triggering or influencing N_2O emissions. Cellular age, cellular viability, mixing, light irradiation, temperature and nutrient supply could be tested as main parameters as they are particularly relevant to microalgal activity and microalgal N_2O synthesis (see Guieysse et al., 2013). The data thus gained could then be used to construct a model than can predict N_2O emissions as a function of relevant operational parameters.

As mentioned in Chapter 3 (Section 3.4), monitoring N_2O emissions from eutrophic ecosystems (e.g. eutrophic lakes) and commercial microalgal cultivation systems (e.g. full scale *Dunaliella salina* cultivation) would be valuable. For this purpose, total biomass concentration, cell number, cell viability, optical density, gas composition (i.e. CO_2, O_2, N_2), pH, dissolved oxygen, and key nutrients concentrations should be regularly monitored to better explain the variability of the N_2O measurements. The findings would also be essential to refine and define new N_2O emissions factors from microalgae-based ecosystems and engineered systems.

Appendices

Appendix A. Description of the cultivation medium used during this PhD

A.1 TAP (Tris-Acetate-Phosphate)

Chemical	Concentration $g \cdot L^{-1}$
Trizma base	2.42
K_2HPO_4	0.108
KH_2PO_4	0.056
$NaNO_3$	0.63
or	
NH_4Cl	0.40
$MgSO_4.7H_2O$	0.10
$CaCl_2.2H_2O$	0.05
Glacial Acetic Acid (Adjust pH to 7.0)	1 mL
Hutner Trace element	**Concentration $mg \cdot L^{-1}$**
$Na_2EDTA, 2H_2O$	50.0
H_3BO_3	11.4
$MnCl_2 \cdot 4H_2O$	5.10
$ZnSO_4, 7H_2O$	22.0
$CuSO_4, 5H_2O$	1.57
$Na_2MoO_4 \cdot 2H_2O$	1.01
$CoCl_2 \cdot 6H_2O$	1.61
$FeSO_4. 7H_2O$	4.99

A.2 Minimal Medium

Chemical	Concentration $g \cdot L^{-1}$
K_2HPO_4	1.08
KH_2PO_4	0.56
$NaNO_3$	0.63
or	
NH_4Cl	0.4
$MgSO_4.7H_2O$	1.0
$CaCl_2.2H_2O$	0.5
Hutner Trace element	**Concentration $mg \cdot L^{-1}$**
$Na_2EDTA, 2H_2O$	50.0
H_3BO_3	11.4
$MnCl_2 \cdot 4H_2O$	5.10
$ZnSO_4, 7H_2O$	22.0
$CuSO_4, 5H_2O$	1.57
$Na_2MoO_4 \cdot 2H_2O$	1.01
$CoCl_2 \cdot 6H_2O$	1.61
$FeSO_4. 7H_2O$	4.99

A.3 BG 11

Chemical	Concentration $g \cdot L^{-1}$
$NaNO_3$	1.5
K_2HPO_4	3.1
KH_2PO_4	1.52
$MgSO_4, 7H_2O$	0.075
$CaCl_2, 2H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
$Na_2EDTA, 2H_2O$	0.00098
Trace element	**Concentration $mg \cdot L^{-1}$**
H_3BO_3	2.86
$MnCl_2, 4H_2O$	1.81
$ZnSO_4, 7H_2O$	0.222
$CuSO_4, 5H_2O$	0.079
$Na_2MoO_4, 2H_2O$	0.39
$CoCl_2, 6H_2O$	0.0404

A.4 Zarrouk medium

Chemical	**Concentration g·L^{-1}**
$NaNO_3$	2.5
K_2HPO_4	0.5
KH_2PO_4	1
NaCl	1
$MgSO_4$, $7H_2O$	0,2
$CaCl_2$, $2H_2O$	0.04
$FeSO_4$, $7H_2O$	0.01
Na_2EDTA, $2H_2O$	0.08
Trace element	**Concentration mg·L^{-1}**
H_3BO_3	2.86
$MnCl_2$, $4H_2O$	1.81
$ZnSO_4$, $7H_2O$	0.222
$CuSO_4$, $5H_2O$	0.079
Na_2MoO_4, $2H_2O$	0.0177

Appendix B. *Chlamydomonas reinhardtii* NOR ami-strains construction

The NOR amiRNA was designed by using the online tool WMD3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Home;project=stdwmd). The selected gene target sequence for the NOR amiRNA was 'TGTACTTGACGAGTTCGTCTC'. Specific primers obtained with WMD3 for the amiRNA generation were:

amiFor_CYP55 5'-CTAGTGAGACGAACTCGTCAACTACATCTCGCTGATCGGCACCATGGGGGTGGTGGTGATCAGCGCTATGTACTTGACGAGTTCGTCTCG-3'

amiRev_CYP55 5'-CTAGCGAGACGAACTCGTCAAGTACATAGCGCTGATCACCACCACCCCCATGGTGCCGATCAGCGAGATGTAGTTGACGAGTTCGTCTCA-3'.

These primers were annealed, phosphorylated and cloned following the protocol described by Molnar et al, (2009). pChlamiRNA3, digested with SpeI, was the vector used for cloning the fragment generated with the primers. pChlamiRNA3-amiNOR was sequenced with the primer AmiRNAprecfor (5'-GGTGTTGGGTCGGTGTTTTTG-3'). A clone without mutations was used for transformation of wild-type strain. 19 transformants were selected and NOR expression was measured by qRT-PCR. Primers used for NOR expression in knock down strains were:

NCYP55-RTrev CGTGGGGTTCCTCTCCTTCTCG and,

CYP55-RTfw CCGCTGCCATTCCGGGTCATC.

Reference

Molnar, A., Bassett, A., Thuenemann, E., Schwach, F., Karkare, S., Ossowski, S., Weigel, D., and Baulcombe, D. (2009). Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. Plant J. **58**: 165–174.

Appendix C. Nitrite toxicity assays

To evaluate the effect of NO_2^- on *Chlamydomonas reinhardtii* growth kinetics a mother culture of *C. reinhardtii* 6145c was axenically grown 5.5 days on TAP medium (NO_3^- as N-source). 2.5 mL of the mother culture was then used to inoculate 4 sets of duplicates flasks. Duplicates 250 mL E-flasks were filled with either 122.5 mL of mL TAP medium with NO_3^- as N-source at usual concentration (7 mM control), or 120 mL TAP medium where NO_3^- was replaced by NO_2^- as N-source (at concentration of 3, 6 and 12 mM). Following inoculation, OD at 683 nm was measured daily. Initial and final DCW were also measured.

As can be seen in Figure C.1 presenting the OD evolution over the 4 days of cultivation *C. reinhardtii* growth kinetic was not affected by NO_2^- at all the concentrations tested (3 – 12 mM). This was confirmed when comparing the initial and final DCW for each culture (Figure C.2).

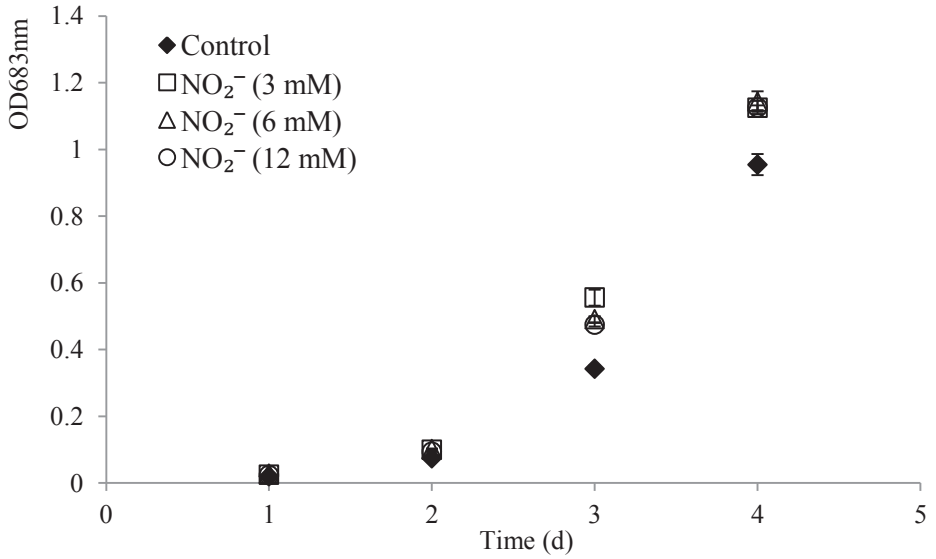


Figure C.1: Optical density (683 nm) of duplicates cultures of *C. reinhardtii* 6145c cultivated with NO_3^- as N-source (7 mM) or NO_2^- as N-source (at 3, 6 and 12 mM).

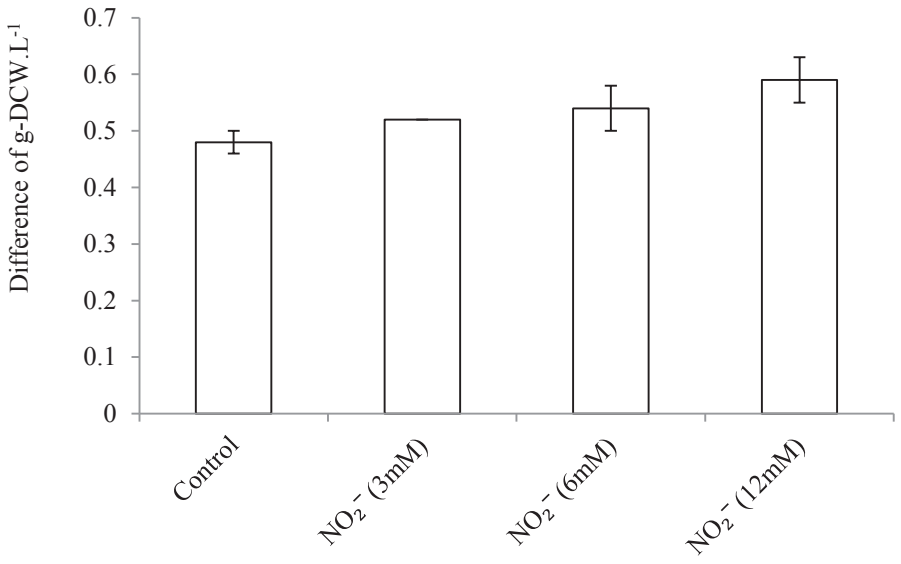


Figure C.2: Difference between the initial (day 1) and final (day 4) DCW (g-DCW·L^{-1}) for the duplicates cultures of *C. reinhardtii* 6145c cultivated with NO_3^- as N-source (7 mM) or NO_2^- as N-source (at 3, 6 and 12 mM).

The results showed that the NO_2^- concentrations tested (3 – 12 mM) were not affecting *C. reinhardtii* 6145c growth kinetics. From the results the occurrence of intracellular stress involving various responses (e.g. NO) cannot however be dismissed.

Appendix D. Polymerase Chain Reaction analysis from *Chlamydomonas reinhardtii* samples

Chlamydomonas reinhardtii was axenically grown 5 days on TAP medium (NO_3^- as N-source) and re-suspended in N-free TAP to perform batch N_2O emission assays as described in Chapter 2 Section 2.2.2 (gas samples were withdrawn 5 h after NO_2^- supply). To provide DNA for PCR analysis, 3 culture samples (2 mL) were aseptically withdrawn in DNA-free Eppendorf tube approximately 1 hr after NO_2^- was supplied to the serum flasks: 1) a sample from the 'normal' culture (TAP with NO_3^-) used to inoculate the batch assay; 2) a sample from the serum flasks used for quantifying N_2O production under NO_2^- supply; and 3) a sample from an additional 'normal' 5 days old culture supplied antibiotics (25 mg streptomycin$\cdot L^{-1}$ and 100 mg penicillin$\cdot L^{-1}$; (Guieysse et al., 2013) to serve as positive control of bacterial-free culture. These samples were stored at -40^{o}C and later thawed prior to DNA extraction. For this purpose, the samples were spun at 13,000 g for 5 min (Eppendorf microcentrifuge) to pellet the algae, the supernatant was discarded, and DNA was extracted using the Bioline isolate Genomic DNA kit according to the manufacturer's protocol (Bioline, UK). After extraction, DNA extracts were fluorometrically quantified using a ND-1000 NanoDrop sampler (Thermo scientific, USA). PCR was carried out on the extracted DNA using the primers and conditions described in Table D.1. Each PCR reaction contained 1× Buffer with 1.5 mM $MgCl_2$ (Roche Diagnostics), 250 µM each dNTP, 10 pmol of each primer, 2 µl of template DNA and 1U Taq polymerase (Roche Diagnostics, Switzerland), in a final volume of 20 µl. Following PCR, 8 µl of reaction mix was analysed on an agarose gel (2% (w/v) agarose in 1× Tris-acetate–EDTA

buffer) and visualized using SYBR-SAFE (Invitrogen) on a gel documentation system (Bio-Rad, USA).

Table D.1: Primers and conditions used during PCR

Gene	Primers	Sequence (5' – 3')	Exp. size (bp)	PCR conditions
Bacteria				
norB	norB1F	CGNGARTTYCTSGARCARCC	670	95°C - 5 min [95°C - 30 s, 54°C - 45 s, 68°C – 45 s] × 35 cycles 68°C -7 min, 10°C hold. (Fagerstone et al., 2011)
	norB8R	CRTADGCVCCRWAGAAVGC		
cnorB	cnorBF	GACAAGNNNTACTGGTGGT	389	
	cnorBR	GAANCCCCANACNCCNGC		
qnorB	qnorBF	GGNCAYCARGGNTAYGA	262	
	qnorBR	ACCCANAGRTGNACNACCCACCA		
16S	F27	AGAGTTTGATCCTGGCTCAG	1000-1500	94 ºC – 3 min [94ºC - 30 s, 50ºC - 30 s, 72ºC – 90 s] × 35 cycles, 72ºC – 5 min, 10 ºC hold (Giovannoni, 1991)
	1492R	TACGGYTACCTTGTTACGACGAC		
Archaea				
amoA	amoAF	STAATGGTCTGGCTTAGACG	635	95°C - 5 min [94°C - 45 s, 53°C - 60 s, 72°C – 60 s] × 30 cycles 72°C -15 min, 10°C hold. (Francis et al., 2005)
	amoAR	GCGGCCATCCATCTGTATGT		
Algae				
Rbcl	RbclAF	ATGTCACCACAAACACAGAGACTAAAGC	1200	94°C - 3 min [94 °C – 30 s, 53°C – 30 s, 72°C – 90 sec] × 35 cycles 72°C – 5 min, 10°C hold (Hasebe et al., 1994).
	RbclAR	GCAGCAGCTAGTTCCGGGCTCCA		

The norB, cnorB and qnorB primer pairs amplify fragments of genes encoding for bacterial NOR (NO-reductase). The amoA F and R pairs amplify a fragment for ammonia monooxygenase in archaea. The 16S primer pairs F27/1492R amplify a region of the 16S rDNA in bacteria. The 16S primers are considered a 'universal' primer pairs for bacteria and archaea. A negative control was included for each primer pairs; this reaction contains all components except the template DNA.

Results from PCR analysis are shown in Table D.2. A PCR product of the expected size was seen for the Rbcl primer pair in all samples except the negative control (Figure D.1). There was however no apparent trace of bacterial and archaeal PCR products in the samples. N_2O production as also confirmed in the serum flasks supplied NO_2^- (2100 ± 20 nmol·g-DCW^{-1} after 5 h incubation).

Table D.2: Results from genomic analysis (Y indicates there was a band of the expected size; N means no band of the expected size). 1: sample from the 'normal' culture (TAP with NO_3^-) used to inoculate the batch assay; 2: sample from the serum flasks used for quantifying N_2O production under NO_2^- supply; and 3: sample from an additional 'normal' 5 days old culture supplied antibiotics.

	16S (*bacteria*)	norB	cnorB	qnorB	amoA (*archaea*)	Rbcl (*algal*)
Negative	N	N	N	N	N	N
1	N	N	N	N	N	Y*
2	N	N	N	N*	N	Y*
3	N	N*	N	N*	N	Y*

* an asterisk indicates the presence of other bands which are PCR artifacts or primer dimers.

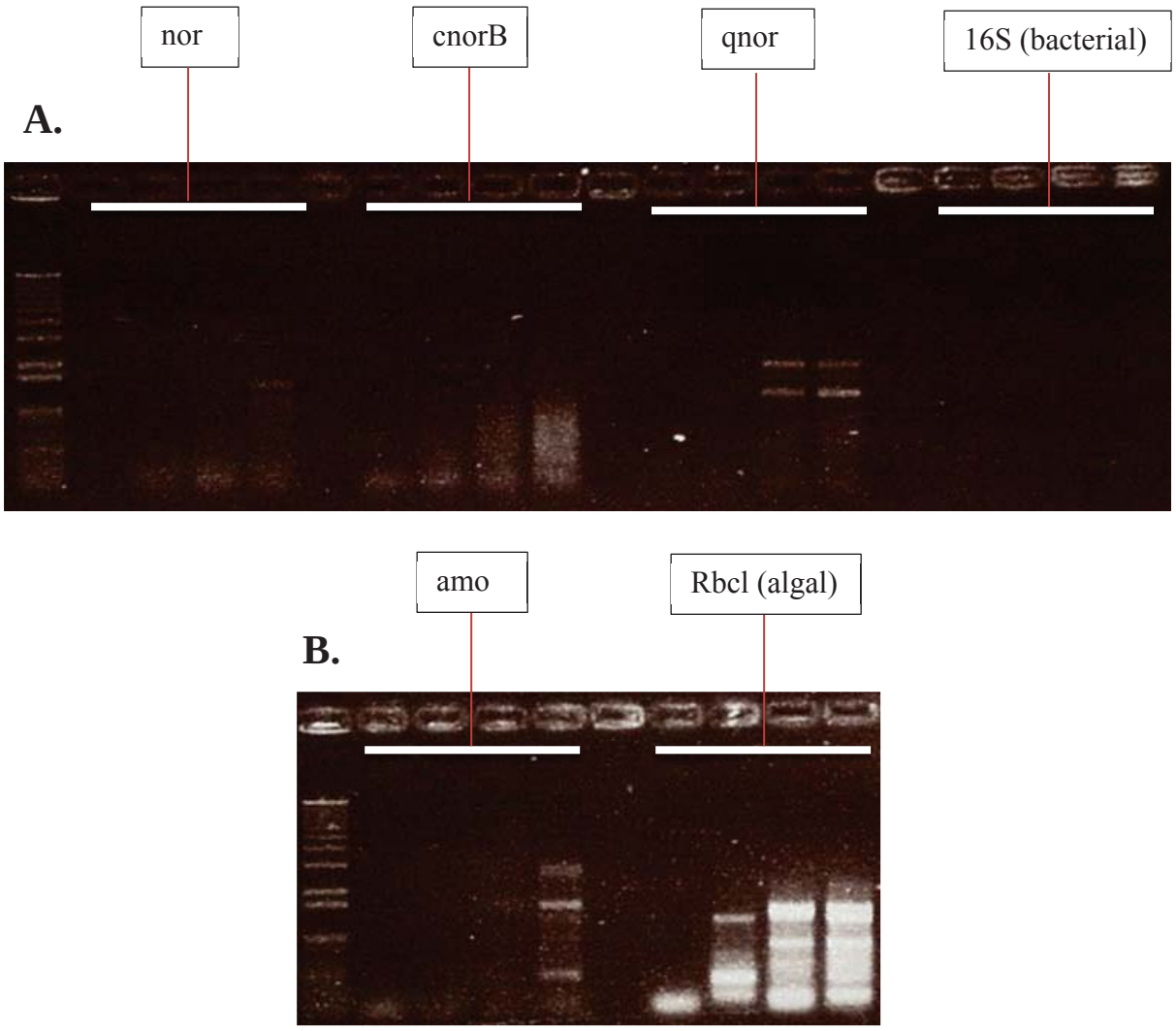


Figure D.1: A. 1% (w/v agarose) gel of 4 primers (norB, cnorB, qnorB, 16S bacterial) amplified by PCR from 4 different samples. B. 1% (w/v agarose) gel of 2 primers (amoA, Rbcl algal) amplified by PCR from 4 different samples. For each pairs of primers left to right: 1, negative control; 2, sample from the 'normal' culture (TAP with NO_3^-) used to inoculate the batch assay; 3, sample from the serum flasks used for quantifying N_2O production under NO_2^- supply; and 4, sample from an additional 'normal' 5 days old culture supplied antibiotics.

References

Fagerstone, K.D., Quinn, J.C., Bradley, T.H., De Long, S.K., and Marchese, A.J. (2011). Quantitative measurement of direct nitrous oxide emissions from microalgae cultivation. Enviro. Sci. Technol. **45**: 9449–9456.

Francis, C. A.; Roberts, K. J.; Beman, J. M.; Santoro, A. E. Oakley, B. B. (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proc. Natl. Acad . Sci. USA. **41**:14683–14688.

Giovannoni, S. J. (1991). Nucleic acid techniques in bacterial systematics, in The Polymerase Chain Reaction: Stackerbrandt, E., Goodfellow, M. (eds), John Wiley and Sons. Chichester, pp 177-203.

Guieysse, B., Plouviez, M., Coilhac, M., and Cazali, L. (2013). Nitrous Oxide (N_2O) production in axenic *Chlorella vulgaris* microalgae cultures: Evidence, putative pathways, and potential environmental impacts. Biogeosciences **10**: 6737–6746.

Hasebe, M.; Omori, T.; Nakazawa, M.; Sano ,T.; Kato, M.; Iwatzuki, K. (1994) rbcL gene sequences provide evidence for the evolutionary lineages of Leptosporangiate ferns. Proc. Natl. Acad . Sci. USA. **91**: 5730 –5734.

Appendix E. RNA sequencing: Optimisation, results summary and supplementary results

E.1 Optimisation and quality controls

The experimental protocol described below was repeated three times before performing the actual RNA sequencing. To prevent any potential genetic drift due to centrifugation and/or re-suspension of the microalgal pellet in N-free medium, 5.5 days old microalgal cultures were directly poured from E-flasks to 6 serum flasks (Batch assays). The cells were then acclimated for 1 h in darkness (under constant temperature and agitation at 25°C and 180 rpm, respectively). Three serum flasks, henceforth referred to as treatment flasks, were randomly picked and supplied with 10 mM NO_2^- (t0), whereas the three other flasks were supplied with autoclaved distilled water to reach the same final volume. Gas and liquid samples were withdrawn from each flask after 1.25 (t1: 15 min after NO_2^- supply), 3 (t2: 3 h after NO_2^- supply) and 24 hours (t3: 24 h after NO_2^- supply) to quantify N_2O and extract RNA, respectively. The initial and final pH, dried cell weight concentrations (DCW), optical densities (OD) and NO_3^-/NO_2^- concentrations were also measured (Béchet et al., 2015; Alcántara et al., 2015). The N_2O gaseous concentrations recorded at each time point between triplicates over the three repeated experiments were normally distributed and the relative standard error of the mean never exceeded 14% (for each time point n = 9, p = 0.05).

E.2 Results summary

Table E.1 and Figure E.1 summarise data from key parameters monitored during the RNA sequencing experiment. Over 24 h of incubation, DCW, OD and pH decreased slightly in both control and treatment groups, but only treatments samples supplied with NO_2^- were producing N_2O.

Table E.1: Initial and final pH, DCW, optical density (OD) and NO_3^-/NO_2^- concentrations from the chosen RNA sequencing experiment. Data shows average ± (max-min)/2 for the triplicate used for RNA extraction (n = 3).

Parameter	Initial	Final	
		Control	Treatment
DCW (g-DCW·L^{-1})	0.47 ± 0.03[a]	0.45 ± 0.02	0.45 ± 0.01
OD	1.03 ± 0.06	0.967 ± 0.01	0.917 ± 0.01
pH [a]	7.62	7.46	7.51
NO_3^- (mM) [a]	5.25 ± 0.20	5.14 ± 0.20	4.82 ± 0.53
NO_2^- (mM) [a]	<LOD*	0.08 ± 0.04	9.8 ± 0.18
N_2O produced (nmole)	1.90 ± 0.08	1.76 ± 0.20 [a]	29.1± 0.92 [a]

[a] As duplicates or triplicates were performed, data represent average ± (max-min)/2.
* Limit of detection (0.05 mg L^{-1}).

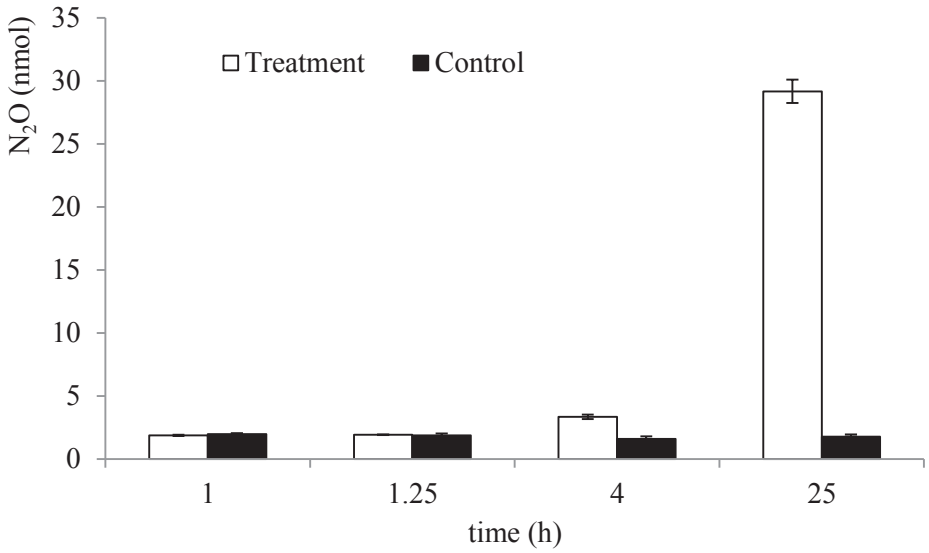


Figure E.1: N_2O (average nmole ± SE) recorded from *C. reinhardtii* treatment (Supplied with 10 mM NO_2^-) and control batch assays from the RNA sequencing experiment.

RNA was extracted from the samples and analyzed using an Agilent bioanalyzer (Agilent, USA). The amount and quality of the RNA recovered was externallty assessed and found satisfactory for RNA sequencing (NZGL ltd, New Zealand). Following RNA sequencing and analysis, it became clear that NO_2^- supply in darkness had an effect on *C. reinhardtii* as shown in the PCA plot (Figure E.2). The plot represents the 'genetic variances' between triplicates and sample type. While for time 1 and 2 control and treamtment 'behave' similarly at time 3 control and treatment diverged considerably showing an effect of NO_2^- supply.

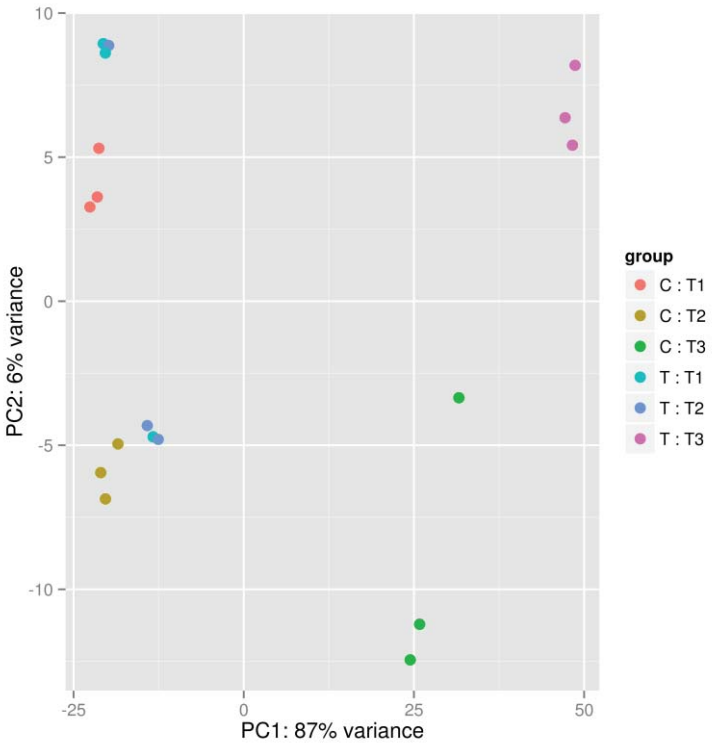


Figure E.2: PCA plot of the variance for control and treatment from the RNA sequencing data.

E.3 RNA-seq data evidence a stress response in NO_2^- supplied samples

Table E.2 shown the differences in the RNA-seq data between controls and treatments for several eukaryotic initiation factors after 24 hours of incubation. A very strong down regulation (on average >5 Log 2 fold change) of eukaryotic initiation factors is generally indicative of a cellular response to stress (Langland et al., 1996; Hinnebusch, 1997; Roy et al., 2010; Pakos-zebrucka et al., 2016).

Table E.2: RNA-seq based differential expression data between T3 control and T3 treatment *C. reinhardtii* for the 19 annotated Eukaryotic initiation factors (EIF) genes. Log2FC values in bold are significant based on a Benjamin-Hochberg adjusted *p-value* < 0.05.

gene name	gene symbol	log2 Fold Change	p-value
Cre03.g199900	EIF4E	**-1.70**	$1.91 \cdot 10^{-09}$
Cre05.g242300	EIF3D	**-1.61**	$8.49 \cdot 10^{-09}$
Cre02.g098450	EIF2G	**-1.56**	$2.45 \cdot 10^{-08}$
Cre16.g676314	EIF3H	**-1.69**	$6.94 \cdot 10^{-08}$
Cre03.g194400	EIF3I	**-1.31**	$5.90 \cdot 10^{-07}$
Cre16.g654500	EIF3F	**-1.73**	$1.32 \cdot 10^{-06}$
Cre12.g529950	EIF4G	**-1.03**	$1.07 \cdot 10^{-05}$
Cre06.g269450	EIF3G	**-1.01**	$1.07 \cdot 10^{-04}$
Cre04.g217550	EIF3C	**-1.23**	0.00207
Cre13.g564250	EIF3A	**-0.77**	0.0110
Cre12.g531550	EIF2B	**-0.640**	0.0117
Cre12.g490000	EIF2A-2	**-1.02**	0.0289
Cre12.g515650	EIF3K	-0.887	0.0561
Cre12.g498100	EIF3E	-0.653	0.0587
Cre03.g190100	EIF3B	-0.860	0.0701
Cre08.g375900	EIF2A-1	-0.625	0.0943
Cre06.g251600	EIF5	0.226	0.487
Cre13.g585150	EIF6	0.104	0.679
Cre03.g199647	EIF4A	0.072	0.790

E.4 Supplementary results (RNA-seq data of associated genes to nitrate assimilation)

The first approach with the RNAseq data was to target genes thought to be potentially involved during N_2O synthesis based on the six putative pathways described in Chapter 1. The Log2 fold changes of associated genes (*i.e.* regulation) related to the genes potentially involved in N_2O synthesis were also targeted. As showed in Table E.3 only NIT2, the gene regulating nitrogen assimilation was differentially expressed at t3 with a Log2 fold change of -0.9. The expression change of NIT2 (*i.e.* down regulation) would confirmed the presence of NO as NIT2 has been suggested to be repressed by guanosine monophosphate (GMP) when GMP is activated in response to NO (de Montaigu et al., 2010).

Table E.3: Log2 fold change (Log2FC) for genes related to nitrate assimilation and cytochrome c oxidase between control (C) and treatment (T) samples (nrc = normalised read counts). Positive Log2FC describes an up-regulation (*i.e.* increase in gene expression) and negative Log2FC describes a down-regulation (*i.e.* decrease in gene expression).

Genes	**annotation**	**Time (h) after exposure to NO_2^-**		
		0.25	**3**	**24**
NIT2	Nitrogen assimilation regulation	0.01 (7)	0.12 (8)	**-0.9 (4)**
NAR1.2	Nitrite/carbonate chloroplast transporter	0.4 (0.4)	0.05 (0.2)	-0.4 (0.1)
NAR1.3	Formate/nitrite transporter (intracellular)	0.2 (4)	0.3 (3)	-0.2 (2)
NAR1.4	Nitrite transporter (Intracellular)	-0.08 (11)	0.18 (12)	0.2 (19)
NAR1.5	Nitrite transporter (intracellular)	-0.04 (1)	-0.1 (1)	-0.7 (0.4)
NAR1.6	Nitrite transporter (intracellular)	-0.09 (1)	-0.6 (1)	-0.5 (0.4)
NRT2.1	Nitrate transporter (membrane)	-0.4 (0.1)	-1.4 (0.04)	-2 (0.02)
NRT2.2	Nitrate specific transporter	-0.4 (1)	-1 (1)	0.8 (0.01)
NRT2.3	Nitrate/Nitrite bi-specific transporter (membrane)	0.6 (0.4)	0.3 (0.3)	0.8 (0.1)

Reference

Alcántara, C., Muñoz, R., Norvill, Z., Plouviez, M., and Guieysse, B. (2015). Nitrous oxide emissions from high rate algal ponds treating domestic wastewater. Bioresour. Technol. **177**: 110–117.

Béchet, Q., Chambonnière, P., Shilton, A., Guizard, G., and Guieysse, B. (2015). Algal productivity modeling: A step toward accurate assessments of full-scale algal cultivation. Biotechnol. Bioeng. **112**: 987–996.

Hinnebusch, A. (1997). Translational regulation of yeast GCN4. J. Biol. Chem. **272**:21661-21664.

Langland, J.O., Langland, L.A., Browning, K.S., and Roth, D.A. (1996). Phosphorylation of plant eukaryotic initiation factor-2 by the pPKR , and inhibition of protein synthesis *in vitro*. J. Biol. Chem. **271**: 4539–4544.

de Montaigu, A., Sanz-Luque, E., Galván, A., and Fernández, E. (2010). A soluble guanylate cyclase mediates negative signaling by ammonium on expression of nitrate reductase in *Chlamydomonas*. Plant Cell **22**: 1532–1548.

Pakos-zebrucka, K., Koryga, I., Mnich, K., Ljujic, M., Samali, A., and Gorman, A.M. (2016). The integrated stress response. EMBO reports. **17**: 1–22.

Roy, B., Vaughn, J.N., Kim, B.-H., Zhou, F., Gilchrist, M.A., and Von Arnim, A.G. (2010). The h subunit of eIF3 promotes reinitiation competence during translation of mRNAs harboring upstream open reading frames. RNA **16**: 748–761.

Appendix F. Gas measurement, pressure correction and GC calibration

F.1 Gas analysis and pressure correction

Assuming N_2O is an ideal gas transferring rapidly between the gaseous and liquid phases with respect to sampling frequency, the total amount of N_2O present (n^t, mole) in a sealed flask at the sampling time t was calculated as the sum of amounts of N_2O present in the gas (n_g^t, mole) and liquid phase (n_l^t, mole) at time t as:

$$n^t = \frac{x_{N2O}^t \cdot P^t \cdot V_g}{R \cdot T} + H_{N2O} \cdot x_{N2O}^t \cdot P^t \cdot V_l \qquad \text{Equation F.1}$$

Where x_{N2O}^t (mole N_2O/mole gas) is the molar fraction of N_2O in the flasks headspace at time t, P^t is the pressure in the gas headspace (atm) at time t, V_g and V_l are the volumes (L) of the gas and liquid phases, respectively (these volumes were assumed to be constant as not liquid sample was withdrawn), R is the ideal gas constant (0.082057 L atm K^{-1} mol^{-1}), T is the temperature in the flaks (298 K in all experiments), and H_{N2O} is the Henry law constant of N_2O at the experimental temperature (2.5×10^{-2} mol· N_2O $L^{-1}\cdot Atm_{N2O}^{-1}$; Sander, (1999); verified experimentally). Withdrawing gas samples with a syringe modified the pressure inside the flasks through gas phase volume expansion from V_g to $V_g + V_s$, where V_s is the sample volume (L). We assumed that this expansion was much faster than N_2O mass transfer so that x_{N2O}^t quantified at time t (via gas chromatography) was representative of the equilibrium achieved at P^t just immediately before the gas sample was withdrawn. The pressure change in the flasks was taken into

account when computing the amount of N_2O present in the flaks at the next sampling time (t+1) as:

$$n^{t+1} = \frac{x_{N2O}^{t+1} \cdot P^{t+1} \cdot V_g}{R \cdot T} + H_{N2O} \cdot x_{N2O}^{t+1} \cdot P^{t+1} \cdot V_l \qquad \text{Equation F.2}$$

Where P^{t+1} (atm) is calculated as:

$$P^{t+1} = P^t \cdot \left(\frac{V_g}{V_g + V_s}\right) \qquad \text{Equation F.3}$$

The total amount of N_2O produced at t +1 (n^{t+1}) was then calculated by accounting for the amount of N_2O withdrawn from the sample at time t as:

$$n^{t+1} = \frac{x_{N2O}^{t+1} \cdot P^{t+1} \cdot V_g}{R \cdot T} + H_{N2O} \cdot x_{N2O}^{t+1} \cdot P^{t+1} \cdot V_l + x_{N2O}^{t+1} \cdot P^t \cdot V_s \qquad \text{Equation F.4}$$

F.2 GC Calibration

GC calibration was performed in using a 100 ppm N_2O in N_2 standard. Serum flasks (122 ± 2 mL) were flushed with neutral gas (N_2) and a known volume of gas was successively withdrawn and replaced by the exact same volume of gas standard (Figure F.1). As described below, the pressure changes incurred when injecting calibration gas and withdrawing gas samples were accounted for (then changes were experimentally monitored in controls and found consistent with the theoretical calculations). An example of a calibration performed during this study is presented in Figure F.2.

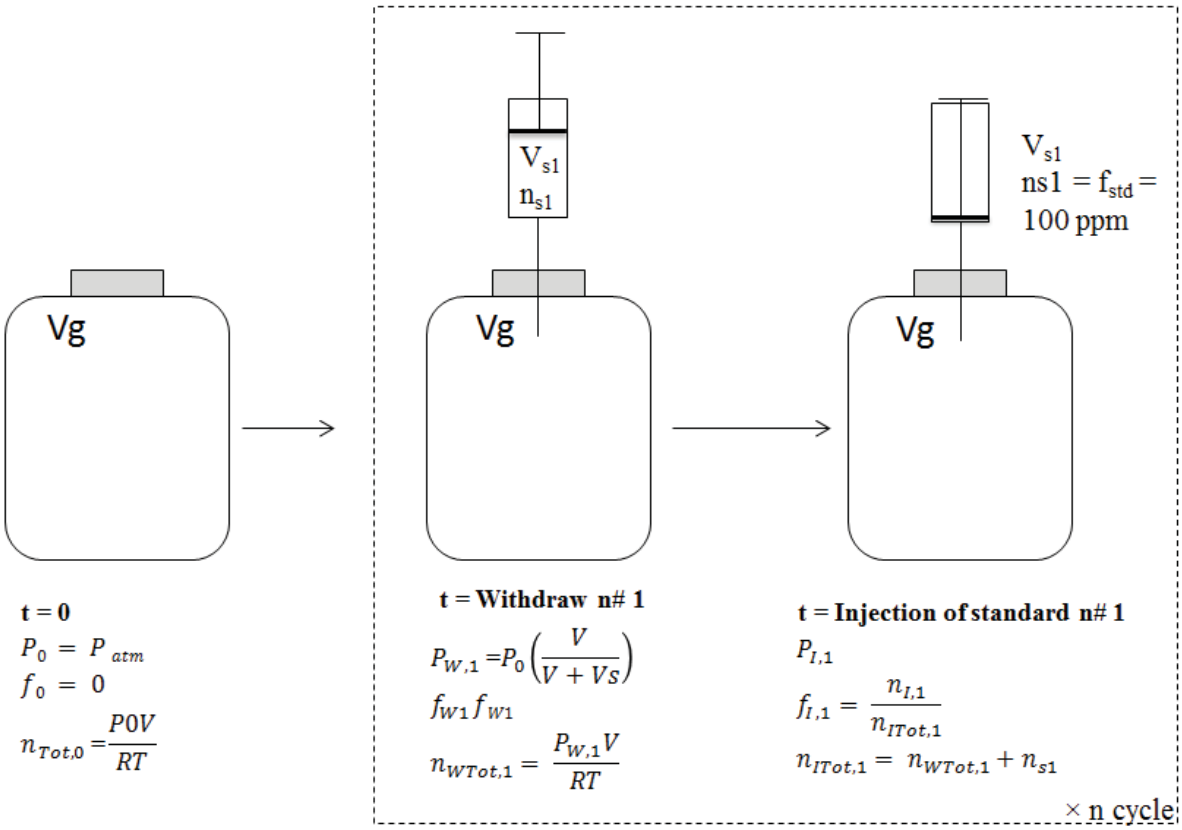


Figure F.1: System used to perform the GC calibration

P = Pressure in atm

$V_g = V$ = Gas volume in the flask (L)

V_s = Gas volume in the syringe (L)

T = Temperature in K

R = Gas constant (0.082057 L atm K^{-1} mol^{-1})

f_{N2O} = N_2O fraction (nmol·mol^{-1})

n = N_2O (mole)

C =N_2O concentration (mol·L^{-1})

n_{Tot} = Total mole of gas

S =Syringe

W = Time after withdraw

$e.g.\ n_{W,1}$ = N_2O (nmole) after withdraw

I = Time after injection

$e.g.\ f_{I,1}$ = N_2O fraction after injection (nmol·mol^{-1})

<u>At t = 1, after the first withdrawn and the first injection of standard:</u>

$$f_{I,1} = \frac{n_{I,1}}{n_{ITot,1}} = \frac{n_{I,1}}{n_{WTot,1} + n_{STot,1}}, \text{ Equation F.5}$$

We have also,

$$n_{I,1} = n_{W,1} + \left(C_{S,1}V_{S,1}\right) \text{ with, } C_{S,1}V_{S,1} = n_{S,1} = \frac{PatmVs}{RT} \times fs, \quad \text{Equation F.6}$$

With the pressure evolution Equation F.3 defined above $\left(P_n = P_{n-1} \times (\frac{Vg}{Vs+Vg})\right)$, $P_{atm} = P_0$, and Equation F.5 and 6 becomes,

$$f_{I,1} = \frac{n_{W,1} + \left(\frac{P_0 V_S}{RT}\right) \times fs}{\frac{P_0\left(\frac{V}{V+V_S}\right)V}{RT} + \frac{P_0 V_S}{RT}}, \text{ Equation F.7}$$

Considering that $n_{W,1}$ = N_2O (nmole) after first withdraw

$$f_{I,1} = f_S \times \frac{V_S}{\frac{V^2}{V + V_S} + V_S} = f_S \times \frac{V_S(V + V_S)}{V^2 + (V_S(V + V_S)}$$

At t = n, several cycle

$f_{I,n} = \frac{n_{I,n}}{n_{ITot,n}}$, Equation F.8

$P_{W,n} = P_{I,n-1} \times \left(\frac{V}{V+V_S}\right)$, Equation F.9

$n_{I,n} = n_{W,n} + \left(\frac{P_0 V_S}{RT}\right) \times f_S$, Equation F.10

$n_{W,n} = n_{I,n-1} - \left(\frac{P_{W,n} \times V_S}{RT}\right) \times f_{W,n}$, Equation F.11

$f_{W,n} = f_{I,n-1}$, Equation F.12

$n_{ITot,n} = n_{WTot,n} + \frac{P_0 V_S}{RT}$, Equation F.13

$n_{WTot,n} = n_{ITot,n-1} - \frac{P_{W,n} \times V_S}{RT}$, Equation F.14

With Equation F.9-14, Equation F.8 becomes,

$$f_{W,n+1} = f_{I,n} = \frac{n_{I,n-1} - \left(\frac{P_{W,n} \times V_S}{RT} \times f_{I,n-1}\right) + \left(\frac{P_0 V_S}{RT}\right) \times f_S}{n_{ITot,n-1} - \left(\frac{P_{W,n} \times V_S}{RT}\right) + \left(\frac{P_0 V_S}{RT}\right)}$$

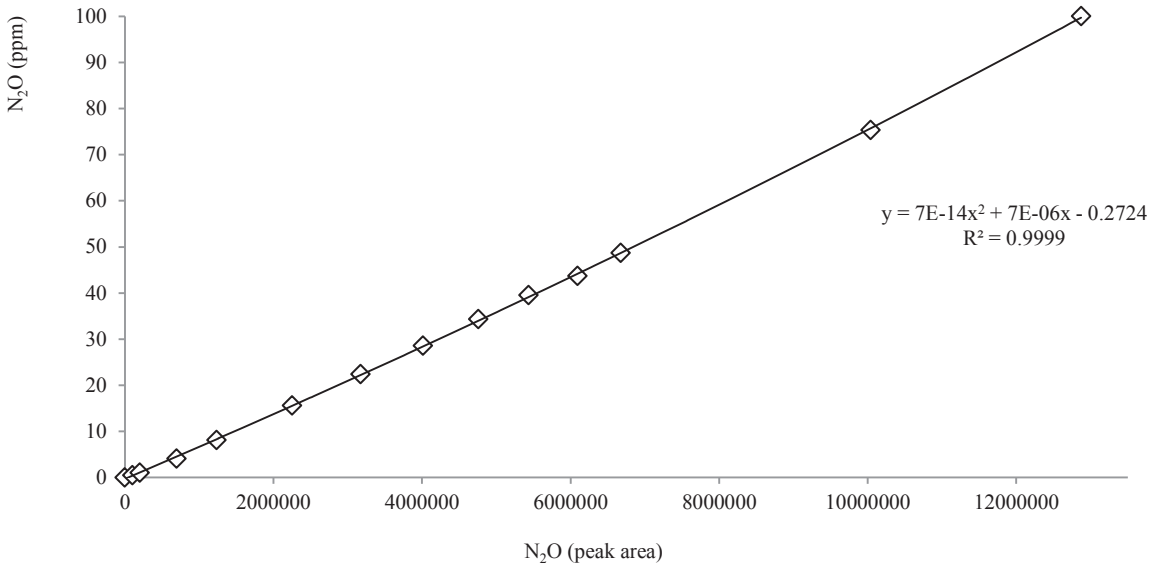


Figure F.2: Example of a calibration curve performed and used during this study.

Reference:

Sander, R. (1999). Compilation of Henry's Law constants for inorganic and organic Species of potential importance in environmental chemistry. http://www.mpch-mainz.mpg.de/~sander/res/henry.html

Appendix G. Data analysis of the positive and negative controls performed during batch assays

G.1 Positive controls

Throughout this study positive controls (cultures of wildtype 6145c supplied 10 mM NO_2^- in darkness) were repeated 40 times. The distribution of the results achieved is shown in Figure G.1. Standard deviations and 95% confidence intervals associated with this data are shown in Table G.1.

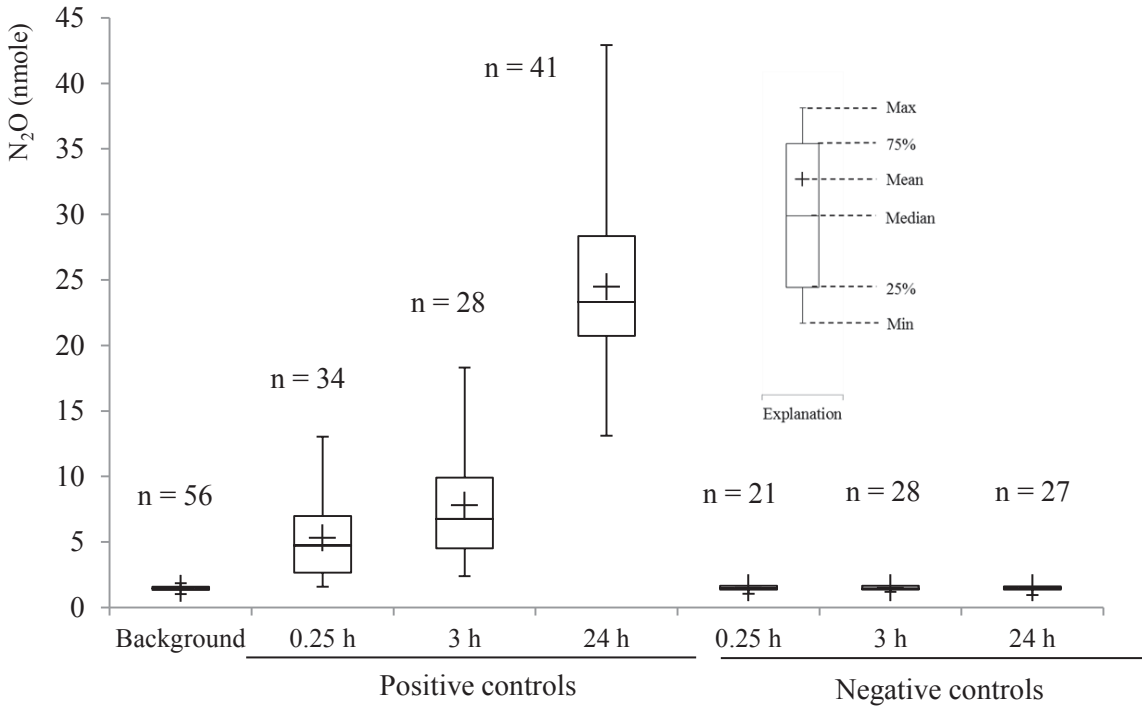


Figure G.1: Box plot of the N_2O production (nmole) recorded in positive and negative controls at various sampling times (0.25, 3 and 24 h).

Table G.1: Statistical analysis of the N_2O produced in positive controls.

	N_2O after 0.25 h (nmole)	N_2O after 3 h (nmole)	N_2O after 24 h (nmole)
n (sample size)	34	28	41
Minimum	1.58	2.39	13.1
Maximum	13.0	18.3	42.9
Mean	5.30	7.80	24.4
Median	4.74	6.76	23.3
Standard deviation	3.01	4.43	6.59
Relative standard error	57	57	27
95% confidence interval	1.01	1.64	2.02

Three gas samples were generally withdrawn (after 15 min, 3 h and 24 h of NO_2^- supply) from duplicates. N_2O was always significantly produced in the positive controls. The N_2O (nmole) recorded in the flasks between the three sampling time was always statistically different (two samples t-test at 95% level of confidence between sampling at 15 min and 3 h, $p < 0.05$; between sampling at 3 h and 24 h, $p < 0.05$; between sampling at 15 min and 24 h, $p < 0.05$) with increasing N_2O production as a function of time.

G.2 Negative controls

In order to dismiss possible abiotic N_2O production, negative controls (N-free medium supplied with 10 mM NO_2^-; or N-free medium supplied with chemical effectors and 10 mM NO_2^-; or autoclaved microalgal suspension supplied with 10 mM NO_2^-) were incubated in similar conditions than the other samples within the same experiment. Gas samples were withdrawn regularly (after 15 min, 3 h and 24 h of NO_2^- supply). Figure G.2 presents an example of the amount of N_2O recorded in duplicates flasks of negative

controls (N-free medium supplied with 10 mM NO_2^-; and autoclaved microalgal suspension supplied with 10 mM NO_2^-) over a 24 h period.

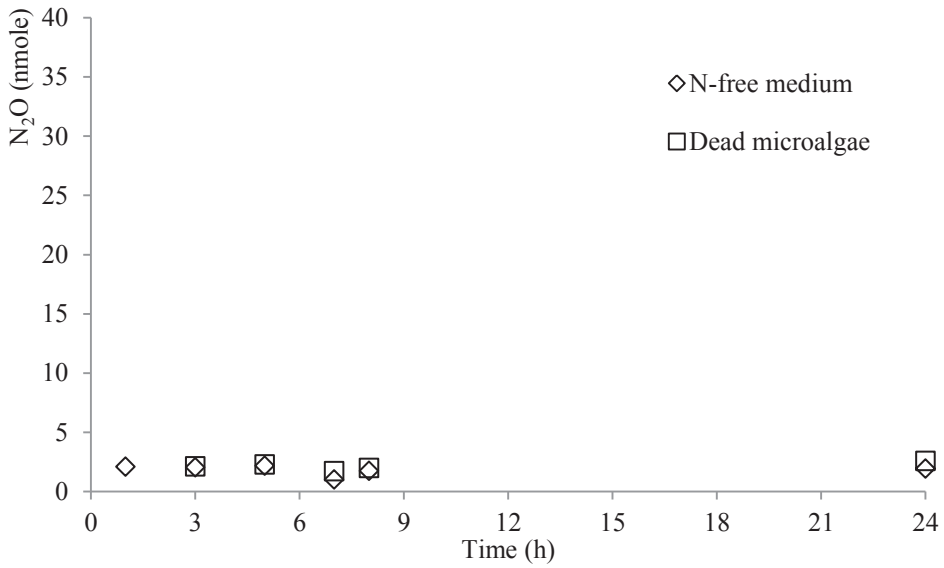


Figure G.2: N_2O production (nmole) in negative controls (N-free medium or dead microalgal suspension) supplied 10 mM NO_2^- in darkness.

N_2O was never significantly produced in the negative controls flasks (Figure G.1) and no statistical difference between the amount of N_2O produced at each sampling time was observed (two samples t-test at 95% level of confidence between sampling at 15 min and 3 h, $p = 0.871$; between sampling at 3 h and 24 h, $p = 0.951$; between sampling at 15 min and 24 h, $p = 0.798$). After 15 min, 3 h and 24 h incubation, the negative control averaged 1.51 ± 0.11 nmol N_2O (average ± standard deviation, n = 21); 1.49 ± 0.08 nmol N_2O (average ± standard deviation, n = 28) and 1.51 ± 0.09 (average ± standard deviation, n = 27) respectively.

G.3 Statistical validity of duplicates

Duplicates tests were used during the batch assays experiments. As seen in Figure G.3 the N_2O production (nmol·g-DCW^{-1}) thus calculated (the amount of N_2O produced a time t = 24 h (nmole) minus the background level (1.45 nmole), divided by the initial cell mass in g-DCW·L^{-1}) between 0-24 h followed a normal distribution (centred at 1784 N_2O nmol·g-DCW^{-1}) and the relative standard error was estimated at 25% (n = 40). Statistical analysis output (two sample t-test, $\alpha = 0.05$) summarised in Table G.2 showed that the observed difference between set of duplicates (from positive controls performed within 1.5 years) did not differ significantly (*p-value* = 0.380) confirming that the use of duplicates was satisfactory.

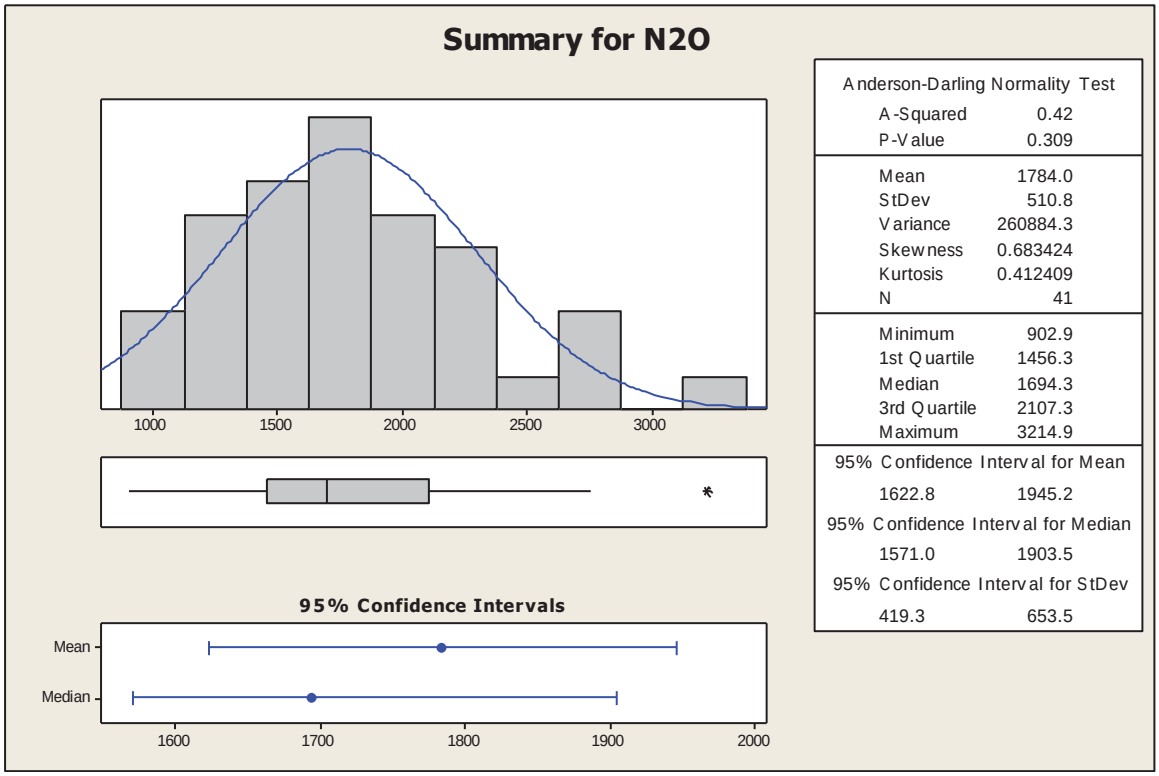


Figure G.3: Summary plot of the normality test obtained from MINITAB software for the positive controls.

Table G.2: Summary of the two samples t-test from the set of duplicate for positive controls

	N	Mean	Std Dev	Std Error
Set of duplicate 1	21	1853	474	103
Set of duplicate 2	20	1711	549	123

p-value = 0.380

Appendix H. Summary of the data analysis from the batch assays experiments

In Tables H1 to H4, results are provided as average total N_2O amount (nmole ± SD) and average specific N_2O production (nmol·g-DCW^{-1} ± SD). Specific N_2O production was calculated by subtracting the amount of N_2O already present in flasks at the time t - 1 to the total amount of N_2O recorded at the sampling time. Results are shown as "< LOQ" when the amount of N_2O in the flasks was below 2.9 nmole. Sample size (n) is provided in parenthesis when n > 3 and data represent average ± (max-min)/2 when n < 4. When n > 4, standard errors (SD) represent combined uncertainty based on standard deviations (the error on dry cell weight measurement was experimentally estimated to 5%).

Table H.1: Total amounts of N_2O (nmole) and specific N_2O production (nmol·g DCW^{-1}) in *C. reinhardtii* 6145c cultures incubated in darkness or illumination (significant production is shown in red). As can be seen, no significant N_2O production was recorded in N-free controls (no supply of N) inoculated *C. reinhardtii* 6145c and incubated in darkness or under illumination.

Conditions	**N source**	**Effector**	**N_2O after 0.25 h**		**N_2O after 3 h**		**N_2O after 24 h**	
			Total	Production	Total	Production	Total	Production
Darkness	-		1.50 ± 0.25 (21)	< LOQ	1.49 ± 0.20 (28)	< LOQ	1.50 ± 0.23 (27)	< LOQ
	-	L-Arg	1.63 ± 0.06	< LOQ	1.52 ± 0.08	< LOQ	1.52 ± 0.07	< LOQ
	NO_2^-		5.30 ± 3.01 (34)	300 ± 230 (34)	7.80 ± 4.43 (28)	490 ± 345 (28)	24.5 ± 6.59 (41)	1770 ± 500 (41)
	NO_2^-	L-Arg	2.14 ± 0.02	< LOQ	2.76 ± 0.15	< LOQ	23.2 ± 0.53	1680 ± 100
	NO_2^-	L-NNA	1.66 ± 0.20	< LOQ	5.49 ± 0.26	310 ± 26.0	40.4 ± 0.60	3015 ± 200
	NO_2^-	L-Arg + L-NNA	1.50 ± 0.08	< LOQ	5.58 ± 0.10	315 ± 8.03	39.4 ± 0.58	2920 ± 400
	NO_2^-	CN^-	1.59 ± 0.04	< LOQ	1.89 ± 0.31	< LOQ	3.78 ± 0.07	180 ± 20.0
	NO_2^- [a]		6.06 ± 1.96	360 ± 150	24.9 ± 6.82	1810 ± 540	5720 ± 1200	[44.0 ± 9.5] × 10^4
	NO_2^- [b]		11.5 ± 1.24	780 ± 100	16.0 ± 1.95	1130 ± 160	29.4 ± 3.40	2170 ± 300
	NO_2^- [c]	Tungstate	5.14 ± 1.03	290 ± 80	8.36 ± 1.94	540 ± 150	34.0 ± 2.61	2530 ± 200
	NO_3^-		1.44 ± 0.03 (4)	< LOQ	1.49 ± 0.12 (4)	< LOQ	1.48 ± 0.11 (4)	< LOQ
	NH_4^+		1.40 ± 0.00 (4)	< LOQ	1.48 ± 0.09 (4)	< LOQ	1.53 ± 0.06 (4)	< LOQ
Illumination	-		1.36 ± 0.00	< LOQ	1.33 ± 0.03	< LOQ	1.47 ± 0.16	< LOQ
	NO_2^-		1.62 ± 0.60 (5)	< LOQ	5.29 ± 0.49 (12)	< LOQ	17.1 ± 6.97 (11)	1210 ± 500 (11)
	NO_3^-		1.39 ± 0.03 (4)	< LOQ	1.52 ± 0.15 (4)	< LOQ	1.55 ± 0.15 (4)	< LOQ
	NH_4^+		1.41 ± 0.01 (4)	< LOQ	1.48 ± 0.11 (4)	< LOQ	1.48 ± 0.14 (4)	< LOQ

[a] Anoxic. Batch assays were prepared as described above. Following NO_2^-supply, the microalgal suspension was flushed with N_2 to create an anoxic atmosphere (Appendix M).

[b] Positive controls of tungstate inhibition experiment: *C. reinhardtii* 6145c was pre-cultivated 10 d in TAP medium, transferred in fresh TAP medium and further cultivated 4 days, then tested for N_2O production as described above (Appendix L).

[c] tungstate inhibition experiment: *C. reinhardtii 6145c* was pre-cultivated 10 d in TAP medium, transferred in 'modified' TAP medium (laden with tungstate) and further cultivated 4 d, then tested for N_2O production as described above (Appendix L).

Table H.2: Total amounts of N_2O (nmole) and specific N_2O production (nmol·g-DCW^{-1}) in *C. reinhardtii* 2929 (NR-lacking mutant) cultures incubated in darkness or illumination (significant production is shown in red). These results show that no 'immediate' N_2O production took place in cultures of the NR-deficient mutant and that late N_2O synthesis is not likely NR-mediated (see Chapter 2 for full discussion).

Conditions	**N source**	**Effector**	**N_2O after 0.25 h**		**N_2O after 3 h**		**N_2O after 24 h**	
			Total	Production	Total	Production	Total	Production
Darkness	NO_2^-		1.16 ± 0.25 (8)	< LOQ	2.30 ± 0.76 (8)	< LOQ	29.0 ± 9.20 (8)	2100 ± 700 (8)
	NO_2^-	CN^- 0.5 mM	-	-	10.0 ± 0.6 [a]	700 ± 100 [a]	12.6 ± 1.11	900 ± 100
	NO_2^-	CN^- 2 mM	-	-	2.71 ± 0.05 [a]	< LOQ	3.16 ± 0.10	130 ± 20
Illumination	NO_2^-		1.26 ± 0.02	< LOQ	1.32 ± 0.06	< LOQ	15.1 ± 0.12	1100 ± 100

[a] after 6 h

Table H.3: Total amounts of N_2O (nmole) and specific N_2O production (nmol·g-DCW^{-1}) in cultures of NiR-deficient mutants *C. reinhardtii* M3 and M4 incubated in darkness or illumination (significant production is shown in red). As can be seen, the NiR mutant synthesised N_2O when supplied NO_2^- and when supplied NO_3^- under illumination. These results demonstrate that NR and NiR activities impact N_2O synthesis by regulating intracellular NO_2^- concentration (see Chapter 2 for full discussion).

Strain	**Conditions**	**N source**	**N_2O after 1 h**		**N_2O after 6 h**		**N_2O after 24 h**	
			Total	Production	Total	Production	Total	Production
M3	Darkness	NO_2^-	11.4 ± 0.26	800 ± 50	55.8 ± 24.1	4200 ± 2000	75.6 ± 11.1	6000 ± 900
		NO_3^-	2.03 ± 0.35	< LOQ	1.97 ± 0.08	< LOQ	4.06 ± 1.45	200 ± 110
	Illumination	NO_2^-	4.64 ± 0.78	250 ± 60.0	370 ± 8.20	[2.8 ± 0.16] × 10^4	3480 ± 60.0	[27.0 ± 1.40] × 10^4
		NO_3^-	4.37 ± 0.00	230 ± 20.0	29.6 ± 3.42	2180 ± 290	720 ± 71.0	[5.60 ± 0.61] × 10^4

Table H.4: Total amounts of N_2O (nmole) and specific N_2O production (nmol·g-DCW^{-1}) in cultures of *C. reinhardtii* 704 and a NOR-knock down mutant (CYP55-silenced) incubated in darkness (significant production is shown in red). These results demonstrate that NOR is critical to late N_2O synthesis (see Chapter 2 for full discussion).

Strain	**N source**	**N_2O after 1 h**		**N_2O after 6 h**		**N_2O after 24 h**	
		Total	Production	Total	Production	Total	Production
704	NO_2^-	2.03 ± 0.06	< LOQ	6.72 ± 0.99	410 ± 80.0	138 ± 9.42	[1.06 ± 0.9] × 10^4
amiCYP55	NO_2^-	1.78 ± 0.02	< LOQ	2.17 ± 0.05	< LOQ	8.41 ± 2.40	540 ± 200

Appendix I. N_2O synthesis of various *Chlamydomonas reinhardtii* strains

C. reinhardtii 6145c, 2929 (NR mutant), M3 (NiR mutant), 409, 217, 112, 530, 124+ and 125+ were cultivated on TAP medium with NH_4^+ as nutrient source (NH_4^+ was used as some strains were mutant and could not grow neither on NO_3^- nor NO_2^-). Strains 409 and 217 are two *Chlamydomonas reinhardtii* wildtype other than 6145c. Strains 112 and 530 are the parents strain of the NR repressed mutant 2929, and strains 124/125+ are two wildtypes deficient in nitrate assimilation enzymes such as NR.

Following 5.5 days growth, aliquot of microalgal suspension (25 – 50 mL) were rinsed and re-suspended in N-free medium supplied with NO_2^- 10 mM. Gas samples were withdrawn after 24 h and as presented in Figure I.1 all *C. reinhardtii* strains had the ability to synthesise N_2O supplied with 10 mM NO_2^- and incubated in darkness. The results confirmed that N_2O synthesis is well-spread in *C. reinhardtii* but the emissions rates appeared to be strain-dependent.

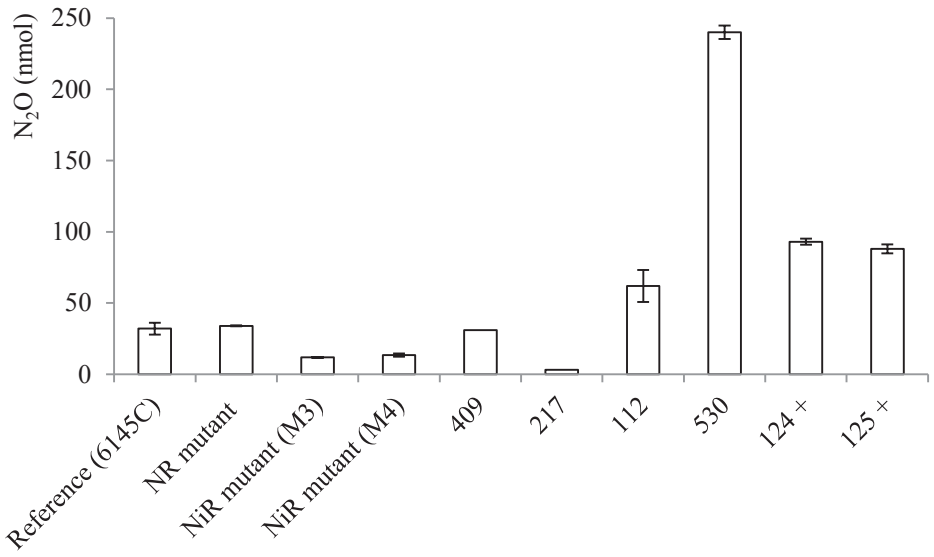


Figure I.1: N_2O produced (nmole) by *C. reinhardtii* 6145c, NR mutant (2929), NiR mutant (M3), 409, 217, 112, 530, 124+ and 125+ (initial DCW of 0.25 g-DCW·L^{-1}) supplied with 10 mM NO_2^- and incubated in darkness. The data represent average ± (Max – Min)/2.

Appendix J. Dynamic experiment with *Chlamydomonas reinhardtii* 6145c cultures in indoor 2 L bench scale tubular photobioreactors

Experiments were carried out in two indoor 2 L bench scale photobioreactors (PBR A and PBR B). Each PBR was made of an acrylic tube hermetically sealed with a metal lid (Figure J.1). An outer tube filled with water allowed for temperature control (25ºC ± 1ºC) using a temperature controller and the PBRs were placed in the centre of 7 "cool white" bulbs. To prevent settling of the culture and carbon limitations, an air/CO_2 (98%/2% v/v) mixture was bubbled at a flow of 1 L.min^{-1}. A hole in the lid was used for sampling. A thermos-syringe was used to flush the gas headspace (i.e. at least 3 times) and gas samples were withdrawn analysed by gas chromatography.

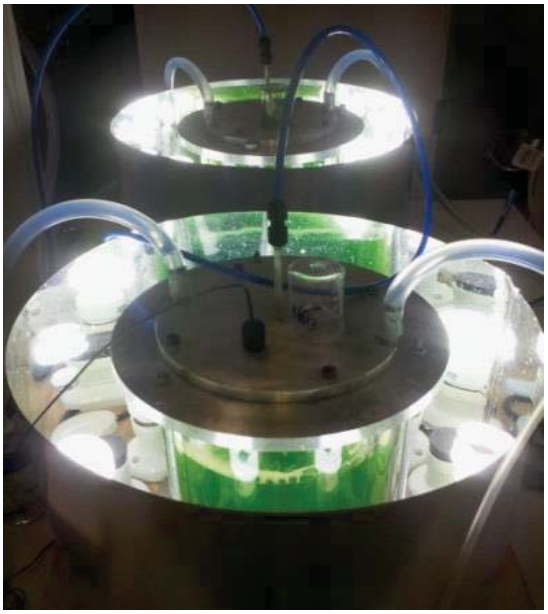


Figure J.1: 2 L bench scale indoor

In both PBRs *C. reinhardtii* was cultivated on TAP with NO_3^- as N source. The day of the experiment PBR A was covered with aluminium foil to simulate darkness which represented the beginning of the experiment (t = 0 min). PBR B was kept illuminated. At t = 160 min NO_2^- was supplied in both reactors.

The results presented in Figure J.2 showed that the supply of NO_2^- triggered N_2O production in actively growing *C. reinhardtii* 6145c cultures. The response was rapid in both reactors; however PBR A, which was in darkness, showed higher N_2O production after the addition of NO_2^-.

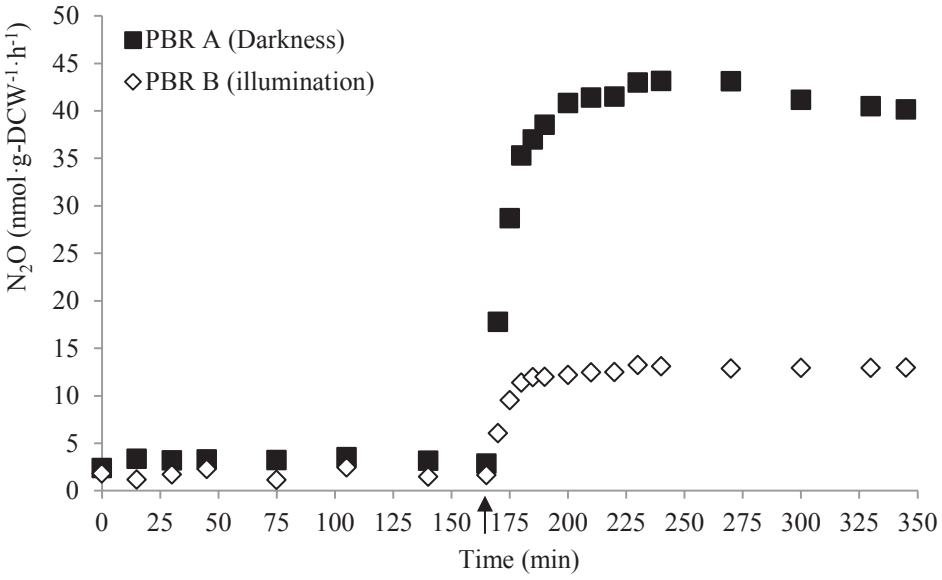


Figure J.2: N_2O production rates (nmol·g-DCW^{-1}·h^{-1}) from *C. reinhardtii* 6145c cultures in 2 L indoor photobioreactor in darkness (PBR A) or illumination (PBR B). 10 mM NO_2^- was supplied in both reactors after 165 min (black arrow).

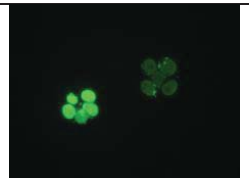
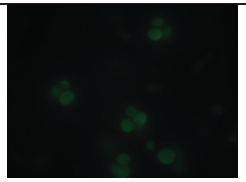
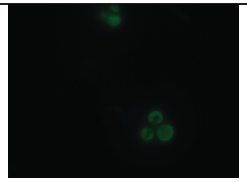
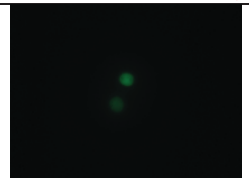
Appendix K. NO generation during N_2O synthesis in *Chlamydomonas reinhardtii*

DAF-FM Diacetate (4-amino-5-methylamino-2`.7`-difluore-fluorescein diacetate) is non-fluorescent and can pass the cell membrane (Wei et al., 2014). Inside cells, DAF-FM diacetate is converted by estherases into the non-permeable DAF-FM that then reacts with NO to form a fluorescent DAF-FM triazol derivative (Wei et al., 2014). To detect NO, 10 μM of DAF-FM Diacetate (≥98%, Sigma-Aldrich, USA) was therefore added to 5.5 days-old *C. reinhardtii* cultures subsequently incubated for 0.5-1 h in serum flasks (25°C, constant agitation at 180 rpm) under low light. Following the protocol described in Chapter 2 Section 2.2.7 the fluorescence in the cells was observed under fluorescence microscopy (Olympus BX51 microscope, Japan) with excitation at 520-550 nm for chlorophyll auto-fluorescence (characterised by a red colour) and at 460 – 490 nm for DAF FM triazol fluorescence (characterised by a green colour) using U-MWIG2 and U-MWIBA2 filters (Olympus, Japan), respectively.

To confirm green fluorescence was indeed caused by intracellular NO generation, a NO donor 0.3 mM (DEA NONOate, Sigma-Aldrich, USA) and NO scavenger (bovine haemoglobin, Sigma-Aldrich, USA) were added to *C. reinhardtii* cells pre-incubated with DAF-FM diacetate. In addition, because HNO has been proposed as a possible N_2O intermediate (Guieysse et al., 2013), the reactivity of DAF-FM diacetate with HNO was tested by adding Angeli`s salt (a HNO donor; Sigma-Aldrich, USA) to pre-incubated *C. reinhardtii* cells at 8 mM. Finally, as N_2O synthesis was inhibited in the presence of the mitochondrial inhibitor cyanide (CN^-), KCN (2 mM) was added to pre-incubated *C. reinhardtii* cells supplied NO_2^-. The results from the microscopic

observations are presented in Table J.1. As can be seen, a strong green fluorescence was recorded when the NO donor was supplied to pre-incubated *C. reinhardtii* cells, confirming efficient NO detection via green fluorescence (Table J.1). Green fluorescence was repressed when the NO scavenger was added to pre-incubated cells supplied either NO_2^- or the NO donor, which provides further evidence of NO generation via NO_2^- reduction. A weak green fluorescence was observed when the HNO donor Angeli's salt was used. The fluorescence may have been caused by DAF-FM reactivity with HNO and/or the generation of NO_2^- from HNO (Shoman and Aly, 2016), meaning the potential involvement of HNO as product of NO_2^- reduction (and HNO subsequent reduction into N_2O) cannot be entirely dismissed. Cyanide was also found to repress NO synthesis in pre-incubated cells supplied NO_2^-. While these results appears to disagree with other findings linking NO_2^- reduction by NR immediately following NO_2^- addition (COX being only involved after approx. 20 h of exposure, see manuscript), NO quenching and/or synthesis repression may have been caused by the broad reactivity of CN^-. If was unfortunately not possible to assess the impact of CN^- on NO following long term (20 h) exposure to NO_2^- because of the high reactivity of DAF-FM in pre-incubated cells. Altogether, the clear differences in green fluorescence strength observed in the positive controls (e.g. when either NO_2^- or the NO donor were added to pre-incubated cells) against all negative controls support the hypothesis of NO formation, in agreement with the literature (Sakihama et al., 2002; Wei et al., 2014; Chamizo-Ampudia et al., 2016).

Table K.1: Microscopic observations of *C. reinhardtii* cells pre-incubated with DAF-FM Diacetate and subsequently supplied with various effectors. Under the hypothesis that NO_2^- biological reduction yielded NO, red text show negative controls and green text shows positive controls. Microscopic photographs were taken with a Micropublisher 5 colour CCD camera (QImaging, Canada).

Conditions (all cells were pre-incubated with DAF-FM Diacetate)	Pre- incubated cells supplied DEA NONOate (NO donor): this test verified the efficiencies of the NO donor and NO detection.	Pre-incubated cells supplied DEA NONOate (NO donor) and haemoglobin (NO quencher): this test verified the efficiency of the NO quencher.	Pre-incubated cells supplied NO_2^- (putative substrate of NO biosynthesis) and haemoglobin (NO quencher): this test provides further evidence of NO_2^- reduction to NO.	Pre-incubated cells supplied NO_2^- (putative substrate of NO biosynthesis) and CN^- (inhibitor of COX-mediated NO_2^- reduction to NO): lack of fluorescence suggests COX involvement in NO synthesis immediately following nitrite addition	Pre-incubated cells supplied Angeli`s salt as HNO donor. The mild fluorescence suggest a 'mild' interfering effect but the low level support the hypothesis of NO as production of NO_2^- reduction (see positive control in Table S5.1).
Microscopic photographs (×100) (excitation wavelengths 460 – 480 nm; emission wavelengths: 510-580 nm)					

The 'positive control' conducted with the wildtype strain was repeated with NR-deficient *C. reinhardtii* 2929 stain. As can be seen below (Figure K.1), red fluorescence was observed (left) but NO production was not detected (right) following NO_2^- supply and incubation in darkness.

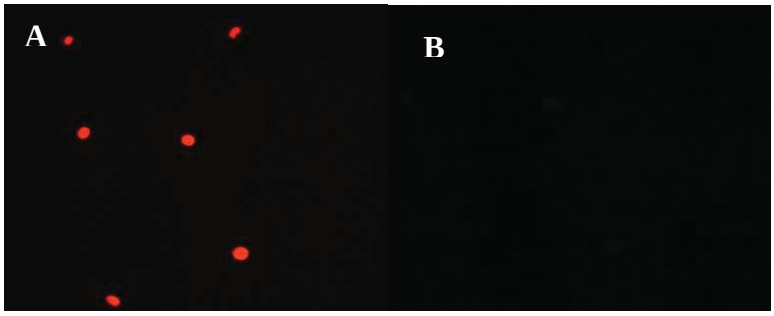


Figure K.1: Microscopic observations (×40) of *C. reinhardtii* 2929 pre-incubated with DAF FM Diacetate and supplied with NO_2^- 10 mM (positive control): A) excitation wavelengths 520 – 550 nm; emission wavelengths: 580 – 797 nm; B) excitation wavelengths 460 – 480 nm; emission wavelengths: 510-580 nm.

References:

Chamizo-Ampudia, A., Sanz-Luque, E., Llamas, Á., Ocaña-Calahorro, F., Mariscal, V., Carreras, A., Barroso, J.B., Galván, A., and Fernández, E. (2016). A dual system formed by the ARC and NR molybdoenzymes mediates nitrite-dependent NO production in *Chlamydomonas*. Plant. Cell Environ.: **39**:2097-2107.

Guieysse, B., Plouviez, M., Coilhac, M., and Cazali, L. (2013). Nitrous Oxide (N_2O) production in axenic *Chlorella vulgaris* microalgae cultures: Evidence, putative pathways, and potential environmental impacts. Biogeosciences **10**: 6737–6746.

Sakihama, Y., Nakamura, S., and Yamasaki, H. (2002). Nitric oxide production mediated by nitrate reductase in the green alga *Chlamydomonas reinhardtii*: an alternative NO production pathway in photosynthetic organisms. Plant Cell Physiol. **43**: 290–297.

Shoman, M.E. and Aly, O.M. (2016). Nitroxyl (HNO): A reduced form of nitric oxide with distinct chemical, pharmacological, and therapeutic properties. Oxid. Med. Cell. Longev. http://dx .doi:10.1155/2016/4867124.

Wei, L., Derrien, B., Gautier, A., Houille-Vernes, L., Boulouis, A., Saint-Marcoux, D., Malnoë, A., Rappaport, F., de Vitry, C., Vallon, O., Choquet, Y., and Wollman, F.-A. (2014). Nitric oxide-triggered remodeling of chloroplast bioenergetics and thylakoid proteins upon nitrogen starvation in *Chlamydomonas reinhardtii*. Plant Cell **26**: 353–372.

Appendix L. N_2O synthesis by *Chlamydomonas reinhardtii* pre-cultivated with tungstate, a molybdenum enzyme inhibitor.

Tungstate generally decreases NR catalytic activity by replacing the enzyme co-factor molybdenum without preventing NR synthesis (Deng et al., 1989). Based on the protocol used by Guieysse et al. (2013), *C. reinhardtii* 6145c was cultivated 10 days in TAP medium. These cultures were then centrifuged and re-suspended in either "normal" TAP or "modified" TAP where molybdate (MoO_2^{4-}) was replaced by orthotungstate (0.253 mg Na_2WO_4 L^{-1}) to chemically inhibit NR. Following 'normal' cultivation during 4 days, aliquot (25 – 50 mL) of these suspensions were withdrawn, centrifuged, re-suspended in N-free TAP medium, and supplied 10 mM NO_2^-. These cultures were incubated in darkness and N_2O production was monitored over a 24 h period.

As seen in Figure L.1, tungstate-treated cells produced less N_2O than control cells during the first 5 h of incubation, although the levels of N_2O production after 24 h of incubation were similar in the treatment and control assays. These results suggested that NR is involved in immediate N_2O production, probably by catalysing the reduction of NO_2^- to NO as suggested in *Chlorella vulgaris* (Guieysse et al., 2013). It is however important to note that tungstate is not specific to NR but also inhibit other enzymes possessing a molybdenum co-factor (MoCo) such as xanthine dehydrogenase/oxidase. Thus, our results also dismissed the possibility that other MoCo enzymes than NR could be involved during later N_2O synthesis under NO_2^- supply.

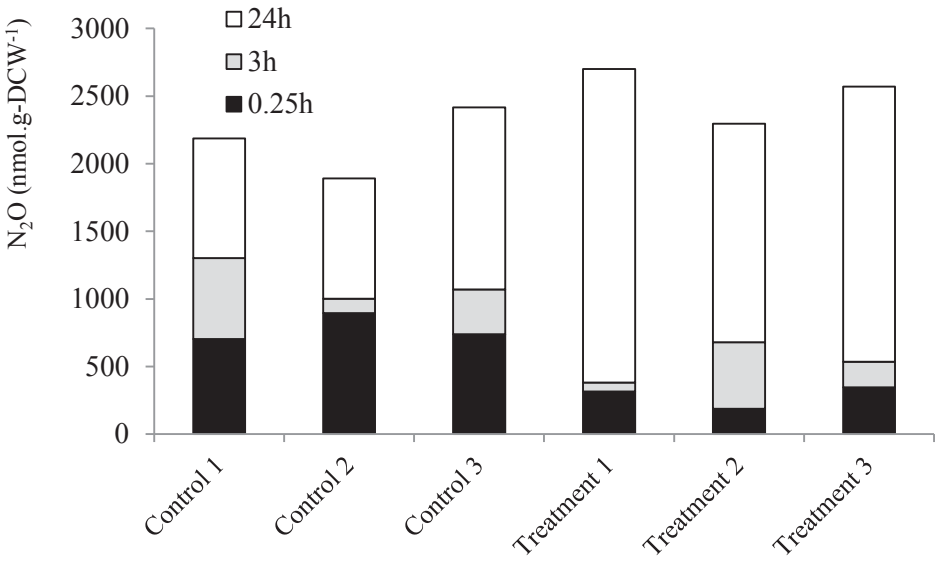


Figure L.1: N_2O specific production (nmol·g-DCW^{-1}) in cultures of *Chlamydomonas reinhardtii* 6145c (0.22 g·L^{-1}) supplied 10 mM NO_2^- and incubated in darkness. The 'treatment' samples were withdrawn from NR-inhibited cell cultures.

References:

Deng, M., Moureaux, T., and Caboche, M. (1989). Tungstate, a molybdate analog inactivating nitrate reductase, deregulates the expression of the nitrate reductase structural gene. Plant Physiol. **91**: 304–309.

Guieysse, B., Plouviez, M., Coilhac, M., and Cazali, L. (2013). Nitrous Oxide (N_2O) production in axenic *Chlorella vulgaris* microalgae cultures: Evidence, putative pathways, and potential environmental impacts. Biogeosciences **10**: 6737–6746.

Appendix M. N_2O synthesis by *Chlamydomonas reinhardtii* under anoxia

Aliquot (25 -50 mL) of 5.5 days old *C. reinhardtii* 6145c and its NR repressed mutant 2929 cells were centrifuged. Supernatant was discarded and pellet was and re-suspended on N-free medium in serum flasks (Batch assays). Following supply of NO_2^- some duplicates of both *C. reinhardtii* strains 6145 and 2929 were flushed with N_2 to create an anoxic atmosphere. A negative control consisting of N free medium supplied with 10 mM NO_2^- and flushed with N_2 was also performed. N_2O was then monitored during a 24 h period. The results are presented in Figure M.1 and M.2.

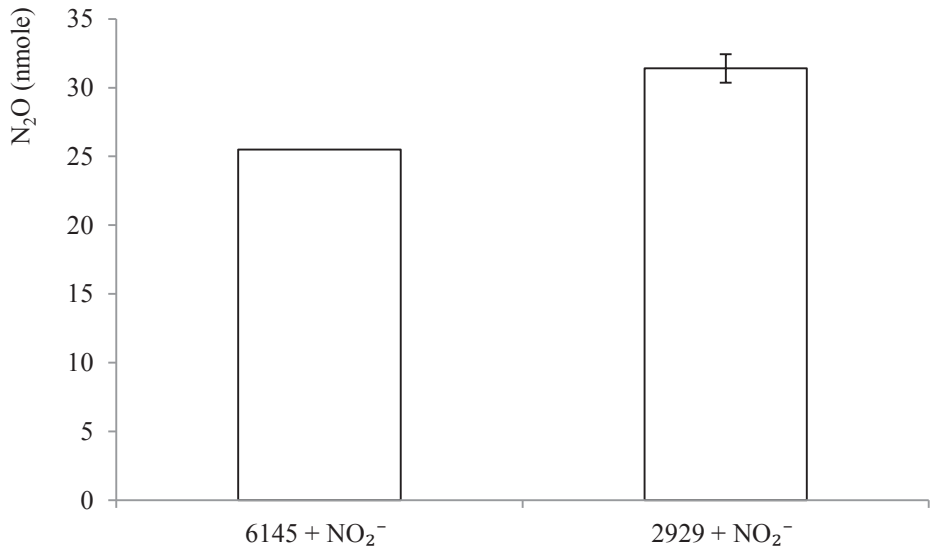


Figure M.1: N_2O produced (nmole) after 24 h by triplicate cultures of *C. reinhardtii* 6145c and 2929 (initial DCW of 0.25 g-DCW·L^{-1}) supplied with 10 mM NO_2^- incubated in darkness under normoxic conditions.

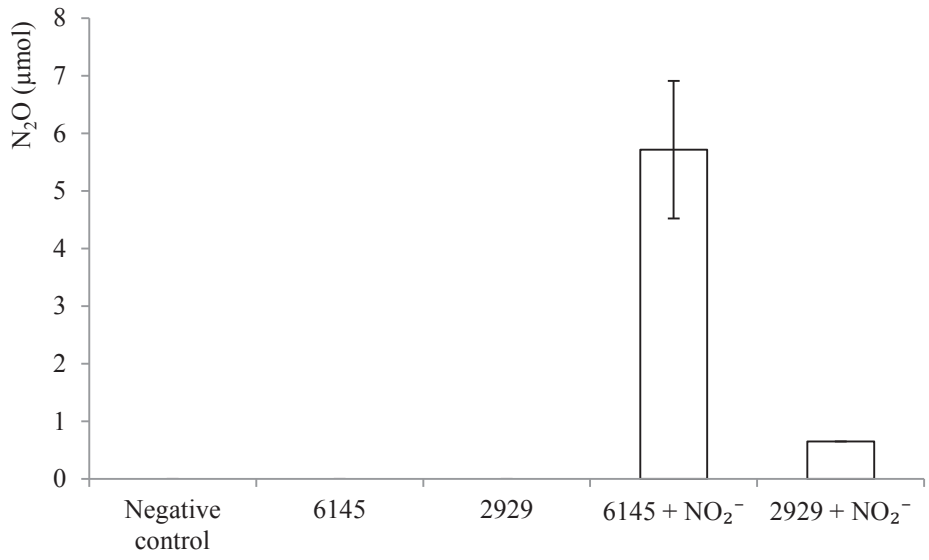


Figure M.2: N_2O produced (µmole) after 24 h by triplicate cultures of *C. reinhardtii* 6145c and 2929 (initial DCW of 0.25 g-DCW·L^{-1}) supplied with 10 mM NO_2^- incubated in darkness under anoxic conditions.

Similarly to normoxic conditions, in anoxic conditions NO_2^- supply triggered N_2O synthesis in *C. reinhardtii* 6145 and 2929. While under normoxia both *C. reinhardtii* strains synthesised N_2O at usual rates (30 nmole equivalent to 2500 nmole·g-DCW^{-1}) under anoxic conditions upon NO_2^- supply, N_2O synthesis sky rocketed (Figure M.2). In light of the potential implication of mitochondria and NO as discussed in this study, such massive increase in N_2O production could be explained by anoxia which is known to favour 1/ NO_2^- reduction into NO by mitochondria (Tischner et al., 2004; Gupta et al., 2016), and 2/ NO availability for N_2O synthesis since NO oxidation is decreased under anoxia. **References:**

Gupta, A.K., Kumari, A., Mishra, S., Wany, A., and Gupta, K.J. (2016). The functional role of nitric oxide in plant mitochondrial metabolism. **77**. http://dx.doi.org/10.1016/bs.abr.2015.10.007.

Tischner, R., Planchet, E., and Kaiser, W.M. (2004). Mitochondrial electron transport as a source for nitric oxide in the unicellular green alga Chlorella sorokiniana. FEBS Lett. **576**: 151–155.

Appendix N. Conferences abstracts

N_2O (Nitrous oxide) emissions during full-scale microalgae cultivation outdoors

M. Plouviez[1], B. Guieysse[1], A. Shilton[1], M. Packer[2], H. Thuret[1], E. Alaux[1]

[1]*SEAT, Massey University, Palmerston North, New Zealand*; [2]*Cawthron Institute, Nelson, New Zealand*

While reported for decades, the ability of microalgae to synthesise nitrous oxide (N_2O) has been the focus of several recent studies highlighting the risks associated with the potential release of this greenhouse gas (GHG) during algae cultivation. However, to date, N_2O emissions have never been systematically quantified during full-scale algae cultivation outdoors.

In order to investigate the potential environmental impact of microalgae cultivation, N_2O emissions were quantified during *Chlorella vulgaris* cultivation in 50-L photobioreactors (PBRs) operated outdoors under temperate climatic conditions (Palmerston North, New Zeland). *C. vulgaris* was used as model algae as this species is widely used for its commercial potential, its ease of cultivation and its ability to produce N_2O. The PBRs were operated semi-continuously by refreshing half the culture volume with fresh medium (buffered BG11) each time biomass concentration reached 1 $g.L^{-1}$. CO_2-enriched air (2% v/v) was continuously bubbled in the reactor to supply carbon and mix the culture. Specific N_2O emissions rates ($nmol.h^{-1}.g\text{-}DW^{-1}$) were calculated based on biomass concentration (as dried weight, DW, per litre) and analysis of effluent gas samples by GC-ECD. N_2O emissions rates were thus recorded over 335 samples withdrawn over 86 days of operation representing all seasons.

Significant positive N_2O emissions were recorded in all samples. N_2O emissions fluctuated greatly (0.49 to 1183 $nmol.h^{-1}.g\text{-}DW^{-1}$) and no clear relation was seen between emission rates and environmental parameters (*e.g.* temperature, light intensity, nitrite concentration). Over all, N_2O emissions of 157-197 nmol $N_2O.h^{-1}.g\text{-}DW^{-1}$ ($p=0.05$, $n=335$) were recorded over the duration of the experiment. To put these results into perspective, such emission rates would represent 77 to 96 kg $N_2O.ha^{-1}.yr^{-1}$ or 23 to 29 t CO_2-eq $ha^{-1}.yr^{-1}$ in 0.25 m deep raceway ponds operated at a hydraulic retention time of 7 days (yielding an average algae concentration of 512 mg $DW.L^{-1}$ based on published prediction of *C. vulgaris* productivity of 66.8 $t.ha^{-1}$ yr^{-1} in Mediterranean climate). This carbon footprint therefore represents 18-23% of the CO_2

fixed by the algae during photosynthesis. Preliminary results also showed nitrogen source and species selection provide efficient mitigation strategies.

Keywords: Microalgae, N_2O (Nitrous oxide), Environmental significance.

References:

1. Nitrous oxide (N_2O) production in axenic *Chlorella vulgaris* microalgae cultures: Evidence, putative pathway, and potential environmental impact. B. Guieysse, M. Plouviez, M. Coilhac, and L. Cazali (2013).

Direct and indirect N_2O emissions during primary domestic wastewater treatment in a pilot-scale high rate algal pond

Maxence Plouviez*, Esther Posadas, Romain Lebrun*, Raul Munoz**, Benoit Guieysse****

* Massey University, New Zealand; b.j.guieysse@massey.ac.nz
** Valladolid University, Valladolid, Spain

Abstract:

A concrete 1000 L high rate algae pond (HRAP) was fed semi-continuously with primary wastewater at an average HRT of 10 days. The HRAP influent and effluent were frequently monitored for pH, DO, temperature, TOC, DOC, TN, TSS, COD, nitrate, nitrite and ammonium and dissolved N_2O. Based on the monitoring, direct and indirect (from NH_3 redeposited and converted in N_2O) N_2O emissions averaged 0.007 g N_2O-N/d (n = 13) and 0.015 ± 0.005 g N_2O-N/d, respectively. Total emissions were thus estimated to 0.48 % of the influent TN input of 4.56 ± 0.36 g N/d. In person equivalent these emissions would represent 29 g N_2O/PE-yr, 9 fold higher than IPCC default emission factor of 3.2 g N_2O/capita-yr for conventional wastewater treatment. N_2O emissions from algae-based wastewater treatment should therefore be carefully monitored and accounted for during impact assessment.

Appendix O. Summary of the operational parameters from the monitoring study in 50 L photobioreactors

Table O.1: N_2O emissions and operational parameters (DCW, temperature, light irradiance and hydraulic retention time (HRT)) measured during microalgae cultivation in 50 L column photobioreactors (n = sampling size for N_2O measurements). a calculated between 28/03 and 11/04.

Date (days of monitoring)	Season	Microalgae species	N-source	n	Number of PBR	DCW (average g-DCW·L^{-1})	Biomass productivity (g·m^{-2}·d^{-1})	Temperature (°C)	Light irradiance (W·m^{-2})	Average HRT (days)
12/06/12 to 18/06/12 (6 days)	Winter	*C. vulgaris*	NO_3^-	76	1 2 3 4	0.41– 0.62 (0.53) 0.53 – 0.77 (0.65 0.29 – 0.42 (0.35) 0.51 – 0.81 (0.64)	1.39 1.60 0.83 1.94	-3.00 – 11.0	0.00 – 330	10
28/03/14, 31/03/14 and 11/04/14 (3 days)	Autumn	*C. vulgaris*	NO_3^-	26	1 2	0.91 – 1.05 (0.95) 0.71 – 0.87 (0.76)	5.00[a] 7.10[a]	14.0 – 21.0	4.75 – 690	8
04/06/14 to 04/07/14 (32 days)	Winter	*S. platensis*	NO_3^-	90	1 2	0.12 – 0.78 (0.44) 0.10 – 1.00 (0.62)	2.30 2.70	2.00 – 17.0	3.00 – 430	10
04/06/14 to 10/07/14 (36 days)	Winter	*Neochloris*	NO_3^-	136	1 2	0.17 – 1.50 (0.73) 0.15 – 1.40 (0.84)	2.06 2.12	2.00 – 17.0	3.00 – 430	11
28/07/14 to 28/08/14 (32 days)	Winter	*C. vulgaris*	NO_3^-	84	1 2	0.10 – 1.30 (0.59) 0.11 – 1.15 (0.63)	3.05 3.22	5.00 – 16.0	0.00 – 490	10
28/07/14 to 28/08/14 (32 days)	Winter	*C. vulgaris*	NH_4^+	84	1 2	0.19 – 1.24 (0.80) 0.20 – 1.18 (0.80)	4.10 4.20	5.00 – 16.0	0.00 – 490	10
13/10/14 to 28/10/14 (15 days)	Spring	*C. vulgaris*	NO_3^-	44	1 2	0.57 – 1.33 (0.80) 0.47 – 1.28 (0.81)	8.05 7.32	13.0 – 20.0	75.0 – 580	8
04/11/14 to 28/11/14 (24 days)	Spring	*C. vulgaris*	NO_3^-	64	1 2	0.10 – 1.24 (0.72) 0.10 – 1.25 (0.68)	7.10 8.92	10.0 – 17.0	120 – 890	7
01/12/14 to 10/12/14 (12 days)	Summer	*C. vulgaris*	NO_3^-	32	1 2	0.50 – 1.01 (0.71) 0.50 – 0.90 (0.72)	12.40 10.0	11.0 – 22.0	100 – 970	7

Appendix P. Controls and statistical analyses performed on N_2O measurements from the N_2O monitoring

The concentrations of N_2O in the surrounding air and in the inlet gas mixture bubbled in the reactors (controls) were regularly quantified and discounted from the photobioreactor (PBR) N_2O exhaust gas concentration when computing net N_2O productivities. Figure P.1 shows the N_2O concentrations measured in the PBRs and controls during *C. vulgaris* cultivation on BG 11 fed with NO_3^-. As can be seen, a clear difference between the N_2O concentrations measured from the PBRs and from the controls was noted.

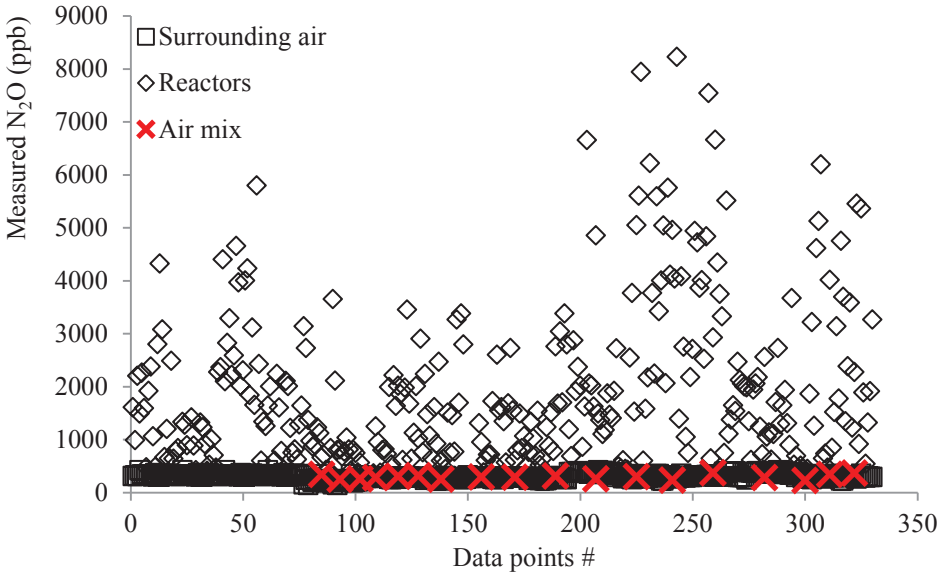


Figure P.1: Measured N_2O concentrations from the PBRs (◊) and controls (□: surrounding air, ×: Air mix) from the monitoring performed when *C. vulgaris* was cultivated on BG 11 fed with NO_3^-.

In order to verify that the difference between the N_2O concentration measured from the PBR exhaust gas and the controls was statistically significant, two samples t-test (α =

0.05) were performed using the statistical software MINITAB 16 (See below for an example of the output obtained in MINITAB after a two sample t-tests between the N_2O measured from one PBR against the N_2O measured in controls).

June 2012 Reactor 1

```
Two-sample T for Reactor 1 2012 (ppb) vs Air (ppb)

                         N   Mean  StDev  SE Mean
Reactor 1 2012 (ppb)  19   1698   1033      237
Air (ppb)             19  330.9   27.1      6.2


Difference = mu (Reactor 1 2012 (ppb)) - mu (Air (ppb))
Estimate for difference:  1367
95% CI for difference:  (886, 1848)
T-Test of difference = 0 (vs not =): T-Value = 5.77  P-Value = 0.000  DF = 36
Both use Pooled StDev = 730.6317
```

A p-value was computed for each analysis. If the p-value of the test was < 0.05 it meant that the difference between the mean of the measured N_2O concentration from the PBR and the mean of the measured N_2O concentration from the controls was significant at the 95% level of confidence; otherwise (*p-value* > 0.05) the difference was determined to not be significant. The results obtained from the different monitorings are presented in Table P.1.

Table P.1: Summary of the two sample t-test ($\alpha = 0.05$) analyses between the measured N_2O concentration from the PBR and the measured N_2O concentration from the controls.

Date, species, medium and N source		N	Mean (N_2O in ppb)	Std Dev	Std Error	p-value (t-test, $\alpha = 0.05$)
12/06/12 to 18/06/12 *C. vulgaris* on BG11/NO_3^-	Reactor 1	19	1698	1033	237	
	Control	19	330.9	27.1	6.2	< 0.05
	Reactor 2	19	793	408	94	
	Control	19	330.9	27.1	6.2	< 0.05
	Reactor 3	19	3056	1143	262	
	Control	19	330.9	27.1	6.2	< 0.05
	Reactor 4	19	1207	640	147	
	Control	19	330.9	27.1	6.2	< 0.05
28/03/14, 31/03/14 and 11/04/14, *C. vulgaris* on BG11/NO_3^-	Reactor 1	13	1182	829	230	
	Control	13	219	49.1	14	< 0.05
	Reactor 2	13	1043	892	247	
	Control	13	219	49.1	14	< 0.05
04/06/14 to 04/07/14, S platensis on Zarrouk/NO_3^-	Reactor 1	45	276.9	37.2	5.6	
	Control	45	265.4	25.5	3.8	0.091
	Reactor 2	45	259.4	86.0	13	
	Control	45	269.4	22.4	247	0.580
04/06/14 to 10/07/14, *Neochloris*, BG11/NO_3^-	Reactor 1	68	1072	839	102	
	Control	68	266.6	24.7	3.0	< 0.05
	Reactor 2	68	1042	858	104	
	Control	68	266.6	24.7	3.0	< 0.05
28/07/14 to 28/28/14, *C. vulgaris* BG11/NO_3^-	Reactor 1	42	1421	875	135	
	Control	42	277.1	21.2	3.3	< 0.05
	Reactor 2	42	1264	801	124	
	Control	42	277.1	21.2	3.3	< 0.05
28/07/14 to 28/28/14, *C. vulgaris* BG11/NH_4^+	Reactor 1	42	266	19.9	3.0	
	Control	42	278.1	21.6	3.2	0.01[a]
	Reactor 2	42	270.1	22.2	3.3	
	Control	42	278.1	21.6	3.2	0.150
13/10/14 to 10/12/14, *C. vulgaris* BG11/NO_3^-	Reactor 1	84	2765	1936	211	
	Control	84	329.1	44.7	4.9	< 0.05
	Reactor 2	68	1861	1401	170	
	Control	68	327.7	45.7	5.5	< 0.05

[a] while the results suggest N_2O consumption in the reactor, the difference in N_2O outlet and inlet concentrations were very small so this particular observation finding should be taken with caution. It is however clear that no significant production occurred when *C. vulgaris* was fed ammonium.

As seen in Table P.1, the difference between the measured N_2O concentration from the PBRs and the measured N_2O concentration from the controls was always significant when *C. vulgaris* and *Neochloris* were cultivated on BG 11 with NO_3^- as N-source. In contrast, when *C. vulgaris* was cultivated on BG 11 with NH_4^+ as N-source and *S. platensis* cultivated on Zarrouk medium fed NO_3^- the difference between the measured N_2O concentration from the PBRs and the measured N_2O concentration from the controls was not significant. These results suggest that N_2O was not generated from these microalgal suspensions. In consequence, net N_2O productivities were not computed and deemed as not statistically significant (i.e. the value obtained would be centred on 0 with variation due to measurement errors).

Appendix Q. Preliminary work with N_2O and NO specific microsensors

Q.1 N_2O microsensor (N_2O-500)

Dissolved N_2O detection was carried out with a N_2O microsensor N_2O-500 (Unisense, Denmark) in 120 ± 1mL Durand bottles closed with a plastic cap and parafilm (Figure Q.1). The probe was connected to a Unisense meter and data were analysed with the software "Sensor Trace" (Unisense, Denmark). Following the manufacturer protocol, the microsensor was pre-amplified for 10 min in applying a voltage level of -1.2 V in oxygen and N_2O free water (first use only). Then a voltage level of -0.8 V was applied until a stable signal below 20 mV. The microsensor was immersed in oxygen and N_2O free buffer or microalgal suspension to perform calibration or measurement, respectively. Calibration was performed by withdrawing a volume of gas and replacing the same volume by N_2O standard (100 ppm; N_2O in N_2). Pressure change and dissolved N_2O concentration were then calculated as given in Appendix F. An example of calibration is presented in Figure Q.2.

Figure Q.1: Set-up used to measure dissolved N_2O with Unisense N_2O microsensor.

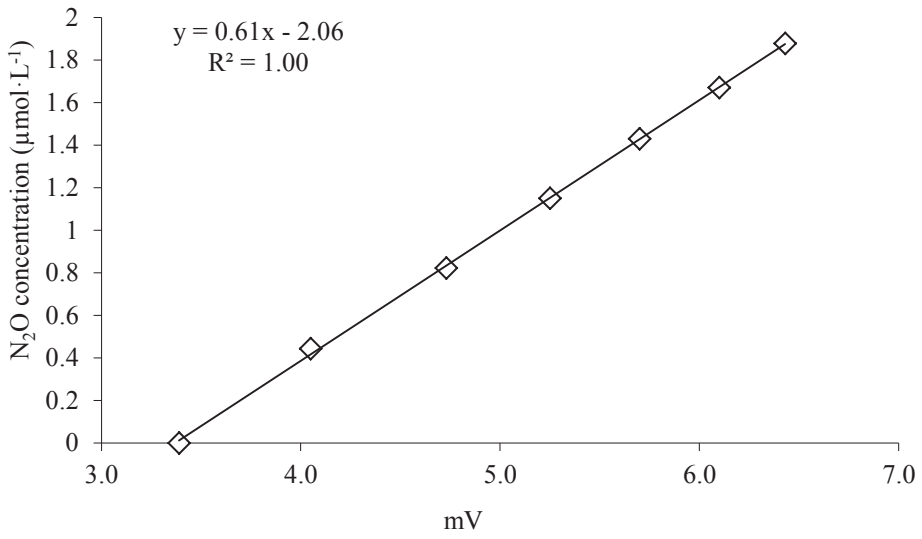


Figure Q.2: N_2O calibration of the microsensor N_2O-500

Q.2 NO microsensor (NO-500)

Dissolved NO detection was carried out with a NO microsensor NO-500 (Unisense, Denmark) in 120 ± 1mL Durand bottles closed with plastic cap and parafilm (Figure Q.1). The probe was connected to a Unisense meter and data were analysed with the software "Sensor Trace" (Unisense, Denmark). Following the manufacturer protocol, the microsensor was amplified for 24 h in applying a voltage level of +1.25 V in oxygen and N_2O free water (first time only). Then, each time of use, the voltage level was kept at +1.25 V until a stable signal below 20 mV. The microsensor was immersed in oxygen and N_2O free buffer at pH 7.4 or microalgal suspension to either perform calibration or measurement. Calibration was performed by adding diethylamine NONOate diethylammonium salt (DEA NONOate ≥98%, Sigma Aldrich, USA) which release NO with $t_{1/2}$ of 16 min at 22°C and pH 7.4. An example of calibration is given in Figure Q.3.

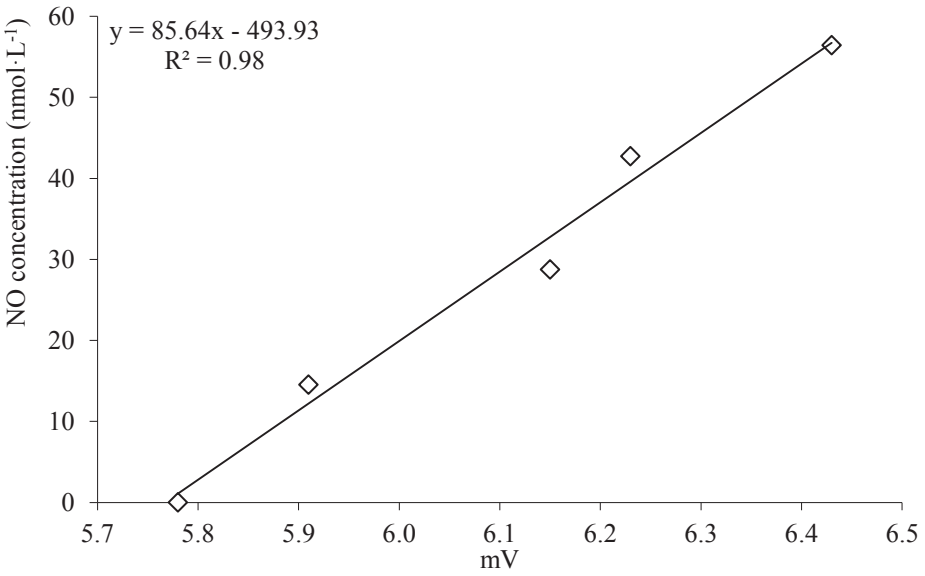


Figure Q.3: NO calibration of the microsensor NO-500

Q.3 Example of microsensor use

The microsensors were tentatively used to continuously follow NO and N_2O generation in microalgal suspension. Prior to any use in outdoor systems, the accuracy of the microsensor was verified by comparing the gaseous N_2O measured with the GC and the gaseous N_2O estimated from the dissolved N_2O measured by the microsensor. Because the microsensor measured the amount of dissolved N_2O, the amount of gaseous N_2O released could be calculated based on Equation Q.1:

$$\Phi_{N_2O} = Kla_{N_2O} \times ([N_2O]^* - [N_2O]), \text{ Equation Q.1}$$

Φ_{N_2O} = N_2O flux ($mol \cdot L^{-1} \cdot s^{-1}$)

Kla_{N_2O} = N_2O mass transfer coefficient (s^{-1})

$[N_2O]^*$ = Dissolved N_2O concentration at equilibrium ($mol \cdot L^{-1}$)

$[N_2O]$ = Dissolved N_2O concentration ($mol \cdot L^{-1}$)

Kla, the volumetric liquid mass transfer coefficient, is define by Kl, the transfer coefficient and a the interfacial area per volume unit of reactor. The Kla value is specific of the system design and operation (e.g. mixing) as well as salinity, temperature and pH. In consequence, during this thesis, mass transfer coefficients were estimated for each cultivation system used. Kla was calculated by integrating Equation Q.2:

$$\frac{d[G]}{dt} = Kla\ ([G]^* - [G]), \text{ Equation Q.2}$$

Giving Equation Q.3:

$$ln\left(\frac{[G]^* - [G]_t}{[G]^* - [G]_0}\right) = -Kla \times t, \text{ Equation Q.3}$$

Kla_{N_2O} = N_2O mass transfer coefficient (s^{-1})

$[N_2O]^*$ = Dissolved N_2O concentration at equilibrium ($mol \cdot L^{-1}$)

$[N_2O]$ = Dissolved N_2O concentration ($mol \cdot L^{-1}$)

t = Time (s)

While only an indirect method was used to calculate Kla from the 50 L PBRs, Kla in 2 L PBRs[19] was calculated in using a direct and an indirect method. For each method used the systems were flushed with N_2. For the direct method N_2O standard was bubbled in the system and dissolved N_2O was measured with the N_2O microsensor and then Kla(N_2O) was computed using equation Q.3. For the indirect method, O_2 was bubbled in the system and dissolved O_2 was measured with a multimeter (Orion Star

[19] As described in Section 3.1.2.1.

A326, Thermo Scientific). The mass transfer coefficient for oxygen, $Kl_a(O_2)$ was then used to compute the mass transfer coefficients of N_2O (Equation Q.4), based on the diffusivities of these gases (Ye et al., 2014). The results are presented in Table Q.1.

$$Kla_{N_2O} = Kla_{O_2} \times \sqrt{\frac{D_{F_{N_2O}}}{D_{F_{O_2}}}}, \text{ Equation Q.4}$$

Kla_{N_2O} = N_2O mass transfer coefficient (s^{-1})

Kla_{O_2} = O_2 mass transfer coefficient (s^{-1})

$D_{F_{O_2}}$ = Molecular diffusivity of O_2 in water ($1.98 \times 10^{-9} \cdot m^{-2} \cdot s^{-1}$ at 20°C)

$D_{F_{N_2O}}$ = Molecular diffusivity of N_2O in water ($1.84 \times 10^{-9} \cdot m^{-2} \cdot s^{-1}$ at 20°C)

Table Q.1: Summary of the calculated and estimated (est) mass transfer coefficients (Kla) for O_2 and N_2O in 2 L and 50 L PBRs.

System	Medium	Temp (°C)	pH	$KlaO_2$ (s^{-1})	$KlaN_2O$_est (s^{-1})	$KlaN_2O$ (s^{-1})
2 L PBR	BG 11	20	7.0	0.0028 – 0.0030	0.0027 – 0.0029	0.0026 – 0.0027
50 L PBR	BG 11*	12.5 – 20	7.2	0.00085 – 0.0009	0.00082 – 0.00087	-

*Service tap water

As seen in Table Q.1, the estimated and calculated values of the N_2O mass transfer coefficients in the 2 L PBRs were similar. These results confirmed that even in a dynamic system (i.e. bubbling) the N_2O microsensor was detecting N_2O accurately. An additional experiment was performed with *C. vulgaris* cultivated in 2 L PBR in order to verify that the N_2O production calculated from the N_2O measured by the GC, or

estimated from the dissolved N_2O concentration measured by the microsensor, would give similar results.

C. vulgaris was cultivated on BG 11 in 2 L PBR at a constant temperature of 20°C. The day of the experiment, 1 mM NO_2^- was supplied in the microalgal suspension after 1 h. Gaseous and dissolved N_2O were measured with the N_2O microsensor and the GC, respectively. As seen in Figure Q.4, the N_2O production calculated from the gaseous N_2O measured by the GC or estimated from the dissolved N_2O concentration obtained by the microsensor were similar. The results suggested that the accuracy of the microsensor was satisfying.

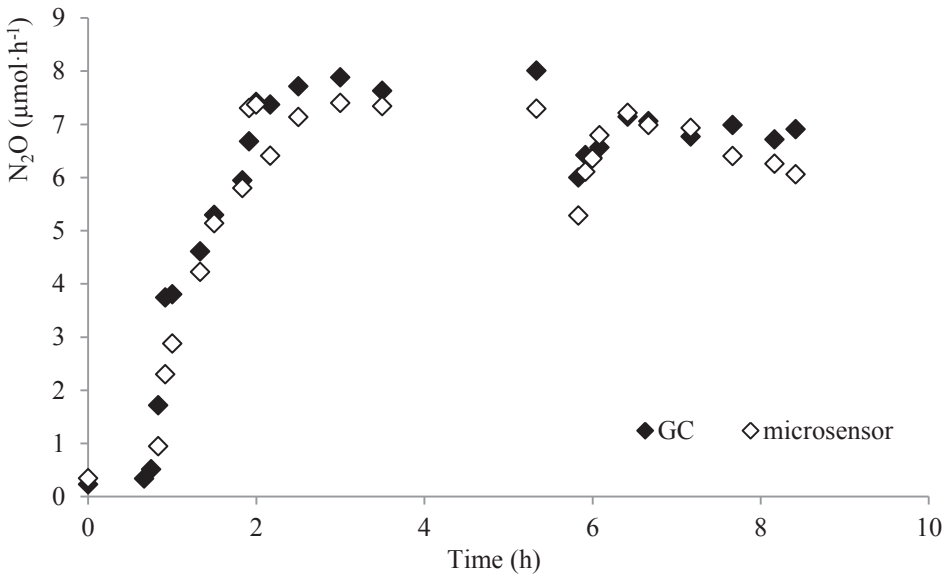


Figure Q.4: *C. vulgaris* N_2O production ($\mu mol \cdot h^{-1}$) calculated from GC measurements or estimated from dissolved N_2O concentrations measured by a N_2O microsensor. 1 mM NO_2^- was supplied after 1h.

Q.4 Main issue encounter with the N_2O and NO microsensor

The preliminary results presented in Figure P.4 seemed really promising. In order to get high temporal resolution of N_2O emission profiles during microalgal cultivation the microsensor would have been a key tool. However, the microsensors appeared to suffer from numerous issues and were unreliable when used in outdoor conditions (50 L PBR). For instance the microsensors suffered from many issues (e.g. irreproducibility between uses, long preparation needed for each use), the main being that due to membrane fragility the high cost / time of use was high (average time of use in our laboratory < 1 month). Besides, the microsensors suffered from high various interferences (e.g. NO interfere with N_2O signal) and were affected by temperature (Jenni et al., 2012). It was thus decided to stop using the microsensors due to the many issues stated above and the consequent lack of accuracy during outdoor monitoring.

References:

Jenni, S., Mohn, J., Emmenegger, L., and Udert, K.M. (2012). Temperature dependence and interferences of NO and N_2O microelectrodes used in wastewater treatment. Environ. Sci. Technol.**46**: 2257–2266.

Ye, L., Ni, B.J., Law, Y., Byers, C., and Yuan, Z. (2014). A novel methodology to quantify nitrous oxide emissions from full-scale wastewater treatment systems with surface aerators. Water Res. **48**: 257–268.

Appendix R. Correlation between dry weight, solar irradiance, ambient air temperature, broth temperature, NO_3^- concentration, NO_2^- concentration and N_2O production during *Chlorella vulgaris* cultivation in 50 L PBRs.

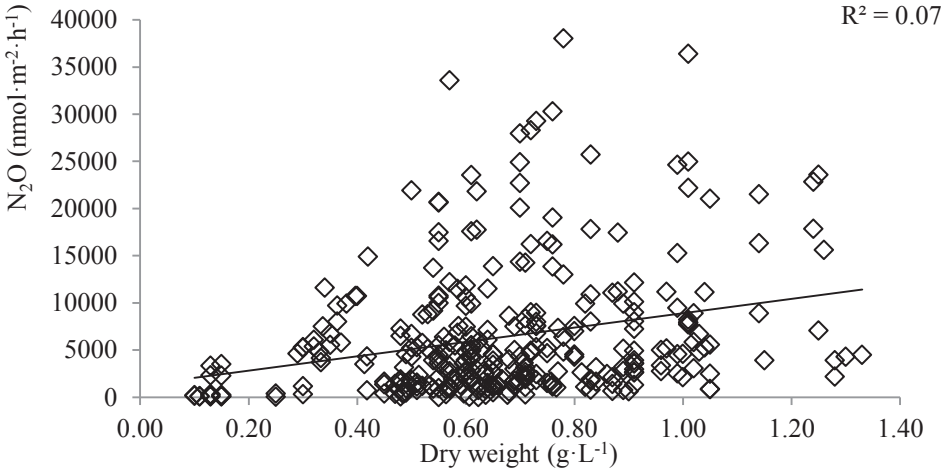


Figure R.1: Dry weight ($g \cdot L^{-1}$) against N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) during *C. vulgaris* cultivation in 50 L photobioreactors.

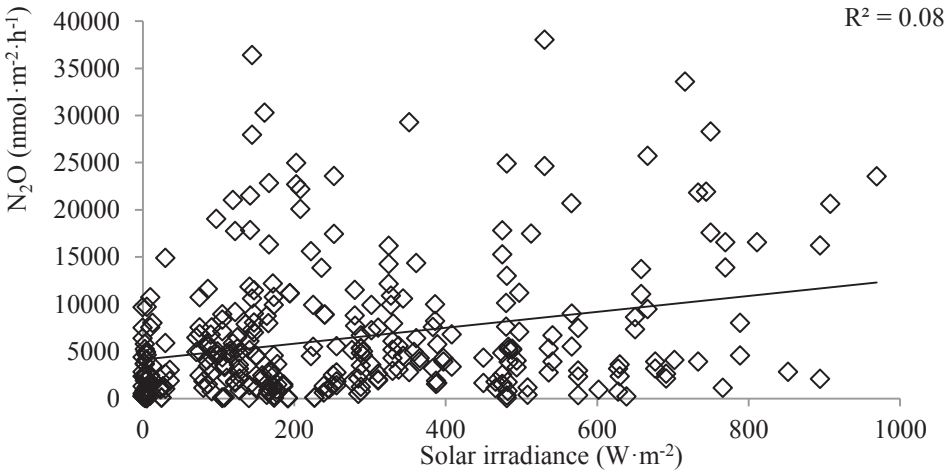


Figure R.2: Solar irradiance ($W \cdot m^{-2}$) against N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) during *C. vulgaris* cultivation in 50 L photobioreactors.

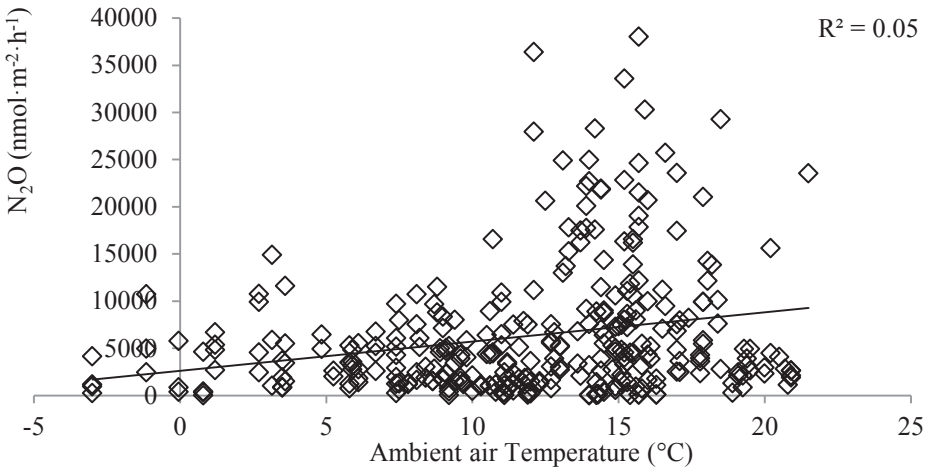


Figure R.3: Ambient air temperature (°C) against N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) during *C. vulgaris* cultivation in 50 L photobioreactors.

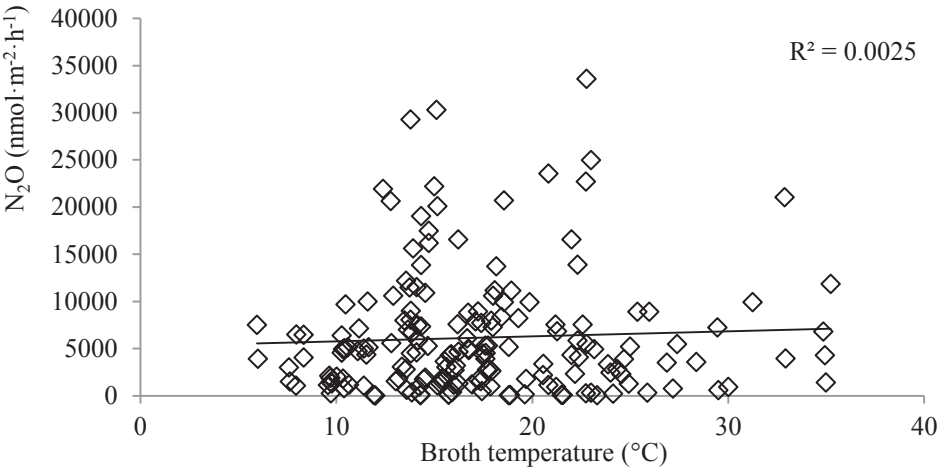


Figure R.4: Broth temperature (°C) against N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) during *C. vulgaris* cultivation in 50 L photobioreactors.

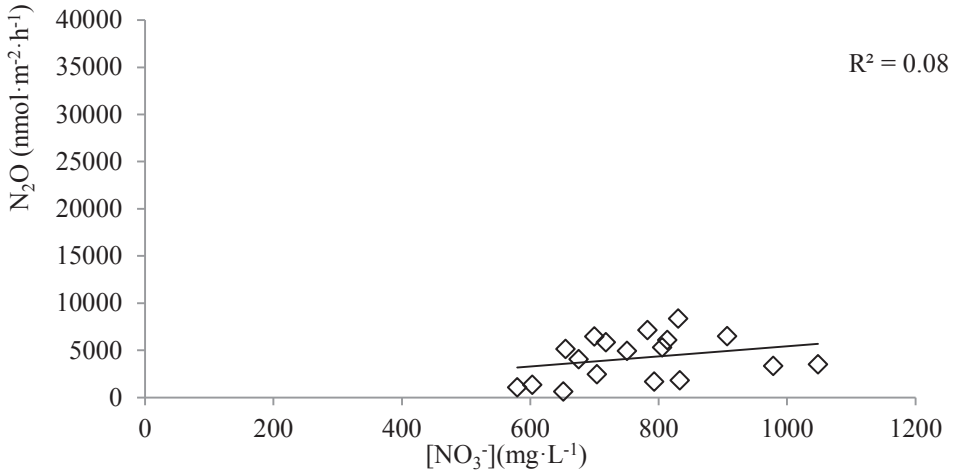


Figure R.5: NO_3^- concentration ($mg \cdot L^{-1}$) against N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) during *C. vulgaris* cultivation in 50 L photobioreactors.

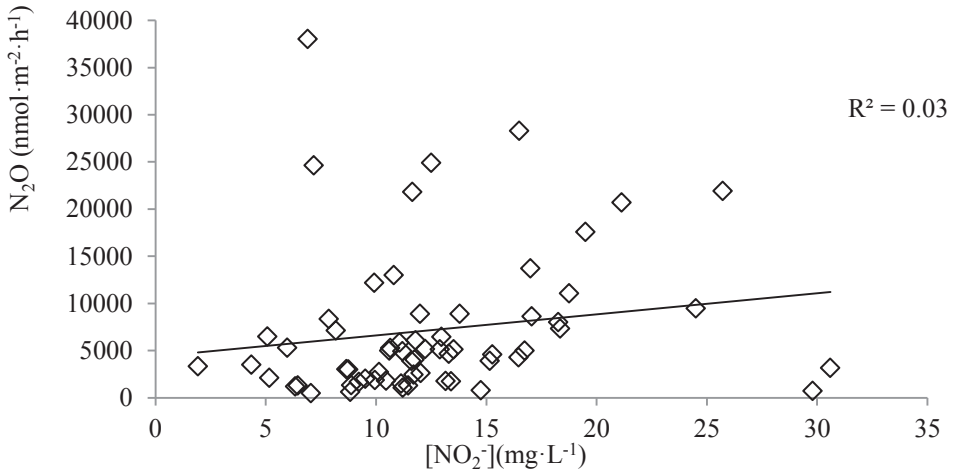


Figure R.6: NO_2^- concentration ($mg \cdot L^{-1}$) against N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) during *C. vulgaris* cultivation in 50 L photobioreactors.

Appendix S. Statistical analysis of the influence of environmental and operational parameters on N_2O emissions from *Chlorella vulgaris* cultivated in 50 L PBRs fed NO_3^-

Table S.1 summarise the variables measured during *C. vulgaris* cultivation on BG 11 with NO_3^- as nitrogen source. To identify the environmental (e.g. solar irradiance, outdoor temperature) and operational (e.g. sampling time, dry weight, broth temperature and NO_2^- concentration) parameters that could significantly influence N_2O emissions, a stepwise linear regression analysis with backward elimination method (α = 0.1) was performed in Minitab (MINITAB 16)[20]. Throughout several steps, this method removed any least significant variable at 95% confidence until the significant variables remain. It must be noted that for some parameters the datasets (e.g. broth temperature, NO_2^- concentration and pH) were incomplete in comparison with the N_2O production dataset. Therefore the missing data could bias the regression analysis output. Statistical techniques such as multiple imputations are available to deal with incomplete datasets (Horton and Lipsitz, 2001). However, when > 20% of data associated with a specific parameter are missing, the estimates of the missing information can be highly variable (Horton and Lipsitz, 2001). Considering that broth temperature, NO_2^- concentration and pH had > 20% missing values these parameters were excluded from the regression analysis to prevent flawing the analysis. All the steps followed (1 to 5) during the regression analysis are described below.

[20] Advised by Dr. Nihal Jayamaha, Massey University.

Table S.1: Summary of measured variables during *C. vulgaris* monitoring cultivated on BG 11 with NO_3^- as nutrient source.

Variables	N	Mean	Min	Max
N_2O (nmol·m^{-2}·h^{-1})	320	6235	9.50	38030
Sampling time (h)	320	N.A	N.A	N.A
DCW (g·L^{-1})	320	0.66	0.10	1.33
Solar irradiance (W·m^{-2})	320	265	0	969
Air Temp (°C)	320	11.8	-3.00	21.5
Broth Temp (°C)	180	17.4	6.00	35.2
[NO_2^-] (mg·L^{-1})	63	12.6	1.92	30.6
pH	65	7.00	6.73	7.43

Step 1-Correlations analysis:

A correlation analysis (Pearson correlation) was performed prior to the stepwise regression analysis. This step was critical in order to verify that some of the variables are not intra-correlated, that could bias the regression output (a Pearson correlation coefficient greater than 0.8 indicates a strong correlation between variables).

Correlations analysis output

	Sampl Time	DW (g·L^{-1})	N_2O (nmol·h^{-1}·m^{-2})
DW (g·L^{-1})	0.095		
N_2O (nmol·h^{-1}·m^{-2})	-0.013	0.255	
Solar IR (W·m^{-2})	-0.046	0.155	0.284
Temp out (ºC)	0.013	0.368	0.250

	Solar IR (W·m^{-2})
Temp out (ºC)	0.494

Cell Contents: Pearson correlation

As seen in the correlation analysis output above, the variables tested were not intra-correlated. The stepwise regression analysis was therefore performed.

Step-2 Stepwise regression

As stated above, a stepwise regression analysis with backward elimination method (α = 0.1) was performed.

Stepwise Regression output

Backward elimination. Alpha-to-Remove: 0.1

Response is N_2O ($nmol \cdot h^{-1} \cdot m^{-2}$) on 4 predictors, with N = 317
N(cases with missing observations) = 3 N(all cases) = 320

Step	1	2	3
Constant	340.76	-210.85	86.52
Sampl Time	-1089		
T-Value	-0.42		
P-Value	0.675		
DW ($g \cdot L^{-1}$)	5884	5816	6395
T-Value	3.46	3.44	4.02
P-Value	0.001	0.001	**0.000**
Solar IR ($W \cdot m^{-2}$)	6.7	6.8	7.7
T-Value	3.62	3.65	4.71
P-Value	0.000	0.000	**0.000**
Temp out (°C)	81	81	
T-Value	1.02	1.02	
P-Value	0.309	0.310	
S	6521	6513	6513
R-Sq	12.95	12.90	**12.61**
R-Sq(adj)	11.83	12.06	12.05
Mallows Cp	5.0	3.2	2.2

As seen in the stepwise regression analysis output, dry weight and solar irradiance were found to be the only significant variables at the 95% confidence (*p-value* < 0.05). Both increasing variables had a positive influence on the N_2O emissions.

The R^2 of the regression equation was however really weak ($R^2 = 0.13$), suggesting that the multiple regression equation generated was inadequate. Moreover, residuals generated from the regression analysis did not follow a normal distribution (Figure S.1) indicating that the regression equation produced was not statistically valid. The

residuals plot showed that residuals were getting larger for large values of the variable. Such pattern is common with highly skewed dataset and a logarithm transformation can be used to normalise the data (Levine et al., 2001).

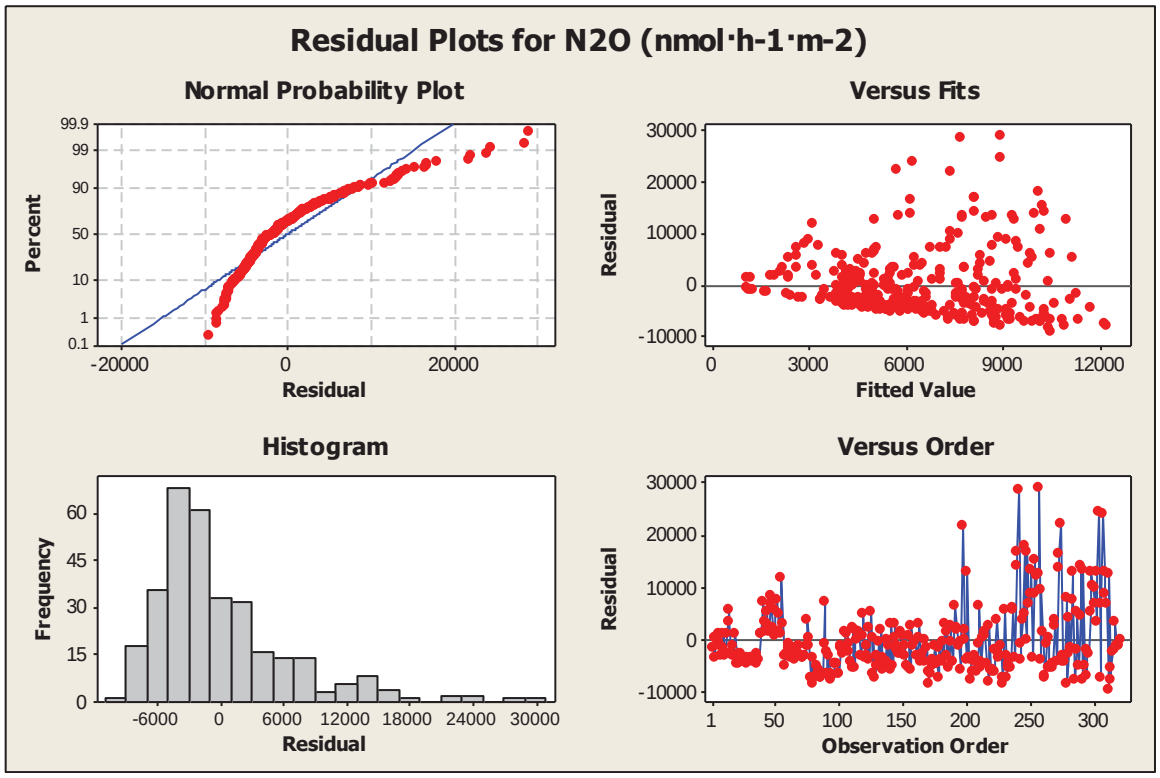


Figure S.1: Residual plots from the stepwise regression analysis.

Step-3 Data transformation (logarithm) and new multiple regression analysis

Following a logarithm transformation of the N_2O production data, another multiple regression analysis was performed.

Multiple regression analysis (#2)

The regression equation is
Ln (N_2O) = 6.50 + 1.95 DW (g/L) + 0.00118 Solar irradiance (W.m-2)

Predictor	Coef	SE Coef	T	P
Constant	6.4960	0.2103	30.89	0.000

DW ($g \cdot L^{-1}$)	1.9545	0.2930	6.67	**0.000**
Solar IR ($W \cdot m^{-2}$)	0.0011760	0.0003121	3.77	**0.000**

S = 1.24181 R-Sq = 18.1% R-Sq(adj) = 17.6%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	108.305	54.152	35.12	0.000
Residual Error	318	490.387	1.542		
Total	320	598.692			

Source	DF	Seq SS
DW (g/L)	1	86.403
Solar irradiance (W.m-2)	1	21.902

Unusual Observations

Obs	DW ($g \cdot L^{-1}$)	Ln (N2O)	Fit	SE Fit	Residual	St Resid
25	0.61	3.7227	7.6850	0.1063	-3.9623	-3.20R
29	0.62	2.2560	7.7208	0.1056	-5.4648	-4.42R
102	0.11	4.0848	6.7404	0.1812	-2.6557	-2.16R
103	0.13	4.4358	7.0171	0.1711	-2.5812	-2.10R
148	0.15	4.1361	6.9934	0.1650	-2.8573	-2.32R
208	1.33	8.4103	9.3565	0.2082	-0.9462	-0.77 X
235	0.55	2.6508	7.7964	0.0792	-5.1456	-4.15R
265	0.10	5.3239	7.4428	0.2310	-2.1189	-1.74 X
267	0.25	3.4774	7.5498	0.1657	-4.0724	-3.31R
268	0.48	4.3808	7.6596	0.0892	-3.2788	-2.65R
279	0.61	7.6429	8.7401	0.2119	-1.0972	-0.90 X
299	0.55	9.9338	8.6388	0.2210	1.2950	1.06 X
312	0.61	10.0670	8.8278	0.2340	1.2392	1.02 X

R denotes an observation with a large standardized residual.
X denotes an observation whose X value gives it large leverage.

Durbin-Watson statistic = 1.26170

The residuals plot showed that the residuals of the regression using logarithm transformed N_2O data were normally distributed. However, some outliers were still present and influenced the distribution (Figure S.2). Most importantly the residuals were found to be positively auto-correlated (Durbin-Watson statistic = 1.26 < the lower Durbin Watson given in the critical value 95% confidence table D_L = 1.81037, n = 320, k (predictors) = 2), biasing the multiple regression output. The regression analysis was consequently not statistically valid (i.e. the independent variables are inefficient, Petit-Bois et al., 2015). Various methods exist to remediate to autocorrelation. For this

analysis, the variables were transformed using the Cochrane–Orcutt procedure (Cochrane and Orcutt, 1949). The Cochrane-Orcutt procedure is an iterative process that adjusts the variables of a regression to suppress autocorrelation.

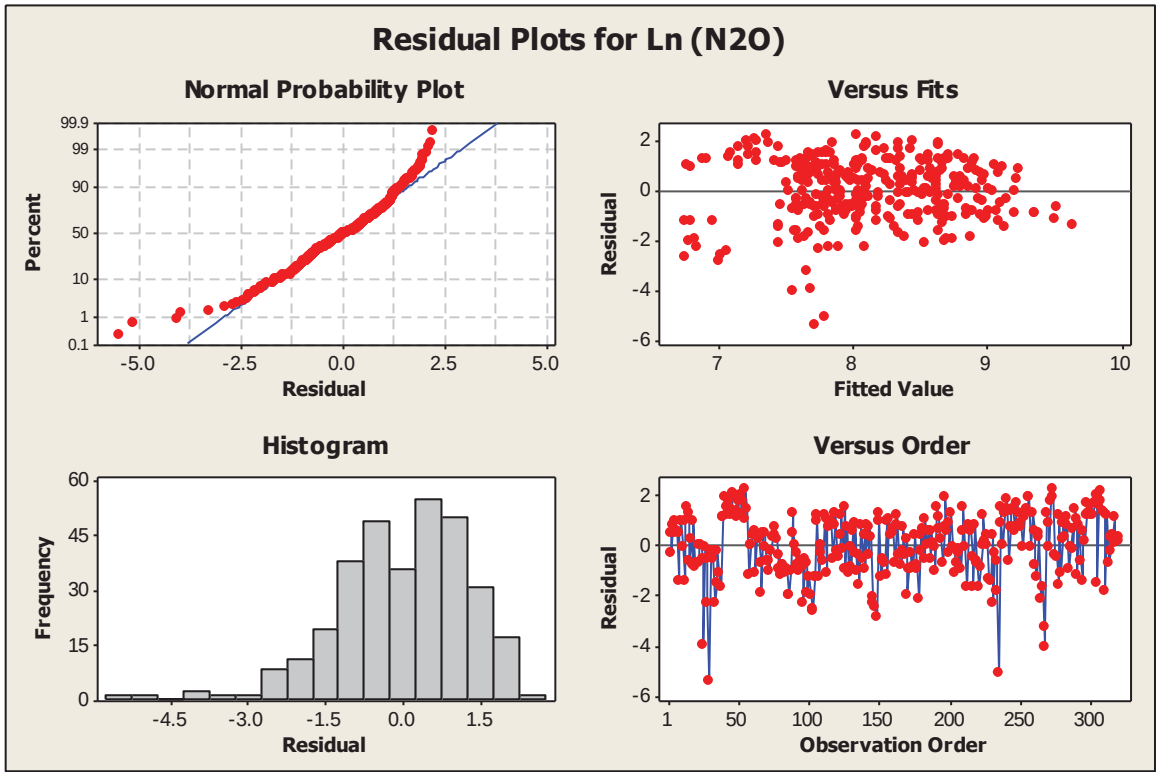


Figure S.2: Residual plots from the regression analysis performed with Logarithm transformed data.

Step-4 Data transformation using the Cochrane-Orcutt procedure

Following data transformation with the Cochrane-Orcutt procedure another multiple regression analysis was performed.

Regression Analysis (#3)

The regression equation is
T_Ln (N_2O)= 4.13 + 2.07 T_DW + 0.000689 T_Solar IR

Predictor	Coef	SE Coef	T	P
Constant	4.1298	0.1639	25.19	0.000
T_DW	2.0661	0.3444	6.00	0.000
T_Solar IR	0.0006892	0.0003283	2.10	0.037

S = 1.15171 R-Sq = 12.1% R-Sq(adj) = 11.5%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	57.888	28.944	21.82	0.000
Residual Error	317	420.482	1.326		
Total	319	478.370			

Source	DF	Seq SS
T_DW	1	52.041
T_Solar IR	1	5.847

Unusual Observations

Obs	DW (g/L)*	Ln (N2O)*	Fit	SE Fit	Residual	St Resid
25	0.35	1.0173	4.8613	0.0869	-3.8440	-3.35R
29	0.38	0.2227	4.9071	0.0845	-4.6844	-4.08R
101	-0.22	2.1593	3.6878	0.2313	-1.5285	-1.35 X
128	1.05	5.0720	6.4878	0.2228	-1.4158	-1.25 X
145	-0.26	1.1985	3.5975	0.2457	-2.3990	-2.13RX
228	1.01	5.0137	6.4907	0.2172	-1.4770	-1.31 X
231	0.01	2.2437	4.4859	0.1990	-2.2421	-1.98 X
235	0.44	0.5219	5.0493	0.0820	-4.5274	-3.94R
236	0.35	8.2904	4.9003	0.0749	3.3901	2.95R
250	0.98	6.2599	6.1674	0.2144	0.0924	0.08 X
252	0.38	5.2865	5.4062	0.1973	-0.1197	-0.11 X
265	-0.22	1.8897	3.9508	0.2505	-2.0612	-1.83 X
267	0.16	1.2876	4.6594	0.1231	-3.3718	-2.94R
269	0.30	7.2741	4.8087	0.0810	2.4653	2.15R
285	0.57	4.8451	5.8126	0.1962	-0.9676	-0.85 X
294	-0.04	3.3180	4.3528	0.2034	-1.0348	-0.91 X

R denotes an observation with a large standardized residual.
X denotes an observation whose X value gives it large leverage.

Durbin-Watson statistic = 2.06644

As seen in Figure R.3, the residuals obtained from the regression using the transformed data were normally distributed. Most importantly, the Durbin-Watson statistic (Durbin-Watson = 2.06644) fell above the upper end of the 95% confidence Durbin-Watson value (D_u = 1.82291, n = 320, k (predictors) = 2) which means that the residuals were

not autocorrelated. The variables were transformed back to their original scale and the fitted regression function thus became:

The regression equation is
Ln (N_2O)= 6.52 + 2.07 DW ($g \cdot L^{-1}$) + 0.000689 Solar IR ($W \cdot m^{-2}$)

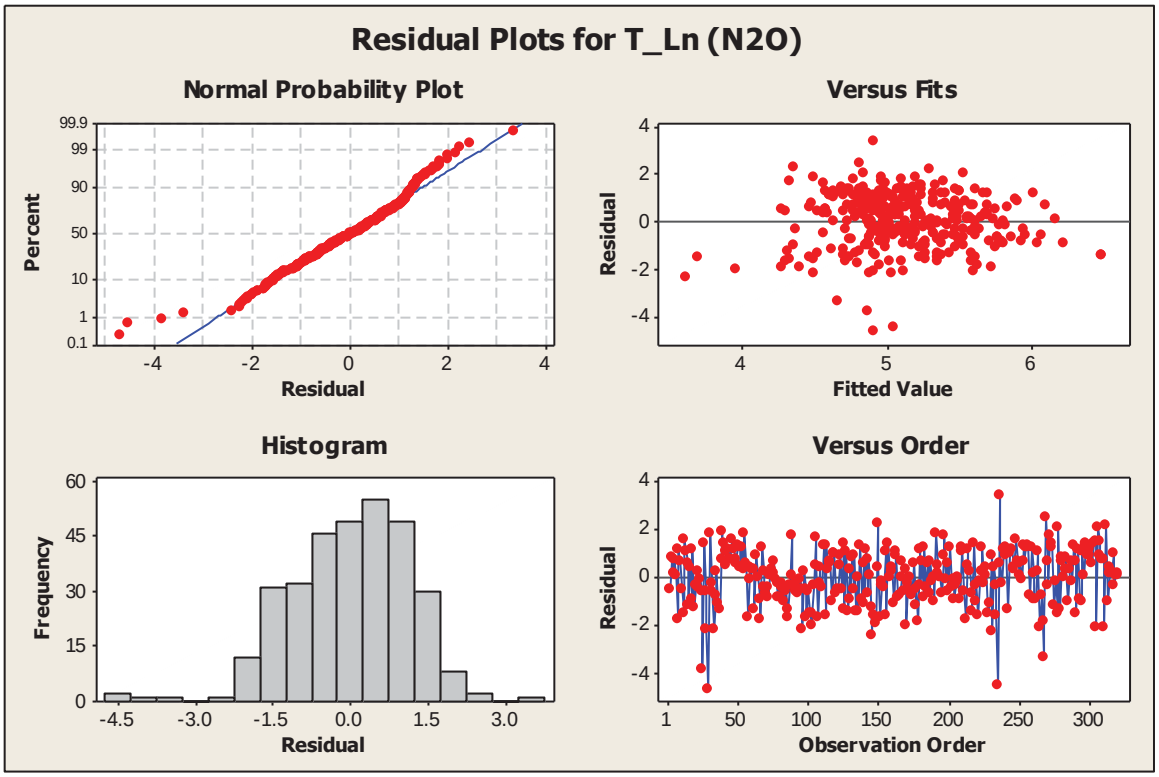


Figure S.3: Residual plots from the regression analysis performed using the transformed (Cochrane-Orcutt) data.

Step-5 Discussion/Conclusions

Stepwise regression analysis showed that dry weight and solar irradiance were found to be the only significant variables at 95% confidence (*p-value* < 0.05). The regressions

outputs (i.e. equation) were however not statistically valid because the residuals from the regression analysis were not normally distributed and auto-correlated. Consequently, data transformations (logarithm and Cochrane-Orcutt) were necessary. Even though both transformations appeared to be beneficial, the R^2 of the final multiple regression was still weak (R^2 = 0.12). Thus, even though dry weight and solar irradiance were significant variables (*p-value* < 0.05) the regression equation was not adequate due to the low R^2 (Figure S.4 is a good illustration of the non-adequacy of the regression equation). It was thus decided to not use the regression output during the evaluation of the influence of operational parameters on N_2O emissions during *C. vulgaris* cultivation. Considering that for some parameters a large number of data were missing further monitoring with more analyses would be required to potentially predict N_2O emissions from microalgal systems.

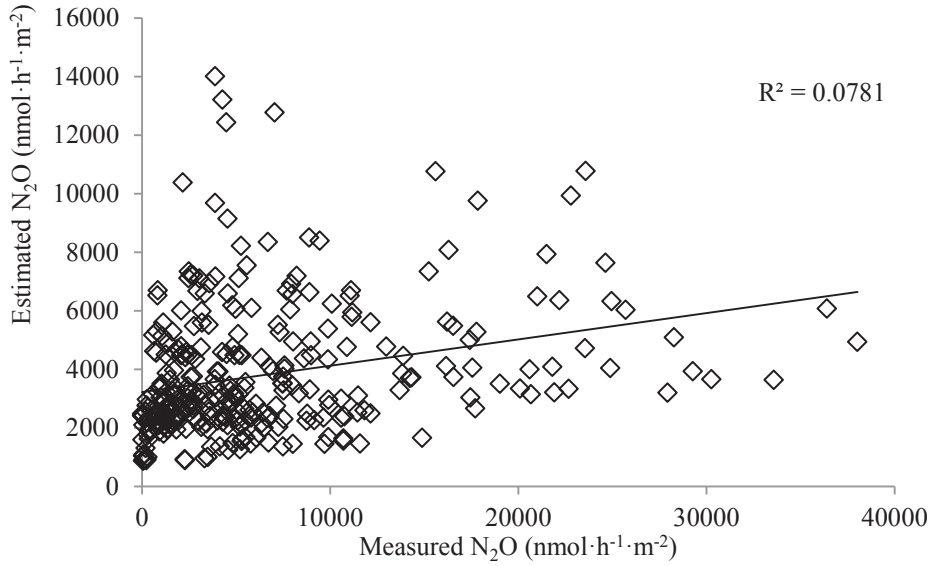


Figure S.4: Scatter plot of measured N_2O ($nmol \cdot h^{-1} \cdot m^{-2}$) against estimated N_2O ($nmol \cdot h^{-1} \cdot m^{-2}$) from multiple regression.

References:

Cochrane, D. and Orcutt, G, H. (1949). Application of Least Squares Regression to Relationships Containing Auto- Correlated Error. J. Am. Stat. Assoc. **44**: 32–61.

Levine, D.M., Ramsey, Smidt, R.K. (2001). Applied Statistics for engineers and scientists: usingmicrosoft Excel and MINITAB. 1st edition, Pearson Ed.

Horton, N.J. and Lipsitz, S.R. (2001). Multiple Imputation in Practice : Comparison of software packages for regression models with missing variables. Taylor & Francis , Ltd . on behalf of the American Statistical Associati. Am. Stat. **55**: 244–254.

Petit-Bois, M., Baek, E.K., Van den Noortgate, W., Beretvas, S.N., and Ferron, J.M. (2015). The consequences of modeling autocorrelation when synthesizing single-case studies using a three-level model. Behav. Res. Methods. **48**:803–812.

Appendix T. Correlation between dry cell weight (DCW), optical density (OD) and cell number from *microalgae* cultures

T.1 Additional experiments to establish potential correlation between DCW, OD and cell number

T.1.1 Materials and Methods

Axenic mother cultures of *C. vulgaris* were prepared in 250 mL Erlenmeyer-flasks (E-flasks) filled with 125 mL of BG 11 and inoculated with a single colony (maintained on a sterilised solid medium containing 15 $g \cdot L^{-1}$ of agar agar in a buffered BG 11) medium. Axenic liquid cultures were incubated in a Minitron incubator (Infors HT, Switzerland) at 25 ± 1°C under continuous agitation (180 rpm), constant illumination (21 W of PAR m^{-2} at the culture surface, using five 18 W Polylux coolwhite tubes), and in an atmosphere of 2% (vol.) CO_2 in air. After 1 week, 25 mL of the mother cultures were used to inoculate three 500 mL E-flasks filled with 225 mL of BG 11. Following inoculation triplicates of DCW, OD and cell counts were measured in each flask over 18 days of cultivation. The analytical methods of DCW and OD can be found in Section 3.1.2.5; cell counting was performed by using a Neubauer chamber (hemocytometer) from Celeromics (Spain) observed under a microscope.

T.1.2 Results

The evolution of DCW, OD and cell number can be found in Figure T.1.

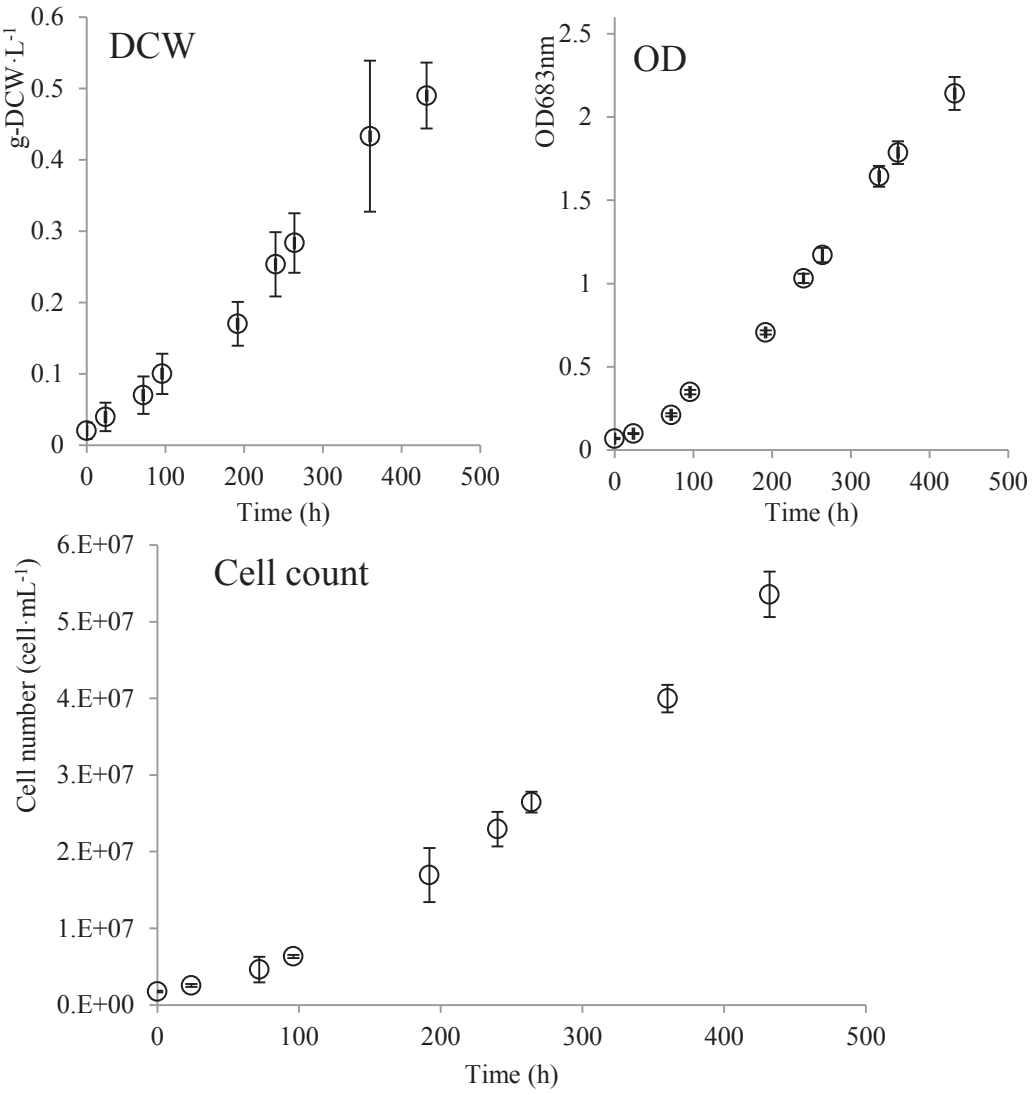


Figure T.1: DCW (average g-DCW·L^{-1} ± SD, n = 9), OD683nm (average ± SD, n = 9) and cell number (average cells·mL^{-1} ± SD, n = 9) evolution from triplicates *C. vulgaris* cultures in an incubator at 25°C under constant illumination for 18 days. The error bars represents the standard deviation.

DCW measurements had the highest standard deviations and were by consequent the least accurate variable. However, as can be seen in Figure T.2, in using averages, DCW and OD were linearly correlated (R^2 = 0.99) as well as OD and cells number (R^2 =

0.99) and DCW and cells number (R^2 = 0.98). These correlations indicate that one variable could be used to accurately estimate one of the other variables. Caution is however needed when assessing these data as microalgal cultivation under 'ideal' laboratory conditions are not representative of microalgal outdoor cultivation.

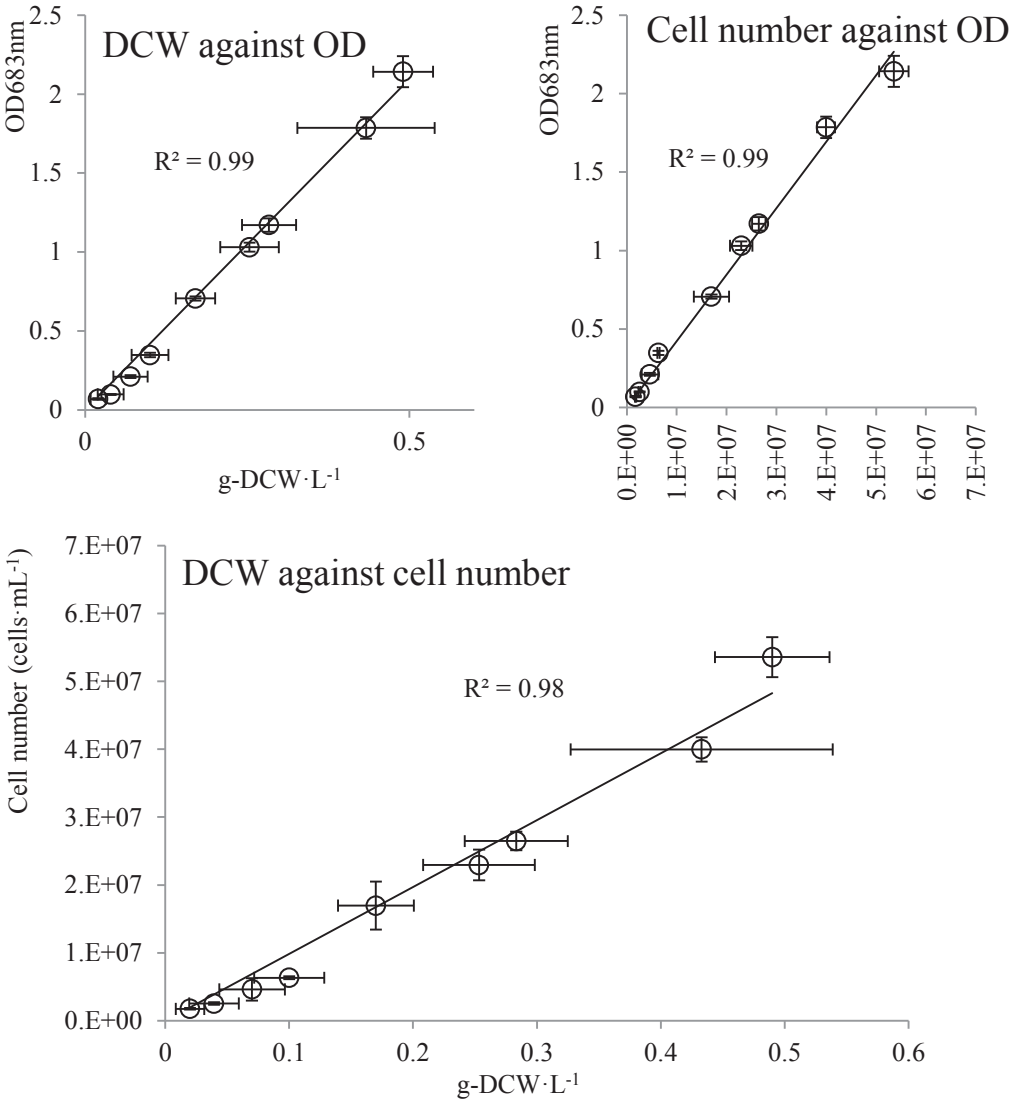


Figure T.2: Correlation between DCW and OD, cell number and OD, and DCW and cell number from the average DCW (g-DCW·L^{-1}), OD (683 nm) and cell number (cells·mL^{-1}) measured from triplicates *C. vulgaris* cultures in an incubator at 25°C under constant illumination for 18 days. The error bars represents the standard deviation.

In order to verify if the DCW measured during the outdoor cultivation of *C. vulgaris* in 50 L PBRs was actually representative of the cell number, cell counting was performed on 10 saved samples (randomly picked) from the monitoring performed in August 2014 (10 mL frozen samples at -20°C in 15 mL falcon tubes). The frozen samples were initially thawed at room temperature and then used to perform cell counting. Figure T.3 presents the DCW, previously measured, against the cell number calculated for each samples.

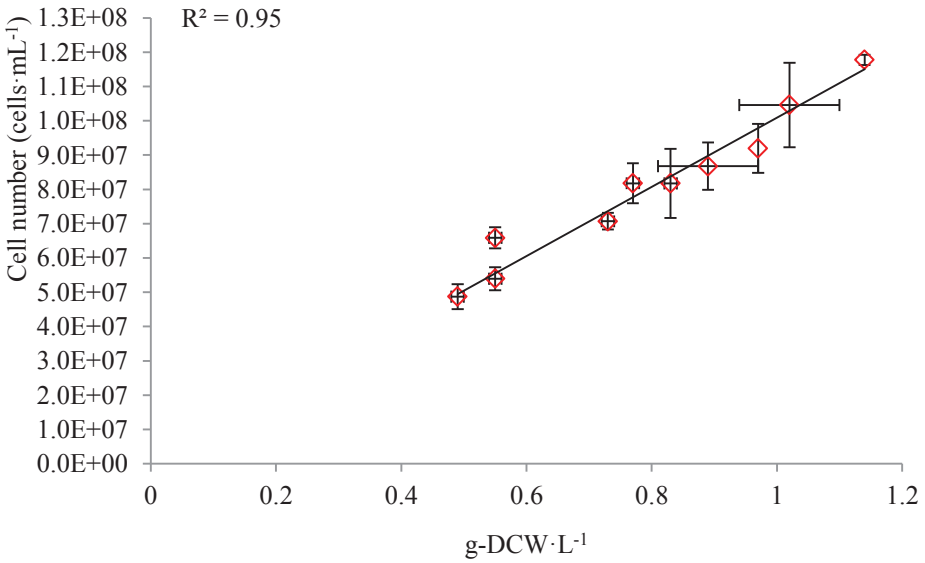


Figure T.3: Correlation between DCW (g-DCW·L^{-1}) and cell number (cells·mL^{-1}) from 10 saved samples from the monitoring performed in August 2014 (*C. vulgaris* cultures fed NO_3^- in 50 L PBRs). The error bars represents the standard deviation.

As can be seen in Figure T.3, DCW and cell number were also found to be linearly correlated during the pilot monitoring (R^2 = 0.95). Altogether, this data provide strong evidence that DCW provided a reliable proxy to cell number during the monitoring performed in our study.

T.2 OD and DCW measurements from the monitoring performed on *Chlorella vulgaris, Neochloris* and *Arthrospira platensis* cultures in 50 L PBRs

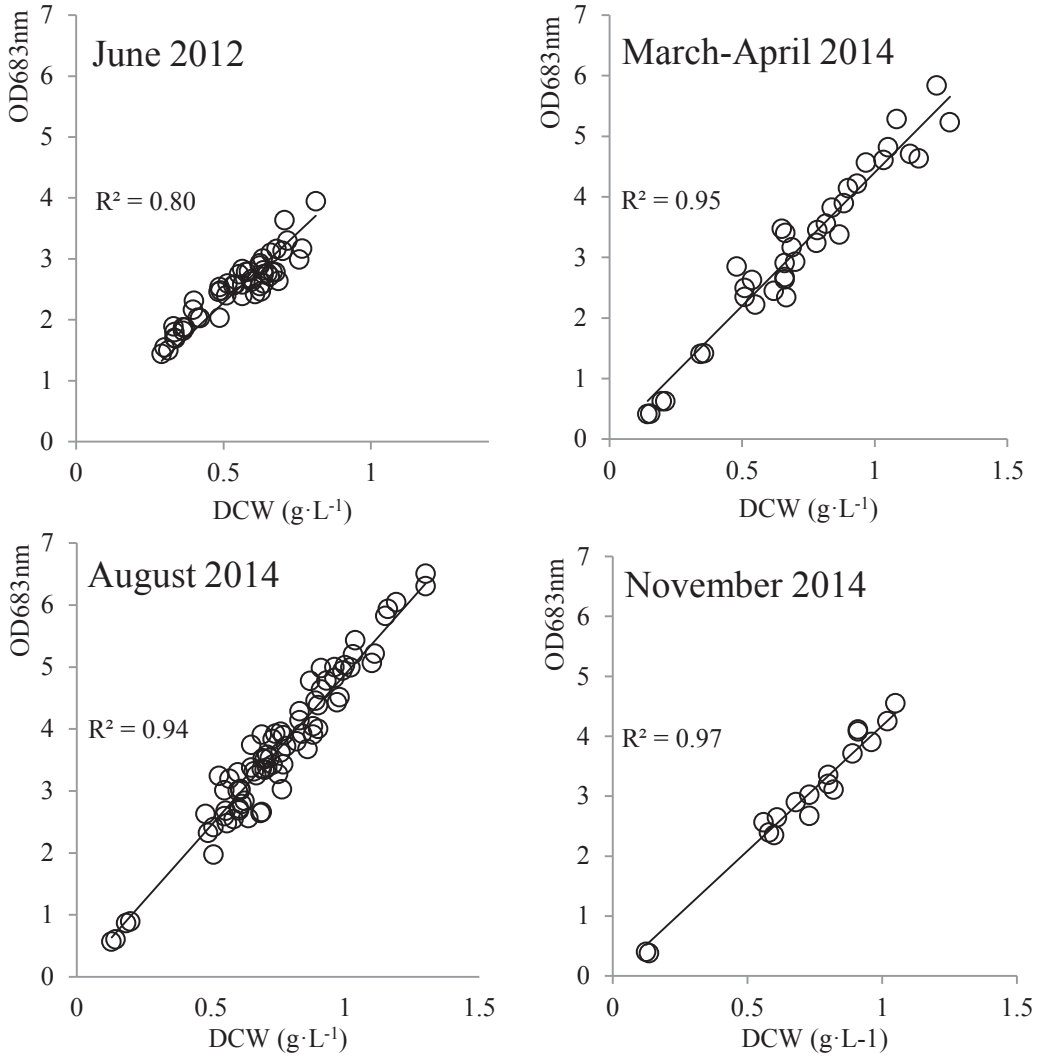


Figure T.4: DCW (g-DCW·L^{-1}) as a function of the optical density (683nm) measured from *C. vulgaris* cultures in 50 L PBRs from 4 different monitoring (June 2012, March-April 2014, August 2014 and November 2014).

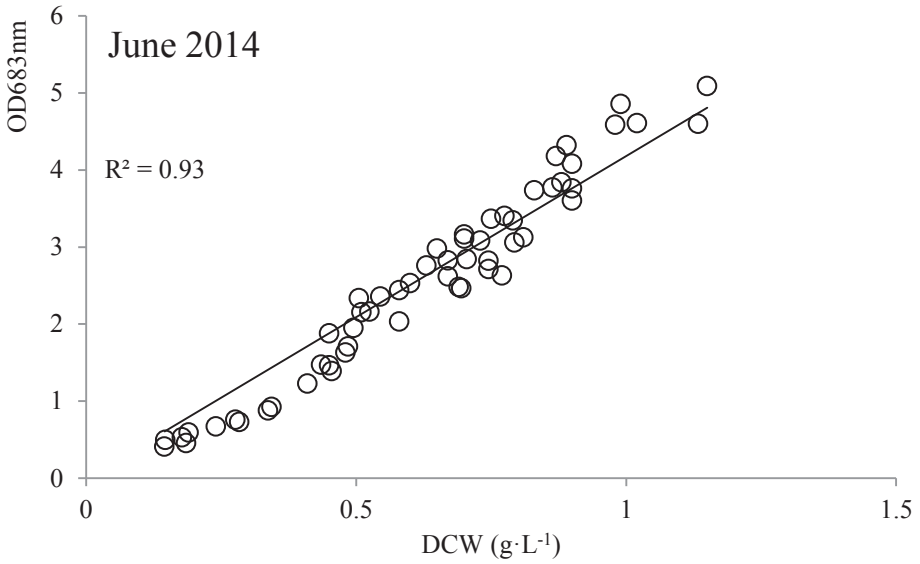


Figure T.5: DCW (g-DCW·L^{-1}) as a function of the optical density (683nm) measured from *Neochloris* cultures in 50 L PBRs from the monitoring performed in June 2014.

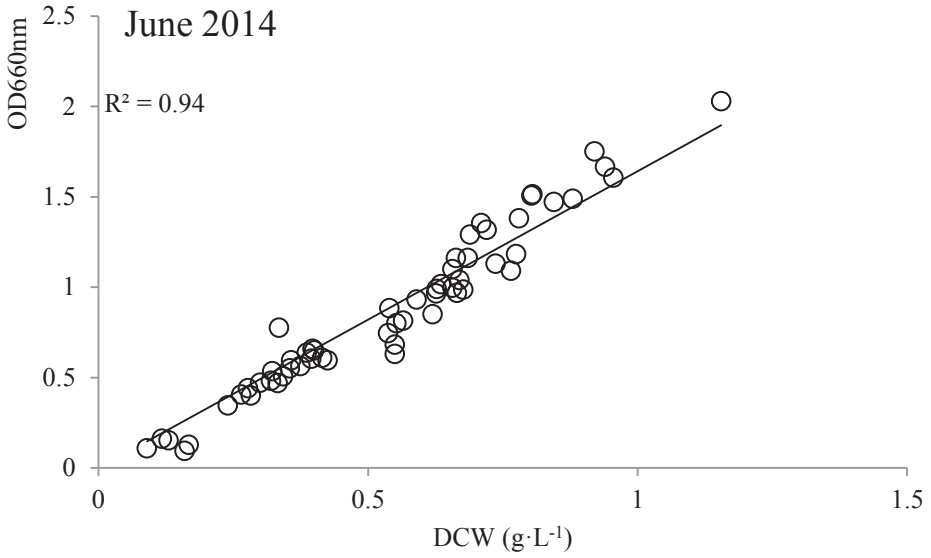


Figure T.6: DCW (g-DCW·L^{-1}) as a function of the optical density (660nm) measured from *A. platensis* cultures in 50 L PBRs from the monitoring performed in June 2014.

As can be seen in Figure T.4, T.5 and T.6, for the three microalgae species (*C. vulgaris, Neochloris and A. platensis*) cultivated in the 50 L PBRs, the measured DCW was always linearly correlated to the optical density at 683nm (R^2 = 0.80 − 0.97).

Appendix U. Daily N_2O evolution from 2 *Chlorella vulgaris* cultures in 50 L photobioreactors in August 2014

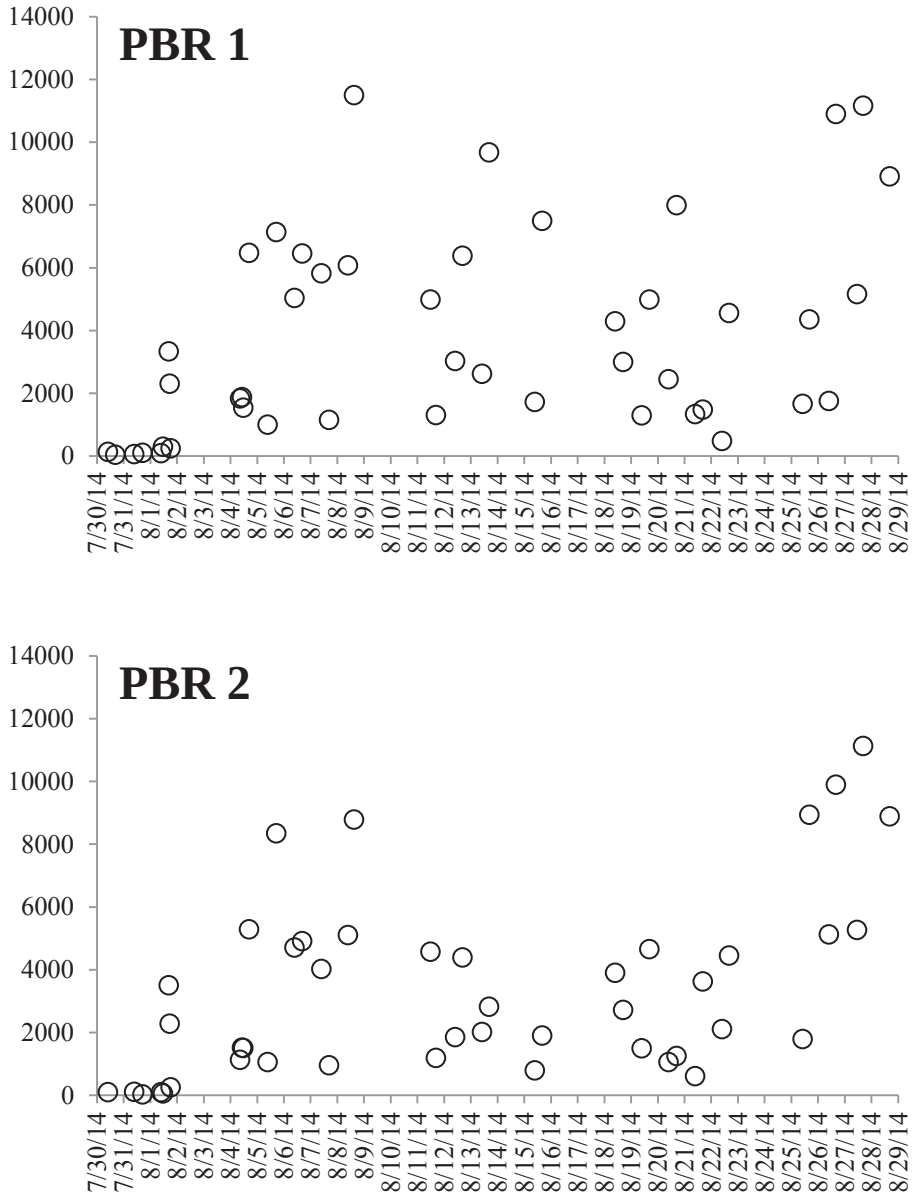


Figure U.1: Daily N_2O production rates ($nmol \cdot m^{-2} \cdot h^{-1}$) from 2 *C. vulgaris* cultures in 50 L PBRs (PBR 1 and 2) over August 2014.

Appendix V. Daily pattern between N2O production and light irradiance recorded during *Neochloris* cultivation in 50 L PBRs

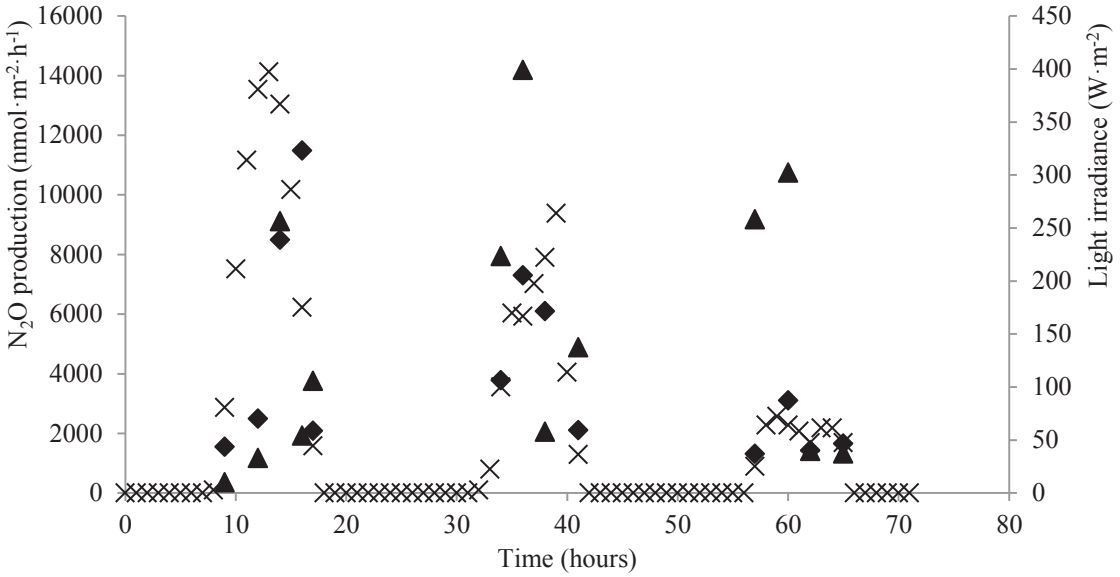


Figure V.1: Change in N_2O production rates ($nmol \cdot m^{-2} \cdot h^{-1}$) and solar irradiance (×, $W \cdot m^{-2}$) from two *Neochloris* cultures in 50 L PBRs (PBR 1: ♦ and PBR 2:▲). Pattern recorded the 23rd, 24th and 25th of June 2014. On those days of monitoring the DCW of PBR 1 were measured at 0.82, 0.90 and 0.51 $g \cdot L^{-1}$ and the DCW of PBR 2 were measured at 1.05, 1.12 and 0.63 $g \cdot L^{-1}$.

Appendix W. Correlation between monthly averaged N_2O production and monthly averaged biomass specific light availability

Following a reviewer suggestion (Algal research), N_2O emissions ($nmol \cdot m^{-2} \cdot h^{-1}$) were plotted against irradiance (I_0, $W \cdot m^{-2}$) divided by biomass concentration ($g\text{-}DCW \cdot m^{-2}$) (with a reactor density of $1 \cdot m^{-2}$ (as described by Béchet et al., 2014). While we found no correlation when analysing the entire data set (Figure W.1), a good linear correlation was found when using monthly averaged data (Figure W.2, $R^2 = 0.92$). While this must be viewed cautiously given the large N_2O variability and unevenly distributed data, this could indicate that NO_2^- intracellular accumulation is closely linked to the photosynthetic output during outdoor cultivation (i.e. N_2O production is correlated to the amount of light actually received). Importantly, while interesting 'fundamentally' this correlation is currently not particularly helpful for environmental assessment as it would require prior knowledge (or prediction) of cell concentration.

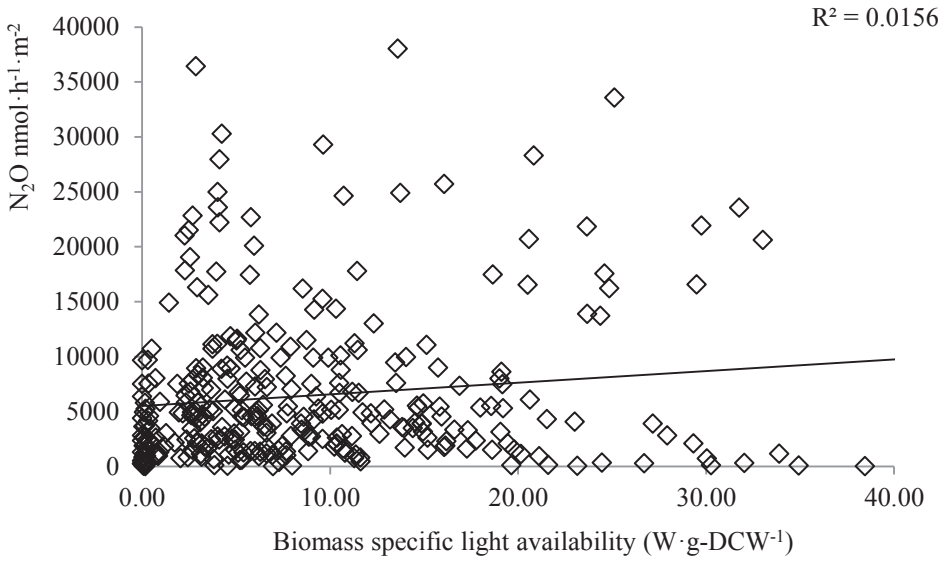


Figure W.1: N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) as a function of the biomass specific light availability ($W \cdot g\text{-}DCW^{-1}$). The biomass specific light availability was calculated by dividing the light impinging over the reactor ground ($W \cdot m^{-2}$) by the biomass concentration per ground area ($g\text{-}DCW \cdot m^{-2}$).

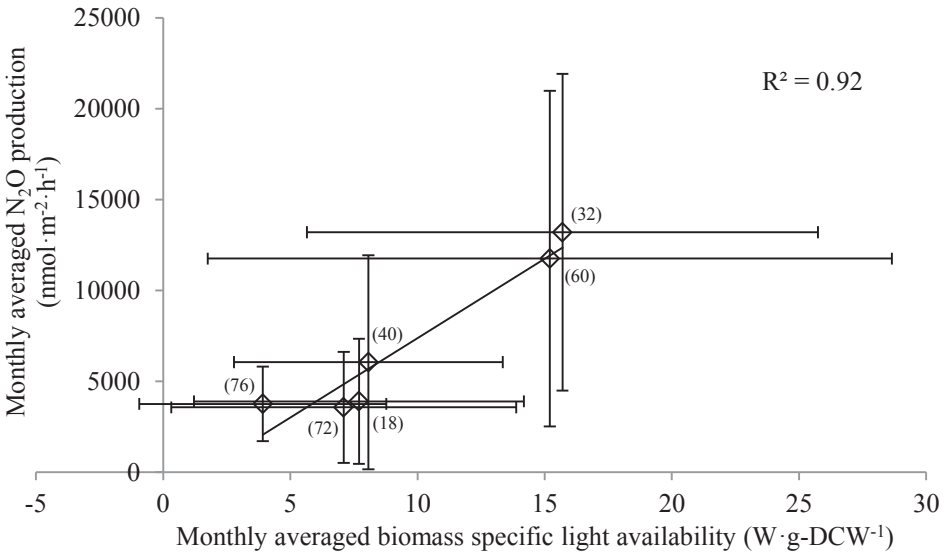


Figure W.2: Monthly averaged N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) as a function of the monthly averaged biomass specific light availability ($W \cdot g\text{-}DCW^{-1}$). Numbers in brackets represent the N_2O sampling size used to calculate the average, March (n = 18); June (n = 76); August (n = 72); October (n = 40); November (n = 60); December (n = 32). The error bars represent the standard deviation.

Appendix X. N_2O production by *Chlorella vulgaris* cultures in 2 L bench scale reactors when fed NH_4^+ or NO_3^- supplied with 0.5 mM NO_2^-

Experiments were carried out in two 2 L bench scale PBRs (PBR A and PBR B)[21]. *C. vulgaris* was cultivated in PBR A fed NO_3^- and PBR B fed NH_4^+ (Same relative concentration of N). The day of the experiment, both reactors were supplied with 0.5 mM NO_2^-. Gas samples were regularly withdrawn with a thermos-syringe from a hole in the lid and analysed by gas chromatography. As seen in Figure X.1, *C. vulgaris* fed NH_4^+ possessed the ability to synthesise N_2O after an addition of 0.5 mM exogenous NO_2^- (PBR B). Although higher N_2O production would be expected with higher NO_2^- supply, the N_2O production were 25 times lower from *C. vulgaris* cultures fed NH_4^+ (PBR B) than from *C. vulgaris* cultures fed NO_3^- (PBR A). See discussion in Chapter 3 Section 3.1.3.4.

[21] As described in Section 3.1.2.1.

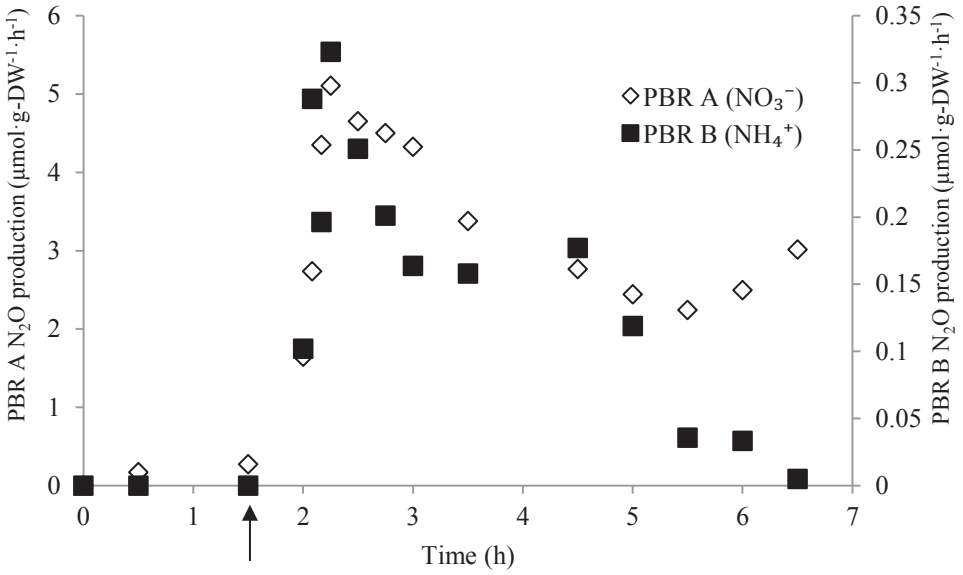


Figure X.1: N_2O production (µmol·g-DCW^{-1}·h^{-1}) in two 2 L indoor PBRs with *C. vulgaris*. PBR A was fed NO_3^- and PBR B was fed NH_4^+. Both PBRs were under continuous illumination and supplemented with 0.5 mM NO_2^- (Arrow).

Appendix Y: Data distribution (dry weight, light intensity, air temperature and N_2O production) over the data range chosen for simulation (first and third quartile of N_2O production during *Chlorella vulgaris* cultivation in 50 L PBRs fed NO_3^-).

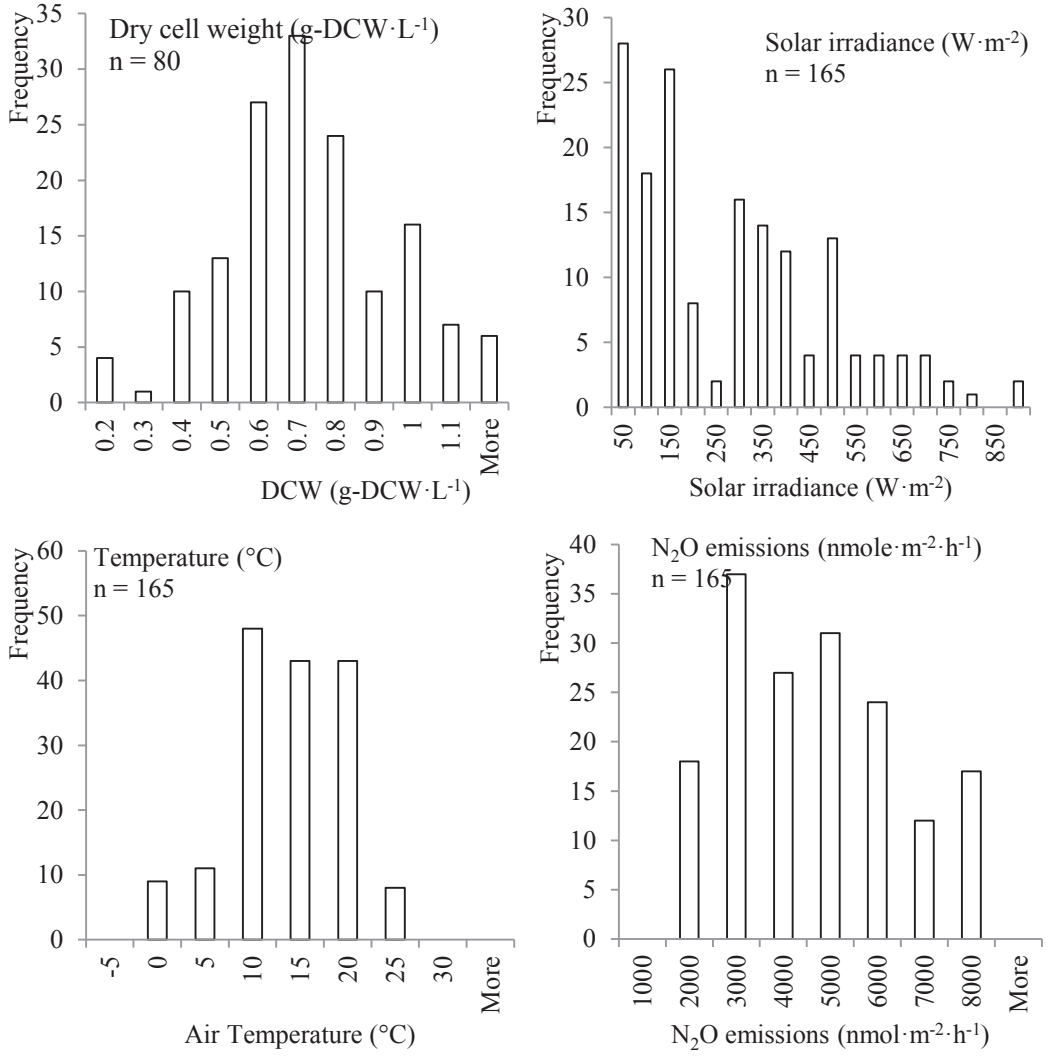


Figure Y.1: Histograms of dry weight ($g \cdot L^{-1}$), light intensity ($W \cdot m^{-2}$), temperature (°C) and N_2O production ($nmole \cdot m^{-2} \cdot h^{-1}$) distribution over the range chosen for simulation (first and third quartile of N_2O production rates, 1500 – 8000 nmol $N_2O \cdot h^{-1} \cdot m^{-2}$).

Appendix Z. N_2O quantification from a HRAP treating domestic wastewater

Z.1 Sampling optimisation

In order to evaluate the best sampling method and test conditions to evaluate N_2O emissions from the HRAP, sampling optimisation was performed. The parameters tested for sampling optimisation are summarised in Table Z.1. Sampling flasks were used to estimate dissolved N_2O concentration in the HRAP and calculate N_2O emissions. Liquid samples were withdrawn from the HRAP with a thermos syringe and poured in gas tight glass bottle to prevent air entrainment. Prior to sampling, flasks were flushed with N_2 in order to create a neutral atmosphere and ease N_2O transfer from the liquid to the gas phase. Under such conditions, an equilibrium between the liquid and the gas phases was rapidly reached (< 20 min) as no statistically difference was observed between the amount of N_2O measured at t = 5 min and t = 10 min as well as t = 25 min (two sample t-test, $\alpha = 0.05$, *p-value* <0.05). It was therefore decided to quantify N_2O in the gas phase 30 minutes after sampling and with biological N_2O production always negligible in this time period (see below). Two different flask sizes, 122 ± 2 mL (referred as serum flasks) or 12.5 ± 1 mL (referred as vials) were tested for sampling. N_2O data obtained (after re-calculation) showed no significant difference between the two containers used (two sample t-test, $\alpha = 0.05$, *p-value* <0.05). It was therefore decided to use vials for dissolved N_2O measurements in the HRAP and serum flasks for batch assays. Due to higher volume, serum flasks indeed allowed for several gas samples/flasks, to evaluate N_2O generation kinetics after a supply of NO_2^-.

HRAP mixed with a simple paddle-well are supposedly well-mixed (Benemann et al., 1987). The HRAP used in this study was well-mixed as evidence by the fact that no significant difference was noted between dissolved N_2O concentration (two sample t-test, $\alpha = 0.05$, *p-value* <0.05) when liquid samples were withdrawn at various depths in the HRAP. Considering that N_2O is produced biologically, a poison can be used to inhibit microbial activity and in consequence to prevent N_2O production between sampling and analysis (Ferrón et al., 2012; Kamp et al., 2013). Therefore, the use of $ZnCl_2$ was tested and after 30 min, no significant difference was observed between the poisoned and live samples (two sample t-test, $\alpha = 0.05$, *p-value* <0.05). This result showed that the N_2O transfer from the liquid to the gas phase was faster than biological N_2O production. Moreover, results from our laboratory showed that heavy metal could cause a stress effect in microalgae, at time boosting N_2O synthesis (data not shown). Consequently the use of poison was not needed and risky with the sampling method used.

Table Z.1: Summary of the methods used for sampling optimization

Optimisation	**Methods**	**Significant: Yes or No**	**Comment**
Equilibration time	N_2O measured regularly in flasks between 0 – 3 h	Yes	An equilibrium between the liquid and the gas phase was rapidly reached (< 20 min). Because between 1 – 3 h N_2O concentration could evolve considerably, 30 min was chosen as being representative.
Depth of sampling	Sampling performed at different depth 0.05-0.25 m in the HRAP	No	Sampling depth had no significant effect on the concentration of dissolved N_2O concentration measured.
Poison the samples	$ZnCl_2$ (50%) was added to inhibit the microbial biomass	No	No significant effect in the 30 min between sampling and analysis.

References:

Benemann, J.R., Tillett, D.M., and Weissman, J.C. (1987). Microalgae biotechnology. Tibtech **5**: 358–363.

Ferrón, S., Ho, D.T., Johnson, Z., I., and Huntley, M., E. (2012). Air − Water fluxes of N_2O and CH_4 during microalgae (*Staurosira sp.*) cultivation in an open raceway pond. Environ. Sci. Technol. **46**: 10842–10848.

Kamp, A., Stief, P., Knappe, J., and De Beer, D. (2013). Response of the ubiquitous pelagic Diatom *Thalassiosira weissflogii* to darkness and anoxia. PLoS One **8**: 1–11.

Appendix AA. Distribution of the N_2O production measured from HRAP microcosms and statistical analysis of the data between operation at 7 and 10 days HRT

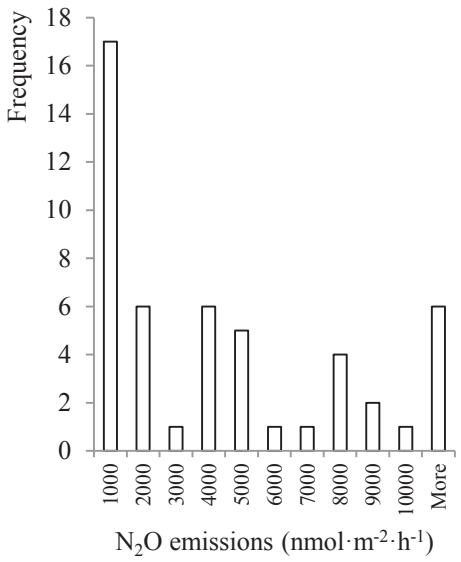


Figure AA.1: N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$) distribution from the HRAP microcosms monitoring.

Table AA.1: Summary of the two sample t-test (α = 0.05) analyses of the data (N_2O production, daily averaged air temperature, broth temperature at sampling, daily averaged solar irradiance and N_2O specific rates recorded from the batch assays) between operation at 7 and 10 days HRT.

	HRT	**N**	**Mean**	**Standard deviation**	**Standard error**	**p-value (t-test, α = 0.05)**
N_2O production ($nmol \cdot m^{-2} \cdot h^{-1}$)	7	22	8400	7230	1540	< 0.05
	10	28	2010	2510	470.0	
Daily averaged air temperature (ºC)	7	22	16.4	3.85	0.82	< 0.05
	10	28	11.2	3.80	0.72	
Broth temperature at sampling (ºC)	7	22	16.1	3.87	0.82	< 0.05
	10	28	10.9	4.31	0.81	
Daily averaged solar irradiance ($W \cdot m^{-2}$)	7	22	181	88.0	19.0	< 0.05
	10	28	125	82.0	15.0	

Statements of contribution to doctoral thesis containing publications

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Maxence Plouviez

Name/Title of Principal Supervisor: Prof. Benoit Guieysse

Name of Published Research Output and full reference:

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

In which Chapter is the Published Work: 1

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  M Plouviez was the main contributor of the literature review.

Maxence Plouviez Digitally signed by Maxence Plouviez Date: 2016.11.28 10:29:21 +13'00'

Candidate's Signature

28/11/2016

Date

Professor Benoit Guieysse, PG Director Digitally signed by Professor Benoit Guieysse, PG Director DN: cn=Professor Benoit Guieysse, PG Director, o=Massey University, ou=SEAT, email=b.j.guieysse@massey.ac.nz, c=NZ Date: 2016.11.28 12:24:11 +13'00'

Principal Supervisor's signature

28/11/2016

Date

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Maxence Plouviez

Name/Title of Principal Supervisor: Prof. Benoit Guieysse

Name of Published Research Output and full reference:

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. (Submitted)

In which Chapter is the Published Work: 2

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  M Plouviez was the main contributor of the manuscript. He carried experimental work, data analysis and he wrote most of the manuscript.

Maxence Plouviez Digitally signed by Maxence Plouviez Date: 2016.11.28 10:29:21 +13'00'

Candidate's Signature

28/11/2016

Date

Professor Benoit Guieysse, PG Director Digitally signed by Professor Benoit Guieysse, PG Director DN: cn=Professor Benoit Guieysse, PG Director, o=Massey University, ou=SEAT, email=b.j.guieysse@massey.ac.nz, c=NZ Date: 2016.11.28 12:24:11 +13'00'

Principal Supervisor's signature

28/11/2016

Date

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Maxence Plouviez

Name/Title of Principal Supervisor: Prof. Benoit Guieysse

Name of Published Research Output and full reference:

Plouviez, M.; Shilton, A.; Packer, M.; Thuret-Benoist H.; Alaux, E.; Guieysse, B. Nitrous oxide (N2O) emissions from microalgae cultures in 50 L photobioreactors. (Ready for submission)

In which Chapter is the Published Work: 3

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  M Plouviez was the main contributor of the manuscript. He carried some of the experimental work, and he supervised H Thuret-Benoist and E Alaux who performed part of the experimental work. M Plouviez also analysed the data and he wrote most of the manuscript.

Maxence Plouviez Digitally signed by Maxence Plouviez Date: 2016.11.28 10:29:21 +13'00'

Candidate's Signature

28/11/2016

Date

Professor Benoit Guieysse, PG Director Digitally signed by Professor Benoit Guieysse, PG Director DN: cn=Professor Benoit Guieysse, PG Director, o=Massey University, ou=SEAT, email=b.j.guieysse@massey.ac.nz, c=NZ Date: 2016.11.28 12:24:36 +13'00'

Principal Supervisor's signature

28/11/2016

Date

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Maxence Plouviez

Name/Title of Principal Supervisor: Prof. Benoit Guieysse

Name of Published Research Output and full reference:

Plouviez, M.; Guieysse, B.; Shilton, A.; Packer, M.; Thuret-Benoist, H.; Alaux, E. N2O (Nitrous oxide) emissions during full-scale microalgae cultivation outdoors. (BFFM 2015, Montpellier Supagro, France)

In which Chapter is the Published Work: 3

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  M Plouviez was the main contributor of the conference paper. He carried some of the experimental work, and he supervised H Thuret-Benoist and E Alaux who performed part of the experimental work. M Plouviez also analysed the data, wrote most of the manuscript and did the oral presentation at the international conference BFFM 2015.

Maxence Plouviez Digitally signed by Maxence Plouviez Date: 2016.11.28 10:29:21 +13'00'

Candidate's Signature

28/11/2016

Date

Professor Benoit Guieysse, PG Director Digitally signed by Professor Benoit Guieysse, PG Director DN: cn=Professor Benoit Guieysse, PG Director, o=Massey University, ou=SEAT, email=b.j.guieysse@massey.ac.nz, c=NZ Date: 2016.11.28 11:55:29 +13'00'

Principal Supervisor's signature

28/11/2016

Date

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Maxence Plouviez

Name/Title of Principal Supervisor: Prof. Benoit Guieysse

Name of Published Research Output and full reference:

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. (Ecotechnologies for wastewater treatment, Cambridge, United Kingdom, 2016)

In which Chapter is the Published Work: 3

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  M Plouviez carried some of the experimental work, and he supervised R Lebrun who performed part of the experimental work. M Plouviez analysed the data, help writing and editing the conference paper.

Maxence Plouviez Digitally signed by Maxence Plouviez Date: 2016.11.28 10:29:21 +13'00'

Candidate's Signature

28/11/2016

Date

Professor Benoit Guieysse, PG Director Digitally signed by Professor Benoit Guieysse, PG Director DN: cn=Professor Benoit Guieysse, PG Director, o=Massey University, ou=SEAT, email=b.j.guieysse@massey.ac.nz, c=NZ Date: 2016.11.28 12:23:34 +13'00'

Principal Supervisor's signature

28/11/2016

Date