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N\textsubscript{2}O synthesis by microalgae: Pathways, significance and mitigations

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

Doctor of Philosophy

in Environmental Engineering

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Abstract

Over the last decades, various studies have reported the occurrence of emissions of nitrous oxide (N$_2$O) from aquatic ecosystems characterised by a high level of algal activity (e.g. eutrophic lakes) as well as from algal cultures representative of the processes used by the algae biotechnology industry. As N$_2$O is a potent greenhouse gas (GHG) and ozone depleting pollutant, these findings suggest that large scale microalgal cultivation (and possibly, eutrophic ecosystems) could contribute to the global N$_2$O budget. Considering the current rapid development of microalgal biotechnologies and the ubiquity of microalgae in the environment, this PhD research was undertaken to determine the biochemical pathway of microalgal N$_2$O synthesis and evaluate the potential significance of microalgal N$_2$O emissions with regard to climate change.

To determine the pathway of N$_2$O 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$_2$O under aerobic conditions. The results evidenced that N$_2$O synthesis involves 1) NO$_2^-$ reduction into nitric oxide (NO), followed by 2) NO reduction into N$_2$O 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$_2$O is
synthesised via NR-ARC-mediated NO$_2^-$ reduction under physiological conditions (i.e. low/moderate intracellular NO$_2^-$) but that under NO$_2^-$ stress (i.e. induced by high intracellular NO$_2^-$), N$_2$O synthesis involves both NR-ARC-mediated and COX-mediated NO$_2^-$ reductions. RNA sequencing analysis on *C. reinhardtii* samples confirmed that the genes encoding ARC, COX and NOR were expressed in NO$_2^-$-laden culture, although NO$_2^-$ addition did not trigger significant transcriptomic regulation of these genes. We therefore hypothesise that the microalgal N$_2$O pathway may be involved in NO regulation in microalgae where NOR acts as a security valve to get rid of excess NO (or NO$_2^-$).

To evaluate N$_2$O emissions during microalgal cultivation, N$_2$O emissions were quantified during the long term outdoor cultivation of commercially relevant microalgae species (*Chlorella vulgaris*, *Neochloris* sp. and *Arthrospira platensis*) in 50 L pilot scale tubular photobioreactors (92 days) and during secondary wastewater treatment in a 1000 L high rate algal pond (365 days). Highly variable N$_2$O emissions were recorded from both systems (0.0 – 38 μmol N$_2$O·m$^{-2}$·h$^{-1}$, n = 510 from the 50 L photobioreactors; 0.008 – 28 μmol N$_2$O·m$^{-2}$·h$^{-1}$, n = 50 from the high rate algal pond). Based on these data, we estimated that the large scale cultivation of microalgae for biofuel production in order to, for example, replace 30% of USA transport fuel with algal-derived biofuel (i.e. a commonly used sustainability target), could generate N$_2$O emissions representing up to 10% of the currently budgeted global anthropogenic N$_2$O emissions. In contrast, N$_2$O emissions from the microalgae-based pond systems commonly used for wastewater treatment would represent less than 2% of the currently budgeted global N$_2$O emissions from wastewater treatment. As emission factors to
predict N\textsubscript{2}O emissions during microalgae cultivation and microalgae-based wastewater
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provide simple mitigation solutions.
Acknowledgments

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

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

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Structure of the thesis

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

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

Chapter 1


Chapter 2


Chapter 3

Plouviez, M.; Shilton, A.; Packer, M.; Thuret-Benoist H.; Alaux, E.; Guieysse, B. Nitrous oxide (N2O) emissions from microalgae cultures in 50 L photobioreactors. (*Accepted (with revisions) in Algal Research*).

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


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

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

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

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

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

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


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

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

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

AOA: Ammonia-oxidizing archaea  
AOB: Ammonia-oxidizing bacteria  
AOX: Alternative oxidase  
ARC: Amidoxime reducing component  
CN\': Cyanide ion  
COX: Cytochrome c oxidase  
DAF FM Diacetate: 4-amino-5-methylamino-2',7'-difluore-fluorescein diacetate  
DEA NONOate: diethylamine NONOate  
DCW: Dry cell weight  
DO: Dissolved oxygen  
E-flasks: Erlenmeyer flasks  
EFs: Emissions factors  
Fd: Ferredoxin  
GC: Gas chromatography  
GHG: Greenhouse gas  
HNO: Nitroxyln  
HRAP: High rate algae pond  
IPCC: Intergovernmental Panel for Climate change  
L-Arg: L-arginine  
L-NNA: N\(\omega\)-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  
NOF NiR: 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
N₂O: 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\textsubscript{2}O synthesis and defined research knowledge gaps. In addition, using simple and conservative estimations this chapter was evaluating if microalgal N\textsubscript{2}O emissions could be a potential issue. The aim was to evaluate if we should consider microalgal N\textsubscript{2}O 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):

ABSTRACT

This chapter discusses the potential significance of nitrous oxide (N₂O) 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 N₂O emissions from eutrophic lakes alone could yield global N₂O emissions equating to 18% of the N₂O 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 N₂O 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 N₂O synthesis considering that i) N₂O emissions have been repeatedly reported from a diverse range of aquatic ecosystems characterised by a high level of algal activity; ii) N₂O synthesis by pure microalgal cultures has been unambiguously demonstrated and current knowledge on N₂O synthesis from a broad range of organisms provides the foundation for six putative microalgal N₂O 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 N₂O 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 (CO₂) 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 (N₂O) has been suspected for decades and unambiguously demonstrated in pure cultures (Table 1.1). Guieysse et al, (2013) for example estimated the rate of N₂O emissions during microalgae cultivation with nitrate (NO₃⁻) as a nitrogen (N)-source to 1.38 – 10.1 kg N-N₂O ha⁻¹·yr⁻¹, which is higher than the rate of N₂O emissions from natural vegetation of 0.7 kg N-N₂O ha⁻¹·yr⁻¹ (Smeets et al., 2009). Microalgal N₂O synthesis is concerning because N₂O 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 N₂O emissions (Table 1.1) in light of recent advances in microalgal biology and current knowledge of microbial pathways for N₂O synthesis. The potential environmental significance of microalgal N₂O 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 N₂O source.
Table 1.1: N$_2$O emissions reported from studies acknowledging algal N$_2$O synthesis (chronological order of publication).

<table>
<thead>
<tr>
<th>Ecosystem</th>
<th>N$_2$O fluxes$^a$</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ocean</td>
<td>115 nmol·m$^{-2}$·h$^{-1}$</td>
<td>Cohen and Gordon, (1978) reported that, in surface water, N$_2$O emissions were correlated to the presence of nitrite (NO$_2^-$) and oxygen (O$_2$). These authors dismissed algae as key contributor.</td>
</tr>
<tr>
<td>Ocean</td>
<td>409 nmol·m$^{-2}$·h$^{-1}$</td>
<td>Pierotti and Rasmussen, (1980) recognised phytoplankton as a significant N$_2$O source in upper layer of upwelling regions.</td>
</tr>
<tr>
<td>Costal wetland</td>
<td>125 – 228 nmol·m$^{-2}$·h$^{-1}$</td>
<td>Smith et al, (1983) noted the potential of algal N$_2$O synthesis and attributed N$_2$O production to bacterial nitrification-denitrification.</td>
</tr>
<tr>
<td>Laboratory assays</td>
<td>933 – 1579 nmol·g-DW$^{-1}$·h$^{-1}$</td>
<td>Weathers, (1984) demonstrated N$_2$O synthesis in axenic microalgae culture supplied with NO$_2^-$.</td>
</tr>
<tr>
<td>Laboratory assays</td>
<td>1500 nmol·g-DW$^{-1}$·h$^{-1}$</td>
<td>Weathers and Niedzielski, (1986) demonstrated N$_2$O synthesis in axenic cyanobacteria cultures supplied with NO$_2^-$.</td>
</tr>
<tr>
<td>Ocean</td>
<td>123 – 132% saturation</td>
<td>Outdot et al, (1990) attributed N$_2$O emissions in upper (euphotic) zone to NO$_3^-$ assimilation by phytoplankton.</td>
</tr>
<tr>
<td>Lakes</td>
<td>300 – 700 nmol·m$^{-2}$·h$^{-1}$</td>
<td>Mengis et al, (1997) attributed N$_2$O production in the upper layer of eutrophic lakes to “actively growing algae”.</td>
</tr>
<tr>
<td>Ocean</td>
<td>88 nmol·m$^{-2}$·h$^{-1}$</td>
<td>Morell et al, (2001) acknowledged the existence of an algal N$_2$O pathway but did not discuss in further.</td>
</tr>
<tr>
<td>Lake</td>
<td>357 – 2450 nmol·m$^{-2}$·h$^{-1}$</td>
<td>Wang et al, (2006) concluded that “excessive N$_2$O emissions during algal bloom periods suggested that algae played a significant role in the emission of N$_2$O.”</td>
</tr>
<tr>
<td>Open pond</td>
<td>580 nmol·m$^{-2}$·h$^{-1}$</td>
<td>Florez-leiva et al., (2010) reported N$_2$O emissions from a Nannochloris culture in an open pond</td>
</tr>
<tr>
<td>Laboratory assays</td>
<td>13 – 145 nmol·g-DW$^{-1}$·h$^{-1}$</td>
<td>Fagerstone et al, (2011) acknowledged the potential of microalgal synthesis but attributed N$_2$O synthesis in</td>
</tr>
</tbody>
</table>
cultures of the microalga *Nannochloropsis salina* to bacteria.

<table>
<thead>
<tr>
<th>System</th>
<th>Flux Range</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raceway pond</td>
<td>320 nmol·L⁻¹</td>
<td>Ferrón et al, (2012) acknowledged the potential of algal synthesis but did not conclude on the sources of N₂O emissions recorded during the outdoor culture of marine diatom <em>Staurosira</em> sp.</td>
</tr>
<tr>
<td>Laboratory assays</td>
<td>109 – 1480 nmol·g-DW⁻¹·h⁻¹</td>
<td>Guieysse et al, (2013) demonstrated N₂O synthesis by axenic <em>Chlorella vulgaris</em> and linked this activity to NO₃⁻ reduction.</td>
</tr>
<tr>
<td>Photobioreactor</td>
<td>240 – 1250 nmol·m⁻²·h⁻¹</td>
<td>Guieysse et al, (2013) reported that N₂O synthesis during <em>Chlorella vulgaris</em> culture was correlated to light intensity.</td>
</tr>
<tr>
<td>Photobioreactor</td>
<td>0 – 36 nmol·g-DW⁻¹·h⁻¹</td>
<td>Mezzari et al, (2013) attributed N₂O emissions to microalgae.</td>
</tr>
<tr>
<td>Photobioreactor</td>
<td>9698 – 10761 nmol·g-DW⁻¹·h⁻¹</td>
<td>Harter et al, (2013) acknowledged the potential of microalgal synthesis but attributed N₂O emissions in <em>Dunaliella salina</em> cultures to anaerobic bacteria.</td>
</tr>
<tr>
<td>Laboratory assay</td>
<td>0.087 – 0.3 nmol·L⁻¹·h⁻¹</td>
<td>Kamp et al, (2013) attributed N₂O emissions from <em>Thalassiotheria weissflogii</em> cultures to NO₃⁻ dissimilatory reduction by this diatom.</td>
</tr>
<tr>
<td>Raceway pond</td>
<td>2 – 5685 nmol·g-DW⁻¹·h⁻¹</td>
<td>Alcántara et al, (2015) linked N₂O emissions to <em>Chlorella vulgaris</em> activity during synthetic wastewater in a laboratory scale raceway pond.</td>
</tr>
</tbody>
</table>

* 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 N₂O·m⁻²·h⁻¹ (natural aquatic and engineered systems) or nmol N₂O·g-DW⁻¹·h⁻¹ (laboratory studies).
1.2 \( \text{N}_2\text{O} \) emissions from microalgal-based (eco)systems

As illustrated in Table 1.1, very few studies have hitherto acknowledged the potential of algal \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) sources: Pierotti and Rasmussen, (1980) thus reported evidence of high productivity of primary producers correlated to \( \text{NO}_3^- \) uptake in oceanic upwelling regions suggesting \( \text{N}_2\text{O} \) was partly produced by phytoplankton, while Lamontagne et al, (2003) established that experimental \( \text{N}_2\text{O} \) concentrations exceeded \( \text{N}_2\text{O} \) estimations based on known biological sources and sinks of \( \text{N}_2\text{O} \) in river, and hypothesised the possibility of missing \( \text{N}_2\text{O} \) sources.

Despite the uncertainty around the origin of \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) emissions have been repeatedly reported for decades. \( \text{N}_2\text{O} \) emissions were for example correlated to \( \text{O}_2 \) 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$_2$O 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$_2$O in the presence of NO$_2^-$ (Weathers, 1984; Weathers and Niedzielski, 1986). Interestingly, while several authors demonstrated or hypothesised N$_2$O 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$_2$O 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$_2$O 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$_2$O 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$_2$O 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$_2$O 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$_2$O biosynthesis (i.e. enzymes and substrates involved) and of microalgal and plant biology, provides the basis for six possible routes for N$_2$O synthesis in microalgae (Box 1.1).
**Figure 1.1:** Common N$_2$O pathways

**a) Bacterial pathways**

Nitrification

\[
\begin{align*}
\text{NH}_4^+ & \rightarrow \text{NO}_2^- & \text{Hao} & \rightarrow \text{NO}_2^- & \rightarrow \text{NO} & \rightarrow \text{N}_2O & \rightarrow \text{N}_2 \\
\text{NO}_3^- & \rightarrow \text{N}_2O & \rightarrow \text{N}_2O & \rightarrow \text{N}_2 \\
\end{align*}
\]

Denitrification

\[
\begin{align*}
\text{NO}_3^- & \rightarrow \text{NO} & \rightarrow \text{N}_2O & \rightarrow \text{N}_2O & \rightarrow \text{N}_2 \\
\text{NO}_2^- & \rightarrow \text{NO} & \rightarrow \text{N}_2O & \rightarrow \text{N}_2O & \rightarrow \text{N}_2 \\
\end{align*}
\]

Nitrifier-Denitrification

\[
\begin{align*}
\text{NH}_4^+ & \rightarrow \text{NO}_2^- & \rightarrow \text{NO} & \rightarrow \text{N}_2O & \rightarrow \text{N}_2O & \rightarrow \text{N}_2 \\
\text{NO}_3^- & \rightarrow \text{NO} & \rightarrow \text{N}_2O & \rightarrow \text{N}_2O & \rightarrow \text{N}_2 \\
\end{align*}
\]

**b) Fungal pathway**

\[
\begin{align*}
\text{NO}_3^- & \rightarrow \text{NO} & \rightarrow \text{N}_2O & \rightarrow \text{N}_2O & \rightarrow \text{N}_2 \\
\text{NO}_2^- & \rightarrow \text{NO} & \rightarrow \text{N}_2O & \rightarrow \text{N}_2O & \rightarrow \text{N}_2 \\
\end{align*}
\]

**Figure 1.1** describes common N$_2$O pathways. **a)** N$_2$O synthesis by bacteria adapted from Wrage et al. (2001) N$_2$O 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$_2$O = Nitrous oxide; Nio = Nitrite oxidoreductase; N$_2$OR = Nitrous oxide reductase).
Box 1.1 Putative pathways of microalgal N\textsubscript{2}O synthesis

\textbf{N\textsubscript{2}O synthesis from nitrate assimilation}: This pathway was first suggested by Hahn and Junge, (1977) who proposed that N\textsubscript{2}O was a degradation product from hyponitrous acid (H\textsubscript{2}N\textsubscript{2}O\textsubscript{2}) or nitroxyl (HNO), two potential intermediates in the pathway of nitrate (NO\textsubscript{3}\textsuperscript{-}) assimilatory reduction into cellular material. N\textsubscript{2}O synthesis was later linked to nitrite (NO\textsubscript{2}\textsuperscript{-}) extracellular supply at high concentration (Weathers, 1984; Guieysse et al., 2013) and Guieysse et al. (2013) refined the putative ‘assimilatory pathway’ in \textit{C. vulgaris} by linking the reduction of intracellular NO\textsubscript{2}\textsuperscript{-} by nitrate reductase (NR) into NO or HNO and the subsequent reduction of these precursors into N\textsubscript{2}O (Figure 1.2), see Guieysse et al., (2013) for further discussion.

Recent findings by Chamizo-Ampudia et al, (2016) showed that in the microalgae \textit{Chlamydomonas reinhardtii} NO\textsubscript{2}\textsuperscript{-} 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\textsubscript{2}\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-} (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\textsubscript{2}O synthesis.

\textbf{N\textsubscript{2}O emissions during microalgal nitrification}: When pre-cultivated using ammonium (NH\textsubscript{4}\textsuperscript{+}) as sole exogenous N-source and subsequently subjected to N-deprivation, various microalgae have the ability to release NO\textsubscript{3}\textsuperscript{-} and NO\textsubscript{2}\textsuperscript{-} concomitantly to an increase in NR activity (Morris and Syrett, 1963, 1965; Kessler and Oesterheld, 1970). The generation of NO\textsubscript{3}\textsuperscript{-} or NO\textsubscript{2}\textsuperscript{-} 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$_2$O synthesis (as discussed above), the possibility of a ‘microalgal N$_2$O-nitrification pathway’ involving NR-mediated reduction of endogenous NO$_2^-$ into NO or HNO deserves consideration.

**N$_2$O 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$_2$O 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$_2$O synthesis in cyanobacteria.

**Other potential pathways leading to N$_2$O synthesis in microalgae:** As discussed above, NO is a potential precursor of N$_2$O in microalgae. Three alternative N$_2$O 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 $N_\omega$-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$_2$O when $N_\omega$-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).
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\textsubscript{2}O emissions from microalgae-based (eco)systems

As shown in Section 1.2, there is significant evidence that microalgae produce N\textsubscript{2}O 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\textsubscript{2}O is a globally significant ozone-depleting pollutant and a GHG. Of particular relevance, the Intergovernmental Panel for Climate Change (IPCC) currently quantifies global N\textsubscript{2}O emissions from anthropogenic and natural sources without consideration that microalgae can produce N\textsubscript{2}O. This section focuses on the impact of N\textsubscript{2}O as a GHG.

1.3.1 Eutrophic lakes

In its gas inventory methodology, the IPCC (Ciais et al., 2013), documents that N\textsubscript{2}O 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\textsubscript{2}O in the IPCC assessments”. Global N\textsubscript{2}O emissions from eutrophic lakes and reservoirs were estimated based on the yearly average production of 0.9 kg N-N\textsubscript{2}O·ha\textsuperscript{-1}·yr\textsuperscript{-1} (357 nmol N\textsubscript{2}O·m\textsuperscript{-2}·h\textsuperscript{-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 km² (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-N₂O·yr⁻¹, which is equivalent to 18% of the current IPCC estimate of 600 kt N-N₂O·yr⁻¹ for N₂O 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-N₂O·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 N₂O·m⁻²·h⁻¹. The role microalgae are playing in these highly variable but poorly understood emissions becomes clear as Wang et al. (2006) quantified N₂O 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 N₂O (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·m⁻²·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 N₂O·h⁻¹·g-DW⁻¹ (Guieysse et al., 2013), microalgae cultivation over 35 million ha would release 24 – 183 kt N-N₂O·yr⁻¹ (0.71 – 5.2 kg N-N₂O·ha⁻¹·yr⁻¹), which represents up to 15% of the 1200 kt N-N₂O·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 at 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 N₂O 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 N₂O 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 N₂O 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 N₂O emissions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microalgae species</td>
<td>The green microalgae <em>Chlorella vulgaris, Chlorella rubescens, Homosphaerica sp., Scenedesmus obliquus, Coelastrum sp., Chlorococcum vacuolarum and Nannochloris</em>, the diatom <em>Thalassiosira weissflogii</em> and the cyanobacteria <em>Aphanocapsa 6308, Aphanocapsa 6714</em>, and <em>Nostoc</em> sp have been shown to synthesise N₂O at species-dependent rates.</td>
<td>(Weathers, 1984; Weathers and Niedzielski, 1986; Florez-Leiva et al., 2010; Guieysse et al., 2013; Kamp et al., 2013; Alcántara et al., 2015)</td>
</tr>
<tr>
<td>Microalgal concentration</td>
<td><em>C. vulgaris</em> N₂O production rates were linearly correlated to microalgal concentration.</td>
<td>(Guieysse et al., 2013)</td>
</tr>
<tr>
<td>Cellular stage</td>
<td>4-days old <em>C. vulgaris</em> cells generated more N₂O than 7-days old cells (over 4 hours of incubation with nitrite under darkness).</td>
<td>(Guieysse et al., 2013)</td>
</tr>
<tr>
<td>Light intensity</td>
<td>N₂O synthesis increased 10 – 15 times when photosynthesis was inactivated in the presence of NO₂⁻ in batch laboratory assays, although daytime N₂O emissions were recorded during outdoor <em>C. vulgaris</em> cultures in column photobioreactors.</td>
<td>(Weathers, 1984; Weathers and Niedzielski, 1986; Guieysse et al., 2013; Kamp et al., 2013; Alcántara et al., 2015)</td>
</tr>
<tr>
<td>Nitrogen supply</td>
<td>Several studies have shown a clear correlation between NO₃⁻ assimilation and/or the presence/supply of NO₂⁻ and N₂O synthesis by microalgae.</td>
<td>(Weathers, 1984; Weathers and Niedzielski, 1986; Guieysse et al., 2013; Kamp et al., 2013; Alcántara et al., 2015)</td>
</tr>
</tbody>
</table>
1.4 Conclusions

N$_2$O emissions have been repeatedly recorded from a diverse range of microalgae-based ecosystems and N$_2$O synthesis by pure microalgae has been demonstrated in the laboratory. Current knowledge provides the foundation for inferring at least six pathways for N$_2$O 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$_2$O 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$_2$O flux that are measured in multiple orders of magnitude, provides strong motivation for further developing and understanding this phenomenon. Acknowledging and understanding N$_2$O emissions from microalgae is also of paramount importance to improve strategic investments in the fields of microalgal biotechnology.
References


Preface

From the critical literature review presented in Chapter 1 we determined six putative microalgal N$_2$O pathways. Consequently, laboratory experiments were performed to study the biochemistry of microalgal N$_2$O synthesis. This study aimed to identify the key enzymes and intermediates involved during microalgal N$_2$O synthesis, and understand why microalgae synthesise N$_2$O. The findings discussed in this chapter are critical to the prediction of environmental or operational conditions that may favour or repress microalgal N$_2$O 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:


Parts of this work were performed with the collaboration of Prof. Emilio Fernández Reyes⁴, Dr. Emanuel Sanz-Luque, Dr. Francisco Ocaña-Calahorro, and Dr. Dave Wheeler⁵. 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.

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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 (N₂O) synthesis by aerobic *Chlamydomonas reinhardtii* cultures supplied with nitrite (NO₂⁻). In darkness, this biosynthesis was characterised by an immediate and short period (0 – 15 min) of N₂O 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 NO₂⁻ 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 N₂O 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 NO₂⁻-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, NO₃⁻, is the main N source, microalgal N₂O synthesis involves 1) NO₃⁻ reduction into NO₂⁻ by nitrate reductase; followed by 2) NO₂⁻ reduction into NO by a dual system of NR and amidoxime-reducing NO-forming nitrite reductase; and 3) NO reduction into N₂O by NOR. NO₂⁻ reduction by COX appears to act as a means to detoxify NO₂⁻ when the intracellular concentration of this compound is high.
2.1 Introduction

Nitrous oxide (N$_2$O) 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$_2$O 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$_2$O 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$_2$O by a NO reductase (NOR) or the spontaneous dimerization of HNO into N$_2$O. However, if NO can indeed be reduced into N$_2$O under aerobic conditions, several alternative NO-generation scenarios could also lead to N$_2$O 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$_2$O 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$_2$O 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$_2$O 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·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·L$^{-1}$ (g-DCW·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).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>6145c</td>
<td>Reference strain used during this study (wild-type); grows on nitrate and nitrite</td>
<td>Navarro et al., (2000)</td>
</tr>
<tr>
<td>M3</td>
<td>NiR deficient; cannot grow on nitrate nor nitrite</td>
<td>Navarro et al., (2000)</td>
</tr>
<tr>
<td>2929</td>
<td>Lack NR; cannot grow on nitrate nor nitrite</td>
<td>Sakihama et al., (2002)</td>
</tr>
<tr>
<td>704</td>
<td>Wild type strain used as parental strain for mutagenesis on NOR (CYP55 gene); grows on nitrate and nitrite</td>
<td>This study</td>
</tr>
<tr>
<td>amiCYP55</td>
<td>Lower NOR expression (46%) than the parent strain 704; grows on nitrate and nitrite</td>
<td>This study</td>
</tr>
<tr>
<td>409</td>
<td>Wild-type; grows on nitrate and nitrite</td>
<td>(Pröschold et al., 2005)</td>
</tr>
<tr>
<td>217</td>
<td>Wild-type; grows on nitrate and nitrite</td>
<td>Sakihama et al., (2002)</td>
</tr>
<tr>
<td>112</td>
<td>Parent strain of the mutant 2929</td>
<td><em>Chlamydomonas</em> centre</td>
</tr>
<tr>
<td>530</td>
<td>Acetate requiring mutant, parent strain of the mutant 2929</td>
<td><em>Chlamydomonas</em> centre</td>
</tr>
<tr>
<td>124</td>
<td>NR, NiR and HANiT deficient, cannot grow on nitrate nor nitrite</td>
<td>(Pröschold et al., 2005)</td>
</tr>
<tr>
<td>125</td>
<td>NR, NiR and HANiT deficient, cannot grow on nitrate nor nitrite</td>
<td>(Pröschold et al., 2005)</td>
</tr>
</tbody>
</table>
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 NO₂⁻, NO₃⁻, or NH₄⁺) or NO₂⁻ at various concentrations (3, 6, 12 and 24 mM).
2. *C. reinhardtii* 409, 217, 112, 530, 124, 125 were supplied 10 mM NO₂⁻ in darkness.
3. *C. reinhardtii* NR mutant 2929 was supplied with 10 mM NO₂⁻ in darkness.
4. *C. reinhardtii* NiR mutant M3 was supplied with 10 mM NO₂⁻ or NO₃⁻ both in darkness and under illumination.
5. *C. reinhardtii* wild-type 704 and amiCYP55 (NOR knock down mutant) were supplied with 10 mM NO₂⁻ 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 NO₂⁻ concentrations chosen were useful to study the N₂O pathway and aimed to easily detect N₂O synthesis. It is however important to note that the concentration of 10 mM NO₂⁻ used in most of our assays is irrelevantly high in comparison to typical microalgal cultivation (NO₃⁻ 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$_4^{2-}$) was replaced by orthotungstate (WO$_4^{2-}$) at the concentration of 0.253 mg·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$_2$O 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 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 NO₂⁻ 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 NO₂· supply), 4 (t₂: 3 h after NO₂⁻ supply) and 25 hours (t₃: 24 h after NO₂⁻ supply) to measure N₂O 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). NO₃⁻/NO₂⁻ 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 N₂O 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 N₂O measurement

The gaseous concentration of N₂O 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 N₂O produced in the flasks assuming the dissolved N₂O concentration in the aqueous phase was in equilibrium with the gas phase. N₂O 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 N₂O standard in N₂ (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·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, NO₂⁻ (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 NO₂⁻) 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
N₂O production, negative controls (N-free medium supplied with 10 mM NO₂⁻; or N-free medium supplied with chemical inhibitors and 10 mM NO₂⁻; or autoclaved microalgal suspension supplied with 10 mM NO₂⁻) were also performed. While positive significant N₂O production was always recorded from the positive controls, no significant N₂O 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’ N₂O 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 N₂O production was higher than this threshold, N₂O production was deemed significant and specific production values (nmol g·DCW⁻¹) were calculated as the amount of N₂O produced a time t (nmole) minus the background level (1.45 nmole), divided by the initial cell mass (g·DCW·L⁻¹). Standard deviations (SD) represent combined uncertainties of N₂O 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 N₂O emissions from axenic *C. reinhardtii* cultures

Prior to this study, the ability of *C. reinhardtii* to synthesise N₂O had never been investigated. Preliminary batch assays were conducted and N₂O synthesis was only recorded in *C. reinhardtii* 6145c cultures supplied with NO₂⁻ (Figure 2.1). In contrast no significant N₂O synthesis was recorded in flasks not supplied with NO₂⁻ or in negative controls (i.e. flasks containing autoclaved cultures or sterile medium in the presence of NO₂⁻).

![Figure 2.1: N₂O 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 NO₂⁻ supplementation and incubated for 24 h in darkness or illumination; sterile N-free medium with or without NO₂⁻ supplementation; and autoclaved culture of *C. reinhardtii* 6145c supplied with 10 mM NO₂⁻ and incubated in darkness.](image-url)
The production of $\text{N}_2\text{O}$ 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 $\text{N}_2\text{O}$ under aerobic conditions. $\text{N}_2\text{O}$ 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 $\text{N}_2\text{O}$.

**Figure 2.2**: $\text{N}_2\text{O}$ 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 \( \text{N}_2\text{O} \) emission from \( \text{NO}_2^- \)-laden \( C. vulgaris \) and \( C. reinhardtii \) cultures. For example, \( C. vulgaris \) has previously been shown to produce \( \text{N}_2\text{O} \) at a constant rate (zero-order) over 48 hours of incubation in darkness (Guieysse et al. 2013). In contrast, \( \text{N}_2\text{O} \) emission from \( C. reinhardtii \) incubated under nearly identical conditions was characterised by a ‘3-phases’ kinetics of \( \text{N}_2\text{O} \) production (Figure 2.3a and b). As seen in Figure 2.3a, a short period of high \( \text{N}_2\text{O} \) production started immediately following \( \text{NO}_2^- \) 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 \( C. reinhardtii \) \( \text{N}_2\text{O} \) synthesis suggests a long-term response triggering \( \text{N}_2\text{O} \) emissions, whereas the shorter response suggests that \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) production after 1 and 4 h shown in Figure 2.3a and 2.3b, respectively), difficult to determine (as \( \text{N}_2\text{O} \) production could not be continuously monitored), and impacted by quantification uncertainty in the low gaseous \( \text{N}_2\text{O} \) 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: $\text{N}_2\text{O}$ 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 $\text{N}_2\text{O}$ 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 N₂O production rates achieved by *C. reinhardtii* 6145c (100 nmol N₂O·h⁻¹·g-DCW⁻¹) were, however, significantly lower than rates previously recorded with *C. vulgaris* (1000 nmol N₂O·h⁻¹·g-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 N₂O was demonstrated (Figure 2.4).

![Diagram summarising the findings from Section 2.3.1: N₂O emissions from axenic *C. reinhardtii* cultures.]

**Figure 2.4:** Diagram summarising the findings from Section 2.3.1: N₂O emissions from axenic *C. reinhardtii* cultures.

### 2.3.2 NO₂⁻ acts as substrate during microalgal N₂O synthesis

As shown in Chapter 1 (Box 1.1: Other potential pathways leading to N₂O synthesis in microalgae), several routes and substrates (i.e. nitrogen in different forms) could lead to
N₂O synthesis in microalgae. Knowing the potential link between N₂O synthesis and nitrate assimilation, batch assays were conducted with NO₂⁻, NO₃⁻, and NH₄⁺. As seen in Figure 2.5, while the supply of NO₂⁻ triggers significant N₂O emissions (1200 nmol·g-DCW⁻¹ after 24 h), the supply of NO₃⁻ or NH₄⁺ did not trigger any significant emissions from axenic *C. reinhardtii* cultures (details are given in Appendix H, Table H1).

**Figure 2.5:** N₂O 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 NO₂⁻ or NO₃⁻ or NH₄⁺ and incubated in darkness or under illumination for 24 h.

Further results showed that N₂O emissions were linearly correlated to extracellular NO₂⁻ concentration (Figure 2.6). The results therefore revealed an association between NO₂⁻ reduction and N₂O 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 NO₂⁻ and N₂O was also confirmed in a different
setting (2 L photobioreactor) when the supply of NO$_2^-$ triggered rapid N$_2$O synthesis by actively growing *C. reinhardtii* 6145c cultures (Appendix J).

**Figure 2.6:** N$_2$O 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$_2$O synthesis from nitric oxide synthase activity (NOS) as hypothesised in Chapter 1 (Box 1.1: Other potential pathways leading to N$_2$O 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$_2$O synthesis observed in our cultures. Firstly, N$_2$O synthesis was not recorded in NO$_2^-$-free cultures supplied with L-arg. Secondly, N$_2$O 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:** N₂O 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 NO₂⁻ 10 mM, or L-arg 10 mM, or L-Arg and NO₂⁻ at 10 mM, or L-arg, NNA and NO₂⁻ at 10 mM or NNA and NO₂⁻ at 10 mM and incubated for 24 h in darkness.

The results confirmed that only NO₂⁻ acted as a substrate during N₂O synthesis in *C. reinhardtii* (Figure 2.8).
2.3.3 NO is a key intermediate during N₂O synthesis

NO has been suggested as the potential intermediate during N₂O 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 N₂O 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 NO₂⁻; cells supplied with NO₂⁻ but not pre-incubated with DAF-FM diacetate; cells pre-incubated with DAF-FM diacetate and subsequently exposed to NO₂⁻. 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 NO₂⁻ (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 NO₂⁻ suggests NO₂⁻ 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 NO₂⁻ (Table K.1, Appendix K), confirming NO synthesis from NO₂⁻. Taken together these results demonstrate that the
conditions triggering N\textsubscript{2}O synthesis (Figure 2.1, 2.5 and 2.6) also trigger NO production in \textit{C. reinhardtii}.
Table 2.2: Fluorescence of *C. reinhardtii* 6145c cells incubated with DAF FM Diaceta
tate and supplied NO$_2^\cdot$. Under the hypothesis that NO$_2^\cdot$ biological reduction yielded NO; red text shows negative control (when DAF FM diacetate and/or NO$_2^\cdot$ were not present), whereas the green text show the treatment (DAF FM diacetate and NO$_2^\cdot$ were both present). Microscopic photographs were taken with a Micropublisher 5 colour CCD camera (QImaging, Canada).

<table>
<thead>
<tr>
<th>Conditions: Control cells (no pre-incubation with DAF-FM Diacetate, no NO$_2^\cdot$ supply): these tests were conducted to characterize the green and red fluorescence backgrounds.</th>
<th>Cells pre-incubated with DAF-FM Diacetate (no NO$_2^\cdot$ supply): These tests were conducted to identify potential interfering effects of the pre-incubation on fluorescence detection.</th>
<th>Cells supplied NO$_2^\cdot$ (10 mM) but no pre-incubated with DAF-FM Diacetate: These tests were conducted to identify potential interfering effects of NO$_2^\cdot$ on fluorescence detection.</th>
<th>Cells pre-incubated with DAF-FM Diacetate and supplied NO$_2^\cdot$ (10 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopic photographs (×40) (excitation wavelengths 520–550 nm; emission wavelengths: 580–797 nm)</td>
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<tr>
<td>Microscopic photographs (×40) (excitation wavelengths 460–480 nm; emission wavelengths: 510-580 nm)</td>
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</table>

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 N₂O synthesis via NO₂⁻ reduction (Guieysse et al, 2013), an additional control was performed where \textit{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 (N₂O₃) and nitrosonium anion (NO⁺). However, the aqueous degradation of Angeli’s salt releases both NO₂⁻ and HNO under physiological condition (Dutton et al., 2004; Miranda et al., 2005), meaning that ‘indirect’ NO₂⁻ supply may have caused NO synthesis in these tests. While our results strongly suggest that NO was generated during N₂O 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).
2.3.4 Short-term N₂O synthesis involves NR but late synthesis involves other enzymes

In microalgae NR normally reduces NO₃⁻ into NO₂⁻ via a 2 electron reduction during the first step of nitrate assimilation. NR has long been suspected to catalyse NO₂⁻ 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

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**Figure 2.9:** Diagram summarising the findings from Section 2.3.3: NO is a key intermediate during N₂O synthesis.
dependent on the amount of $\text{NO}_2^-$ accumulated in cell cytoplasm and to be often occurring at low levels (0.1–1% of NR total activity) due to competition between $\text{NO}_3^-$ and $\text{NO}_2^-$ 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 $\text{NO}_2^-$ 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 $\text{NO}_2^-$ increases in the presence of $\text{NO}_3^-$ (Chamizo-Ampudia et al., 2016). In *C. vulgaris*, the involvement of NR during N$_2$O synthesis was suggested by evidence that N$_2$O 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$_2$O 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$_2$O was not recorded in $\text{NO}_2^-$-laden cultures of the NR-deficient strain, although similar 0 – 24 h N$_2$O emissions were recorded in $\text{NO}_2^-$-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 $\text{NO}_2^-$ 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 $\text{NO}_2^-$-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 \( \text{N}_2\text{O} \) synthesis involved \( \text{NO}_2^- \) reduction into NO by the dual system NR-ARC immediately following \( \text{NO}_2^- \) addition (0 – 15 min), whereas another enzymatic system was involved in the late \( \text{N}_2\text{O} \) response. The possible involvement of other molybdoenzymes during the late \( \text{N}_2\text{O} \) production phase (e.g. xanthine oxidase) was also dismissed based on the tungstate inhibition assays.

\[ \text{N}_2\text{O} \text{(nmol·g-DCW-1)} \]

**Figure 2.10:** \( \text{N}_2\text{O} \) 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 \( \text{NO}_2^- \) 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 $\text{N}_2\text{O}$ 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$_2$O synthesis. As NO$_2^-$ ‘fuels’ N$_2$O synthesis via NO, the activities of NR and NiR are likely to impact N$_2$O 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$_2$O under illumination (Figure 2.11).

![Figure 2.11: N$_2$O 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.](image-url)
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 $\text{NO}_3^-$ (Kaiser and Huber, 2001), this NiR mutant was able to carry out the stoichiometric reduction of $\text{NO}_3^-$ into $\text{NO}_2^-$ (Navarro et al., 2000) but could not reduce $\text{NO}_2^-$ into $\text{NH}_4^+$. Nitrite therefore over-accumulated in illuminated NiR-deficient cells (Navarro et al., 2000), which probably resulted in increased NO turnover boosting $\text{N}_2\text{O}$ emissions. Nitrite-laden cultures of NiR-deficient mutants were also shown to release $\text{N}_2\text{O}$ both in darkness and under illumination (Figure 2.11).

The indirect impact of NR and NiR on intracellular $\text{NO}_2^-$ accumulation suggests that microalgal $\text{N}_2\text{O}$ emissions should mainly occur under illumination when $\text{NO}_3^-$ is the dominant N source, as reported by Guieysse et al. (2013) during the outdoor cultivation of *C. vulgaris* supplied $\text{NO}_3^-$ 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 $\text{N}_2\text{O}$ synthesis: In the presence of $\text{NO}_3^-$, NR supplies $\text{NO}_2^-$ as the substrate for $\text{N}_2\text{O}$ synthesis and if NiR is lacking or inactivated (i.e. during a period of low photosynthetic activity) it favours $\text{NO}_2^-$ accumulation, potentially leading to $\text{N}_2\text{O}$ synthesis (Figure 2.12).
4. Is NR involved during N$_2$O synthesis in *C. reinhardtii*?

**Wild-type (reference strain):**
*Chlamydomonas reinhardtii* 6145c (NR inhibited by tungstate)

- NO$_2^-$ supplied in darkness
- N$_2$O

**Mutant:**
*Chlamydomonas reinhardtii* 2929 (lacks NR)

- NO$_2^-$ supplied in darkness
- N$_2$O

**Mutant:**
*Chlamydomonas reinhardtii* M3 (NiR-deficient but possess an active NR)

- NO$_2^-$ supplied in light
- NO$_2^-$ produced and accumulated
- N$_2$O

NR is not necessarily involved during N$_2$O synthesis in *C. reinhardtii*.

NR function is critical during N$_2$O synthesis as it is supplying NO$_2^-$ under normal growth conditions.

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**Figure 2.12:** Diagram summarising the findings from section 2.3.4: Short-term N$_2$O synthesis involves NR but late synthesis involves other enzymes in *C. reinhardtii*, and 2.3.5: NR and NiR activities impacts N$_2$O synthesis under NO$_3^-$ supply.
2.3.6 Late N$_2$O 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$_2$O 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$_2$O (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$_2$O 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$_2$O production.
Figure 2.13: N₂O 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 NO₂⁻ and 2 mM KCN (“CN⁻”) against CN⁻ free cultures (“control”); all cultures were incubated for 24 h in darkness.

Given that N₂O synthesis by both *C. reinhardtii* strains was not completely abolished following KCN addition, AOX may also be partially responsible for NO₂⁻ 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 NO₂⁻ 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 N₂O from NO₂⁻ (44 ± 8 nmole N₂O in abiotic tests after 3 h). It should also be noted that NO₂⁻ 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 N₂O synthesis in *C. reinhardtii*

Plant hemoglobins are known to reduce NO₂⁻ 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 NO₂⁻ detoxification (Sturms et al., 2011) or survival under anoxia (Hemschemeier et al., 2013). *C. reinhardtii* possesses 12 truncated hemoglobins (THB1-12) and some of these proteins could be able to reduce NO₂⁻ (Huwald et al., 2015; Ciaccio et al., 2015). *C. reinhardtii* THB1 is in turn involved in NO signalling, NO₃⁻ 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 NO₃⁻, 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 N₂O synthesis is needed.

The results discussed in Section 2.3.6 showed that mitochondrial COX was probably the enzyme catalysing the reduction of NO₂⁻ into NO during the late phase of N₂O 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 N₂O synthesis involves NO₂⁻ reduction to NO by mitochondrial COX and Section 2.3.7: Possible role of hemoglobins during N₂O synthesis in *C. reinhardtii*.

2.3.8 N₂O synthesis by *C. reinhardtii* involves NO reduction into N₂O by NOR

Although concurrent NO and N₂O syntheses in response to NO₂⁻ supply were repeatedly observed in *C. reinhardtii* cultures (Figure 2.1; 2.5; 2.6; Table 2.2), NO
reduction into N₂O is difficult to explain because NO is typically oxidized into nitrogen dioxide (NO₂) 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 N₂O under hypoxia (Morozkina and Kurakov, 2007; Shoun et al., 2012). When supplied with NO₂⁻, a *C. reinhardtii* NOR-knock down mutant (amiCYP55 strain with gene CYP55 silenced using artificial micro iRNA) synthesised 70 – 90% less N₂O⁷ than its respective parent (Figure 2.15).

![Figure 2.15: N₂O 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 NO₂⁻ and incubated for 24 h in darkness.](image)

⁷ N₂O 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 N₂O via NOR-mediated NO reduction under aerobic conditions (Figure 2.16). Interestingly, N₂O synthesis increased when NO₂⁻-laden *C. reinhardtii* 6145c was incubated under anoxic conditions (Appendix M). This finding may be explained by the absence of competition between NO₂⁻ and O₂ 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 N₂O by NOR.

**Figure 2.16:** Diagram summarising the findings from Section 2.3.8: N₂O synthesis by *C. reinhardtii* involves NO reduction into N₂O by NOR.
2.3.9 RNA-seq based transcriptomics suggest that microalgal N\textsubscript{2}O 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\textsubscript{2}O production in \textit{C. reinhardtii}, RNA-seq was carried out on triplicate samples collected at 0.25, 3, and 24 hours after the addition of NO\textsubscript{2}\textsuperscript{-} in the “treatment” cultures. These particular time points were chosen based on the three phases N\textsubscript{2}O kinetic in \textit{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 \textit{C. reinhardtii} genome (\textit{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\textsubscript{2}\textsuperscript{-} in darkness had an effect on \textit{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\textsubscript{2}O production in \textit{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\textsubscript{2}O synthesis in \textit{C. reinhardtii}. The differential expression is then given by a log2 fold change (log2FC) which indicate the up (\textit{i.e.} increase) or down (\textit{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 \textit{p-value} threshold of 0.05).
**Table 2.3:** Log2FC between control and treatment groups of candidate genes potentially involved in microalgal N₂O synthesis. Numbers in parenthesis represent mean normalised counts.

<table>
<thead>
<tr>
<th>Genes</th>
<th>annotation</th>
<th>Time (h) after exposure to NO₂⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>NIT1</td>
<td>Nitrate reductase (NR)</td>
<td>0.0 (1)</td>
</tr>
<tr>
<td>NII 1</td>
<td>Nitrite reductase (NiR)</td>
<td>-0.2 (2)</td>
</tr>
<tr>
<td>CYP55</td>
<td>Nitric oxide reductase (NOR)</td>
<td>0.2 (1500)</td>
</tr>
<tr>
<td>ARC</td>
<td>Amidoxime reducing component</td>
<td>0.0 (10)</td>
</tr>
<tr>
<td>THB1</td>
<td>Truncated Hemoglobin</td>
<td>0.7* (1142)</td>
</tr>
<tr>
<td>THB2</td>
<td>Truncated Hemoglobin</td>
<td>-0.6* (133)</td>
</tr>
<tr>
<td>COX2a</td>
<td>Cytochrome c oxidase subunit II</td>
<td>0.1 (945)</td>
</tr>
<tr>
<td>COX3</td>
<td>Cytochrome c oxidase subunit III</td>
<td>-0.1 (1583)</td>
</tr>
<tr>
<td>COX5c</td>
<td>Cytochrome c oxidase subunit</td>
<td>-0.1 (1445)</td>
</tr>
<tr>
<td>MT-CO1</td>
<td>Mitochondrial cytochrome c oxidase subunit 1</td>
<td>0.0 (2)</td>
</tr>
<tr>
<td>COX2b</td>
<td>Cytochrome c oxidase subunit</td>
<td>-0.1 (3801)</td>
</tr>
<tr>
<td>COX11</td>
<td>Involved in the insertion of Copper into the CuB center of subunit Cox1p</td>
<td>-0.1 (2)</td>
</tr>
<tr>
<td>COX13</td>
<td>Cytochrome c oxidase subunit 10</td>
<td>-0.1 (2219)</td>
</tr>
<tr>
<td>COX15</td>
<td>Cytochrome c oxidase assembly factor</td>
<td>0.2 (2)</td>
</tr>
<tr>
<td>COX16</td>
<td>Cytochrome c oxidase assembly factor</td>
<td>0.0 (2)</td>
</tr>
<tr>
<td>COX17</td>
<td>Involved in the delivery of Copper to mitochondria</td>
<td>0.2 (8)</td>
</tr>
<tr>
<td>COX18</td>
<td>Cytochrome c oxidase assembly factor</td>
<td>0.2 (4)</td>
</tr>
<tr>
<td>COX19</td>
<td>Cytochrome c oxidase assembly factor</td>
<td>0.2 (21)</td>
</tr>
<tr>
<td>COX191</td>
<td>Involved in cytochrome c oxidase expression</td>
<td>0.1 (3)</td>
</tr>
<tr>
<td>COX23</td>
<td>Cytochrome c oxidase assembly factor involved in Copper homeostasis</td>
<td>0.0 (49)</td>
</tr>
<tr>
<td>COX90</td>
<td>Cytochrome c oxidase subunit</td>
<td>-0.1 (910)</td>
</tr>
<tr>
<td>AOX1</td>
<td>Alternative oxidase</td>
<td>0.5* (1050)</td>
</tr>
<tr>
<td>AOX2</td>
<td>Alternative oxidase</td>
<td>-0.2 (17)</td>
</tr>
</tbody>
</table>

* 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 $\text{NO}_2^-$ reduction into NO leading to ‘late’ $\text{N}_2\text{O}$ 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 $\text{NO}_2^-$ exposure (Table 2.3). *C. reinhardtii* THB1, which was significantly upregulated in the treatment cells after 0.25 and 24 h of $\text{NO}_2^-$ exposure (Log2FC of 0.7 and 0.5, respectively), is also involved in NO signaling, $\text{NO}_3^-$ assimilation, NR activity regulation and, of particular relevance, NO scavenging under normoxia via its dioxygenase activity (Sanz-Luque et al., 2015b). Consequently, during $\text{NO}_3^-$ assimilation, THB1 may help to regulate NO intracellular accumulation by promoting the transformation of NO into $\text{NO}_3^-$ in parallel to NO reduction to $\text{N}_2\text{O}$. 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 $\text{N}_2\text{O}$ 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\textsubscript{2}\textsuperscript{-} 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\textsubscript{2}O synthesis. The data also showed that the transcriptional regulation of NR, ARC, NiR, and NOR genes was potentially not required under NO\textsubscript{2}\textsuperscript{-} 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\textsubscript{2}\textsuperscript{-}-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$_2$O under aerobic conditions via NO$_2^-$ reduction into NO followed by NO reduction into N$_2$O. 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$_2$O 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\textsubscript{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\textsubscript{2}O synthesis was lower under aerobic conditions (0.03 \% g-N-N\textsubscript{2}O synthesised - g N-input\textsuperscript{-1} after 24 h of incubation in darkness for strain 6145c) than under anoxia (0.8 \% g-N-N\textsubscript{2}O synthesised - g N-input\textsuperscript{-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\textsubscript{x} generation, Lamattina et al., 2003). Clearly, our understanding of the function and regulation of the enzymes and substrates involved in N\textsubscript{2}O synthesis in microalgae remains limited. Nevertheless, the evidence herein presented (such as the ability of \textit{C. reinhardtii} to reduce NO into N\textsubscript{2}O 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\textsubscript{2}O synthesis under physiological conditions is linked to NO\textsubscript{3} assimilation in \textit{C. reinhardtii} (Figure 2.17) in a mechanism similar to the fungal N\textsubscript{2}O denitrification pathway (Shoun et al., 2012), with the significant difference that the microalgae carry out N\textsubscript{2}O synthesis under aerobic conditions. This finding has broad implications because the pathway of NO\textsubscript{3} assimilation is conserved among microalgal 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\(_2\)O (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\(_2\)O could be found in numerous microalgae species. This ability may also explain why correlations between primary productivity and N\(_2\)O 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\(_2\)O 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\(_2\)O emissions have been reported. For example, nitrite-laden *C. vulgaris* was previously shown to constantly produce N\(_2\)O 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\(_2\)O emissions during algal cultivation.
**Figure 2.17:** N₂O 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\textsubscript{2}O. Microalgal N\textsubscript{2}O synthesis is related to nitrate assimilation and is a consequence of the intracellular accumulation of NO\textsubscript{2}\textsuperscript{−}. Under physiological conditions NO\textsubscript{2}\textsuperscript{−} is reduced to NO by the dual system NR-ARC. When NO\textsubscript{2}\textsuperscript{−} is present in high concentrations, its reduction into NO is also mediated by mitochondrial COX. The NO generated is then reduced into N\textsubscript{2}O by NOR. We therefore hypothesise that N\textsubscript{2}O synthesis may be a biochemical route to regulate NO (or NO\textsubscript{2}\textsuperscript{−}) levels in microalgae.
References


Chapter 3.

Evaluation of potential microalgal N$_2$O emissions in engineered systems

Preface

As discussed in Chapter 1, microalgal N$_2$O emissions are currently not recognised in international greenhouse gas inventories. In Chapter 2 we suggested that the ability of microalgae to synthesise N$_2$O is wide-spread among microalgae species, which implies that microalgal N$_2$O synthesis could be a global significant N$_2$O 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$_2$O emissions. These particular variables were chosen due to their relevance to microalgal activity and N$_2$O emissions (Chapter 1 and 2). The data obtained would have been useful to construct a mechanistic model to predict N$_2$O 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$_2$O), 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$_2$O 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$_2$O emissions from large scale outdoor microalgae culture. For this purpose, two well studied engineered systems were chosen and monitored for N$_2$O emissions (See Box 3.1 for the selection of cultivation systems).

In the light of the findings presented in Chapter 2 (i.e. N$_2$O 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$_2$O 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$_2$O, 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$_2$O 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$_2$O 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$_2$O emissions” rather than “What is the magnitude of microalgal
Finally, tools for estimating N$_2$O 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$_2$O 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$_2$O emissions had never been investigated long-term in any outdoor systems at pilot scale, so N$_2$O 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$_2$O monitoring performed by Guieysse

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$^8$ 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$_2$O 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$_2$O) 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$_2$O monitoring performed during *C. vulgaris* cultivation) and Sub-section 2 (i.e. Part (6 months) of the N$_2$O 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).


3.1 N$_2$O emissions from commercial microalgae species cultivated in 50 L photobioreactors

ABSTRACT

This study investigated the potential environmental significance of N$_2$O 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$_2$O 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$_2$O·m$^{-2}$·h$^{-1}$ (36 days of cultivation, n = 136, median = 2000 nmol N$_2$O·m$^{-2}$·h$^{-1}$) and 9.60 – 38000 nmol N$_2$O·m$^{-2}$·h$^{-1}$ (90 days of cultivation, n = 332, median = 4100 nmol N$_2$O·m$^{-2}$·h$^{-1}$), respectively, when NO$_3^-$ was supplied as N-source. Based on the emissions of 1500 – 8000 nmol N$_2$O·m$^{-2}$·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$_2$O emissions were positively correlated to biomass concentration ($R^2 = 0.77$) and light intensity ($R^2 = 0.57$). No N$_2$O 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$_2$O mitigation strategies.
3.1.1 Introduction

While N₂O 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). N₂O 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, N₂O emissions have been reported at pilot or full scale during the cultivations of Nannochloris in a 48 m³ open pond (up to 580 nmol N₂O·m⁻²·h⁻¹; Florez-Leiva et al., 2010); Staurosira sp. in a 64 m³ raceway pond (up to 320 nmol N₂O·m⁻²·h⁻¹ over 15 h; Ferrón et al., 2012); Chlorella vulgaris in a 50 L column photobioreactor (240 – 1250 nmol N₂O·m⁻²·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 N₂O production and N cycling in algae cultures, especially in non-axenic cultures where N₂O may be produced by associated microorganisms. N₂O can indeed be generated during nitrite (NO₂⁻) reduction to N₂O by algae (Chapter 2), which can occur during the assimilatory denitrification of nitrate (NO₃⁻) into ammonium (NH₄⁺) in algae, and/or during NO₂⁻ reduction into N₂O or NH₄⁺ oxidation into NO₂⁻ by associated microorganisms (Alcántara et al., 2015). These mechanisms thus involves common substrates, products, and intermediates (e.g. NO₂⁻ is reduced in nitric oxide, NO, which is then reduced into N₂O) 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$_2$O synthesis during microalgae cultivation, this study investigates the potential environmental significance of N$_2$O 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$_2$O 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$_2$O monitoring (Guieysse et al., 2013). As C. vulgaris ability to synthesise N$_2$O is well documented (Weathers, 1984; Guieysse et al., 2013; Alcántara et al., 2015), the influence of N supply on N$_2$O emissions during the outdoor cultivation of this species was also tested. Finally, the potential significance of microalgal N$_2$O 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·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·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 (± 1°C) 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·m$^{-2}$) at 18 ± 1°C and CO$_2$-enriched air bubbling (2% vol., 1 L·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·L⁻¹, and then the cultures were operated 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·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 CO₂ enriched (2% v/v) air at a flow rate of approximatively 1 L·min⁻¹. Gas flow rates were
controlled using rotameters (air: Ki Air Instruments: 0 – 20 L·min⁻¹; CO₂: Aalborg PMR 1-013799: 0 – 500 mL·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 m² 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·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$_2$O 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$_2$O in these samples was quantified using a Shimadzu GC-2010 gas-chromatography system (Shimadzu, Japan). The concentrations of N$_2$O 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$_2$O exhaust gas concentration when computing net N$_2$O 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).
\[
\Phi_{N_2O} = \frac{1}{A} \times \left( \frac{(fN_2O_{reactor} - fN_2O_{air}) \times P_{atm} \times \varphi_{gas}}{R \times T} \right), \text{ Equation 3.1}
\]

Where:

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

\(P_{atm}\) = Atmospheric pressure (Pa)

\(\varphi_{gas}\) = 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 \(\mu\)m, 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 \( \text{NO}_3^- \) and \( \text{NO}_2^- \) 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·min\(^{-1}\) with a 13 mM KOH aqueous solution. Ammonium (\( \text{NH}_4^+ \)) was measured using AQUAfast ammonia thermal digestion kit (Thermo Scientific, USA).

3.1.2.6 Microsensors for dissolved N\(_2\)O measurement

Dissolved N\(_2\)O was measured by amperic detection with N\(_2\)O 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\(_2\)O in the aqueous phase. The reduction of N\(_2\)O at the metal cathode surface is detected as an electric signal and the reduction rate of N\(_2\)O is directly proportional to N\(_2\)O concentration. Although the use of these sensors seemed promising for continuous N\(_2\)O 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\(_2\)O signal) and high temperature dependence (Jenni et al., 2012). Thus, although this probe confirmed N\(_2\)O synthesis in microalgal suspension, their use was discontinued.
3.1.3 Results and discussion

3.1.3.1 N\textsubscript{2}O emissions from microalgae cultivated in 50 L PBRs

In order to evaluate N\textsubscript{2}O emissions from commercial microalgae cultures, N\textsubscript{2}O was measured from \textit{C. vulgaris} cultures fed either NO\textsubscript{3}\textsuperscript{-} or NH\textsubscript{4}\textsuperscript{+}, \textit{Neochloris} cultures fed NO\textsubscript{3}\textsuperscript{-}, and \textit{A. platensis} cultures fed NO\textsubscript{3}\textsuperscript{-} (Table 3.1).
Table 3.1: N$_2$O emissions recorded during microalgae cultivation in 50 L column photobioreactors (n = sampling size for N$_2$O measurements). Summary of operational parameters can be found in Appendix O.

<table>
<thead>
<tr>
<th>Date (days of monitoring)</th>
<th>Season</th>
<th>Microalgae species</th>
<th>Nitrogen Source</th>
<th>n</th>
<th>Number of PBR operated</th>
<th>N$_2$O emissions (nmol·m$^{-2}$·h$^{-1}$)</th>
<th>Median N$_2$O emissions (nmol·m$^{-2}$·h$^{-1}$)$^a$</th>
<th>N$_2$O emissions (nmol N$_2$O·g-DCW$^{-1}$·h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12/06/12 to 18/06/12 (6 days)</td>
<td>Winter</td>
<td><em>C. vulgaris</em></td>
<td>NO$_3^-$</td>
<td>76</td>
<td>1</td>
<td>400.0 – 10800</td>
<td>3400</td>
<td>10.0 – 270</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>9.55 – 2940</td>
<td>1400</td>
<td>0.25 – 73.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>3700 – 14900</td>
<td>6100</td>
<td>92.0 – 370</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>290.0 – 5150</td>
<td>2500</td>
<td>7.30 – 130</td>
</tr>
<tr>
<td>28/03/14, 31/03/14 and 11/04/14 (3 days)</td>
<td>Autumn</td>
<td><em>C. vulgaris</em></td>
<td>NO$_3^-$</td>
<td>26</td>
<td>1</td>
<td>840.0 – 12150</td>
<td>2600</td>
<td>16.0 – 270</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>270.0 – 14250</td>
<td>2300</td>
<td>7.70 – 400</td>
</tr>
<tr>
<td>04/06/14 to 04/07/14 (32 days)</td>
<td>Winter</td>
<td><em>S. platensis</em></td>
<td>NO$_3^-$</td>
<td>90</td>
<td>1</td>
<td>Not significant*</td>
<td>Not significant*</td>
<td>Not significant*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>Not significant*</td>
<td>Not significant*</td>
<td>Not significant*</td>
</tr>
<tr>
<td>04/06/14 to 10/07/14 (36 days)</td>
<td>Winter</td>
<td><em>Neochloris</em></td>
<td>NO$_3^-$</td>
<td>136</td>
<td>1</td>
<td>50.5 – 11600</td>
<td>2100</td>
<td>2.30 – 280</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>130.0 – 14200</td>
<td>1900</td>
<td>3.50 – 340</td>
</tr>
<tr>
<td>28/07/14 to 28/08/14 (32 days)</td>
<td>Winter</td>
<td><em>C. vulgaris</em></td>
<td>NO$_3^-$</td>
<td>84</td>
<td>1</td>
<td>85.0 – 11490</td>
<td>2600</td>
<td>13.0 – 510</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>63.0 – 11100</td>
<td>2300</td>
<td>9.60 – 540</td>
</tr>
<tr>
<td>28/07/14 to 28/08/14 (32 days)</td>
<td>Winter</td>
<td><em>C. vulgaris</em></td>
<td>NH$_4^+$</td>
<td>84</td>
<td>1</td>
<td>Not significant*</td>
<td>Not significant*</td>
<td>Not significant*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>Not significant*</td>
<td>Not significant*</td>
<td>Not significant*</td>
</tr>
<tr>
<td>13/10/14 to 28/10/14 (15 days)</td>
<td>Spring</td>
<td><em>C. vulgaris</em></td>
<td>NO$_3^-$</td>
<td>44</td>
<td>1</td>
<td>910.0 – 29300</td>
<td>6100</td>
<td>32.0 – 800</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>330.0 – 15600</td>
<td>4000</td>
<td>14.0 – 350</td>
</tr>
<tr>
<td>04/11/14 to 28/11/14 (24 days)</td>
<td>Spring</td>
<td><em>C. vulgaris</em></td>
<td>NO$_3^-$</td>
<td>64</td>
<td>1</td>
<td>14.2 – 38000</td>
<td>12800</td>
<td>0.50 – 980</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>32.0 – 27900</td>
<td>7000</td>
<td>2.60 – 800</td>
</tr>
<tr>
<td>01/12/14 to 10/12/14 (12 days)</td>
<td>Summer</td>
<td><em>C. vulgaris</em></td>
<td>NO$_3^-$</td>
<td>32</td>
<td>1</td>
<td>930.0 – 35600</td>
<td>11200</td>
<td>33.0 – 1180</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>580.0 – 11400</td>
<td>8700</td>
<td>33.0 – 350</td>
</tr>
</tbody>
</table>

$^a$Significance was statistically validated using two sample t-test, $\alpha = 0.05$ (Appendix P).

a N$_2$O 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 N₂O emissions (up to 38000 and 14200 nmole·m⁻²·h⁻¹, respectively) were recorded from *C. vulgaris* and *Neochloris* cultures fed NO₃⁻. In contrast, N₂O was never detected from *C. vulgaris* cultures fed NH₄⁺ or *A. platensis* cultures fed NO₃⁻.

While 10 microalgae species have been indicated to generate N₂O emissions in the literature (Chapter 1), this ability had not previously been investigated in *Neochloris* and *A. platensis*. While the lack of N₂O emissions from *A. platensis* cultures evidences N₂O 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, N₂O 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 N₂O 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, N₂O emissions could thus potentially be associated with NO₂⁻ reduction by algae (see further discussion below), NH₄⁺ oxidation by bacteria or archaea, or NO₂⁻ reduction by bacteria and fungi. N₂O production was however never detected in cultures fed NH₄⁺, suggesting N₂O synthesis by ammonium oxidizing bacteria (AOA) and archaea (AOB) was not significant in this study. In comparison, the ability of *C. vulgaris* to synthesise N₂O is relatively well documented (Weathers, 1984; Guieysse et al., 2013; Alcántara et al., 2015) and has been linked to NO₂⁻ reduction under aerobic conditions (Guieysse et al., 2013). Finally, N₂O emission during NO₂⁻ 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 N₂O emissions measured from *C. vulgaris* culture fed NO₃⁻ (320 data points over 92 days of cultivation) covering a broad range of algae concentrations, dissolved NO₂⁻ 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·m⁻²·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 N₂O production (nmol·m⁻²·h⁻¹) measured during C. vulgaris cultivation in 50 L photobioreactors.
Numerous parameters influencing algal activity could potentially impact N\textsubscript{2}O emissions during algae cultivation. In particular, Guieysse et al. (2013) reported that N\textsubscript{2}O emissions by axenic \textit{C. vulgaris} in laboratory NO\textsubscript{2}\textsuperscript{-}-laden batch cultures were positively correlated to extracellular NO\textsubscript{2}\textsuperscript{-} concentration (albeit not linearly) and cell mass, and negatively correlated to light intensity. In the present study, \textit{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\textsubscript{2}O 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\textsubscript{2}O emissions. In analysing raw data sets (Figures R1-6, Appendix R), no clear statistical correlation could be found between N\textsubscript{2}O emissions and daily DCW (R\textsuperscript{2} = 0.070), solar irradiance (R\textsuperscript{2} = 0.080), nitrite concentration (R\textsuperscript{2} = 0.030), and air or broth temperature (R\textsuperscript{2} = 0.050 and 0.0030, respectively). However, a stepwise linear regression analysis with backward elimination (personal communication\textsuperscript{11}) showed that DCW and solar irradiance were significant variables at 95% confidence (\textit{p-value} < 0.05) with increasing biomass concentration and solar irradiance having a positive effect on N\textsubscript{2}O 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\textsuperscript{2} (R\textsuperscript{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\textsubscript{2}O emissions. In order to circumvent the effect of noise, data were

\textsuperscript{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 N₂O 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 N₂O productions (nmol N₂O·g-DCW⁻¹·h⁻¹) are also given in Table 3.1 to compare with other monitoring studies.

**Figure 3.3:** N₂O 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\(^\text{12}\) (Béchet et al., 2015b) was also quantified in June 2012 and during the entire period of monitoring, *C. vulgaris* viability always remained high ($96 \pm 1.0$; $98 \pm 0.8$; $97 \pm 0.9$; and $98 \pm 1.0$ % 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\(^\text{13}\) 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

\(^\text{12}\) Testing performed by another operator, Dr Quentin Béchet.
\(^\text{13}\) 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$_2$O production (nmol·m$^{-2}$·h$^{-1}$) as a function of predicted instantaneous biomass productivities (kg-DW·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$_2$O 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) $\text{N}_2\text{O}$ production (nmol·m$^{-2}$·h$^{-1}$) as a function of solar irradiance (W·m$^{-2}$) for each solar irradiance population decile (in each decile $n = 32$). b) Weekly averaged $\text{N}_2\text{O}$ production (nmol·m$^{-2}$·h$^{-1}$) as a function of weekly averaged solar irradiance (W·m$^{-2}$).

A causality between light supply and $\text{N}_2\text{O}$ synthesis was also indicated during the outdoor cultivation of *Neochloris* (Appendix V) and *C. vulgaris* fed $\text{NO}_3^-$ (Figure 3.6). As can be seen $\text{N}_2\text{O}$ 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 ($\text{NO}_3^-$ as N-source) in May 2011 (Guieysse et al., 2013), but contrast the results from the same authors (and from Chapter 2) who showed $\text{N}_2\text{O}$ synthesis by axenic *C. vulgaris* supplied with $\text{NO}_2^-$ was boosted under darkness during *in vitro* laboratory assays.
Figure 3.6: Changes in N₂O 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 N₂O emissions under NO₃⁻ outdoors (this study) and NO₂⁻ indoors supply (Guieysse et al., 2013) may be explained by light-dependent mechanisms influencing NO₃⁻ assimilation and therefore impacting intracellular NO₂⁻ accumulation (e.g. imbalances between the rate of NO₃⁻ reduction into NO₂⁻ and the rates of NO₂⁻ reduction into NH₄⁺ and N₂O, 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 NO₂⁻ and NO generation). It is therefore possible that the rate of N₂O synthesis in light-limited (e.g. outdoors) C. vulgaris cultures fed NO₃⁻ is limited by the light-dependent reduction of NO₃⁻ into NO₂⁻, while the rate of N₂O synthesis in C. vulgaris fed NO₂⁻ increases when photosynthesis-repression prevents NO₂⁻ reduction into NH₄⁺ by nitrite reductase. Consequently, if N₂O production from outdoor C. vulgaris cultures fed NO₃⁻ 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$_2$O 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$_2$O 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$_2$O synthesis and, indeed, as showed in Figure 3.6 NO$_2^-$ addition significantly boosted N$_2$O emissions from outdoors *C. vulgaris* cultures fed NO$_3^-$ (this was confirmed during a parallel indoor experiment, Appendix X).
Surprisingly, no statistical correlation between extracellular NO$_2^-$ concentration and N$_2$O 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$_2$O emissions (63.0 – 11100 nmol N$_2$O·m$^{-2}$·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$_2$O 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$_2$O
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 (Alcântara et al., 2015) and not evidenced by N$_2$O 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$_2$O under a broad range of broth temperatures (10 – 30°C). Even when clustering the data, no correlation could be established between N$_2$O 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$_2$O emissions.
Figure 3.8: a) N$_2$O production (nmol·m$^{-2}$·h$^{-1}$) as a function of air temperature (°C) for each air temperature population decile (in each decile n = 32). b) N$_2$O emissions (nmol·m$^{-2}$·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$_2$O 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$_2$O emissions (up to 15% of the N$_2$O 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$_2$O emissions rates

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$^{14}$ Exponential fit is generally used to fit data sets involving temperature. Considering the weak correlation between N$_2$O 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$_2$O 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 1$^{st}$ and 3$^{rd}$ quartile of the monitoring data (1500 – 8000 nmol N$_2$O·m$^{-2}$·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$_2$O·m$^{-2}$·h$^{-1}$). Based on these rates, the cultivation of algae over 35 million ha of raceway ponds$^{15}$ would release 132 – 685 kt N-N$_2$O·yr$^{-1}$, which is equivalent to 2 – 10% of the amount of N$_2$O globally emitted by all anthropogenic sources each year (6.9 Tg N-N$_2$O·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$_2$O emissions during microalgal cultivation.

### 3.1.3.3 N$_2$O emission factors

N$_2$O 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$_2$O (kg N-N$_2$O) by using specific N$_2$O emission factors (EFs, kg N-N$_2$O·kg N$^{-1}$, De Klein et al., 2006; Doorn et al., 2006). Based on this approach, the emissions of 1500 – 8000 nmol N$_2$O·m$^{-2}$·h$^{-1}$ (equivalent to 0.02 – 0.11 g N-N$_2$O·m$^{-3}$·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 N₂O 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 N₂O emissions during algae cultivation. Similarly, Weathers and Niedzielski, (1986) reported that the cyanobacteria _Anacystis nidulans_ did not produce N₂O when supplied NO₃⁻ (or NO₂⁻) 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 N₂O synthesised can be different according to microalgae species. Consequently, further monitoring would be required to evaluate the potential ability and the magnitude of N₂O 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 NH₄⁺ as N-source may provide a simple and economical solution to prevent N₂O emissions from microalgal cultivation, as shown here when _C. vulgaris_ was fed NH₄⁺. In addition, N₂O emission from _C. vulgaris_ cultures fed with NH₄⁺ was triggered by artificially raising the NO₂⁻ concentration by 0.5 mM and the N₂O 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$_2$O 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$_2$O emissions reported during commercially relevant algae cultivation outdoors. Significant and variable N$_2$O emissions were detected during the outdoor cultivation of *C. vulgaris* (9.60 – 38000 nmol N$_2$O·m$^{-2}$·h$^{-1}$) and *Neochloris* sp. (50.5 – 14200 nmol N$_2$O·m$^{-2}$·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$_2$O 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$_2$O emissions such as growth kinetics which were lacking in our study. Based on the rates reported of 1500 – 8000 nmol N$_2$O·h$^{-1}$·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$_2$O emissions. Based on these rates, N$_2$O 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 microalgal species that do not appear to synthesise N$_2$O (e.g. *A. platensis*) offer possible mitigation solutions. Nevertheless, N$_2$O emissions from microalgal production should be carefully monitored and accounted for in impact assessment such as life cycle analysis.
3.2 N₂O emissions from HRAP fed real domestic wastewater

ABSTRACT

While the intergovernmental panel for climate change (IPCC) currently estimate that 200 kt of N₂O are indirectly generated from wastewater effluent discharge, direct N₂O emissions during centralised wastewater treatment are considered negligible. In order to determine if direct N₂O emissions from microalgae-based pond systems could also be considered negligible, N₂O 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 (NO₃⁻), nitrite (NO₂⁻), ammonium (NH₄⁺), and N₂O. N₂O emissions were found to range from 5.10 to 19000 μg N-N₂O·m⁻²·d⁻¹ (median of 2300 μg N-N₂O·m⁻²·d⁻¹, n = 50). Based on the emissions of 450 – 4600 μg N-N₂O·m⁻²·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·d⁻¹ (n = 50). Using these emissions we estimated global direct N₂O emissions during secondary microalgae-based wastewater treatment at 0.36 – 3.8 kt N-N₂O·yr⁻¹. This range represents less than 2% of global N₂O emissions budgeted by the IPCC for wastewater treatment. Therefore, N₂O 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\textsubscript{2}O 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\textsubscript{2}O emissions from microalgae-based systems for wastewater treatment. To date, two studies have focused on N\textsubscript{2}O 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\textsubscript{2}O), 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-N\textsubscript{2}O·m\textsuperscript{-2}·d\textsuperscript{-1} in a stabilisation pond located in Mexico, Glaz et al, (2016) measured N\textsubscript{2}O emissions up to 0.001 μg N-N\textsubscript{2}O·m\textsuperscript{-2}·d\textsuperscript{-1} and 0.5 μg N-N\textsubscript{2}O·m\textsuperscript{-2}·d\textsuperscript{-1} from stabilisation ponds in Western Australia and Quebec, respectively.

Due to the large variation in the N\textsubscript{2}O 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\textsubscript{2}O from microalgae-based pond systems. In addition, the global significance of N\textsubscript{2}O 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 N₂O emissions from microalgae-based pond systems could be massive.

In order to evaluate the potential significance of N₂O emissions from microalgae-based systems for WWT, N₂O 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 N₂O 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·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·s⁻¹).

![Figure 3.9: 1000 L HRAP fed primary wastewater](image-url)
3.2.2.2 Sampling and N₂O 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 N₂O 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 N₂ (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 N₂O concentration was reached in the vial. Because the equilibrium was rapid and not affected by significant N₂O production (or consumption) it was assumed that the N₂O measured at t = 30 min was representative of the N₂O 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 N₂O concentration was then back calculated from the gaseous N₂O concentration using Henry’s law. The amount of atmospheric N₂O emitted was calculated based on Equation 3.2:
\[ \Phi_{N_2O} = Kla_{N_2O} \times ([N_2O]^* - [N_2O]), \text{ Equation 3.2} \]

\[ \Phi_{N_2O} = \text{N}_2\text{O flux (mol·L}^{-1}\cdot\text{s}^{-1}) \]

\[ Kla_{N_2O} = \text{N}_2\text{O mass transfer coefficient (s}^{-1}) \]

\[ [N_2O]^* = \text{Dissolved N}_2\text{O concentration at equilibrium (mol·L}^{-1}) \]

\[ [N_2O] = \text{Dissolved N}_2\text{O concentration (mol·L}^{-1}) \]

\( Kla_{(O_2)} \), was experimentally determined by filling the HRAP with 1000 L of tap water, under continuous mixing with the paddle wheel and bubbling with \( \text{N}_2 \) to decrease \( \text{O}_2 \) concentration below 1 mg·L\(^{-1}\). Bubbling was then stopped and \( \text{O}_2 \) concentration was measured every minute for 4 hours. The logged data were then used to estimate \( Kla_{(O_2)} = 0.18 \text{ h}^{-1} \) in tap water by integrating \( \text{O}_2 \) concentration as a function of time (Akita and Yoshida, 1973). The mass transfer coefficient for oxygen, \( Kla_{(O_2)} \) was used to compute the mass transfer coefficients of \( \text{N}_2\text{O} \), \( Kla_{(N_2O)} \) 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).

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

\[ Kla_{N_2O} = \text{N}_2\text{O mass transfer coefficient (s}^{-1}) \]

\[ Kla_{O_2} = \text{O}_2 \text{ mass transfer coefficient (s}^{-1}) \]

\[ D_{F_{O_2}} = \text{Molecular diffusivity of O}_2 \text{ in water (1.98 } \times 10^{-9}\text{·m}^{-2}\cdot\text{s}^{-1} \text{ at 20ºC)} \]

\[ D_{F_{N_2O}} = \text{Molecular diffusivity of N}_2\text{O in water (1.84 } \times 10^{-9}\text{·m}^{-2}\cdot\text{s}^{-1} \text{ at 20ºC)} \]
3.2.2.3 Analytical procedures

See Section 3.1.2.4
3.2.3 Results and discussion

3.2.3.1 N$_2$O emissions from a HRAP

This study presents the first long-term investigation of direct N$_2$O 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$_2$O with direct emissions fluctuating from $7.60 - 28000$ nmol N$_2$O·m$^{-2}$·h$^{-1}$ (Figure 3.10). These significant and highly variable N$_2$O emissions represented $5.10 - 19000$ μg N-N$_2$O·m$^{-2}$·d$^{-1}$ with a median value of $2300$ μg N-N$_2$O·m$^{-2}$·d$^{-1}$. N$_2$O 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$_2$O emissions recorded under 7 days HRT operation ($70 - 19000$ μg N-N$_2$O·m$^{-2}$·d$^{-1}$ with a median value of $4500$ μg N-N$_2$O·m$^{-2}$·d$^{-1}$) were significantly higher (two sample t-test, $α = 0.05$, $p$-value < 0.05; See Appendix AA) than N$_2$O emissions recorded under 10 days HRT operation ($5.1 - 6000$ μg N-N$_2$O·m$^{-2}$·d$^{-1}$ with a median value of $700$ μg N-N$_2$O·m$^{-2}$·d$^{-1}$).
Figure 3.10: a) $\text{N}_2\text{O}$ production (nmol·m$^{-2}$·h$^{-1}$) from weekly samples when the HRAP was operated at 10 days HRT. b) $\text{N}_2\text{O}$ production (nmol·m$^{-2}$·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, $\alpha = 0.05$, \textit{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$_2$O emissions was related to the change in HRT or the change in temperature and solar irradiance.

Figure 3.11 showed N$_2$O 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$_2$O 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 $> 4^\circ$C (Braker et al., 2010; Béchet et al., 2015a). N$_2$O 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$_2$O 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$_2$O emissions recorded may also be explained by changes in microbial population in the HRAP.
Figure 3.11: N₂O production (nmol·m⁻²·h⁻¹) against TSS (mg·L⁻¹), NO₂⁻ (mg·L⁻¹), pH, solar irradiance (W·m⁻²), T (°C), and DO (mg·L⁻¹) during wastewater treatment in a HRAP operated at 10 and 7 days HRT.
While identifying the actual source of N$_2$O in the HRAP was beyond the scope of only measuring N$_2$O emissions for impact assessment (e.g. greenhouse gas inventories), various mechanisms can be proposed to explain the N$_2$O emissions. In wastewater N$_2$O can be biologically generated via bacterial nitrification, bacterial denitrification, bacterial nitrification-denitrification, archaeal ammonium oxidation (Limpiyakorn et al., 2011), and microalgal N$_2$O 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·L$^{-1}$ at night to super saturated values at day time). On the other hand, denitrification could explain some of the N$_2$O emissions recorded when the HRAP was operated at 7 days HRT because the culture was experiencing anoxia (< 1 mg·L$^{-1}$ during night time). In addition, bacterial and archaeal nitrifying activity may explain the production of NO$_3^-$ (48.5 ± 7.50 mg·L$^{-1}$, 95% confidence, n = 49) and NO$_2^-$ (2.42 ± 0.63 mg·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$_2$O in the HRAP.
Figure 3.12: $\text{NO}_3^-$ (mg·L$^{-1}$), $\text{NO}_2^-$ (mg·L$^{-1}$) and $\text{NH}_4^+$ (mg·L$^{-1}$) concentrations in the filtered HRAP microalgal/bacterial suspension.

Nitrite is known to fuel microalgal $\text{N}_2\text{O}$ synthesis. Consequently, the $\text{NO}_2^-$ produced in the HRAP could be utilised by the microalgae (but also potentially nitrifying bacteria), leading to $\text{N}_2\text{O}$ production. Overall the co-occurrence of mechanisms leading to $\text{N}_2\text{O}$ emissions could not be totally disregarded. Further work is required (i.e. metagenomics) to determine the source(s) of $\text{N}_2\text{O}$ in the HRAP and to improve our understanding of $\text{N}_2\text{O}$ emissions in HRAPs fed wastewater$^{16}$.

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$^{16}$ 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 N₂O emission factors

The direct N₂O emissions measured during this study ranged from 5.1 to 19000 μg N-N₂O·m⁻²·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-N₂O·m⁻²·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-N₂O·m⁻²·d⁻¹ (equivalent to 0.0018 – 0.018 g N-N₂O·m⁻³·d⁻¹) represented 0.04 – 0.45% of the TN influent load of 4.5 g TN·d⁻¹ fed to the HRAP. As seen in Figure 3.13a, this range is significantly higher than the 0.0047% g N-N₂O·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).
3.2.3.3 Significance of N₂O emissions from a pilot HRAP fed with domestic wastewater

As illustrated in Figure 3.13b, the IPCC currently does not account for direct N₂O 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-N₂O·yr⁻¹ are globally generated from wastewater effluent (Ciais et al., 2013).

In order to evaluate whether direct N₂O 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$_2$O emissions measured (450 – 4600 μg N-N$_2$O·m$^{-2}$·d$^{-1}$), global N$_2$O emissions from microalgae-based ponds were estimated at 0.36 – 3.8 kt N-N$_2$O·yr$^{-1}$. These emissions represent 0.18 – 1.9% of global N$_2$O emissions budgeted for wastewater treatment discharge by the IPCC (200 kt N-N$_2$O·yr$^{-1}$). Consequently, global direct N$_2$O 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$_2$O emissions from pond systems are likely to be lower than the emissions of 0.36 – 3.8 kt N-N$_2$O·yr$^{-1}$ for the following reasons:

1. N$_2$O 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$_2$O, has generally a lower productivity in stabilisation ponds than in HRAP (Craggs et al., 2014).
3. Potential seasonal and temporal effects on N$_2$O 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).

\[
\lambda_s = 350(1.107 - 0.002T)^{7.25}, \text{ Equation 3.4}
\]

\[\lambda_s = \text{Surface BOD loading rate (kg·ha}^{-1}·d^{-1})\]

\[T = \text{Mean air temperature (°C)}\]

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$_2$O emissions of 5.1 – 19000 $\mu$g N-N$_2$O·m$^{-2}$·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 $\mu$g N-N$_2$O·m$^{-2}$·d$^{-1}$, representing 25 – 75% of the data) N$_2$O emissions factors were estimated at 0.04 – 0.45% of the N load for wastewater treatment. Using this data, we estimated that N$_2$O emissions from microalgae-based wastewater treatment ponds would have a relatively minor environmental impact, representing 0.18 – 1.9% of the global N$_2$O 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$_2$O emissions.
3.3 Relevance of the monitoring studies

Section 3.1 and 3.2 presented the first long-term monitoring of direct N\textsubscript{2}O emissions from microalgae cultivated in photobioreactors fed synthetic media and a high rate algal pond fed real domestic wastewater.

Tools for estimating N\textsubscript{2}O emissions from microalgal cultivation were lacking. Based on the methodology described by the IPCC for GHG inventory\textsuperscript{17}, emissions factors (EFs) were calculated in order to estimate N\textsubscript{2}O emissions from microalgal cultivation. EFs of 0.1 – 0.4% of the N load were estimated for microalgae culture fed NO\textsubscript{3}\textsuperscript{-}-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 microalgal-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\textsubscript{2}O emissions can occur during microalgae cultivation and that consequently, N\textsubscript{2}O emissions should be considered for accurate environmental assessments of microalgal cultivation. Importantly, as discussed in Chapter 1, algal-mediated N\textsubscript{2}O emissions may occur from a range of microalgal-dominated engineered and natural ecosystems and the global magnitude of N\textsubscript{2}O emissions from these systems is currently unknown.

\textsuperscript{17} As described in Section 3.1.3.4, N\textsubscript{2}O 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\textsubscript{2}O, by using specific N\textsubscript{2}O emission factors (kg N\textsubscript{2}O·kg N\textsuperscript{-1}) which are based on field N\textsubscript{2}O 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$_2$O 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$_2$O 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$_2$O. 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$_2$O (especially if eutrophication is of anthropogenic origin).
References


Chapter 4.

Conclusions and future prospects
4.1 Conclusions

While the synthesis of N$_2$O by microalgae was suggested and documented for 40 years, the ‘microalgal N$_2$O bio-origin’ was rarely acknowledged in studies evaluating N$_2$O emissions from microalgae-based (eco)systems. From the critical literature review, it became clear that N$_2$O synthesis by microalgae needed further investigation. Firstly, basic calculations using available data showed that N$_2$O 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$_2$O synthesis were poorly understood. Only two putative microalgal N$_2$O pathways were described in the literature, but based on the knowledge of the ‘conventional mechanisms’ leading to N$_2$O emissions in biological systems we were able to suggest six putative microalgal N$_2$O pathways. Using this knowledge as a basis, the synthesis of N$_2$O 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$_2$O 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$_2$O 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$_2$O 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 \( \text{NO}_2^- \) accumulation (i.e. low/moderate intracellular \( \text{NO}_2^- \)), we hypothesised that \( \text{N}_2\text{O} \) synthesis should mainly occur under illumination and via NR-ARC-mediated \( \text{NO}_2^- \) reduction under physiological conditions (i.e. when \( \text{NO}_3^- \) is the dominant N source). By contrast, \( \text{N}_2\text{O} \) should be synthesised via both NR-ARC and COX-mediated \( \text{NO}_2^- \) reduction under \( \text{NO}_2^- \) stress (i.e. induced by high intracellular \( \text{NO}_2^- \)). High throughput RNA sequencing of \textit{C. reinhardtii} 6145c samples showed that the genes encoding NR, ARC, NiR, NOR, COXs, AOXs, and THBs (proteins potentially involved during microalgal \( \text{N}_2\text{O} \) synthesis) were expressed in \textit{C. reinhardtii} 6145c samples supplied with \( \text{NO}_2^- \) and incubated in darkness. While \( \text{NO}_2^- \) 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 \( \text{NO}_2^- \)-supplied tests. Overall, the RNA sequencing data were consistent with the biochemical evidence that under high \( \text{NO}_2^- \) loading:

1. NO is generated during \( \text{N}_2\text{O} \) 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 \( \text{NO}_2^- \).
2. \( \text{NO}_2^- \) reduction into NO leading to ‘late’ \( \text{N}_2\text{O} \) synthesis is not mediated by NR, as \( \text{NO}_2^- \) supply did not trigger any significant transcriptional regulation of NR gene in the tests.
3. \( \text{NO}_2^- \) stress was evidenced by the significantly different up-regulation of initiation factors genes in the tests (with \( \text{NO}_2^- \)) versus controls (no \( \text{NO}_2^- \)).

In light of the findings obtained from the laboratory \textit{in vitro} batch assays performed, we concluded that \( \text{N}_2\text{O} \) synthesis may represent a means of regulating NO (and \( \text{NO}_2^- \))
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$_2$O 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$_2$O pathway provides new advances in microalgal biology.

- Environmental implications: given that nitrate assimilation is highly conserved in some microalgae, the ability to synthesise N$_2$O could be widely spread among algal species. Knowing the ubiquity of microalgae in the environment, the aerobic microalgal N$_2$O pathway could be a globally significant N$_2$O source.

To the best of our knowledge, the potential environmental significance of N$_2$O emissions from microalgal cultivation was unknown and N$_2$O emissions during microalgal cultivation had never been thoroughly assessed. We thus presented the first long-term N$_2$O monitoring in outdoors microalgal engineered systems. N$_2$O 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$_2$O monitoring performed during commercially-relevant microalgae species cultivation, we showed that: significant and variable N$_2$O emissions were recorded from *C. vulgaris* and *Neochloris sp.* cultures fed NO$_3^-$ (9.60 – 38000 nmol N$_2$O·m$^{-2}$·h$^{-1}$ n = 332, and 50 – 14200 nmol N$_2$O·m$^{-2}$·h$^{-1}$ n = 136, respectively). While further monitoring of *C. vulgaris* cultures indicated that N$_2$O 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 $\text{NO}_3^-$ is the main N source, $\text{N}_2\text{O}$ emissions occur during illumination. Also, a $\text{NO}_2^-$ supply in $C. \text{ vulgaris}$ cultures fed $\text{NO}_3^-$ triggered $\text{N}_2\text{O}$ emissions, supporting the relation between nitrate assimilation, $\text{NO}_2^-$, and $\text{N}_2\text{O}$ synthesis. Based on the conservative $\text{N}_2\text{O}$ emissions recorded (1500 – 8000 nmol $\text{N}_2\text{O} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$, representing 25 – 75% of the data), we estimated that $C. \text{ vulgaris}$ as feedstock for replacing 30% of all US transport fuels could generate $\text{N}_2\text{O}$ emissions representing up to 10% of the global anthropogenic $\text{N}_2\text{O}$ emissions currently budgeted by the IPCC. Because no significant emissions were recorded from $A. \text{ platensis}$ cultures fed $\text{NO}_3^-$ or $C. \text{ vulgaris}$ cultures fed $\text{NH}_4^+$ we were able to propose the use of $\text{NH}_4^+$ or species management as potential mitigation solutions.

- From the $\text{N}_2\text{O}$ monitoring performed during wastewater treatment in a HRAP, we showed that significant and variable $\text{N}_2\text{O}$ emissions of 5.1 to 19000 $\mu$g $\text{N} \cdot \text{N}_2\text{O} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ (equivalent to 8 – 28000 nmol $\text{N}_2\text{O} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ n= 50) were recorded. Because $\text{N}_2\text{O}$ can be produced by various microorganisms in HRAP fed wastewater, it was difficult to explain the actual source and the variability in the $\text{N}_2\text{O}$ emissions recorded. Nevertheless, based on the conservative $\text{N}_2\text{O}$ emissions rates measured (450 – 4600 $\mu$g $\text{N} \cdot \text{N}_2\text{O} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$, representing 25 – 75% of the data), we estimated that microalgae-based wastewater treatment ponds would generate minor global $\text{N}_2\text{O}$ emissions representing less than 2% of the global $\text{N}_2\text{O}$ emissions from wastewater treatment currently budgeted by the IPCC.
Based on the N₂O productions recorded we also estimated the first emission factors of 0.1 – 0.4% of the N load for microalgal cultivation fed NO₃⁻-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 N₂O 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 N₂O synthesis and considering microalgal N₂O emissions.

Overall, from the work performed within this thesis we clearly showed that 1) microalgae have the ability to synthesise N₂O and 2) N₂O can be generated during microalgal cultivation. The variability of N₂O emissions and the poor correlations between operational parameters generated uncertainty in our estimations. Nevertheless, we advised that microalgal N₂O emissions should be monitored and accounted for in greenhouse gas inventories and life cycle analysis of microalgal cultivation. Microalgal biotechnology is still at its infancy so there is an opportunity to understand and anticipate the ‘N₂O 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 N₂O emissions variability. In addition, because our research did not include the evaluation of potential N₂O 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 N₂O 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 N₂O biochemical pathway. For instance, *C. reinhardtii* could be incubated in darkness or under illumination, and with or without NO₂⁻ 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 N₂O synthesis according to the growth conditions. The results would be valuable for understanding how microalgal cells modulate NO₂⁻, NO, and N₂O 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 N₂O 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 N₂O 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$_2$O (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$_2$O source’$^{18}$, and that the N$_2$O pathway is probably widely spread among microalgae species, a search for homologs of the genes involved in microalgal N$_2$O 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$_2$O pathway in the environment, and if this pathway is conserved in microalgae. Additionally, in order to investigate the microbial N$_2$O 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$_2$O emissions recorded during pilot scale cultivation. To better evaluate the impact of operational parameters on N$_2$O emissions, controlled experiments with the commercially relevant microalgae C. vulgaris could be performed. Considering the complexity of the N$_2$O pathway and the high number of variables that can potentially

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$^{18}$ Based on the current knowledge of the N cycle, various studies (and personal communication) that focused on the N cycle and N$_2$O synthesis, have noted some counter-intuitive pattern (e.g. unexplained N losses; N$_2$O 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)

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**Hutner Trace element**

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**Trace element**

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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  
CTAGTGAGACGAACTCGTCAACTACATCTCGCTGATCGGCACCATGGGGGTGGTGGTGATCAGCGCTATGTACTTGACGAGTTCGTCTC-3'

amiRev_CYP55  
CTAGCGAGACGAACTCGTCAAGTACATAGCGCTGATCACCACCCCCCATGGTGCCGATCAGCGAGATGTAGTTGACGAGTTCGTCTC-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'-GGTGTTGGGTGGTCGGTGTTTTTG-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

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).](image-url)
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$_2$O 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$_2$O production under NO$_2^-$ supply; and 3) a sample from an additional ‘normal’ 5 days old culture supplied antibiotics (25 mg streptomycin·L$^{-1}$ and 100 mg penicillin·L$^{-1}$; (Guieysse et al., 2013) to serve as positive control of bacterial-free culture. These samples were stored at -40°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>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Sequence (5’ – 3’)</th>
<th>Exp. size (bp)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>norB</td>
<td>norB1F</td>
<td>CGNGARTTYCTSGARCARCC</td>
<td>670</td>
<td>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)</td>
</tr>
<tr>
<td></td>
<td>norB8R</td>
<td>CRTADGCVCCRWAGAAAVGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cnorB</td>
<td>cnorBF</td>
<td>GACAAGNNNTACTGGTGT</td>
<td>389</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cnorBR</td>
<td>GAANCCCCACNCNCNGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>qnorB</td>
<td>qnorBF</td>
<td>GNCAVACRGNTAYGA</td>
<td>262</td>
<td></td>
</tr>
<tr>
<td></td>
<td>qnorBR</td>
<td>ACCCANAGRTGNACNACCCACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S</td>
<td>F27</td>
<td>AGAGTTTGATCCTGGCTCAG</td>
<td>1000-1500</td>
<td>94 ºC – 3 min [94°C - 30 s, 50°C - 30 s, 72°C – 90 s] × 35 cycles, 72°C – 5 min, 10 ºC hold (Giovannoni, 1991)</td>
</tr>
<tr>
<td></td>
<td>1492R</td>
<td>TACGGYTACCTTGTTACGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Archaea</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amoA</td>
<td>amoAF</td>
<td>STAATGGTCTGGCTTAGACG</td>
<td>635</td>
<td>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)</td>
</tr>
<tr>
<td></td>
<td>amoAR</td>
<td>GCGGCCATCCATCTGTATGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Algae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rbcl</td>
<td>RbclAF</td>
<td>ATGTCACCACAACACAGAGACTAAAGC</td>
<td>1200</td>
<td>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).</td>
</tr>
<tr>
<td></td>
<td>RbclAR</td>
<td>GCAGCAGCTAGTTCCGGGCTCCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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. N₂O production as also confirmed in the serum flasks supplied NO₂⁻ (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 NO₃⁻) used to inoculate the batch assay; 2: sample from the serum flasks used for quantifying N₂O production under NO₂⁻ supply; and 3: sample from an additional ‘normal’ 5 days old culture supplied antibiotics.

<table>
<thead>
<tr>
<th>Sample</th>
<th>16S</th>
<th>norB</th>
<th>cnorB</th>
<th>qnorB</th>
<th>amoA</th>
<th>Rbcl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(bacteria)</td>
<td>(archaea)</td>
<td>(algal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>1</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y*</td>
</tr>
<tr>
<td>2</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N*</td>
<td>N</td>
<td>Y*</td>
</tr>
<tr>
<td>3</td>
<td>N</td>
<td>N*</td>
<td>N</td>
<td>N*</td>
<td>N</td>
<td>Y*</td>
</tr>
</tbody>
</table>

* 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 NO₃⁻) used to inoculate the batch assay; 3, sample from the serum flasks used for quantifying N₂O production under NO₂⁻ supply; and 4, sample from an additional ‘normal’ 5 days old culture supplied antibiotics.
References


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$_2$O 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$_2$O 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$_2$O.

**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).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Initial</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
</tr>
<tr>
<td>DCW (g-DCW·L$^{-1}$)</td>
<td>0.47 ± 0.03$^a$</td>
<td>0.45 ± 0.02</td>
</tr>
<tr>
<td>OD</td>
<td>1.03 ± 0.06</td>
<td>0.967 ± 0.01</td>
</tr>
<tr>
<td>pH$^a$</td>
<td>7.62</td>
<td>7.46</td>
</tr>
<tr>
<td>NO$_3^-$ (mM)$^a$</td>
<td>5.25 ± 0.20</td>
<td>5.14 ± 0.20</td>
</tr>
<tr>
<td>NO$_2^-$ (mM)$^a$</td>
<td>&lt;LOD$^*$</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>N$_2$O produced (nmole)</td>
<td>1.90 ± 0.08</td>
<td>1.76 ± 0.20$^*$</td>
</tr>
</tbody>
</table>

$^a$ As duplicates or triplicates were performed, data represent average ± (max-min)/2.

* Limit of detection (0.05 mg L$^{-1}$).
Figure E.1: N\textsubscript{2}O (average nmole ± SE) recorded from *C. reinhardtii* treatment (Supplied with 10 mM NO\textsubscript{2}\textsuperscript{−}) 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 externally assessed and found satisfactory for RNA sequencing (NZGL ltd, New Zealand). Following RNA sequencing and analysis, it became clear that NO\textsubscript{2}\textsuperscript{−} 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 treatment ‘behave‘ similarly at time 3 control and treatment diverged considerably showing an effect of NO\textsubscript{2}\textsuperscript{−} 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 NO₂⁻ 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.

<table>
<thead>
<tr>
<th>gene name</th>
<th>gene symbol</th>
<th>log2 Fold Change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre03.g199900</td>
<td>EIF4E</td>
<td>-1.70</td>
<td>1.91·10⁻⁰⁹</td>
</tr>
<tr>
<td>Cre05.g242300</td>
<td>EIF3D</td>
<td>-1.61</td>
<td>8.49·10⁻⁰⁹</td>
</tr>
<tr>
<td>Cre02.g098450</td>
<td>EIF2G</td>
<td>-1.56</td>
<td>2.45·10⁻⁰⁸</td>
</tr>
<tr>
<td>Cre16.g676314</td>
<td>EIF3H</td>
<td>-1.69</td>
<td>6.94·10⁻⁰⁸</td>
</tr>
<tr>
<td>Cre03.g194400</td>
<td>EIF3I</td>
<td>-1.31</td>
<td>5.90·10⁻⁰⁷</td>
</tr>
<tr>
<td>Cre16.g654500</td>
<td>EIF3F</td>
<td>-1.73</td>
<td>1.32·10⁻⁰⁶</td>
</tr>
<tr>
<td>Cre12.g529950</td>
<td>EIF4G</td>
<td>-1.03</td>
<td>1.07·10⁻⁰⁵</td>
</tr>
<tr>
<td>Cre06.g269450</td>
<td>EIF3G</td>
<td>-1.01</td>
<td>1.07·10⁻⁰⁴</td>
</tr>
<tr>
<td>Cre04.g217550</td>
<td>EIF3C</td>
<td>-1.23</td>
<td>0.00207</td>
</tr>
<tr>
<td>Cre13.g564250</td>
<td>EIF3A</td>
<td>-0.77</td>
<td>0.0110</td>
</tr>
<tr>
<td>Cre12.g531550</td>
<td>EIF2B</td>
<td>-0.640</td>
<td>0.0117</td>
</tr>
<tr>
<td>Cre12.g490000</td>
<td>EIF2A-2</td>
<td>-1.02</td>
<td>0.0289</td>
</tr>
<tr>
<td>Cre12.g515650</td>
<td>EIF3K</td>
<td>-0.887</td>
<td>0.0561</td>
</tr>
<tr>
<td>Cre12.g498100</td>
<td>EIF3E</td>
<td>-0.653</td>
<td>0.0587</td>
</tr>
<tr>
<td>Cre03.g190100</td>
<td>EIF3B</td>
<td>-0.860</td>
<td>0.0701</td>
</tr>
<tr>
<td>Cre08.g375900</td>
<td>EIF2A-1</td>
<td>-0.625</td>
<td>0.0943</td>
</tr>
<tr>
<td>Cre06.g251600</td>
<td>EIF5</td>
<td>0.226</td>
<td>0.487</td>
</tr>
<tr>
<td>Cre13.g585150</td>
<td>EIF6</td>
<td>0.104</td>
<td>0.679</td>
</tr>
<tr>
<td>Cre03.g199647</td>
<td>EIF4A</td>
<td>0.072</td>
<td>0.790</td>
</tr>
</tbody>
</table>
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$_2$O 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$_2$O 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).

<table>
<thead>
<tr>
<th>Genes</th>
<th>annotation</th>
<th>Time (h) after exposure to NO$_2^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>NIT2</td>
<td>Nitrogen assimilation regulation</td>
<td>0.01</td>
</tr>
<tr>
<td>NAR1.2</td>
<td>Nitrite/carbonate chloroplast transporter</td>
<td>0.4 (0.4)</td>
</tr>
<tr>
<td>NAR1.3</td>
<td>Formate/nitrite transporter (intracellular)</td>
<td>0.2 (4)</td>
</tr>
<tr>
<td>NAR1.4</td>
<td>Nitrite transporter (Intracellular)</td>
<td>-0.08 (11)</td>
</tr>
<tr>
<td>NAR1.5</td>
<td>Nitrite transporter (intracellular)</td>
<td>-0.04 (1)</td>
</tr>
<tr>
<td>NAR1.6</td>
<td>Nitrite transporter (intracellular)</td>
<td>-0.09 (1)</td>
</tr>
<tr>
<td>NRT2.1</td>
<td>Nitrate transporter (membrane)</td>
<td>-0.4 (0.1)</td>
</tr>
<tr>
<td>NRT2.2</td>
<td>Nitrate specific transporter</td>
<td>-0.4 (1)</td>
</tr>
<tr>
<td>NRT2.3</td>
<td>Nitrate/Nitrite bi-specific transporter (membrane)</td>
<td>0.6 (0.4)</td>
</tr>
</tbody>
</table>
Reference


Appendix F. Gas measurement, pressure correction and GC calibration

F.1 Gas analysis and pressure correction

Assuming N\textsubscript{2}O is an ideal gas transferring rapidly between the gaseous and liquid phases with respect to sampling frequency, the total amount of N\textsubscript{2}O present (n\textsubscript{f}, mole) in a sealed flask at the sampling time t was calculated as the sum of amounts of N\textsubscript{2}O present in the gas (n\textsubscript{g}, mole) and liquid phase (n\textsubscript{l}, mole) at time t as:

\[ n_f = \frac{x_{N2O}^t \cdot V_g}{R \cdot T} + H_{N2O} \cdot x_{N2O}^t \cdot P \cdot V_l \]  

Equation F.1

Where \( x_{N2O}^t \) (mole N\textsubscript{2}O/mole gas) is the molar fraction of N\textsubscript{2}O in the flasks headspace at time t, \( P \) 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\textsuperscript{-1} mol\textsuperscript{-1}), \( T \) is the temperature in the flaks (298 K in all experiments), and \( H_{N2O} \) is the Henry law constant of N\textsubscript{2}O at the experimental temperature (2.5×10\textsuperscript{-2} mol· N\textsubscript{2}O L\textsuperscript{-1}·Atm N\textsubscript{2}O\textsuperscript{-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\textsubscript{2}O mass transfer so that \( x_{N2O}^t \) quantified at time t (via gas chromatography) was representative of the equilibrium achieved at \( P \) just immediately before the gas sample was withdrawn. The pressure change in the flasks was taken into
account when computing the amount of N₂O present in the flaks at the next sampling
time (t+1) as:

\[ n^{t+1} = \frac{x_{N_2O}^{t+1} p^{t+1} - V_g}{R \cdot T} + H_{N_2O} \cdot x_{N_2O}^{t+1} \cdot p^{t+1} \cdot V_t \]  
Equation F.2

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

\[ p^{t+1} = p^t \cdot \left( \frac{V_g}{V_g + V_s} \right) \]  
Equation F.3

The total amount of N₂O produced at \( t+1 \) (\( n^{t+1} \)) was then calculated by accounting for
the amount of N₂O withdrawn from the sample at time \( t \) as:

\[ n^{t+1} = \frac{x_{N_2O}^{t+1} p^{t+1} - V_g}{R \cdot T} + H_{N_2O} \cdot x_{N_2O}^{t+1} \cdot p^{t+1} \cdot V_t + x_{N_2O}^{t+1} \cdot p^t \cdot V_s \]  
Equation F.4
F.2 GC Calibration

GC calibration was performed in using a 100 ppm N₂O in N₂ standard. Serum flasks (122 ± 2 mL) were flushed with neutral gas (N₂) 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
\[ P = \text{Pressure in atm} \]
\[ V_g = V = \text{Gas volume in the flask (L)} \]
\[ V_s = \text{Gas volume in the syringe (L)} \]
\[ T = \text{Temperature in K} \]
\[ R = \text{Gas constant (0.082057 L atm K}^{-1} \text{ mol}^{-1}) \]
\[ f_{N_2O} = \text{N}_2\text{O fraction (nmol} \cdot \text{mol}^{-1}) \]
\[ n = \text{N}_2\text{O (mole)} \]
\[ C = \text{N}_2\text{O concentration (mol} \cdot \text{L}^{-1}) \]
\[ n_{\text{Tot}} = \text{Total mole of gas} \]
\[ S = \text{Syringe} \]
\[ W = \text{Time after withdraw} \]
\[ e.g. \ n_{W,1} = \text{N}_2\text{O (nmole) after withdraw} \]
\[ I = \text{Time after injection} \]
\[ e.g. \ f_{I,1} = \text{N}_2\text{O fraction after injection (nmol} \cdot \text{mol}^{-1}) \]

**At \ t = 1, after the first withdrawn and the first injection of standard:**

\[
\begin{align*}
f_{I,1} &= \frac{n_{I,1}}{n_{I,1}} = \frac{n_{I,1}}{n_{W,1} + n_{S,1}}, \text{ Equation F.5} \\
\end{align*}
\]

We have also,

\[
\begin{align*}
n_{I,1} &= n_{W,1} + (C_{S,1}V_{S,1}) \text{ with, } C_{S,1}V_{S,1} = n_{S,1} = \frac{P_{\text{atm}}V_S}{RT} \times f_S, \text{ Equation F.6} \\
\end{align*}
\]

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

\[
\begin{align*}
f_{I,1} &= \frac{n_{W,1} + (P_{\text{atm}}V_s)}{P_0(V_g + V_s)} \times \frac{f_S}{RT}, \text{ Equation F.7} \\
\end{align*}
\]

Considering that \( n_{W,1} = \text{N}_2\text{O (nmole) after first withdraw} \)
At \( t = n \), several cycle

\[
f_{i,n} = \frac{n_{i,n}}{n_{ITot,n}}, \text{ Equation F.8}
\]

\[
P_{W,n} = P_{i,n-1} \times \left( \frac{V}{V + V_S} \right), \text{ Equation F.9}
\]

\[
n_{i,n} = n_{W,n} + \left( \frac{P_0 V_S}{RT} \right) \times f_S, \text{ Equation F.10}
\]

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

\[
f_{W,n} = f_{i,n-1}, \text{ Equation F.12}
\]

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

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

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:

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 NO₂⁻ 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](image)

**Figure G.1:** Box plot of the N₂O 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 N₂O produced in positive controls.

<table>
<thead>
<tr>
<th></th>
<th>N₂O after 0.25 h (nmole)</th>
<th>N₂O after 3 h (nmole)</th>
<th>N₂O after 24 h (nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (sample size)</td>
<td>34</td>
<td>28</td>
<td>41</td>
</tr>
<tr>
<td>Minimum</td>
<td>1.58</td>
<td>2.39</td>
<td>13.1</td>
</tr>
<tr>
<td>Maximum</td>
<td>13.0</td>
<td>18.3</td>
<td>42.9</td>
</tr>
<tr>
<td>Mean</td>
<td>5.30</td>
<td>7.80</td>
<td>24.4</td>
</tr>
<tr>
<td>Median</td>
<td>4.74</td>
<td>6.76</td>
<td>23.3</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>3.01</td>
<td>4.43</td>
<td>6.59</td>
</tr>
<tr>
<td>Relative standard error</td>
<td>57</td>
<td>57</td>
<td>27</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>1.01</td>
<td>1.64</td>
<td>2.02</td>
</tr>
</tbody>
</table>

Three gas samples were generally withdrawn (after 15 min, 3 h and 24 h of NO₂⁻ supply) from duplicates. N₂O was always significantly produced in the positive controls. The N₂O (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 N₂O production as a function of time.

G.2 Negative controls

In order to dismiss possible abiotic N₂O production, negative controls (N-free medium supplied with 10 mM NO₂⁻; or N-free medium supplied with chemical effectors and 10 mM NO₂⁻; or autoclaved microalgal suspension supplied with 10 mM NO₂⁻) 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 NO₂⁻ supply). Figure G.2 presents an example of the amount of N₂O recorded in duplicates flasks of negative
controls (N-free medium supplied with 10 mM NO₂⁻; and autoclaved microalgal suspension supplied with 10 mM NO₂⁻) over a 24 h period.

**Figure G.2**: N₂O production (nmole) in negative controls (N-free medium or dead microalgal suspension) supplied 10 mM NO₂⁻ in darkness.

N₂O was never significantly produced in the negative controls flasks (Figure G.1) and no statistical difference between the amount of N₂O 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 N₂O (average ± standard deviation, \( n = 21 \)); 1.49 ± 0.08 nmol N₂O (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$_2$O production (nmol·g-DCW$^{-1}$) thus calculated (the amount of N$_2$O 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$_2$O 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.

![Summary for N2O](image)

**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

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Std Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set of duplicate 1</td>
<td>21</td>
<td>1853</td>
<td>474</td>
<td>103</td>
</tr>
<tr>
<td>Set of duplicate 2</td>
<td>20</td>
<td>1711</td>
<td>549</td>
<td>123</td>
</tr>
</tbody>
</table>

*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 N₂O amount (nmole ± SD) and average specific N₂O production (nmol·g-DCW⁻¹ ± SD). Specific N₂O production was calculated by subtracting the amount of N₂O already present in flasks at the time t - 1 to the total amount of N₂O recorded at the sampling time. Results are shown as “< LOQ” when the amount of N₂O 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 N₂O (nmole) and specific N₂O 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 N₂O production was recorded in N-free controls (no supply of N) inoculated *C. reinhardtii* 6145c and incubated in darkness or under illumination.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>N source</th>
<th>Effector</th>
<th>N₂O after 0.25 h</th>
<th>N₂O after 3 h</th>
<th>N₂O after 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total Production</td>
<td>Total Production</td>
<td>Total Production</td>
</tr>
<tr>
<td>Darkness</td>
<td>-</td>
<td>L-Arg</td>
<td>1.50 ± 0.25 (21)</td>
<td>&lt; LOQ</td>
<td>1.50 ± 0.23 (27)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>L-Arg</td>
<td>1.63 ± 0.06</td>
<td>&lt; LOQ</td>
<td>1.52 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>L-Arg</td>
<td>5.30 ± 3.01 (34)</td>
<td>300 ± 230 (34)</td>
<td>7.80 ± 4.43 (28)</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>L-Arg</td>
<td>2.14 ± 0.02</td>
<td>&lt; LOQ</td>
<td>2.76 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>L-NNA</td>
<td>1.66 ± 0.20</td>
<td>5.49 ± 0.26</td>
<td>310 ± 26.0</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>L-Arg + L-NNA</td>
<td>1.50 ± 0.08</td>
<td>&lt; LOQ</td>
<td>5.58 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>CN⁻</td>
<td>1.59 ± 0.04</td>
<td>&lt; LOQ</td>
<td>1.89 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>CN⁻</td>
<td>6.06 ± 1.96</td>
<td>360 ± 150</td>
<td>24.9 ± 6.82</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>CN⁻</td>
<td>11.5 ± 1.24</td>
<td>780 ± 100</td>
<td>16.0 ± 1.95</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>CN⁻</td>
<td>5.14 ± 1.03</td>
<td>290 ± 80</td>
<td>8.36 ± 1.94</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>CN⁻</td>
<td>1.44 ± 0.03 (4)</td>
<td>&lt; LOQ</td>
<td>1.49 ± 0.12 (4)</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>CN⁻</td>
<td>1.40 ± 0.00 (4)</td>
<td>&lt; LOQ</td>
<td>1.48 ± 0.09 (4)</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>CN⁻</td>
<td>1.36 ± 0.00</td>
<td>&lt; LOQ</td>
<td>1.33 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>CN⁻</td>
<td>1.62 ± 0.60 (5)</td>
<td>&lt; LOQ</td>
<td>1.52 ± 0.15 (4)</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>CN⁻</td>
<td>1.39 ± 0.03 (4)</td>
<td>&lt; LOQ</td>
<td>1.55 ± 0.15 (4)</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>CN⁻</td>
<td>1.41 ± 0.01 (4)</td>
<td>&lt; LOQ</td>
<td>1.48 ± 0.14 (4)</td>
</tr>
</tbody>
</table>

*a* Anoxic. Batch assays were prepared as described above. Following NO₂⁻ supply, the microalgal suspension was flushed with N₂ 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 N₂O 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 N₂O production as described above (Appendix L).
Table H.2: Total amounts of N₂O (nmole) and specific N₂O 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’ N₂O production took place in cultures of the NR-deficient mutant and that late N₂O synthesis is not likely NR-mediated (see Chapter 2 for full discussion).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>N source</th>
<th>Effector</th>
<th>N₂O after 0.25 h</th>
<th>N₂O after 3 h</th>
<th>N₂O after 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total Production</td>
<td>Total Production</td>
<td>Total Production</td>
</tr>
<tr>
<td><strong>Darkness</strong></td>
<td>NO₂⁻</td>
<td>-</td>
<td>1.16 ± 0.25 (8)</td>
<td>&lt; LOQ</td>
<td>29.0 ± 9.20 (8)</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>CN⁻ 0.5 mM</td>
<td>-</td>
<td>10.0 ± 0.6 a</td>
<td>700 ± 100 a</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>CN⁻ 2 mM</td>
<td>-</td>
<td>2.71 ± 0.05 a</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td><strong>Illumination</strong></td>
<td>NO₂⁻</td>
<td>-</td>
<td>1.26 ± 0.02 &lt; LOQ</td>
<td>1.32 ± 0.06 &lt; LOQ</td>
<td>15.1 ± 0.12 1100 ± 100</td>
</tr>
</tbody>
</table>

a after 6 h
Table H.3: Total amounts of N$_2$O (nmole) and specific N$_2$O 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$_2$O when supplied NO$_2^-$ and when supplied NO$_3^-$ under illumination. These results demonstrate that NR and NiR activities impact N$_2$O synthesis by regulating intracellular NO$_2^-$ concentration (see Chapter 2 for full discussion).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Conditions</th>
<th>N source</th>
<th>N$_2$O after 1 h</th>
<th>N$_2$O after 6 h</th>
<th>N$_2$O after 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td>Production</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NO$_2^-$</td>
<td>11.4 ± 0.26</td>
<td>800 ± 50</td>
<td>55.8 ± 24.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NO$_3^-$</td>
<td>2.03 ± 0.35</td>
<td>&lt; LOQ</td>
<td>1.97 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Illumination</td>
<td>NO$_2^-$</td>
<td>4.64 ± 0.78</td>
<td>250 ± 60.0</td>
<td>370 ± 8.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NO$_3^-$</td>
<td>4.37 ± 0.00</td>
<td>230 ± 20.0</td>
<td>29.6 ± 3.42</td>
</tr>
</tbody>
</table>
Table H.4: Total amounts of N₂O (nmole) and specific N₂O 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 N₂O synthesis (see Chapter 2 for full discussion).

<table>
<thead>
<tr>
<th>Strain</th>
<th>N source</th>
<th>N₂O after 1 h</th>
<th>N₂O after 6 h</th>
<th>N₂O after 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Production</td>
<td>Total</td>
</tr>
<tr>
<td>704</td>
<td>NO₂⁻</td>
<td>2.03 ± 0.06</td>
<td>&lt; LOQ</td>
<td>6.72 ± 0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>138 ± 9.42</td>
</tr>
<tr>
<td>amiCYP55</td>
<td>NO₂⁻</td>
<td>1.78 ± 0.02</td>
<td>&lt; LOQ</td>
<td>2.17 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.41 ± 2.40</td>
</tr>
</tbody>
</table>
Appendix I. N₂O 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 NH₄⁺ as nutrient source (NH₄⁺ was used as some strains were mutant and could not grow neither on NO₃⁻ nor NO₂⁻).

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 NO₂⁻ 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 N₂O supplied with 10 mM NO₂⁻ and incubated in darkness. The results confirmed that N₂O synthesis is well-spread in *C. reinhardtii* but the emissions rates appeared to be strain-dependent.
Figure I.1: N₂O 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 NO₂⁻ 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 (25°C ± 1°C) 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/CO₂ (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$_2$O 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$_2$O production after the addition of NO$_2^-$.

**Figure J.2:** N$_2$O 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\textsubscript{2}O 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 esterases 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\textsubscript{2}O 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\textsubscript{2}O 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\textsubscript{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$_2$O) 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).

<table>
<thead>
<tr>
<th>Conditions (all cells were pre-incubated with DAF-FM Diacetate)</th>
<th>Pre-incubated cells supplied DEA NONOate (NO donor): this test verified the efficiencies of the NO donor and NO detection.</th>
<th>Pre-incubated cells supplied DEA NONOate (NO donor) and haemoglobin (NO quencher): this test verified the efficiency of the NO quencher.</th>
<th>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.</th>
<th>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.</th>
<th>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).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopic photographs (×100) (excitation wavelengths 460 – 480 nm; emission wavelengths: 510-580 nm)</td>
<td><img src="image1" alt="Microscopic photographs" /></td>
<td><img src="image2" alt="Microscopic photographs" /></td>
<td><img src="image3" alt="Microscopic photographs" /></td>
<td><img src="image4" alt="Microscopic photographs" /></td>
<td><img src="image5" alt="Microscopic photographs" /></td>
</tr>
</tbody>
</table>
The ‘positive control’ conducted with the wildtype strain was repeated with NR-deficient \textit{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.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure-k1.png}
\caption{Microscopic observations (×40) of \textit{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.}
\end{figure}

\textbf{References:}


Appendix L. N₂O 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 (MoO₄²⁻) was replaced by orthotungstate (0.253 mg Na₂WO₄ 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 NO₂⁻. These cultures were incubated in darkness and N₂O production was monitored over a 24 h period.

As seen in Figure L.1, tungstate-treated cells produced less N₂O than control cells during the first 5 h of incubation, although the levels of N₂O production after 24 h of incubation were similar in the treatment and control assays. These results suggested that NR is involved in immediate N₂O production, probably by catalysing the reduction of NO₂⁻ 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 N₂O synthesis under NO₂⁻ supply.
Figure L.1: N₂O specific production (nmol·g-DCW⁻¹) in cultures of *Chlamydomonas reinhardtii* 6145c (0.22 g·L⁻¹) supplied 10 mM NO₂⁻ and incubated in darkness. The ‘treatment’ samples were withdrawn from NR-inhibited cell cultures.

References:


Appendix M. N₂O 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 NO₂⁻ some duplicates of both *C. reinhardtii* strains 6145 and 2929 were flushed with N₂ to create an anoxic atmosphere. A negative control consisting of N free medium supplied with 10 mM NO₂⁻ and flushed with N₂ was also performed. N₂O was then monitored during a 24 h period. The results are presented in Figure M.1 and M.2.

![N₂O production graph](image-url)

**Figure M.1:** N₂O 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 NO₂⁻ incubated in darkness under normoxic conditions.
**Figure M.2:** N₂O 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 NO₂⁻ incubated in darkness under anoxic conditions.

Similarly to normoxic conditions, in anoxic conditions NO₂⁻ supply triggered N₂O synthesis in *C. reinhardtii* 6145 and 2929. While under normoxia both *C. reinhardtii* strains synthesised N₂O at usual rates (30 nmole equivalent to 2500 nmole·g-DCW⁻¹) under anoxic conditions upon NO₂⁻ supply, N₂O 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 N₂O production could be explained by anoxia which is known to favour 1/ NO₂⁻ reduction into NO by mitochondria (Tischner et al., 2004; Gupta et al., 2016), and 2/ NO availability for N₂O synthesis since NO oxidation is decreased under anoxia. References:


N₂O (Nitrous oxide) emissions during full-scale microalgae cultivation outdoors

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

While reported for decades, the ability of microalgae to synthesise nitrous oxide (N₂O) 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, N₂O emissions have never been systematically quantified during full-scale algae cultivation outdoors.

In order to investigate the potential environmental impact of microalgae cultivation, N₂O emissions were quantified during *Chlorella vulgaris* cultivation in 50-L photobioreactors (PBRs) operated outdoors under temperate climatic conditions (Palmerston North, New Zealand). *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 N₂O. 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. CO₂-enriched air (2% v/v) was continuously bubbled in the reactor to supply carbon and mix the culture. Specific N₂O emissions rates (nmol.h⁻¹.g-DW⁻¹) were calculated based on biomass concentration (as dried weight, DW, per litre) and analysis of effluent gas samples by GC-ECD. N₂O emissions rates were thus recorded over 335 samples withdrawn over 86 days of operation representing all seasons.

Significant positive N₂O emissions were recorded in all samples. N₂O emissions fluctuated greatly (0.49 to 1183 nmol.h⁻¹.g-DW⁻¹) and no clear relation was seen between emission rates and environmental parameters (e.g. temperature, light intensity, nitrite concentration). Over all, N₂O emissions of 157-197 nmol N₂O.h⁻¹.g-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 N₂O.ha⁻¹.yr⁻¹ or 23 to 29 t CO₂-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 CO₂
fixed by the algae during photosynthesis. Preliminary results also showed nitrogen source and species selection provide efficient mitigation strategies.

**Keywords:** Microalgae, N₂O (Nitrous oxide), Environmental significance.

**References:**


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**Direct and indirect N₂O emissions during primary domestic wastewater treatment in a pilot-scale high rate algal pond**


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** 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 N₂O. Based on the monitoring, direct and indirect (from NH₃ redeposited and converted in N₂O) N₂O emissions averaged 0.007 g N₂O-N/d (n = 13) and 0.015 ± 0.005 g N₂O-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 N₂O/PE-yr, 9 fold higher than IPCC default emission factor of 3.2 g N₂O/capita-yr for conventional wastewater treatment. N₂O 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$_2$O 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$_2$O measurements). a calculated between 28/03 and 11/04.

<table>
<thead>
<tr>
<th>Date (days of monitoring)</th>
<th>Season</th>
<th>Microalgae species</th>
<th>N-source</th>
<th>n</th>
<th>Number of PBR</th>
<th>DCW (average g-DCW·L$^{-1}$)</th>
<th>Biomass productivity (g·m$^{-2}$·d$^{-1}$)</th>
<th>Temperature (°C)</th>
<th>Light irradiance (W·m$^{-2}$)</th>
<th>Average HRT (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12/06/12 to 18/06/12 (6 days)</td>
<td>Winter</td>
<td>C. vulgaris</td>
<td>NO$_3^-$</td>
<td>76</td>
<td>1 2 3 4</td>
<td>0.41 – 0.62 (0.53)</td>
<td>0.53 – 0.77 (0.65)</td>
<td>0.29 – 0.42 (0.35)</td>
<td>0.51 – 0.81 (0.64)</td>
<td>1.39 1.60 0.83 1.94</td>
</tr>
<tr>
<td>28/03/14, 31/03/14 and 11/04/14 (3 days)</td>
<td>Autumn</td>
<td>C. vulgaris</td>
<td>NO$_3^-$</td>
<td>26</td>
<td>1 2</td>
<td>0.91 – 1.05 (0.95)</td>
<td>0.71 – 0.87 (0.76)</td>
<td>1.39 1.60 0.83 1.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>04/06/14 to 04/07/14 (32 days)</td>
<td>Winter</td>
<td>S. platensis</td>
<td>NO$_3^-$</td>
<td>90</td>
<td>1 2</td>
<td>0.12 – 0.78 (0.44)</td>
<td>0.10 – 1.00 (0.62)</td>
<td>5.00a 7.10a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>04/06/14 to 10/07/14 (36 days)</td>
<td>Winter</td>
<td>Neochloris</td>
<td>NO$_3^-$</td>
<td>136</td>
<td>1 2</td>
<td>0.17 – 1.50 (0.73)</td>
<td>0.15 – 1.40 (0.84)</td>
<td>2.06 2.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28/07/14 to 28/08/14 (32 days)</td>
<td>Winter</td>
<td>C. vulgaris</td>
<td>NO$_3^-$</td>
<td>84</td>
<td>1 2</td>
<td>0.10 – 1.30 (0.59)</td>
<td>0.11 – 1.15 (0.63)</td>
<td>3.05 3.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28/07/14 to 28/08/14 (32 days)</td>
<td>Winter</td>
<td>C. vulgaris</td>
<td>NH$_4^+$</td>
<td>84</td>
<td>1 2</td>
<td>0.19 – 1.24 (0.80)</td>
<td>0.20 – 1.18 (0.80)</td>
<td>4.10 4.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13/10/14 to 28/10/14 (15 days)</td>
<td>Spring</td>
<td>C. vulgaris</td>
<td>NO$_3^-$</td>
<td>44</td>
<td>1 2</td>
<td>0.57 – 1.33 (0.80)</td>
<td>0.47 – 1.28 (0.81)</td>
<td>8.05 7.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>04/11/14 to 28/11/14 (24 days)</td>
<td>Spring</td>
<td>C. vulgaris</td>
<td>NO$_3^-$</td>
<td>64</td>
<td>1 2</td>
<td>0.10 – 1.24 (0.72)</td>
<td>0.10 – 1.25 (0.68)</td>
<td>7.10 8.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>01/12/14 to 10/12/14 (12 days)</td>
<td>Summer</td>
<td>C. vulgaris</td>
<td>NO$_3^-$</td>
<td>32</td>
<td>1 2</td>
<td>0.50 – 1.01 (0.71)</td>
<td>0.50 – 0.90 (0.72)</td>
<td>12.40 10.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Appendix P. Controls and statistical analyses performed on N$_2$O measurements from the N$_2$O monitoring

The concentrations of N$_2$O 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$_2$O exhaust gas concentration when computing net N$_2$O productivities. Figure P.1 shows the N$_2$O 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$_2$O concentrations measured from the PBRs and from the controls was noted.

![Figure P.1](image)

**Figure P.1:** Measured N$_2$O 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$_2$O concentration measured from the PBR exhaust gas and the controls was statistically significant, two samples t-test (α =
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$_2$O concentration from the PBR and the mean of the measured N$_2$O 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$_2$O concentration from the PBR and the measured N$_2$O concentration from the controls.

<table>
<thead>
<tr>
<th>Date, species, medium and N source</th>
<th>N</th>
<th>Mean (N$_2$O in ppb)</th>
<th>Std Dev</th>
<th>Std Error</th>
<th>p-value (t-test, $\alpha = 0.05$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12/06/12 to 18/06/12 C. vulgaris on BG11/NO$_3^-$</td>
<td>19</td>
<td>1698</td>
<td>1033</td>
<td>237</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Reactor 1</td>
<td>19</td>
<td>330.9</td>
<td>27.1</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>19</td>
<td>793</td>
<td>408</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>Reactor 2</td>
<td>19</td>
<td>330.9</td>
<td>27.1</td>
<td>6.2</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Control</td>
<td>19</td>
<td>3056</td>
<td>1143</td>
<td>262</td>
<td></td>
</tr>
<tr>
<td>Reactor 3</td>
<td>19</td>
<td>1207</td>
<td>640</td>
<td>147</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Control</td>
<td>19</td>
<td>330.9</td>
<td>27.1</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>28/03/14, 31/03/14 and 11/04/14, C. vulgaris on BG11/NO$_3^-$</td>
<td>13</td>
<td>1182</td>
<td>829</td>
<td>230</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Reactor 1</td>
<td>13</td>
<td>219</td>
<td>49.1</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>13</td>
<td>1043</td>
<td>892</td>
<td>247</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Reactor 2</td>
<td>13</td>
<td>219</td>
<td>49.1</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>13</td>
<td>259.4</td>
<td>86.0</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Reactor 3</td>
<td>13</td>
<td>269.4</td>
<td>22.4</td>
<td>247</td>
<td>0.580</td>
</tr>
<tr>
<td>Control</td>
<td>13</td>
<td>266.6</td>
<td>24.7</td>
<td>3.0</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>04/06/14 to 04/07/14, S platensis on Zarrouk/NO$_3^-$</td>
<td>45</td>
<td>276.9</td>
<td>37.2</td>
<td>5.6</td>
<td>0.091</td>
</tr>
<tr>
<td>Reactor 1</td>
<td>45</td>
<td>265.4</td>
<td>25.5</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>45</td>
<td>259.4</td>
<td>86.0</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Reactor 2</td>
<td>45</td>
<td>269.4</td>
<td>22.4</td>
<td>247</td>
<td>0.580</td>
</tr>
<tr>
<td>Control</td>
<td>45</td>
<td>266.6</td>
<td>24.7</td>
<td>3.0</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>04/06/14 to 10/07/14, Neochloris, BG11/NO$_3^-$</td>
<td>68</td>
<td>1072</td>
<td>839</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>Reactor 1</td>
<td>68</td>
<td>266.6</td>
<td>24.7</td>
<td>3.0</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Control</td>
<td>68</td>
<td>1042</td>
<td>858</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>Reactor 2</td>
<td>68</td>
<td>266.6</td>
<td>24.7</td>
<td>3.0</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Control</td>
<td>68</td>
<td>1421</td>
<td>875</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>Reactor 1</td>
<td>42</td>
<td>277.1</td>
<td>21.2</td>
<td>3.3</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Control</td>
<td>42</td>
<td>1264</td>
<td>801</td>
<td>124</td>
<td></td>
</tr>
<tr>
<td>Reactor 2</td>
<td>42</td>
<td>277.1</td>
<td>21.2</td>
<td>3.3</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Control</td>
<td>42</td>
<td>266</td>
<td>19.9</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>Reactor 1</td>
<td>42</td>
<td>278.1</td>
<td>21.6</td>
<td>3.2</td>
<td>0.01$^a$</td>
</tr>
<tr>
<td>Control</td>
<td>42</td>
<td>270.1</td>
<td>22.2</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Reactor 2</td>
<td>42</td>
<td>278.1</td>
<td>21.6</td>
<td>3.2</td>
<td>0.150</td>
</tr>
<tr>
<td>Control</td>
<td>42</td>
<td>2765</td>
<td>1936</td>
<td>211</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Reactor 1</td>
<td>84</td>
<td>329.1</td>
<td>44.7</td>
<td>4.9</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Control</td>
<td>84</td>
<td>1861</td>
<td>1401</td>
<td>170</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Reactor 2</td>
<td>68</td>
<td>327.7</td>
<td>45.7</td>
<td>5.5</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

$^a$ while the results suggest N$_2$O consumption in the reactor, the difference in N$_2$O 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$_2$O concentration from the PBRs and the measured N$_2$O 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$_2$O concentration from the PBRs and the measured N$_2$O concentration from the controls was not significant. These results suggest that N$_2$O was not generated from these microalgal suspensions. In consequence, net N$_2$O 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$_2$O and NO specific microsensors

Q.1 N$_2$O microsensor (N$_2$O-500)

Dissolved N$_2$O detection was carried out with a N$_2$O microsensor N$_2$O-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$_2$O 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$_2$O 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$_2$O standard (100 ppm; N$_2$O in N$_2$). Pressure change and dissolved N$_2$O 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$_2$O with Unisense N$_2$O microsensor.](image)
Figure Q.2: N₂O calibration of the microsensor N₂O-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 N₂O 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 N₂O 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 t₁/₂ 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 N₂O generation in microalgal suspension. Prior to any use in outdoor systems, the accuracy of the microsensor was verified by comparing the gaseous N₂O measured with the GC and the gaseous N₂O estimated from the dissolved N₂O measured by the microsensor. Because the microsensor measured the amount of dissolved N₂O, the amount of gaseous N₂O released could be calculated based on Equation Q.1:

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

Equation Q.1

\[ \Phi_{N_2O} = \text{N}_2\text{O flux (mol·L}^{-1}·\text{s}^{-1}) \]

\[ Kla_{N_2O} = \text{N}_2\text{O mass transfer coefficient (s}^{-1}) \]

\[ [N_2O]^* = \text{Dissolved N}_2\text{O concentration at equilibrium (mol·L}^{-1}) \]
\[ [N_2O] = \text{Dissolved N}_2\text{O concentration (mol·L}^{-1}) \]

Kla, the volumetric liquid mass transfer coefficient, is defined by KI, 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:

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

\( Kla_{N_2O} = \text{N}_2\text{O mass transfer coefficient (s}^{-1}) \)

\( [N_2O]^* = \text{Dissolved N}_2\text{O concentration at equilibrium (mol·L}^{-1}) \)

\( [N_2O] = \text{Dissolved N}_2\text{O concentration (mol·L}^{-1}) \)

\( t = \text{Time (s)} \)

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\(_2\)O standard was bubbled in the system and dissolved N\(_2\)O was measured with the N\(_2\)O microsensor and then Kla(N\(_2\)O) 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, \( K_{la(O_2)} \) was then used to compute the mass transfer coefficients of N\(_2\)O (Equation Q.4), based on the diffusivities of these gases (Ye et al., 2014). The results are presented in Table Q.1.

\[
K_{la_{N_2O}} = K_{la_{O_2}} \times \sqrt{\frac{D_{F_{N_2O}}}{D_{F_{O_2}}}}, \text{ Equation Q.4}
\]

\( K_{la_{N_2O}} \) = N\(_2\)O mass transfer coefficient (s\(^{-1}\))

\( K_{la_{O_2}} \) = O\(_2\) mass transfer coefficient (s\(^{-1}\))

\( D_{F_{O_2}} \) = Molecular diffusivity of O\(_2\) in water (1.98 × 10\(^{-9}\)·m\(^{-2}\)·s\(^{-1}\) at 20°C)

\( D_{F_{N_2O}} \) = Molecular diffusivity of N\(_2\)O in water (1.84 × 10\(^{-9}\)·m\(^{-2}\)·s\(^{-1}\) at 20°C)

**Table Q.1**: Summary of the calculated and estimated (est) mass transfer coefficients (Kla) for O\(_2\) and N\(_2\)O in 2 L and 50 L PBRs.

<table>
<thead>
<tr>
<th>System</th>
<th>Medium</th>
<th>Temp (°C)</th>
<th>pH</th>
<th>( K_{la_{O_2}} ) (s(^{-1}))</th>
<th>( K_{la_{N_2O}_{est}} ) (s(^{-1}))</th>
<th>( K_{la_{N_2O}} ) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 L PBR</td>
<td>BG 11</td>
<td>20</td>
<td>7.0</td>
<td>0.0028</td>
<td>0.0027</td>
<td>0.0026</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0030</td>
<td>0.0029</td>
<td>0.0027</td>
</tr>
<tr>
<td>50 L PBR</td>
<td>BG 11*</td>
<td>12.5 – 20</td>
<td>7.2</td>
<td>0.00085</td>
<td>0.00082</td>
<td>0.00087</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0009</td>
<td>0.00087</td>
<td></td>
</tr>
</tbody>
</table>

*Service tap water

As seen in Table Q.1, the estimated and calculated values of the N\(_2\)O mass transfer coefficients in the 2 L PBRs were similar. These results confirmed that even in a dynamic system (i.e. bubbling) the N\(_2\)O microsensor was detecting N\(_2\)O accurately. An additional experiment was performed with *C. vulgaris* cultivated in 2 L PBR in order to verify that the N\(_2\)O production calculated from the N\(_2\)O measured by the GC, or
estimated from the dissolved N\textsubscript{2}O concentration measured by the microsensor, would give similar results.

\textit{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\textsubscript{2}\textsuperscript{-} was supplied in the microalgal suspension after 1 h. Gaseous and dissolved N\textsubscript{2}O were measured with the N\textsubscript{2}O microsensor and the GC, respectively. As seen in Figure Q.4, the N\textsubscript{2}O production calculated from the gaseous N\textsubscript{2}O measured by the GC or estimated from the dissolved N\textsubscript{2}O concentration obtained by the microsensor were similar. The results suggested that the accuracy of the microsensor was satisfying.

\textbf{Figure Q.4:} \textit{C. vulgaris} N\textsubscript{2}O production (\textmu mol·h\textsuperscript{-1}) calculated from GC measurements or estimated from dissolved N\textsubscript{2}O concentrations measured by a N\textsubscript{2}O microsensor. 1 mM NO\textsubscript{2}\textsuperscript{-} was supplied after 1h.
Q.4 Main issue encounter with the N₂O and NO microsensor

The preliminary results presented in Figure P.4 seemed really promising. In order to get high temporal resolution of N₂O 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 N₂O 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:


Appendix R. Correlation between dry weight, solar irradiance, ambient air temperature, broth temperature, NO$_3^-$ concentration, NO$_2^-$ concentration and N$_2$O production during *Chlorella vulgaris* cultivation in 50 L PBRs.

**Figure R.1:** Dry weight (g·L$^{-1}$) against N$_2$O production (nmol·m$^{-2}$·h$^{-1}$) during *C. vulgaris* cultivation in 50 L photobioreactors.

**Figure R.2:** Solar irradiance (W·m$^{-2}$) against N$_2$O production (nmol·m$^{-2}$·h$^{-1}$) during *C. vulgaris* cultivation in 50 L photobioreactors.
Figure R.3: Ambient air temperature (°C) against N₂O production (nmol·m⁻²·h⁻¹) during *C. vulgaris* cultivation in 50 L photobioreactors.

Figure R.4: Broth temperature (°C) against N₂O production (nmol·m⁻²·h⁻¹) during *C. vulgaris* cultivation in 50 L photobioreactors.
**Figure R.5:** NO$_3^-$ concentration (mg·L$^{-1}$) against N$_2$O production (nmol·m$^{-2}$·h$^{-1}$) during *C. vulgaris* cultivation in 50 L photobioreactors.

**Figure R.6:** NO$_2^-$ concentration (mg·L$^{-1}$) against N$_2$O production (nmol·m$^{-2}$·h$^{-1}$) during *C. vulgaris* cultivation in 50 L photobioreactors.
Appendix S. Statistical analysis of the influence of environmental and operational parameters on N$_2$O 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$_2$O emissions, a stepwise linear regression analysis with backward elimination method ($\alpha = 0.1$) 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$_2$O 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.

<table>
<thead>
<tr>
<th>Variables</th>
<th>N</th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>N$_2$O (nmol·m$^{-2}$·h$^{-1}$)</td>
<td>320</td>
<td>6235</td>
<td>9.50</td>
<td>38030</td>
</tr>
<tr>
<td>Sampling time (h)</td>
<td>320</td>
<td>N.A</td>
<td>N.A</td>
<td>N.A</td>
</tr>
<tr>
<td>DCW (g·L$^{-1}$)</td>
<td>320</td>
<td>0.66</td>
<td>0.10</td>
<td>1.33</td>
</tr>
<tr>
<td>Solar irradiance (W·m$^{-2}$)</td>
<td>320</td>
<td>265</td>
<td>0</td>
<td>969</td>
</tr>
<tr>
<td>Air Temp (°C)</td>
<td>320</td>
<td>11.8</td>
<td>-3.00</td>
<td>21.5</td>
</tr>
<tr>
<td>Broth Temp (°C)</td>
<td>180</td>
<td>17.4</td>
<td>6.00</td>
<td>35.2</td>
</tr>
<tr>
<td>[NO$_2^-$] (mg·L$^{-1}$)</td>
<td>63</td>
<td>12.6</td>
<td>1.92</td>
<td>30.6</td>
</tr>
<tr>
<td>pH</td>
<td>65</td>
<td>7.00</td>
<td>6.73</td>
<td>7.43</td>
</tr>
</tbody>
</table>

**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).

<table>
<thead>
<tr>
<th>Correlations analysis output</th>
<th>Sampl Time</th>
<th>DW (g·L$^{-1}$)</th>
<th>N$_2$O (nmol·h$^{-1}$·m$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW (g·L$^{-1}$)</td>
<td>0.095</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N$_2$O (nmol·h$^{-1}$·m$^{-2}$)</td>
<td>-0.013</td>
<td>0.255</td>
<td></td>
</tr>
<tr>
<td>Solar IR (W·m$^{-2}$)</td>
<td>-0.046</td>
<td>0.155</td>
<td>0.284</td>
</tr>
<tr>
<td>Temp out (°C)</td>
<td>0.013</td>
<td>0.368</td>
<td>0.250</td>
</tr>
<tr>
<td>Solar IR (W·m$^{-2}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temp out (°C)</td>
<td>0.494</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 ($\alpha = 0.1$) was performed.

<table>
<thead>
<tr>
<th>Stepwise Regression output</th>
</tr>
</thead>
<tbody>
<tr>
<td>Backward elimination. Alpha-to-Remove: 0.1</td>
</tr>
</tbody>
</table>

Response is N$_2$O (nmol·h$^{-1}$·m$^{-2}$) on 4 predictors, with N = 317
N(cases with missing observations) = 3 N(all cases) = 320

<table>
<thead>
<tr>
<th>Step</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>340.76</td>
<td>-210.85</td>
<td>86.52</td>
</tr>
<tr>
<td>Sample Time</td>
<td>-1089</td>
<td>-0.42</td>
<td>0.675</td>
</tr>
<tr>
<td>DW (g·L$^{-1}$)</td>
<td>5884</td>
<td>5816</td>
<td>6395</td>
</tr>
<tr>
<td>Solar IR (W·m$^{-2}$)</td>
<td>6.7</td>
<td>6.8</td>
<td>7.7</td>
</tr>
<tr>
<td>Temp out (°C)</td>
<td>81</td>
<td>81</td>
<td>81</td>
</tr>
<tr>
<td>S</td>
<td>6521</td>
<td>6513</td>
<td>6513</td>
</tr>
<tr>
<td>R-Sq</td>
<td>12.95</td>
<td>12.90</td>
<td><strong>12.61</strong></td>
</tr>
<tr>
<td>R-Sq(adj)</td>
<td>11.83</td>
<td>12.06</td>
<td>12.05</td>
</tr>
<tr>
<td>Mallows Cp</td>
<td>5.0</td>
<td>3.2</td>
<td>2.2</td>
</tr>
</tbody>
</table>

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$_2$O 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).

![Residual Plots for N2O (nmol·h⁻¹·m⁻²)](image)

**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 N₂O production data, another multiple regression analysis was performed.

**Multiple regression analysis (#2)**

The regression equation is

\[ \ln (\text{N}_2\text{O}) = 6.50 + 1.95 \text{ DW (g/L)} + 0.00118 \text{ Solar irradiance (W.m}^{-2}) \]

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coef</th>
<th>SE Coef</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>6.4960</td>
<td>0.2103</td>
<td>30.89</td>
<td>0.000</td>
</tr>
<tr>
<td>Source</td>
<td>DF</td>
<td>SS</td>
<td>MS</td>
<td>F</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>----</td>
<td>--------</td>
<td>--------</td>
<td>-----</td>
</tr>
<tr>
<td>Regression</td>
<td>2</td>
<td>108.305</td>
<td>54.152</td>
<td>35.12</td>
</tr>
<tr>
<td>Residual Error</td>
<td>318</td>
<td>490.387</td>
<td>1.542</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>320</td>
<td>598.692</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

Unusual Observations

<table>
<thead>
<tr>
<th>Obs</th>
<th>DW (g·L⁻¹)</th>
<th>Ln (N₂O)</th>
<th>Fit SE Fit</th>
<th>Residual St Resid</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.61</td>
<td>3.7227</td>
<td>7.6850</td>
<td>0.1063</td>
</tr>
<tr>
<td>29</td>
<td>0.62</td>
<td>2.2560</td>
<td>7.7208</td>
<td>0.1056</td>
</tr>
<tr>
<td>102</td>
<td>0.11</td>
<td>4.0848</td>
<td>6.7404</td>
<td>0.1812</td>
</tr>
<tr>
<td>103</td>
<td>0.13</td>
<td>4.4358</td>
<td>7.0171</td>
<td>0.1711</td>
</tr>
<tr>
<td>148</td>
<td>0.15</td>
<td>4.1361</td>
<td>6.9934</td>
<td>0.1650</td>
</tr>
<tr>
<td>208</td>
<td>1.33</td>
<td>8.4103</td>
<td>9.3565</td>
<td>0.2082</td>
</tr>
<tr>
<td>235</td>
<td>0.55</td>
<td>2.6508</td>
<td>7.7964</td>
<td>0.0792</td>
</tr>
<tr>
<td>265</td>
<td>0.10</td>
<td>5.3239</td>
<td>7.4428</td>
<td>0.2310</td>
</tr>
<tr>
<td>267</td>
<td>0.25</td>
<td>3.4774</td>
<td>7.5498</td>
<td>0.1657</td>
</tr>
<tr>
<td>268</td>
<td>0.48</td>
<td>4.3808</td>
<td>7.6596</td>
<td>0.0892</td>
</tr>
<tr>
<td>279</td>
<td>0.61</td>
<td>7.6429</td>
<td>8.7401</td>
<td>0.2119</td>
</tr>
<tr>
<td>299</td>
<td>0.55</td>
<td>9.9338</td>
<td>8.6388</td>
<td>0.2210</td>
</tr>
<tr>
<td>312</td>
<td>0.61</td>
<td>10.0670</td>
<td>8.8278</td>
<td>0.2340</td>
</tr>
</tbody>
</table>

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 N₂O 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

$$T_{\text{Ln } (\text{N}_2\text{O})} = 4.13 + 2.07 \, T_{\text{DW}} + 0.000689 \, T_{\text{Solar IR}}$$

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coef</th>
<th>SE Coef</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>4.1298</td>
<td>0.1639</td>
<td>25.19</td>
<td>0.000</td>
</tr>
<tr>
<td>$T_{\text{DW}}$</td>
<td>2.0661</td>
<td>0.3444</td>
<td>6.00</td>
<td>0.000</td>
</tr>
<tr>
<td>$T_{\text{Solar IR}}$</td>
<td>0.0006892</td>
<td>0.0003283</td>
<td>2.10</td>
<td>0.037</td>
</tr>
</tbody>
</table>

$S = 1.15171$  \hspace{1cm} $R^2 = 12.1\%$  \hspace{1cm} $R^2(\text{adj}) = 11.5\%$

### Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>2</td>
<td>57.888</td>
<td>28.944</td>
<td>21.82</td>
<td>0.000</td>
</tr>
<tr>
<td>Residual Error</td>
<td>317</td>
<td>420.482</td>
<td>1.326</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>319</td>
<td>478.370</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Seq SS</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{DW}}$</td>
<td>1</td>
<td>52.041</td>
</tr>
<tr>
<td>$T_{\text{Solar IR}}$</td>
<td>1</td>
<td>5.847</td>
</tr>
</tbody>
</table>

### Unusual Observations

<table>
<thead>
<tr>
<th>Obs</th>
<th>DW (g/L)*</th>
<th>Ln (N2O)*</th>
<th>Fit</th>
<th>SE Fit</th>
<th>Residual</th>
<th>St Resid</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.35</td>
<td>1.0173</td>
<td>4.8613</td>
<td>0.0869</td>
<td>-3.8440</td>
<td>-3.35 R</td>
</tr>
<tr>
<td>29</td>
<td>0.38</td>
<td>0.2227</td>
<td>4.9071</td>
<td>0.0845</td>
<td>-4.6844</td>
<td>-4.08 R</td>
</tr>
<tr>
<td>101</td>
<td>-0.22</td>
<td>2.1593</td>
<td>3.6878</td>
<td>0.2313</td>
<td>-1.5285</td>
<td>-1.35 X</td>
</tr>
<tr>
<td>128</td>
<td>1.05</td>
<td>5.0720</td>
<td>6.4878</td>
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</table>

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 ($D_0 = 1.82291$, $n = 320$, $k$ (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:

\[
\text{The regression equation is} \quad \ln(N_2O) = 6.52 + 2.07 \text{ DW (g·L}^{-1}) + 0.000689 \text{ Solar IR (W·m}^{-2})
\]

**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·h$^{-1}$·m$^{-2}$) against estimated $N_2O$ (nmol·h$^{-1}$·m$^{-2}$) from multiple regression.
References:


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·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 (R² = 0.99) as well as OD and cells number (R² =
0.99) and DCW and cells number (R² = 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 NO₃⁻ 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).
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 ($R^2 = 0.80 \rightarrow 0.97$).
Appendix U. Daily N$_2$O evolution from 2 *Chlorella vulgaris* cultures in 50 L photobioreactors in August 2014

**Figure U.1:** Daily N$_2$O production rates (nmol·m$^{-2}·$h$^{-1}$) from 2 *C. vulgaris* cultures in 50 L PBRs (PBR 1 and 2) over August 2014.

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

![Graph](image.png)

**Figure V.1:** Change in N$_2$O production rates (nmol·m$^{-2}$·h$^{-1}$) and solar irradiance (×, W·m$^{-2}$) from two *Neochloris* cultures in 50 L PBRs (PBR 1: ♦ and PBR 2: ▲). Pattern recorded the 23$^{	ext{rd}}$, 24$^{	ext{th}}$ and 25$^{	ext{th}}$ of June 2014. On those days of monitoring the DCW of PBR 1 were measured at 0.82, 0.90 and 0.51 g·L$^{-1}$ and the DCW of PBR 2 were measured at 1.05, 1.12 and 0.63 g·L$^{-1}$. 
Appendix W. Correlation between monthly averaged N\textsubscript{2}O production and monthly averaged biomass specific light availability

Following a reviewer suggestion (Algal research), N\textsubscript{2}O emissions (nmol·m\textsuperscript{-2}·h\textsuperscript{-1}) were plotted against irradiance (I\textsubscript{0}, W·m\textsuperscript{-2}) divided by biomass concentration (g-DCW·m\textsuperscript{-2}) (with a reactor density of 1·m\textsuperscript{-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\textsuperscript{2} = 0.92). While this must be viewed cautiously given the large N\textsubscript{2}O variability and unevenly distributed data, this could indicate that NO\textsubscript{2}\textsuperscript{-} intracellular accumulation is closely linked to the photosynthetic output during outdoor cultivation (i.e. N\textsubscript{2}O 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: N₂O production (nmol·m⁻²·h⁻¹) as a function of the biomass specific light availability (W·g-DCW⁻¹). The biomass specific light availability was calculated by dividing the light impinging over the reactor ground (W·m⁻²) by the biomass concentration per ground area (g-DCW·m⁻²).
Figure W.2: Monthly averaged N\textsubscript{2}O production (nmol·m\textsuperscript{-2}·h\textsuperscript{-1}) as a function of the monthly averaged biomass specific light availability (W·g-DCW\textsuperscript{-1}). Numbers in brackets represent the N\textsubscript{2}O 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. N₂O production by Chlorella vulgaris cultures in 2 L bench scale reactors when fed NH₄⁺ or NO₃⁻ supplied with 0.5 mM NO₂⁻

Experiments were carried out in two 2 L bench scale PBRs (PBR A and PBR B). C. vulgaris was cultivated in PBR A fed NO₃⁻ and PBR B fed NH₄⁺ (Same relative concentration of N). The day of the experiment, both reactors were supplied with 0.5 mM NO₂⁻. 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 NH₄⁺ possessed the ability to synthesise N₂O after an addition of 0.5 mM exogenous NO₂⁻ (PBR B). Although higher N₂O production would be expected with higher NO₂⁻ supply, the N₂O production were 25 times lower from C. vulgaris cultures fed NH₄⁺ (PBR B) than from C. vulgaris cultures fed NO₃⁻ (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 (\( \mu \text{mol} \cdot \text{g-DCW}^{-1} \cdot \text{h}^{-1} \)) in two 2 L indoor PBRs with \textit{C. vulgaris}. PBR A was fed \( \text{NO}_3^- \) and PBR B was fed \( \text{NH}_4^+ \). Both PBRs were under continuous illumination and supplemented with 0.5 mM \( \text{NO}_2^- \) (Arrow).
Appendix Y: Data distribution (dry weight, light intensity, air temperature and N₂O production) over the data range chosen for simulation (first and third quartile of N₂O production during *Chlorella vulgaris* cultivation in 50 L PBRs fed NO₃⁻).

**Figure Y.1:** Histograms of dry weight (g·L⁻¹), light intensity (W·m⁻²), temperature (°C) and N₂O production (nmole·m⁻²·h⁻¹) distribution over the range chosen for simulation (first and third quartile of N₂O production rates, 1500 – 8000 nmol N₂O·h⁻¹·m⁻²).
Appendix Z. N₂O quantification from a HRAP treating domestic wastewater

Z.1 Sampling optimisation

In order to evaluate the best sampling method and test conditions to evaluate N₂O 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 N₂O concentration in the HRAP and calculate N₂O 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 N₂ in order to create a neutral atmosphere and ease N₂O 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 N₂O measured at t = 5 min and t = 10 min as well as t = 25 min (two sample t-test, α = 0.05, p-value <0.05). It was therefore decided to quantify N₂O in the gas phase 30 minutes after sampling and with biological N₂O 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. N₂O data obtained (after re-calculation) showed no significant difference between the two containers used (two sample t-test, α = 0.05, p-value <0.05). It was therefore decided to use vials for dissolved N₂O 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 N₂O generation kinetics after a supply of NO₂⁻.
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\textsubscript{2}O concentration (two sample t-test, $\alpha = 0.05$, \textit{p-value} <0.05) when liquid samples were withdrawn at various depths in the HRAP. Considering that N\textsubscript{2}O is produced biologically, a poison can be used to inhibit microbial activity and in consequence to prevent N\textsubscript{2}O production between sampling and analysis (Ferrón et al., 2012; Kamp et al., 2013). Therefore, the use of ZnCl\textsubscript{2} was tested and after 30 min, no significant difference was observed between the poisoned and live samples (two sample t-test, $\alpha = 0.05$, \textit{p-value} <0.05). This result showed that the N\textsubscript{2}O transfer from the liquid to the gas phase was faster than biological N\textsubscript{2}O production. Moreover, results from our laboratory showed that heavy metal could cause a stress effect in microalgae, at time boosting N\textsubscript{2}O synthesis (data not shown). Consequently the use of poison was not needed and risky with the sampling method used.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|c|l|}
\hline
Optimisation & Methods & Significant: & Comment \\
\hline
Equilibration time & N\textsubscript{2}O 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\textsubscript{2}O concentration could evolve considerably, 30 min was chosen as being representative. \\
\hline
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\textsubscript{2}O concentration measured. \\
\hline
Poison the samples & ZnCl\textsubscript{2} (50%) was added to inhibit the microbial biomass & No & No significant effect in the 30 min between sampling and analysis. \\
\hline
\end{tabular}
\caption{Summary of the methods used for sampling optimization}
\end{table}
References:


Appendix AA. Distribution of the N\textsubscript{2}O production measured from HRAP microcosms and statistical analysis of the data between operation at 7 and 10 days HRT

**Figure AA.1:** N\textsubscript{2}O production (nmol·m\textsuperscript{-2}·h\textsuperscript{-1}) distribution from the HRAP microcosms monitoring.
Table AA.1: Summary of the two sample t-test (α = 0.05) analyses of the data (N₂O production, daily averaged air temperature, broth temperature at sampling, daily averaged solar irradiance and N₂O specific rates recorded from the batch assays) between operation at 7 and 10 days HRT.

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Statements of contribution to doctoral thesis containing publications
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Please indicate either:
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  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

Candidate’s Signature

28/11/2016

Date

Professor Benoit
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Principal Supervisor’s signature

28/11/2016

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  M Plouviez was the main contributor of the manuscript. He carried experimental work, data analysis and he wrote most of the manuscript.

---

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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)

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  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.

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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.
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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)

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  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.

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Date: 2016.11.28 10:29:21 +13’00’

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Date

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Date

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