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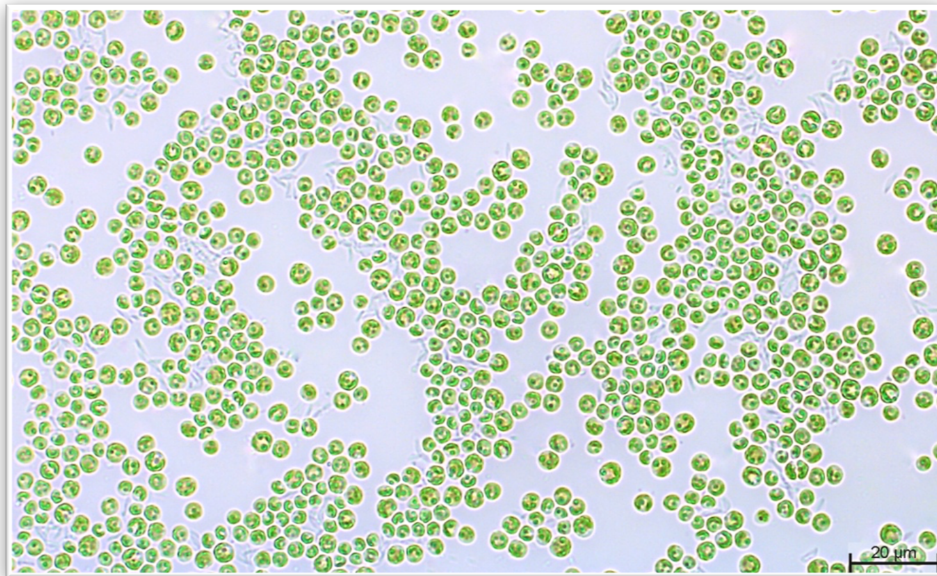

Nitrous Oxide Emissions during Microalgae Cultivation

Master Thesis

of

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Imprint

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Abstract

This thesis investigated the effect of nitrogen source and strain selection on nitrous oxide (N_2O) emissions from *Chlorella vulgaris* cultures. Furthermore, nitrous oxide quantification using Fourier transform infrared spectroscopy (FTIR). This method was successful and suitable. It was found that light had a clear effect: Nitrous oxide production of *Chlorella vulgaris* was significantly increased when algae were incubated in the dark (1'300 nmol gDW^{-1} h^{-1}) and supplied with nitrite as a nitrogen source compared to the cultures in light (130 nmol gDW^{-1} h^{-1}). Moreover, the form of the nitrogen source plays a role: *Chlorella vulgaris* emitted negligible nitrous oxide when supplied with urea or ammonium as nitrogen sources (<18 nmol gDW^{-1} h^{-1}). However, with nitrate as nitrogen source, the nitrous oxide emissions were around 205 nmol gDW^{-1} h^{-1}. The largest emissions showed the cultures with nitrite as nitrogen sources and were about 1'314 nmol gDW^{-1} h^{-1}. Despite considerable differences of nitrous oxide emissions depending on the *Chlorella vulgaris* strain used, these were statistically not significant. Strains SAG 9.88 and CCALA 924 emitted 2.5 to 5 times more nitrous oxide than the others.

In dieser Arbeit wurden die Auswirkung der Stickstoffquelle und die Auswahl des Algenstammes auf die Lachgasemissionen (N_2O) von *Chlorella vulgaris*-Kulturen untersucht. Ausserdem war der Nachweis von Lachgas mittels Fourier-Transformations-Infrarot-Spektroskopie (FTIR) erfolgreich und geeignet. Es wurde festgestellt, dass Licht einen klaren Einfluss hat: Die Lachgasproduktion von *Chlorella vulgaris* war deutlich erhöht, wenn die Algen im Dunkeln (1'300 nmol gDW^{-1} h^{-1}) kultiviert wurden als bei den beleuchteten Kulturen (130 nmol gDW^{-1} h^{-1}). Bei beiden Kulturen wurde Nitrit als Stickstoffquelle verwendet. Ausserdem wurde herausgefunden, dass die Form des Stickstoffdüngers eine Rolle spielt: *Chlorella vulgaris* emittierte vernachlässigbar wenig Lachgas, wenn Harnstoff oder Ammonium als Stickstoffquelle verwendet wurden (<18 nmol gDW^{-1} h^{-1}). Mit Nitrat als Stickstoffquelle lagen die Lachgasemissionen jedoch bei 205 nmol gDW^{-1} h^{-1}. Die größten Emissionen wiesen die mit Nitrit gedüngten Kulturen auf und lagen bei rund 1'314 nmol gDW^{-1} h^{-1}. Trotz der gemessenen Unterschiede bei den Lachgasemissionen von verschiedenen *Chlorella vulgaris* Stämmen, waren diese statistisch nicht signifikant. Die Stämme SAG 9.88 und CCALA 924 emittierten 2.5 bis 5-mal mehr Lachgas als die anderen.

Table of Content

Imprint I
Abstract II
Table of Content III
Introduction 1
Material and Methods 3
- Microalgae and Media 3
- Laboratory Analyses 3
- Experimental Setup 4
- Data analysis 5

Results 6
- Nitrous oxide quantification using FTIR 6
- Effect of nitrogen source on nitrous oxide emissions from *C. vulgaris* cultures 7
- Effect of strain selection on nitrous oxide emissions from *C. vulgaris* cultures 8

Discussion 9
Acknowledgements 11
References 12
Appendix 16
- Fourier Transform Infrared Spectroscopy (FTIR) 16
- FTIR System Settings 16
- Raw Data for Calibration Curve 18
- Rotation–Vibration Spectra of Nitrous Oxide (N_2O) 19
- Chlorella vulgaris Strains 20
- Growth and Nitrogen Consumption 21

Introduction

Efficient management of material flows is central in circular economy (Robles et al., 2020). Nitrogen (N) is of particular concern as it is used as fertiliser and fodder but can also be harmful to the environment: N leaching can lead to eutrophication (as NO_3^- or NH_3) in water bodies (Bergström & Jansson, 2006; Krupa, 2003) and contribute to climate change as greenhouse gases (as N_2O, NO_x, NH_3, NO) (Robles et al., 2020). It is estimated that in agriculture alone, about half of all anthropogenic N inputs are not taken up by the harvested crops (Smil, 1999). At a market price of 300 CHF/t nitrogen fertiliser (Germany Baltic ports, granulated urea, January 2021 (Agrarpreise, 2022)) and a European consumption of 10.5 million t nitrogen fertiliser (European Union, 2015), this would result in a loss of 1.5 billion CHF. Therefore, it is economically and ecologically important to increase the efficiency of which nitrogen flows are managed.

The cultivation of microalgae represents a great opportunity to recycle surplus nitrogen and thus contributes to a circular economy (Kholssi et al., 2021). Microalgae are increasingly used for the commercial production of animal feed, food additives, cosmetics, and biofuels and wastewater treatment (Spolaore et al., 2006). Algae cultivation (macro- and microalgae) accounts for nearly 30 % of global aquaculture production, of which 70 % is macroalgae and 30 % is microalgae (Cai et al., 2021; Raja et al., 2008). In the Asia-Pacific region, there are several commercial producers of microalgae with capacities between 3 and 500 tonnes/year. About nine-tenths of algae cultivation take place in Asia (Sathasivam et al., 2019). The market price for algal biomass ranges from 100 CHF/kg for human consumption and cosmetics, 5 to 20 CHF/kg for animal and fish feed, 1 to 5 CHF/kg for chemicals, and 0.40 CHF/kg for biofuel (Wijffels, 2008).

Nitrogen is an important nutrient that microalgae need. Nitrogen assimilation is required for the formation of genetic material, energy transfer molecules, proteins, enzymes, chlorophylls and peptides (Usher et al., 2014). Thus, *Chlorella vulgaris* consists of 6.2 to 7.7 % elemental nitrogen (by weight) (Mandalam & Palsson, 1998). Nitrogen fertilisers for algae cultures consist mainly of ammonium (NH_4^+), or urea ($(NH_2)_2CO$) (Arumugam et al., 2013). Most microalgae assimilate nitrogen in the form mentioned above, but microalgae can also use nitrate (NO_3^-) and nitrite (NO_2^-) (Wu et al., 2012). However, it was found that a fraction of the nitrogen uptaken by microalgae does not convert into biomass, but is instead released as gaseous emission (20-60 % of the N input) (Mariotto, 2022; Pulgarin et al., 2021). Algal cultures emit mainly gaseous nitrogen (N_2), but also the greenhouse gases nitrous oxide (N_2O) and

nitrogen monoxide (NO) and the air pollutant ammonia (NH_3) (Walsh et al., 2016). Nitrous oxide, ammonia and nitrogen dioxide are among the most persistent gases in the atmosphere and contribute significantly to climate change and biodiversity loss (Vuuren et al., 2011). Because of the great potential of microalgae in the future, it is important to investigate gaseous nitrogen emissions – in particular nitrous oxide – in more detail.

There are two main pathways for nitrous oxide production during the cultivation of microalgae under non-axenic conditions: i) by autotrophic bacteria that can use hydrogen or sulphur compounds as electron donors (Mampaey et al., 2013) or ii) by autotrophic bacteria using ammonium as electron donor (Guieysse et al., 2013) or iii) by heterotrophic denitrifiers using organic compounds instead (Poth & Focht, 1985). It has also been shown that in *Chlorella vulgaris* the intracellular accumulation of nitrite and nitrate can trigger minor nitrous oxide emissions (Batan et al., 2010; Guieysse et al., 2013; Weathers, 1984). The intracellular nitrite is reduced into NO or HNO by nitrate reductase and emitted as nitrous oxide (Plouviez, Shilton, et al., 2019). To prevent nitrous oxide emissions from algal cultures ammonium should be preferred over nitrate as a nitrogen source for *Chlorella vulgaris* cultures (Plouviez, Chambonnière, et al., 2019). In addition, the lighting regime may have an impact on nitrous oxide emissions. With nitrate as nitrogen source, emissions increase when algae are in darkness and decrease with illuminance (Guieysse et al., 2013). The opposite effects of light supply on nitrous oxide emissions can be explained by light-dependent mechanisms. In intracellular NO_2^- production, the rate of NO_3^- reduction to NO_2^- and the rates of NO_2^- reduction to NH_4^+ and N_2O are light-dependent (Guieysse et al., 2013).

The current state of knowledge is too limited to conclude on the best microalgae cultivation conditions; more monitoring and research are needed. Thus, the aim of this master thesis is to measure nitrous oxides emissions in microalgae cultivation. For this purpose, a reliable measurement method for gaseous nitrogen emissions is to be established. To verify the reliability of the measuring method as well as the repeatability of the observed effects, experiments are carried out by changing the type of nitrogen source. This parameter can influence and even reduce nitrous oxide emissions in algae cultures (Kamp et al., 2011; Plouviez, Shilton, et al., 2019). Once this has been confirmed, different strains of algae are used. Previous studies on *Chlorella vulgaris* strains indicated that there may be variances in the genome within the species causing physiological differences (Kessler et al., 1991; Přibyl et al., 2012; Takeshita et al., 2014). Observing such within-species variation is important, as it means that strain selection must be done carefully and thus can reduce nitrous oxide emissions.

Material and Methods

Microalgae and Media

The experiments were performed with seven axenic strains of *Chlorella vulgaris*: SAG 211-11b, SAG 211-11f, SAG 211-11p and SAG 9.88 were obtained from the Culture Collection of Algae at Göttingen University (SAG), Germany. CCALA 256(1), CCALA 256(2) and CCALA 924 were obtained from the Culture Collection of Autotrophic Organism of the Institute for Botany in Třeboň (CCALA), Czech Republic.

Strains of *Chlorella vulgaris* were cultivated in different variations of BG11 medium with the following main elements (g/L): as nitrogen sources 0.85 sodium nitrate ($NaNO_3$) or 0.69 sodium nitrite ($NaNO_2$) or 0.53 ammonium chloride (NH_4Cl) or 0.6 urea (CH_4N_2O); 0.04 dipotassium phosphate (K_2HPO_4), 0.0741 magnesium sulphate heptahydrate ($MgSO_4 \cdot 7\ H_2O$), 0.03528 calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$), 0.00603 ferric citrate ($C_6H_5FeO_7$), 0.00603 citric acid ($HOC(COOH)(CH_2COOH)_2$), 0.001 ethylenediaminetetraacetic acid tetrasodium salt dihydrate ($(NaOOCCH_2)_2NCH_2CH_2N(CH_2COONa)_2 \cdot 2\ H_2O$) and 0.02 sodium carbonate (Na_2CO_3). The trace metal solution had the following concentration (mg/L): 2.86 boric acid (H_3BO_3), 1.81 manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4\ H_2O$), 0.22 zinc sulphate heptahydrate ($ZnSO_4 \cdot 7\ H_2O$), 0.39 sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2\ H_2O$), 0.08 copper(II) sulphate pentahydrate ($CuSO_4 \cdot 5\ H_2O$), 0.05 cobalt(II) nitrate hexahydrate ($Co(NO_3)_2 \cdot 6\ H_2O$). All components were dissolved in distilled water and autoclaved. The pH was set to 7 with a 0.27 M HCl solution.

Laboratory Analyses

Growth of the algae was measured by biomass dry weight. For this purpose, the dry weight (g/L) was measured with a moisture analyser (HC103/01, Mettler Toledo, Switzerland) from a 20 ml sample.

The total amount of dissolved nitrogen in the cultures was measured from the supernatant of centrifuged (3 min at 4'000 rpm and 20 °C) 20 ml samples with a photometric test kit (total Nitrogen, LCK138, Hach-Lange, Rheineck, Switzerland).

The nitrous oxide content in gas samples (as absorbance values) was analysed by Fourier transform infrared spectroscopy (FTIR Tensor 37, Bruker, USA). Every sample was analysed in triplicate. Pure nitrogen (N_2) was used as carrier gas and after each measurement, the gas cell was washed with it. The exact system settings are

described in the appendix. The absorbance values from the FTIR were converted into nmol gDW^{-1} h^{-1}. For this, a calibration curve was constructed with a nitrous oxide/nitrogen gas mixture with known concentrations. Further details are available in the appendix. The linear regression formula (R^2 = 0.99) of the calibration curve is as follows:

$$\text{Nitrous oxide concentration} = 1.002 \cdot 10^{-3} + \text{Absorbance maximum} / 2.98 \cdot 10^{-6}$$

The nitrous oxide concentration is given in nanomoles (nmol) and the absorbance maximum is the maximum of the absorbance value between 2130 and 2241 cm^{-1}. The nitrous oxide concentration was multiplied with the FTIR gas chamber volume (0.2601 L) and divided by the injected gas sample volume (30 ml) to determine the nitrous oxide concentration in the headspace of the algae cultures. This concentration was multiplied by the headspace volume (200 ml) to obtain the total amount of nitrous oxide emitted from the culture. By dividing this number by the total dry weight of the culture and the known duration of the experiment, the nitrous oxide concentration is given in nanomoles and per gram dry weight and hour (nmol gDW^{-1} h^{-1}). Potential dissolved nitrous oxide in the algae cultures as well as sample loss and the effects of sampling on the pressure in the sealed bottles were not taken into account.

Experimental Setup

The *Chlorella vulgaris* precultures were cultivated during 14 days in BG11 medium (nitrate as nitrogen source) in Erlenmeyer flasks at 25 °C, 2 % CO_2, 120 rpm agitation and continuous illumination (4'800 lux) (HT Multitron Pro, Infors, Switzerland).

Three experiments were carried out: First, the suitability of nitrous oxide quantification using FTIR was investigated. Therefore, three replicates of the *Chlorella vulgaris* strain CCALA 256(1) were cultivated with nitrite as nitrogen source in darkness or in light (4'800 lux) conditions. To exclude possible influences of nitrogen gas, air and the sterile medium on the measurements, these microalgae-free abiotic assays were analysed as control groups. Second, three replicates of the *Chlorella vulgaris* strain CCALA 256 (1) were cultivated with either nitrate, nitrite, ammonium, or urea as nitrogen source in darkness (nutrient experiment). Third, seven *Chlorella vulgaris* strains (three replicates per strain) were cultivated with nitrite as nitrogen source in darkness (strain experiment).

For the three experiments described above, the algae were cultivated in a volume of 400 ml BG11 medium in a 0.5 L Schott flask. The flasks were sealed with a septum (PTFE-Silikon, Duran Group, Germany) and incubated for three to four days at 25 °C, and 120 rpm agitation. For those cultures incubated in light, an illumination of

4’800 lux was set. Those cultures incubated in darkness were wrapped in aluminium foil to block the light.

At the start and the end of the experiments, a 20 ml sample from each replicate was transferred to sterile tubes and was stored at 4 °C for further analysis (dry weight and total nitrogen in the medium).

Gas in the headspace was sampled at the end of the experiment with a needle (20 G Sterican, B. Braun Medical, Switzerland) and a syringe (50 ml Omnifix, B. Braun Medical, Switzerland). The syringe was sealed with a three-way valve (Discofix C, B. Braun Medical, Switzerland). The sample volume was 30 ml.

Data analysis

The overall growth over the entire experiment was calculated as the difference between the dry weight at the end and at the beginning of the experiment. Dissolved nitrogen consumption over the entire experiment was calculated as the difference between the dissolved nitrogen concentration in the medium at the end and at the beginning of the experiment.

Statistical analysis and data visualisation were performed with R (version 4.2.2) in RStudio (version 2022.07.2). The significance level was set at $p < 0.05$. The data is presented as the mean and standard error of the mean, wherever there were replicates in the experimental design. If the data met the conditions of homoscedasticity (Levene’s test) and normal distribution of the residuals (Shapiro-Wilk test), they were tested with a one-way ANOVA followed by a Tukey's post hoc test. Otherwise, a Kruskal-Wallis H test was performed. Data on the effect of nitrogen source on nitrous oxide emissions were log-transformed prior to a one-way ANOVA to conform to the assumptions.

Results

Nitrous oxide quantification using FTIR

Nitrous oxide quantification using FTIR was successful and suitable. With this method, nitrous oxide emissions could be detected in the range from 2130 to 2270 cm^{-1} at concentrations above 130 nmol. *Chlorella vulgaris* cultures cultivated with nitrite in darkness emitted 1’300 nmol N_2O gDW^{-1} h^{-1} of nitrous oxide (Figure 1). *Chlorella vulgaris* cultures cultivated with nitrite under light emitted 110 nmol N_2O gDW^{-1} h^{-1} of nitrous oxide. No nitrous oxide could be detected in the microalgae-free abiotic assays of pure nitrogen, air and sterile BG11-media without algae.

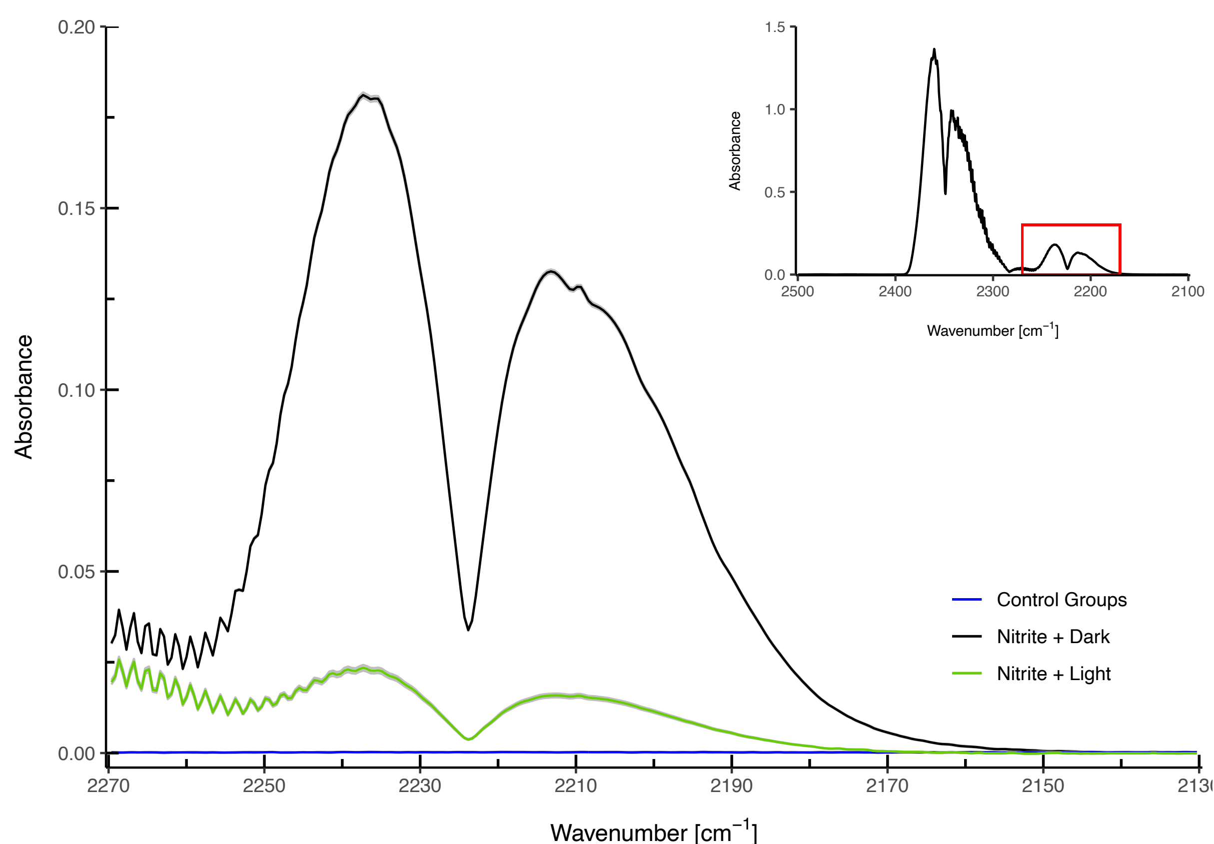

Figure 1: Absorption spectrum from FTIR analyses of *Chlorella vulgaris* cultures cultivated with nitrite in darkness or light. Microalgae-free assays as control groups (pure nitrogen, air and sterile BG11-medium without algae). The absorption spectrum of nitrous oxide is marked in red in the inset figure. The data shown represent averages from triplicate ± SEM (in grey).

Effect of nitrogen source on nitrous oxide emissions from *C. vulgaris* cultures

Nitrous oxide emissions varied depending on the nitrogen sources used ($F_{3,8} = 33.8$, $p < 0.001$). *Chlorella vulgaris* emitted negligible nitrous oxide when supplied with urea or ammonium as nitrogen sources (<18 nmol gDW^{-1} h^{-1}, Figure 2). When nitrate was used as nitrogen source, the nitrous oxide emissions were around 205 nmol gDW^{-1} h^{-1} (Tukey's range test: $p < 0.02$). The largest nitrous oxide emissions showed the cultures with nitrite as nitrogen sources and are about 1'314 nmol gDW^{-1} h^{-1} (Tukey's range test: $p < 0.02$). Differences in nitrous oxide emissions are not an artefact: Neither growth ($F_{3,8} = 1.339$, $p = 0.33$) nor nutrient consumption ($F_{3,8} = 1.821$, $p = 0.22$) did differ regardless of the nitrogen source. For further information, see appendix.

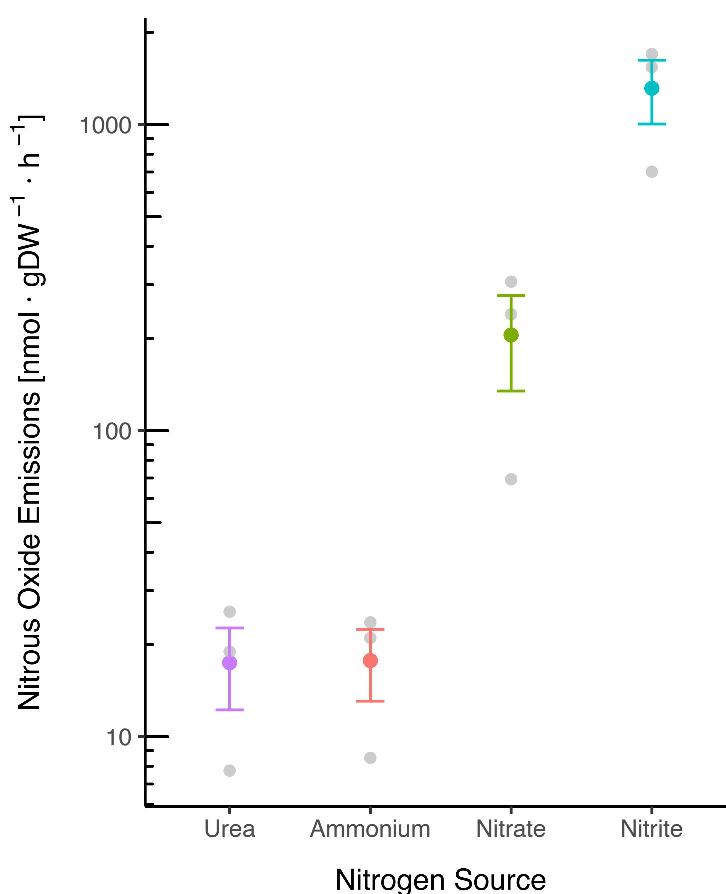

Figure 2: Influence of nitrogen sources in *Chlorella vulgaris* cultures on nitrous oxide emissions in nanomoles and per gram dry weight and hour (nmol gDW^{-1} h^{-1}). The data shown represent averages from triplicate ± SEM at a logarithmic scale.

Effect of strain selection on nitrous oxide emissions from *C. vulgaris* cultures

Despite considerable differences of nitrous oxide emissions depending on the *Chlorella vulgaris* strain used, these were not statistically significant (Chi square = 8.918, $p = 0.18$, $df = 6$). Strains SAG 9.88 and CCALA 924 emitted slightly more nitrous oxide than the others. The standard deviation of the mean in all cultures was very high (SEM ranges from 87.9 to 562). The overall growth did not differ, regardless of the nitrogen source ($F_{3,8} = 0.302$, $p = 0.93$). Differences in nitrous oxide emissions are not an artefact: Neither growth nor nutrient consumption did differ regardless of the strain used ($F_{3,8} = 0.302$, $p = 0.93$ respectively $F_{3,8} = 0.352$, $p = 0.90$). For further information, see appendix.

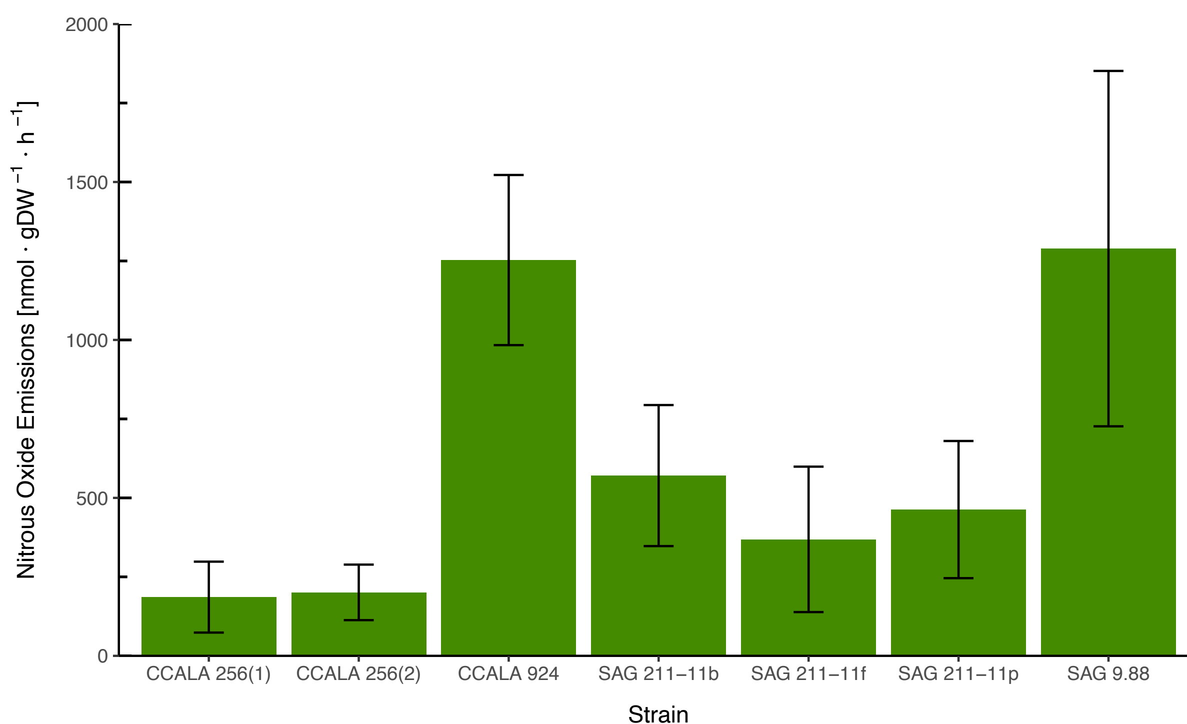

Figure 3: Influence of different *Chlorella vulgaris* strains on nitrous oxide emissions in nanomoles and per gram dry weight and hour (nmol gDW^{-1} h^{-1}). The data shown represent averages from triplicate ± SEM.

Discussion

The quantification of nitrous oxide emissions form microalgae cultures using FTIR-analysis was successful. Furthermore, I was able to confirm that nitrous oxide emissions varied depending on the nitrogen sources used and, thus, confirmed prior results by Guieysse et al., 2013. *Chlorella vulgaris* emitted negligible nitrous oxide when supplied with urea or ammonium as nitrogen sources. However, with nitrate as nitrogen source, nitrous oxide emissions could be detected (205 nmol gDW^{-1} h^{-1}). The largest nitrous oxide emissions were detected in the cultures with nitrite as nitrogen sources (1’314 nmol gDW^{-1} h^{-1}). Despite considerable differences of nitrous oxide emissions, these were not statistically significant.

FTIR spectroscopy is a fast and precise method for measuring nitrous oxide. The advantages are its precision and speed of analysis (several minutes), and heat-stable molecules can be measured (Kagann, 1982). A disadvantage are the high costs of operation (46 to 78 CHF/sample) (MSE Supplies LLC, 2023). In contrast, gas chromatography is a reliable, cheaper (23 to 60 CHF/sample) (University of Florida, 2023) but challenging and time-consuming (30 to 60 minutes) analytical method (M. Edelmann, personal communication, 2. Januar 2023). It is only suitable for non-heat-stable molecules (Struppe, 1995). So far, only one paper is known that used FTIR for measuring nitrous oxide from algae cultures (Fagerstone et al., 2011). However, a different FTIR methodology was used (liquid nitrogen cooled MCT-A detector with ZnSe window). The technical functionality of the used FTIR spectroscopy and further information about the nitrous oxide spectra are described in the appendix.

Experiments on nitrous oxide emissions depending on the nitrogen source in *Chlorella vulgaris* cultures seem to be very replicable. The nitrous oxide emissions reported in this thesis range from 180 to 1’314 nmol gDW^{-1} h^{-1}. The values found in the literature also lie in this range: 109 to 1’480 nmol gDW^{-1} h^{-1} (0.53 g/L nitrite, 25 °C, 5.5 days incubation time, PBR) (Guieysse et al., 2013) and 933 to 1’579 nmol gDW^{-1} h^{-1} (1.2 to 3.0 g/L nitrite, 25 °C, 0.83 day incubation time, PBR) (Weathers, 1984) and 70 to 18’300 nmol gDW^{-1} h^{-1} (median 4’200 nmol gDW^{-1} h^{-1}, 50 gTN/L, 20 °C, 7.5 days incubation time, HRAP) (Plouviez et al., 2019). Furthermore, in all papers mentioned above, the distribution of the emission values is broad. The magnitude and dispersion of nitrous oxide emissions reported by Plouviez et al., 2019 could be caused by the weather and high nitrogen concentration used. As mentioned above, the nitrous oxide production of *Chlorella vulgaris* was significantly increased (12 times higher) when the algae were incubated in the dark and supplied with nitrite as a nitrogen source. These observations are also confirmed by the results of Weathers (1984), who linked

the nitrous oxide emissions of microalgae and cyanobacteria to nitrite reduction in the dark. Furthermore, Guieysse et al. (2013) were able to show that when *Chlorella vulgaris* is cultivated under artificial light and in the presence of 10 µM DCMU ($C_9H_{10}Cl_2N_2O$) as a specific photosystem II inhibitor, the nitrous oxide production was increased. This indicates that the nitrous oxide production is negative linked with the photosynthesis and thus light dependent.

Nitrous oxide emissions in algae cultures are related to the selection of the nitrogen source. In this thesis, the highest nitrous oxide emissions occurred in the cultures with nitrite (NO_2^-) as nitrogen source. This observation is consistent with several studies suggesting that nitrite is the substrate of nitrous oxide synthesis by microalgae (Alcántara et al., 2015; Guieysse et al., 2013; Kamp et al., 2013; Weathers, 1984). However, a part of the emissions could also be caused by bacterial denitrification in low-oxygen environments (Harter et al., 2013). This cannot be completely excluded for the results in this thesis either. This bacterial denitrification could be reduced by an antibiotic treatment, but this would lead to losses in water quality and antibiotic resistance in bacteria (Fagerstone et al., 2011). A possible antibiotic treatment could be as followed: Addition of penicillin and streptomycin would effectively kill both Gram-positive and Gram-negative bacteria, while the two antibiotics would have minimal impact on microalgal cultures (Plouviez, Shilton, et al., 2019).

The comparison of nitrous oxide emissions depending on the strain used found broad distribution within the same strain yet was not statistically significant. However, there is some evidence that differences could have been found with more replicates. Differences within the genome of microalgae strains have been found to correspond to differences in habitat or geographical locations (e.g. in the green algae *Scenedesmus*) (Lewis & Flechtner, 2004). In addition, previous studies on *Chlorella vulgaris* strains (same strains used in this paper) indicated that there may be physiological differences (excretion and emission) within the species, for example excretion of various sugars (SAG 211-11b) (Kessler et al., 1991), growth in the presence of heavy metals (SAG 211-11b and SAG 211-11f) (Kessler, 1986) or lipid productivity (CCALA 256) (Přibyl et al., 2012). The influence of the geographical location (see appendix) and the genome difference of the *Chlorella vulgaris* strains used (Müller et al., 2005) could play a role in this thesis. The high nitrous oxide producing strains SAG 9.88 and CCALA 924 have their origin in Mediterranean countries, the other strains come from oceanic or temperate climatic zone. However, no other experiments investigating the influence of the choice of the *Chlorella vulgaris* strain on their nitrous oxide production are described in the literature to confirm this possibility.

In order to evaluate the relevance of the described nitrous oxide emissions, a simple extrapolation can be made. If *Chlorella vulgaris* would be cultivated in an open thin layer photobioreactor (224 m^2, 2'000 L), using a mineral medium with 1.85 g/L nitrate as nitrogen source, about an average biomass concentration of 4.3 gDW L^{-1} could be expected (Doucha & Lívanský, 2009). If this biomass is now compared with the emission values described in this paper (205 nmol gDW^{-1} h^{-1} = 4'920 nmol gDW^{-1} d^{-1}) and assumed that the reactor is running for 7 days and is exposed to equal-length light and dark cycles (12:12 hours), the result is $15 \cdot 10^6$ nmol of nitrous oxide emissions. The nitrous oxide emissions from a petrol-powered car are estimated to be around $143 \cdot 10^3$ nmol/km (Hoekman, 2020). Thus, the nitrous oxide emissions of the photobioreactor are comparable to a 104 km road trip with a petrol-powered car. Although these estimates do not provide exact numbers, they demonstrate the need of further research on nitrous oxide emissions from algae cultures.

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References

Agrarpreise. (2022, Juli). Agrarpreise Harnstoff. https://www.agrarpreise.at/deutschland-ostseehaefen-geliefert-i-kassamarkt-i-harnstoff-granuliert-i-eur-t+2500+1000230

Alcántara, C., Muñoz, R., Norvill, Z., Plouviez, M., & Guieysse, B. (2015). Nitrous oxide emissions from high rate algal ponds treating domestic wastewater. Bioresource Technology, 177, 110–117. https://doi.org/10.1016/j.biortech.2014.10.134

Arumugam, M., Agarwal, A., Arya, M. C., & Ahmed, Z. (2013). Influence of nitrogen sources on biomass productivity of microalgae Scenedesmus bijugatus. Bioresource Technology, 131, 246–249. https://doi.org/10.1016/j.biortech.2012.12.159

Batan, L., Quinn, J., Willson, B., & Bradley, T. (2010). Net energy and greenhouse gas emission evaluation of biodiesel derived from microalgae. Environmental Science & Technology, 44(20), 7975–7980. https://doi.org/10.1021/es102052y

Bergström, A.-K., & Jansson, M. (2006). Atmospheric nitrogen deposition has caused nitrogen enrichment and eutrophication of lakes in the northern hemisphere: Eutrophication of unproductive northern lakes. Global Change Biology, 12(4), 635–643. https://doi.org/10.1111/j.1365-2486.2006.01129.x

Brendel, D., Skoog, D. A., Hoffstetter-Kuhn, S., & Leary, J. J. (2013). Instrumentelle Analytik: Grundlagen – Geräte – Anwendungen (4d Aufl., Bd. 1). Springer. https://doi.org/10.1007/978-3-662-07916-4

Bryant, M. S., Reeve, S. W., & Burns, W. A. (2008). Observation and analysis of N_2O rotation–vibration spectra. Journal of Chemical Education, 85(1), 121. https://doi.org/10.1021/ed085p121

Cai, J., Lovatelli, A., Aguilar-Manjarrez, J., Cornish, L., Dabbadie, L., Desrochers, A., Diffey, S., Garrido Gamarro, E., Geehan, J., Hurtado, A., Lucente, D., Mair, G., Miao, W., Potin, P., Przybyla, C., Reantaso, M., Roubach, R., Tauati, M., & Yuan, X. (2021). Seaweeds and microalgae: An overview for unlocking their potential in global aquaculture development. Food and Agriculture Organization (FAO). https://doi.org/10.4060/cb5670en

Doucha, J., & Lívanský, K. (2009). Outdoor open thin layer microalgal photobioreactor: Potential productivity. Journal of Applied Phycology, 21(1), 111–117. https://doi.org/10.1007/s10811-008-9336-2

Edelmann, M. (2023, Januar 2). Analytical time of FTIR vs. GC [personal communication].

European Union. (2015). Statistiken über den Düngemittelverbrauch und Nährstoffbilanz (S. 1). eurostat. https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Archive:Fertiliser_consumption_and_nutrient_balance_statistics/de&oldid=222304#Weitere_Informationen_von_Eurostat

Fagerstone, K. D., Quinn, J. C., Bradley, T. H., De Long, S. K., & Marchese, A. J. (2011). Quantitative measurement of direct nitrous oxide emissions from microalgae cultivation. Environmental Science & Technology, 45(21), 9449–9456. https://doi.org/10.1021/es202573f

Guelachvili, G., Birk, M., Bordé, Ch. J., Brault, J. W., & Brown, L. R. (1996). High resolution wavenumber standards for the infrared. International Union of Pure and Applied Chemistry, 68, 193–208. http://pac.iupac.org/publications/pac/authors/G.Guelachvili/

Guieysse, B., Plouviez, M., Coilhac, M., & Cazali, L. (2013). Nitrous oxide (N_2O) production in axenic *Chlorella vulgaris* microalgae cultures: Evidence, putative pathways, and potential environmental impacts. Biogeosciences, 10(10), 6737–6746. https://doi.org/10.5194/bg-10-6737-2013

Harter, T., Bossier, P., Verreth, J., Bodé, S., Van der Ha, D., Debeer, A.-E., Boon, N., Boeckx, P., Vyverman, W., & Nevejan, N. (2013). Carbon and nitrogen mass balance during flue gas treatment with *Dunaliella salina* cultures. Journal of Applied Phycology, 25(2), 359–368. https://doi.org/10.1007/s10811-012-9870-9

Hase, F., Frey, M., Blumenstock, T., Groß, J., Kiel, M., Kohlhepp, R., Mengistu Tsidu, G., Schäfer, K., Sha, M. K., & Orphal, J. (2015). Application of portable FTIR spectrometers for detecting greenhouse gas emissions of the major city Berlin. Atmospheric Measurement Techniques, 8(7), 3059–3068. https://doi.org/10.5194/amt-8-3059-2015

Hoekman, S. K. (2020). Review of nitrous oxide (N_2O) emissions from motor vehicles. SAE International Journal of Fuels and Lubricants, 13(1), 79–98. https://www.jstor.org/stable/27034495

Kagann, R. H. (1982). Infrared absorption intensities for N_2O. Journal of Molecular Spectroscopy, 95(2), 297–305. https://doi.org/10.1016/0022-2852(82)90130-8

Kamp, A., de Beer, D., Nitsch, J. L., Lavik, G., & Stief, P. (2011). Diatoms respire nitrate to survive dark and anoxic conditions. Proceedings of the National Academy of Sciences, 108(14), 5649–5654. https://doi.org/10.1073/pnas.1015744108

Kamp, A., Stief, P., Knappe, J., & de Beer, D. (2013). Response of the ubiquitous pelagic diatom *Thalassiosira weissflogii* to darkness and anoxia. PLoS ONE, 8(12), e82605. https://doi.org/10.1371/journal.pone.0082605

Kessler, E. (1986). Limits of growth of five *Chlorella* species in the presence of toxic heavy metals. Archiv Für Hydrobiologie, Supplementband (Monographische Beiträge), 123–128.

Kessler, E., Kauer, G., & Rahat, M. (1991). Excretion of sugars by *Chlorella* species capable and incapable of symbiosis with *Hydra viridis*. Botanica Acta, 104(1), 58–63. https://doi.org/10.1111/j.1438-8677.1991.tb00194.x

Kholssi, R., Ramos, P. V., Marks, E. A. N., Montero, O., & Rad, C. (2021). Biotechnological uses of microalgae: A review on the state of the art and challenges for the circular economy. Biocatalysis and Agricultural Biotechnology, 36, 102114. https://doi.org/10.1016/j.bcab.2021.102114

Krupa, S. V. (2003). Effects of atmospheric ammonia (NH_3) on terrestrial vegetation: A review. Environmental Pollution, 124(2), 179–221. https://doi.org/10.1016/S0269-7491(02)00434-7

Lewis, L. A., & Flechtner, V. R. (2004). Cryptic species of *Scenedesmus* (chlorophyta) from desert soil communities of Western North America. Journal of Phycology, 40(6), 1127–1137. https://doi.org/10.1111/j.1529-8817.2004.03235.x

Mampaey, K. E., Beuckels, B., Kampschreur, M. J., Kleerebezem, R., van Loosdrecht, M. C. M., & Volcke, E. I. P. (2013). Modelling nitrous and nitric oxide emissions by autotrophic ammonia-oxidizing bacteria. Environmental Technology, 34(12), 1555–1566. https://doi.org/10.1080/09593330.2012.758666

Mandalam, R. K., & Palsson, B. (1998). Elemental balancing of biomass and medium composition enhances growth capacity in high-density *Chlorella vulgaris* cultures. Biotechnology and Bioengineering, 59(5), 605–611. https://doi.org/10.1002/(SICI)1097-0290(19980905)59:5<605::AID-BIT11>3.0.CO;2-8

Mariotto, M. (2022). Cultivation of PHB-producing cyanobacteria in wastewater on laboratory and pilot-scale [Master Thesis, ZHAW]. https://digitalcollection.zhaw.ch/bitstream/11475/25201/1/2022_Mariotto_Mariana_MAS_ENR.pdf

MSE Supplies LLC. (2023). MSE Analytical Services. FTIR Testing Services. https://www.msesupplies.com/collections/materials-characterization-analytical-services-best-value/products/ftir-testing-services-fourier-transform-infrared-spectroscopy-analysis?variant=23496486846522

Müller, J., Friedl, T., Hepperle, D., Lorenz, M., & Day, J. G. (2005). Distinction between multiple isolates of *Chlorella vulgaris* (chlorophyta, trebouxiophyceae) and testing for conspecificity using amplified fragment length polymorphism and its rDNA sequences. Journal of Phycology, 41(6), 1236–1247. https://doi.org/10.1111/j.1529-8817.2005.00134.x

National Institute of Standarts and Technology, U. D. of C. (2020, Mai 27). NIST Chemistry WebBook. Nitrous Oxide. https://webbook.nist.gov/cgi/cbook.cgi?ID=C10024972&Units=SI&Type=IR-SPEC&Index=1#IR-SPEC

Plouviez, M., Chambonnière, P., Shilton, A., Packer, M. A., & Guieysse, B. (2019). Nitrous oxide (N_2O) emissions during real domestic wastewater treatment in an outdoor pilot-scale high rate algae pond. Algal Research, 44, 101670. https://doi.org/10.1016/j.algal.2019.101670

Plouviez, M., Shilton, A., Packer, M. A., & Guieysse, B. (2019). Nitrous oxide emissions from microalgae: Potential pathways and significance. Journal of Applied Phycology, 31(1), 1–8. https://doi.org/10.1007/s10811-018-1531-1

Poth, M., & Focht, D. D. (1985). ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea*: An examination of nitrifier denitrification. Applied and Environmental Microbiology, 49(5), 1134–1141.

Přibyl, P., Cepák, V., & Zachleder, V. (2012). Production of lipids in 10 strains of *Chlorella* and *Parachlorella*, and enhanced lipid productivity in *Chlorella vulgaris*. Applied Microbiology and Biotechnology, 94(2), 549–561. https://doi.org/10.1007/s00253-012-3915-5

Pulgarin, A., Kapeller, A. G., Tarik, M., Egloff, S., Mariotto, M., Ludwig, C., & Refardt, D. (2021). Cultivation of microalgae at high-density with pretreated liquid digestate as a nitrogen source: Fate of nitrogen and improvements on growth limitations. Journal of Cleaner Production, 324, 129238. https://doi.org/10.1016/j.jclepro.2021.129238

Raja, R., Hemaiswarya, S., Kumar, N. A., Sridhar, S., & Rengasamy, R. (2008). A perspective on the biotechnological potential of microalgae. Critical Reviews in Microbiology, 34(2), 77–88. https://doi.org/10.1080/10408410802086783

Robles, Á., Aguado, D., Barat, R., Borrás, L., Bouzas, A., Giménez, J. B., Martí, N., Ribes, J., Ruano, M. V., Serralta, J., Ferrer, J., & Seco, A. (2020). New frontiers from removal to recycling of nitrogen and phosphorus from wastewater in the Circular Economy. Bioresource Technology, 300, 122673. https://doi.org/10.1016/j.biortech.2019.122673

Sathasivam, R., Radhakrishnan, R., Hashem, A., & Abd_Allah, E. F. (2019). Microalgae metabolites: A rich source for food and medicine. Saudi Journal of Biological Sciences, 26(4), 709–722. https://doi.org/10.1016/j.sjbs.2017.11.003

Smil, V. (1999). Nitrogen in crop production: An account of global flows. Global Biogeochemical Cycles, 13(2), 647–662. https://doi.org/10.1029/1999GB900015

Spolaore, P., Joannis-Cassan, C., Duran, E., & Isambert, A. (2006). Commercial applications of microalgae. Journal of Bioscience and Bioengineering, 101(2), 87–96. https://doi.org/10.1263/jbb.101.87

Struppe, H. G. (1995). Gaschromatographie. Nachrichten aus der Chemie, 43(3), 14. https://doi.org/10.1002/nadc.19950430335

Takeshita, T., Ota, S., Yamazaki, T., Hirata, A., Zachleder, V., & Kawano, S. (2014). Starch and lipid accumulation in eight strains of six *Chlorella* species under comparatively high light intensity and aeration culture conditions. Bioresource Technology, 158, 127–134. https://doi.org/10.1016/j.biortech.2014.01.135

University of Florida. (2023). Center for Envirmonmental and Human Toxicology. Analytical Services Pricing. https://toxicology.vetmed.ufl.edu/core-laboratories/analytical-services-pricing/

Usher, P. K., Ross, A. B., Camargo-Valero, M. A., Tomlin, A. S., & Gale, W. F. (2014). An overview of the potential environmental impacts of large-scale microalgae cultivation. Biofuels, 5(3), 331–349. https://doi.org/10.1080/17597269.2014.913925

Vuuren, D. P. van, Bouwman, L. F., Smith, S. J., & Dentener, F. (2011). Global projections for anthropogenic reactive nitrogen emissions to the atmosphere: An assessment of scenarios in the scientific literature. Current Opinion in Environmental Sustainability, 3(5), 359–369. https://doi.org/10.1016/j.cosust.2011.08.014

Walsh, M. J., Gerber Van Doren, L., Sills, D. L., Archibald, I., Beal, C. M., Lei, X. G., Huntley, M. E., Johnson, Z., & Greene, C. H. (2016). Algal food and fuel coproduction can mitigate greenhouse gas emissions while improving land and water-use efficiency. Environmental Research Letters, 11(11), 114006. https://doi.org/10.1088/1748-9326/11/11/114006

Weathers, P. J. (1984). N_2O evolution by green algae. Applied and Environmental Microbiology, 48(6), 1251–1253.

Wijffels, R. H. (2008). Potential of sponges and microalgae for marine biotechnology. Trends in Biotechnology, 26(1), 26–31. https://doi.org/10.1016/j.tibtech.2007.10.002

Wu, L. F., Chen, P. C., Huang, A. P., & Lee, C. M. (2012). The feasibility of biodiesel production by microalgae using industrial wastewater. Bioresource Technology, 113, 14–18. https://doi.org/10.1016/j.biortech.2011.12.128

Appendix

Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectrometers are used for chemical analyses in the MIR range (mid-infrared: 400 - 4’000 cm^{-1}). A Michelson interferometer modulates the light from an infrared radiation source. With a Fourier transform analysis, the recorded intensity-time curve can be mathematically transformed into a frequency-dependent signal (Brendel et al., 2013).

FTIR spectroscopy works like all IR methods: The gas to be investigated absorbs radiation components with a certain wavenumber and intensity between an emitter and a detector. The gas and concentration can be determined from this, which is described by the Beer–Lambert’s law (Hase et al., 2015). The standard IR detector is a DTGS (deuterated triglycine sulphate) detector. A converter translates the analogue signal of the DLATGS detector into a digital signal. The data is then transmitted to the OPUS software, which selects the appropriate measurement parameters.

FTIR System Settings

The gas samples were analysed by Fourier transform infrared spectroscopy with the following material:

Material	Specifications
FTIR	Tensor 37, Bruker, USA
OPUS Software	6,5,97 (20090227)
Gas Chamber	0.2601 L, CaF_2, Schott, Germany
Nitrous Oxide	CAS 10024-97-2, ≥99.5%, PanGas, Switzerland
Nitrogen	CAS 7727-37-9, ≥99.99 %, PanGas, Switzerland

The FTIR measuring instrument had following system settings:

Parameter	Setting
Source	MIR
Beam Splitter	KBr
Aperture	6 mm
Detector	RT-DLaTGS (Internal)
Mirror Velocity	10 Hz
Spectral Range	370-7500 cm^{-1}
Resolution	1 cm^{-1}
Required Upper Frequency	15’000 cm^{-1}
Required Lower Frequency	0 cm^{-1}
Laser Wavenumber	15’800.17 cm^{-1}
High Pass Filter	Open
Low Pass Filter	10 KHz
Acquisition Mode	Double Sided, Forward-Backward
Measuring Time	4 Scans
Phase Resolution	32
Phase Correction Mode	Mertz
Apodisation Function	Blackman-Harris 3-Term
Zerofilling Factor	2

Raw Data for Calibration Curve

The linear regression formula (R^2 = 0.99) of the calibration curve is as followed:

Nitrous oxide concentration = $1.00177859237 \cdot 10^{-3}$ + Absorbance maximum / $2.97679959837 \cdot 10^{-6}$

Whereby the nitrous oxide concentration content is given in nanomoles (nmol) and the absorbance maximum is the maximum of the absorbance value between 2130 and 2241 cm^{-1}. The y-dispersion of the linear regression is 0.0016. The used values for the are calibration curve shown in the table below (Absorbance value as mean of three technical replicates):

Nitrous Oxide Concentration [nmol]	**Absorbance [cm^{-1}]**
161	0.0007
323	0.0009
484	0.0013
646	0.0015
807	0.0018
969	0.0022
1291	0.0029
1614	0.0030
3229	0.0060
4843	0.0094
8071	0.0248
9686	0.0276
11300	0.0344
12914	0.0379
14529	0.0416

Rotation–Vibration Spectra of Nitrous Oxide (N_2O)

The linear molecule N_2O has three normal vibrations called v_1 (symmetric stretching vibration), v_2 (kink vibration) and v_3 (antisymmetric stretching vibration), where v_2 is doubly degenerate (v_{2a} and v_{2b}). The doubly degenerate bending vibration $v_{2a,b}$ and the asymmetric stretching vibration v_3 are found in the mid-infrared range. The symmetric stretching vibration v_1 is Raman-active (Bryant et al., 2008). In the gas phase, nitrous oxide can exhibit the four typical vibrations shown in Figure a below. Besides the main absorption peak between 2'130 and 2'270 cm^{-1}, nitrous oxide also absorbs in the wavelength range of 1'200 – 1'300 cm^{-1} (Figure b). The vibrational frequencies of selected nitrous oxide isomers in the gas state such as $v_{(N \equiv N - O)}$ and $v_{(N = N = O)}$ are 2235 cm^{-1} respectively 2213 cm^{-1} (Guelachvili et al., 1996; National Institute of Standarts and Technology, 2020).

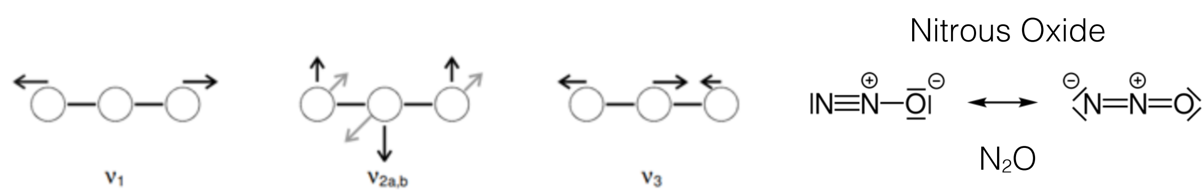

Figure a: The typical vibrations v_1, $v_{2a,b}$, v_3 and the isotopomers of nitrous oxide.

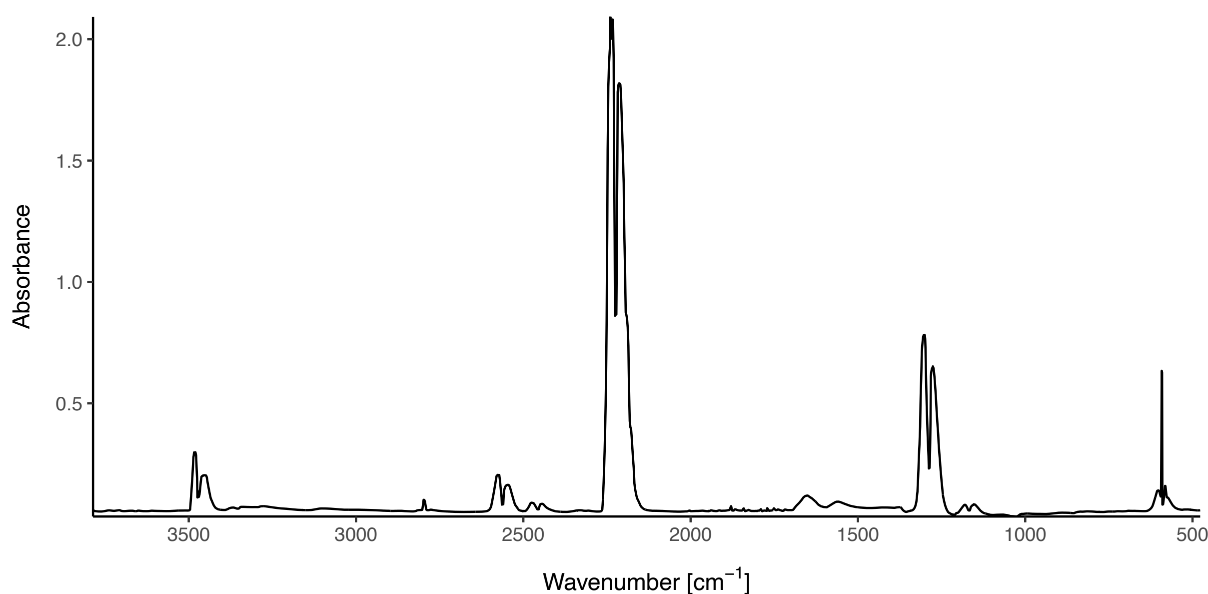

Figure b: Reference absorbance-spectrum of nitrous oxide from the NIST database.

Chlorella vulgaris Strains

The experiments were performed with seven different strains of *Chlorella vulgaris*. SAG 211-11b, SAG 211-11f, SAG 211-11p and SAG 9.88 were obtained from the Culture Collection of Algae at Göttingen University (SAG). CCALA 256(1), CCALA 256(2) and CCALA 924 was obtained from the Culture Collection of Autotrophic Organism of the Institute for Botany in Třeboň (CCALA). Overview of the *Chlorella vulgaris* algae strains used (National Institute of Standarts and Technology, 2020):

Strain/Culture Information	SAG 211-11b	SAG 211-11f	SAG 211-11p	SAG 9.88	CCALA 256	CCALA 924
Origin	Netherlands	United Kingdom	Sweden	Spain	New Zealand	Greece
Year isolated	1889	1939	1942	1986	1982	2005
Climatic Zone	Oceanic	Oceanic	Oceanic	Mediterranean	Temperate	Mediterranean
Collection Site	Eutrophic shallow pond near Delft	From an old mushroom in Dorking	Pond in municipal park in Lund	Wastewater off a sugar refinery in Madrid	Lake Pupuke, Auckland	-
General Habitat	Freshwater	Freshwater	Freshwater	Freshwater	Freshwater	Freshwater
Equivalent Strains	CCAP 211/11B, UTEX 259	CCAP 211/11F	CCAP 211/11P	-	CCAP 211/52, UTEX 2219	-

Growth and Nitrogen Consumption

Overview of the biomass dry weight and growth of the used *Chlorella vulgaris* cultures:

Nitrogen Source	Start dry weight [gDM/L]	End dry weight [gDM/L]	Total Growth [gDM/L]	SEM
Ammonium	3.95	4.38	0.425	0.241
Nitrate	6.93	7.28	0.350	0.189
Nitrite	4.35	4.32	-0.033	0.101
Urea	4.03	4.24	0.211	0.131

Strain	Start dry weight [gDM/L]	End dry weight [gDM/L]	Total Growth [gDM/L]	SEM
CCALA 256(1)	3.88	4.17	0.283	0.093
CCALA 256(2)	4.67	4.95	0.283	0.093
CCALA 924	3.77	4.40	0.633	0.741
SAG 211-11b	3.33	3.60	0.267	0.136
SAG 211-11f	4.20	4.38	0.183	0.044
SAG 211-11p	3.58	3.95	0.367	0.159
SAG 9.88	3.42	4.23	0.817	0.792

Overview of the biomass dry weight and growth of the used *Chlorella vulgaris* cultures:

Nitrogen Source	**Start dry weight [gDM/L]**	**End dry weight [gDM/L]**	**Total Growth [gDM/L]**	**SEM**
Ammonium	264	249	-15	48
Nitrate	647	597	-49	34
Nitrite	267	215	-52	25
Urea	412	304	-108	76

Strain	**Start dry weight [gDM/L]**	**End dry weight [gDM/L]**	**Total Growth [gDM/L]**	**SEM**
CCALA 256(1)	235	207	-27	24
CCALA 256(2)	253	224	-28	27
CCALA 924	252	226	-27	19
SAG 211-11b	245	228	-18	17
SAG 211-11f	288	249	-39	61
SAG 211-11p	231	223	-8	15
SAG 9.88	249	215	-34	21