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Carbon and nitrogen mass balance during flue gas treatment with *Dunaliella salina* cultures

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Abstract The biotreatment of flue gases with algae cultures is a promising option to sequestrate CO_2, yet the emission of other greenhouse gases (GHG) from the cultures can hamper their environmental benefit. Quantitative data on the sequestration potential for CO_2 and NO_x in relation to the direct production of CH_4 and N_2O are urgently required. The present study assessed the flows of carbon (C) and nitrogen (N) through cultures of the green alga *Dunaliella salina*, supplied with biodiesel flue gas, by means of mass balancing. *D. salina* was grown in artificially lighted, field- (42-L bubble column reactor) and laboratory-scale cultures (23 °C, pH 7.5). In the bubble column reactor, algae grew with an average specific growth rate of 0.237 day^{-1} under flue gas supplementation (6.3 % (v/v) CO_2, 1.2 ppmv NO_x), and CO_2 was retained to 39 % in the system. The specific sequestration rate for CO_2 was low, with 0.13 g CO_2 L^{-1} day^{-1}. Cultures emitted up to 13.03 μg CH_4 L^{-1} day^{-1} and 4261 μg N_2O L^{-1} day^{-1}. The moderate retention of NO_x-N was outweighed by emissions of N_2O-N, and total N in the system decreased by 15.48 % during the 9-day trial. Results suggest that GHG production was mainly the outcome of anaerobic microbial processes and their emission was lower in pre-sterilized cultures. Under the tested conditions, up to six times more CO_2 equivalents were emitted during flue gas treatment. Therefore, the direct GHG emissions of algae culture systems, intended for flue gas treatment (i.e. open ponds) need to be reviewed critically.

Keywords Bioremediation · Climate change · Co-sequestration · Life cycle assessment · Microalgae · Photobioreactor

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Introduction

It is widely acknowledged that human activity has led to increasing atmospheric carbon dioxide (CO_2) concentrations, in the past 200 years (Vitousek 1994; Tomkiewicz 2006; Simeonova and Diaz-Bone 2005). The combustion of fossil fuels has been identified as one of the major anthropogenic sources of this gas, contributing to global warming by the greenhouse effect (IPCC 2007; Johnston et al. 2003). Besides the avoidance of emissions, one of the most promising approaches to alleviate this problem is to fix emitted CO_2 in photoautotrophic biomass, ideally in a commercially valuable form (Amin 2009; Stewart and Hessami 2005; Lee

and Lee 2003). This prerequisite is met by microalgae biomass, since it allows the production of a large variety of chemical compounds, therefore offering numerous commercial applications (Sasaki et al. 2002; Spolaore et al. 2006; Del Campo et al. 2007). The halophilic microalga *Dunaliella salina* is considered one of the most efficient biological sources of highly priced β-carotenes and is therefore an interesting candidate (Ben-Amotz and Avron 1990; García-González et al. 2003).

In order to grow microalgae in high density photoautotrophic cultures, supplying the media with additional CO_2 is essential (Benemann 1993), and low priced sources, such as flue gas, can be a commercially interesting alternative to the chemically graded gas (Kadam 1997; Douskova et al. 2009). In addition, CO_2-credits represent a strong economic incentive for the sequestration of flue gas carbon (C). Therefore, a combination of C-sequestration from industrial flue gases and the supply of algae cultures with a low cost C-source represents a sound complementary approach. This has been the focus of numerous studies in the past decades, and promising results have been achieved (Maeda et al. 1995; Brown 1996; Kadam 2002; Borkenstein et al. 2010; Chae et al. 2006; Doucha et al. 2005; Douskova et al. 2009). Depending on the local legislation, also NO_x-credits are granted for the retention of nitrogen (N) oxides (Muylaert et al. 2011), mostly in order to mitigate the adverse effects of acid precipitations (Packer 2009). If legal limits for the CO_2 and NO_x concentration of the off gas need to be met, a co-sequestration can avoid the costs for separate treatment steps (Yoshihara et al. 1996; Nagase et al. 1997; Packer 2009; Lee 2010).

The bulk of *D. salina* is grown in shallow outdoor ponds in Australia and Israel (Del Campo et al. 2007), but nowadays, artificially lighted indoor photobioreactors (PBRs) offer an interesting alternative. For growing bulk biomass, these systems have not managed to displace the simpler open approaches, due to their high investment and operating costs. Yet, PBRs offer a better control over the consistency, quality and composition of the end product (Chrismadha and Borowitzka 1994). Moreover, volumetric and areal productivity can be maximized, and a steady, year-round production is possible, even under central European climatic conditions.

The aim of the present study was to determine the feasibility of growing *D. salina* biomass in a bubble column PBR, supplied with biodiesel flue gas. A mass balance for C and N was performed, to assess whether algae cells can use the CO_2 and NO_x provided with the flue gas for biomass growth. Therefore, the flows of CO_2, CO, C_2H_2 and NO_x through the reactor were assessed, yielding quantitative data on the systems retention potential for C and N. In the recent past, numerous life cycle assessments (LCA) have been performed on microalgae cultures, for example to determine their suitability for the production of biofuels (Batan et al. 2010; Aresta et al. 2005; Lardon et al. 2009; Clarens et al. 2011; Luo et al. 2010; Stephenson et al. 2010; Campbell et al. 2011). Since currently only limited data on the direct emission of greenhouse gases (GHG) from microalgae cultures are available, these are seldom taken into account for LCAs or are estimated using very broad assumptions (Fagerstone et al. 2011). From previous studies we know that algae cultures are potential sources of methane (CH_4) (Florez-Leiva et al. 2010) and nitrous oxide (N_2O) (Florez-Leiva et al. 2010; Fagerstone et al. 2011; Weathers 1984), two of the most important anthropogenic GHG. Their chemical lifetime in the atmosphere is in the time scale of decades for CH_4 and centuries for N_2O, and it is this persistence which determines their strong global warming potential. The emissions of different GHG are commonly compared by expressing their global warming potential as carbon dioxide equivalents (CDE). Based on this concept, 1 g of CH_4 has 25 times the direct global warming potential of 1 g of CO_2 over a 100-year period, and N_2O even 298 times (Solomon et al. 2007). The present mass balance approach for C and N allowed quantifying the direct GHG emissions in a culture of *D. salina* supplied with "real" biodiesel flue gas, yielding quantitative data on the environmental sanity of the applied system.

Materials and methods

Dunaliella salina (Dunal) Teodoresco (SAG 184.80) was grown in an autoclaved medium (120 °C for 20 min): 16.0 mM KNO_3, 1.0 mM $NaH_2PO_4{\cdot}2H_2O$, 61.6 μM $Na_2EDTA{\cdot}2H_2O$, 23.3 μM $FeCl_3{\cdot}6H_2O$, 0.210 μM $CuSO_4{\cdot}7H_2O$, 0.303 μM $ZnSO_4{\cdot}7H_2O$, 0.085 μM $CoCl_2{\cdot}6H_2O$, 1.83 μM $MnCl_2{\cdot}4H_2O$ and 0.052 μM Na_2MoO_4, at 1 M NaCl.

Field scale trial

An internally lighted, airtight bubble column reactor (h=1200 mm, d=200 mm, v=42 L) was inoculated with a pre-concentrated (500×g, 5 min, 20 °C) algae inoculum at a density of 0.12×10^6 cells mL^{-1}. Previously described media was UV- and filter-sterilized (MLS T22676, 0.20 μm), and the reactor was disinfected with a hydrogen peroxide and peroxi-acetic acid solution (5 %) for 24 h. The reactor temperature was kept constant at 23.0±0.5 °C by an external temperature controlled cooling system. Continuous lighting was provided by four white fluorescent tube lights (Phillips Master TL5 HO TOP 54 W/840 1SL) at 550 μmol photons m^{-2} s^{-1}. The reactor was sparged with ambient air, in a continuous way (1.00±0.05 L min^{-1}) to keep the culture mixed and in suspension. Flue gas supply

(first 24 h after inoculation) into the PBR was pH controlled; injection started at pH 7.55 and stopped at pH 7.45. The flue gas flow rate (3.06±0.15 L min^{-1}) was controlled by thermal mass flow controllers (Mass View® MV-304, Bronkhorst High-Tech®, The Netherlands), which recorded the used mass of gas. Added to the constant air flow, total gas flow rate during flue gas injection was 4.06 L min^{-1}. Both air and flue gas were water saturated before injection.

Flue gas was supplied by a 5.7-kW diesel generator (Oreiko DG5500LDE, Machelen, Belgium; 3000 rpm, 406 cc, forced air cooled, direct injected, four stroke) fuelled with rapeseed biodiesel RadiaCid 7961® (Oleon, Ertvelde, Belgium). The generator was operated at a power output of 4 kW during the trial. Flue gas was collected at the muffler exhaust, passed through a screen filter and cooled to room temperature. The average flue gas composition was 6.3±1.9 % (v/v) CO_2, 16.4±2.4 % (v/v) O_2 and 1.2±0.4 ppmv NO_x, with traces of CH_4 (3.3±0.9 ppmv) and C_2H_2 (23.5±7.5 ppmv); the remaining fraction was N_2.

In order to establish a mass balance for exponentially growing *D. salina*, the growth period was subdivided into two distinct phases, determined by the culture density: phase I, 0.25–1.00×10^6 cells mL^{-1}; phase II, 1.00–2.00×10^6 cells mL^{-1} (Fig. 1). The algae culture was sampled at the beginning and the end of each phase, and all mass balance calculations are based on these data. Gas samples were collected in Tedlar bags® at the reactor gas-inlet and -outlet during baseline aeration, as well as during flue gas injection. The bags were filled over a period of 1 h, until approximately 5 L of gas was collected.

Lab scale trial

In order to determine the ability of *D. salina* to use NO_x-N from the gas stream for biomass growth, algae cultures were supplied with an artificial flue gas, and their performance was compared to a positive and negative control in triplicate. In the positive control (i), algae were grown using the previously mentioned media with KNO_3 as N-source, and sparging was performed with a mix of 8 % (v/v) CO_2 in N_2. In treatment "Gas-N" (ii), algae were grown without KNO_3, and sparging was performed with an artificial flue gas (8 % (v/v) CO_2 and 300 ppmv NO in N_2). In the negative control (iii), algae were grown without KNO_3, while sparging was performed with a mix of 8 % (v/v) CO_2 in N_2. All media in this trial were autoclaved before inoculation, while pH and temperature probes were sterilized with ethanol.

D. salina was grown in borosilicate glass bottles; the culture volume was 2 L, and mixing was provided by magnetic stirrers (l=60 mm, 120 rpm). Bottles were placed in a heated water bath (23.0±0.5 °C) with one transparent Plexiglas® wall on the illuminated side. Continuous lighting was provided by cool white fluorescent tubes (100 μmol photons m^{-2} s^{-1}) from outside. As previously described for the field scale trial, the supply of gas was pH controlled (pH 7.5±0.05), the flow rate was set to 0.250 g min^{-1} and the used mass of gas was recorded. The bottles were inoculated with a sterile, pre-concentrated (625×g, 4 min, 20 °C) inoculum, at a density of 0.25×10^6 cells mL^{-1}. Algae and gas samples were taken at a culture density of 1.00×10^6 cells mL^{-1}. Gases were sampled at the gas-inlet and -outlet of the bottles, by filling Tedlar bags® during a 12-h period, collecting approximately 5 L of gas.

Analysis

Growth rates were assessed in terms of cell density with a Beckman Coulter Z1 (Beckman Coulter, USA), with a 70-μm aperture. Algae dry weight (DW) was measured according to Zhu and Lee (1997), and samples were washed with 15 mL ammonium formate (1 M) after filtration (GF/C). Algae samples of 20 mg DW were used for C and N analysis according to the Dumas method (Pregl 1949) with a Carlo-Erba NA-1500 elemental analyser (Thermo Fischer Scientific, USA). Prior to analysis, samples were lypophilised overnight and ground to a homogeneous mixture. After drying in a desiccator, DW was determined for further calculations in tin capsules according to Verardo et al. (1990). The C content of the algae biomass was then used as particulate organic C (POC) in mass balance calculations. For the measurement of dissolved organic C (DOC), algae samples were centrifuged (2000×g, 4 min, 20 °C), pre-acidified and sparged with high purity air. Measurements were performed on the supernatant with a Shimadzu POC-5000A elemental analyser (Shimadzu, Japan). Total inorganic C (TIC) was calculated assuming ideal C speciation; equilibrium absorption of CO_2 was calculated as described by Henry's Law using the constants for the applied conditions according to Raven (1984). Total carbon (TC) was calculated as the sum of POC, DOC and TIC. Sequestrated C was calculated as total retained C according to: $\sum C_{in} - \sum C_{out}$.

After filtering culture samples over a 0.45 μm filter to obtain the dissolved fraction and diluting with Milli-Q water to an appropriate concentration, media samples were analysed for NO_2^- and NO_3^- using a Metrohm IC 761 Compact ion chromatograph (Metrohm, Switzerland) with a Metrosep A supp 5–150 column and a Metrosep A Supp 4/5 guard column. NH_4^+ was measured by colorimetric analysis based on the Nessler reaction, according to Greenberg et al. (1992). Total N (TN) was calculated as the sum of NH_4^+-N, NO_3^--N, NO_2^--N and the N present in the algae biomass.

Gas samples were transferred to vacutainers, previously flushed with He and finally evacuated. CO_2, CH_4, O_2, CO and C_2H_2 were measured using a Finnigan Trace GC Ultra

(Thermo Fischer Scientific, USA), with a Hayesep Q (80–100, 0.25 m+2 m×1/8″) and a Molsive 5A (80–100, 2 m×1/8″) column and He at 90 °C as carrier gas. For the N-mass balance, only reactive gaseous N-compounds were considered, according to N_yO_x=NO+NO_2+N_2O. N_2O was measured using a Shimadzu GC-14B GC (Shimadzu, Japan) with a Porapak Q column (80–100, 1 m+2 m×1/8″) and N_2 as carrier gas at 55 °C. NO was analysed using an Eco Physics CLD 77 AM (Eco Physics, Switzerland), as described by Boon et al. (2009). All NO_x (NO+NO_2) was converted to NO using a Thermo Environmental M35 (Thermo Fischer Scientific, USA), based on the stainless steel catalytic conversion of NO_x to NO at 650 °C. For the calculation of GHG balances, the emissions of CH_4 and N_2O were converted into carbon dioxide equivalents (CDE) assuming 25 CDE per g of CH_4 and 298 CDE per g of N_2O (Solomon et al. 2007).

Statistics

Data was analysed using the SPSS statistics software v19.0.0.1 (SPSS IBM, USA). In the field scale trial, regression analysis was used to determine the relationship between N_2O emission and culture density (n=6). This was performed by pooling the data of several growth trials, under the same experimental conditions (data not shown). The overall error of the C- and N-mass balance during the field scale trial was calculated as: (∑ in−∑ out)−measured values in the reactor (i.e. TC and TN), for the entire experimental period. In the lab scale trial, means were compared by one-way ANOVA (n=3, Tukey, p<0.05). All results of the lab scale trial are given as means±standard deviation (SD).

Results

Field scale trial

Dunaliella salina was grown in a bubble column reactor supplied with biodiesel flue gas, and a mass balance for C and N was established during two phases of exponential algae growth. Results for culture growth are depicted in Fig. 1. After inoculation and flue gas injection, an extended lag phase was observed, and the measurements for the mass balance could only start after 9 days, when 0.25×10^6 cells mL^{-1} was reached (start of phase I). Thereafter, the culture grew exponentially reaching 1.00×10^6 cells mL^{-1} on day 15 (start of phase II) and 2.00×10^6 cells mL^{-1} on day 18 (end of phase II) and an algae biomass of 0.46 g DW L^{-1}.

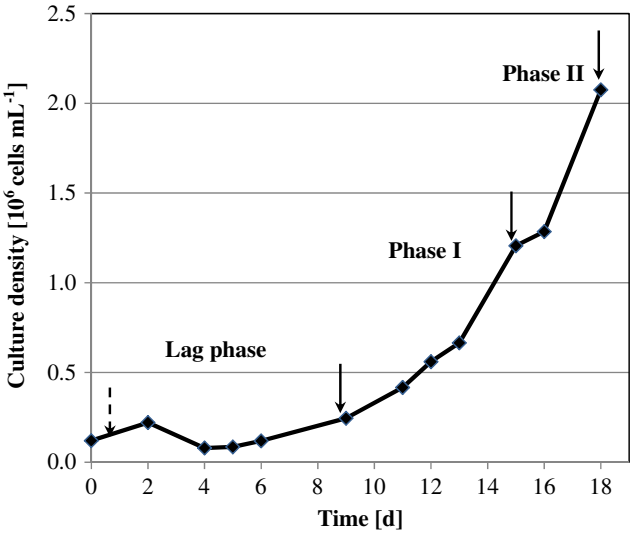

Fig. 1 Culture density [10^6 cells mL^{-1}] of *Dunaliella salina* grown in a 42-L bubble column reactor supplied with biodiesel flue gas. The point of first flue gas injection is depicted by the *dashed arrow*. Sampling points are indicated by *solid arrows*, demarking the two phases (I and II) considered for C- and N-mass balance calculations

Averaged over phases I and II, the specific growth rate of *D. salina* was 0.237 day^{-1}. Results for the C-mass balance indicate that during this time 124 g of CO_2 (33.9 g C) was supplied to the reactor with the flue gas, of which 48.92 g CO_2 (39 %) was retained in the system. CO_2 was the largest contribution of C to the system and accounted for 98 % and 99 % of total supplied C, during phases I and II, respectively. The remaining fraction was supplied as CO, CH_4 and C_2H_2. The only net outflux of C from the reactor system was CH_4-C, with 8.59 μg C L^{-1} day^{-1} and 9.77 μg C L^{-1} day^{-1} in phases I and II, respectively. Overall, the influx of

Table 1 Fluxes of reactive gaseous nitrogen compounds in a culture of *Dunaliella salina* grown in a 42-L bubble column reactor during two phases of exponential growth

Treatment	Time (days)	NO_x (μg N L^{-1} day^{-1})	N_2O (μg N L^{-1} day^{-1})	Net balance[c] (μg N L^{-1} day^{-1})
Phase I[a]	6	−0.05	1108	1108±50
Phase II[b]	3	−0.44	2712	2711±133

Negative values indicate net influxes to the system, positive values net outfluxes

[a] From 0.25 to 1.00×10^6 cells mL^{-1}

[b] From 1.00 to 2.00×10^6 cells mL^{-1}

[c] Overall gaseous mass balance ± error

Table 2 Specific emission of greenhouse gases and carbon dioxide equivalent balance per experimental phase, in a culture of *Dunaliella salina* grown in a 42-L bubble column reactor during two phases of exponential growth

	CO_2		CH_4		N_2O		Net balance
	g CO_2 L^{-1} day^{-1}	g	μg CH_4 L^{-1} day^{-1}	CDE[c]	μg N_2O L^{-1} day^{-1}	CDE	CDE
Phase I[a]	−0.08	−18.95	11.45	0.07	1741	130.76	111.88
Phase II[b]	−0.24	−29.97	13.03	0.04	4261	160.04	130.07

Negative values indicate net influxes to the system, positive values net outfluxes

[a] From 0.25 to 1.00×10^6 cells mL^{-1}

[b] From 1.00 to 2.00×10^6 cells mL^{-1}

[c] Carbon dioxide equivalent assuming 25 CDE per g of CH_4 and 298 CDE per g of N_2O (Solomon et al. 2007)

gaseous C-compounds into the reactor was higher than the outflux, and summed over both phases a total of 13.5 g C was retained in the system. After phase II, this TC was present in the system as 23 % POC, 56 % DOC and 18 % TIC. The total C-mass balance error was 3.85 %. The specific sequestration rate for CO_2, averaged over both phases, was 0.13 g CO_2 L^{-1} day^{-1}. According to subjective observations, biofilm growth was reported on the inner walls of the cylinder and on the submerged light source. Furthermore, the development of bioflocs, partly comprised by aggregated algae, was observed at later stages of the growth trial.

Results for the N-mass balance indicate that summed over both phases, 7.46 % of the originally provided NO_3^--N was used for algae biomass growth. In all samples, the measured concentrations of NH_4^+ and NO_2^- were below the detection limit of 0.5 mg L^{-1}. The measured fluxes of reactive gaseous N-compounds, during the trial, are depicted in Table 1. A net influx of NO_x-N of 0.05 μg N L^{-1} day^{-1} and 0.44 μg N L^{-1} day^{-1} was recorded in phases I and II, respectively. The numerically largest gaseous N-flux in the balance was a net outflux of N_2O-N. The emissions of N_2O-N were 1108 μg N L^{-1} day^{-1} in phase I and increased to 2712 μg N L^{-1} day^{-1} in phase II. This outflux outweighed the moderate net influx of NO_x-N, and therefore overall N-losses to the gas phase were 1108±50 μg N L^{-1} day^{-1} in phase I and 2711± 133 μg N L^{-1} day^{-1} in phase II. As a consequence, TN in the system decreased by 15.48 %, from 0.22±0.01 g N L^{-1} (start of phase I) to 0.19±0.01 g N L^{-1} (end of phase II).

Table 3 Specific growth rate and C/N ratio of *Dunaliella salina* grown under different nitrogen regimes in a 9-day small-scale batch trial (n=3)

Treatment	Growth rate (day^{-1})	C/N ratio
Positive control	0.167±0.015[a]	6.27±0.24[a]
Gas-N	0.146±0.008[ab]	17.98±0.59[b]
Negative control	0.126±0.026[b]	21.69±6.34[b]

Means ± SD

Means within columns marked with different letters are significantly different from each other (p<0.05)

The emissions of GHG during both phases of the field scale trial are summarized in Table 2. The total retention of CO_2 by the system (48.92 g) was composed of 18.95 g CO_2 in phase I and 29.97 g CO_2 in phase II, which corresponds to a specific sequestration of 0.08 and 0.24 g CO_2 L^{-1} day^{-1} in phases I and II, respectively. CH_4 emissions were 11.45 μg CH_4 L^{-1} day^{-1} in phase I and 13.03 μg CH_4 L^{-1} day^{-1} in phase II. Therefore, 0.07 and 0.04 CDE were emitted as CH_4 during phases I and II, respectively. N_2O emissions were 1741 μg N_2O L^{-1} day^{-1} in phase I and 4261 μg N_2O L^{-1} day^{-1} in phase II. Therefore, 130.76 and 160.04 CDE were emitted as N_2O during phases I and II, respectively. The GHG balance revealed a net emission of 111.88 CDE and 130.07 CDE in phases I and II, respectively.

The data of several growth trials (data not shown), under similar experimental conditions, was pooled, and regression analysis yielded a significant (p=0.010, n=6) linear relationship between the emission of N_2O (E in μg N_2O L^{-1} day^{-1}) and culture density (CD in 10^6 cells $milliliter^{-1}$), according to:

$$E = -1561\,(\mathrm{SE}\ 343) + 1653\,(\mathrm{SE}\ 214) \times \mathrm{CD}\ \left(R^2 = 0.94\right).$$

The slope of the regression line was tested significantly different from zero (p=0.002), which corresponds to a significant increase in N_2O emission with increasing culture density.

Lab scale experiment

By using an artificial flue gas, it was assessed whether *D. salina* is able to utilize NO_x-N from the gas stream for biomass growth. Three treatments supplied with different sources of N were tested under lab scale conditions: Treatment positive control received the highest input of N (0.219±0.00 g N L^{-1}), consisting mainly of dissolved KNO_3; in treatment Gas-N the main input of N was supplied as NO with an artificial flue gas (1.35×10^{-3}±1.31×10^{-4} g N L^{-1}), and the negative control received only residual amounts of N originating from the inoculum.

Table 4 Fluxes of reactive gaseous nitrogen compounds in cultures of *Dunaliella salina* grown under different nitrogen regimes in a 9-day small-scale batch trial (n=3)

Treatment	NO_x (μg N L^{-1} day^{-1})	N_2O (μg N L^{-1} day^{-1})	Net balance (μg N L^{-1} day^{-1})
Positive control	0.11±0.03 [a]	0.99±0.61[a]	1.10±0.63[a]
Gas-N	−52.73±9.38[b]	15.13±7.87[b]	−37.60±2.13[b]
Negative control	0.00±0.00[a]	0.06±0.03[a]	0.06±0.03[a]

Means ± SD

Means within columns marked with different letters are significantly different from each other (p<0.05)

Negative values indicate net influxes to the system, positive values net outfluxes

Treatment positive control reached the threshold cell density of 1.00×10^6 cells mL^{-1} after 9 days, thereby ending the trial. Results for specific growth rate and C/N ratio of the biomass are depicted in Table 3. A significantly higher specific growth performance was observed in the positive control compared to the negative control; Gas-N was intermediate and not significantly different from any other treatment. The C/N ratio of the algae biomass was significantly lower in the positive control compared to the treatments Gas-N and negative control, which were not significantly different from each other.

The fluxes of reactive gaseous N-compounds through the systems are depicted in Table 4. Treatments positive and negative control received only trace influxes of reactive gaseous N-compounds with the sparging gas, which were outweighed by a net outflux of N_2O-N and small amounts of NO_x-N, leading to a net loss of N from the system. In treatment Gas-N, a net retention of NO_x-N took place and outweighed the losses of N_2O-N. However, the outflux of N_2O-N was significantly higher in Gas-N, compared to the positive or the negative control.

The measured emissions of N_2O and CH_4 during the lab scale trial are summarized in Table 5. No significant differences between treatments were observed in the emission of CH_4, while treatment Gas-N emitted significantly more N_2O than the positive or the negative control. The GHG balance showed a net retention of CDE in all treatments of the lab scale trial.

Discussion

Algae growth was achieved in a 42-L bubble column reactor using flue gas as only C source. The observed specific growth rate of approximately 0.237 day^{-1} corresponds well with reported values for *D. salina*, which range from 0.142 to 0.200 day^{-1} (Grobbelar 1995; García-González et al. 2005; 2003). Typically, 39 % of the supplied flue gas CO_2 was retained by the system. However, only 23 % of TC was taken up by the algae, and DOC values in the culture media exceeded POC measurements by a factor 2. DOC values in algae cultures found in literature are typically in the range of POC measurements (Nguyen et al. 2005). The high DOC values attained in this trial are likely the outcome of glycerol production by the algae and its accumulation in the media (Tafreshi and Shariati 2009; Hullat and Thomas 2010; Shiladitya DasSarma 2001). Furthermore, the growth of heterotrophic bacteria is considered likely, due to non-axenity during the field scale trial and the availability of glycerol as a substrate. The inclusion of some bacterial biomass in the DOC fraction is possible since DOC was measured in the supernatant after centrifuging at 2000×g for 4 min. A low amount of

Table 5 Specific emission of greenhouse gases and carbon dioxide equivalent balance in cultures of *Dunaliella salina* grown under different nitrogen regimes in a 9-day small-scale batch trial (n=3)

Treatment	CO_2		CH_4		N_2O		Net balance
	g CO_2 L^{-1} day^{-1}	g	μg CH_4 L^{-1} day^{-1}	CDE*	μg N_2O L^{-1} day^{-1}	CDE	CDE
Positive control	−0.07±0.00[a]	−1.35±0.03	0.20±0.03[a]	0.00±0.00	1.56±0.96[a]	0.01±0.01	−1.34±0.03[a]
Gas-N	−0.06±0.01[b]	−1.03±0.15	0.33±0.10[a]	0.00±0.00	23.78±12.36[b]	0.13±0.07	−0.90±0.09[b]
Negative control	−0.03±0.00[c]	−0.55±0.04	0.34±0.08[a]	0.00±0.00	0.10±0.04[a]	0.00±0.00	−0.55±0.04[c]

Means ± SD

Means within columns marked with different letters are significantly different from each other (p<0.05)

Negative values indicate net influxes to the system, positive values net outfluxes

*Carbon dioxide equivalent assuming 25 CDE per g of CH_4 and 298 CDE per g of N_2O (Solomon et al. 2007)

C fixed as algae biomass has to be considered critically in systems for C sequestration, since only this fraction can be harvested efficiently. The specific sequestration rate for CO_2 was 0.13 g CO_2 L^{-1} day^{-1}. This is low compared to other studies, which reported values of 0.65–4.40 g CO_2 L^{-1} day^{-1}, using different culture systems and species (Kurano et al. 1995; Yoshihara et al. 1996; Douskova et al. 2009; Murakami and Ikenouchi 1997). In the present setup, flue gas could not be injected continuously, and 25 % of the CO_2 previously supplied with the flue gas was stripped from the media during air sparging. In agreement with the results of Borkenstein et al. (2010), who used a comparable experimental setup, the used system is effective for biomass growth, but is considered unsuitable to attain a maximum sequestration of CO_2 from the flue gas.

In the present trials, the combustion of biodiesel yielded a low NO_x concentration (1.2 ppmv) in the flue gas, compared to other studies, which used flue gases with 30 ppmv NO_x (Maeda et al. 1995), 100–300 ppmv NO_x (Yoshihara et al. 1996), 100 ppmv NO_x (Nagase et al. 1997) and 300 ppmv (Nagase et al. 2001). Yet comparisons are difficult since the composition of the flue gas is strongly dependent on the used fuel and the combustion process. During the entire field scale experiment, a net influx of NO_x-N from the flue gas took place, contributing up to 0.44 μg N L^{-1} day^{-1}, in phase II (Table 1). NO_x is constituted of two gases, namely NO and NO_2, which are both subjected to distinct solution kinetics upon injection into an aqueous media. NO_2 is converted into nitric acid (HNO_3), while NO will react to form nitrous acid (HNO_2). These reactions yield NO_3^- and smaller amounts of NO_2^-, which are both potentially available for uptake by the algae. This is substantiated by the findings of Yoshihara et al. (1996) who described NO removal by algae cultures of up to 50 % (when using a flue gas with 100 or 300 ppmv NO). In the present study, the net influx of NO_x-N was outweighed by large emissions of N_2O-N (2712 μg N L^{-1} day^{-1} in phase II), and TN in the system decreased by 15.48 % during the 9-day trial. Therefore, supplying cultures of *D. salina* with flue gas as an additional N-source led to a net loss of N from the system, likely originated from the KNO_3^- in the media.

Results of the lab scale trial seem to support the assumption that algae can make use of provided NO_x. The negative control, which did not receive any additional N-source, was strongly N-limited resulting in a lower specific growth rate and significantly higher C/N ratio of the biomass, compared to the positive control (Table 3). The specific growth rate of Gas-N was intermediate and not significantly different from the other two treatments. The observed trend might indicate that *D. salina* in treatment Gas-N had access to an additional source of N, compared to the negative control. The only additional source of N available to the algae was the NO-N supplied with the artificial flue gas. Nevertheless, algae in Gas-N did not match the growth performance of the positive control, and the significantly higher C/N ratio of the biomass indicates N-limited growth, although much higher NO_x concentrations were used compared to the biodiesel flue gas in the field scale trial (300 ppmv vs. 1.2 ppmv NO_x). Therefore, it is suggested that supplying cultures of *D. salina* with the low NO_x concentrations present in biodiesel flue gas as a sole N-source will not yield any commercially interesting algae growth.

N_2O emissions were detected in all experimental algae cultures. In the field scale experiment, emissions of 1741 μg N_2O L^{-1} day^{-1} were measured during phase I and increased to almost 4261 μg N_2O L^{-1} day^{-1} in phase II (Table 2). The microbial processes of anaerobic denitrification and aerobic nitrification are considered the most common sources of natural N_2O emissions (Firestone and Davidson 1989; EPA 2010; IPCC 2007; Forster et al. 2007). In a recent study, Fagerstone et al. (2011) generated strong evidence that the emission of N_2O from a culture of *Nannochloropsis salina* was produced by microbial denitrification under low dissolved oxygen (DO) conditions. Anaerobic conditions are usually not associated with continuously lit PBRs, since these systems are subjected to the accumulation of photosynthetically produced O_2, which needs to be actively removed by sparging or stirring. As suggested by Fagerstone et al. (2011), the formation of biofilms and bioflocs can lead to low local O_2 concentrations, also in otherwise highly oxygenic environments, due to slow diffusion of O_2 into these structures and respiratory O_2 consumption by heterotrophic bacterial growth. Schreiber et al. (2009) described the production of N_2O by denitrifiers in a complex biofilm, under anoxic conditions. Latter findings are in line with the present results, since NO_3^- was readily available as a substrate for denitrification and the formation of biofilms was observed. Many denitrifiers are facultative aerobes, and incomplete anaerobiosis will first inhibit the N_2O reductase in the denitrification process, resulting in an increased emission of N_2O (Takaya et al. 2003). Also C_2H_2 is well known to inhibit the microbial reduction of N_2O (Yoshinari and Knowles 1976; Balderston et al. 1976), and its presence in the inlet gas of the field scale reactor (20 ppmv) might have increased the N_2O/N_2 ratio of the denitrification process (Yu et al. 2008). The increased emission of N_2O in phase II is likely the result of an increased biofilm growth at later time points and therefore a larger availability of anaerobic microsites to sustain denitrifier growth.

Under aerobic conditions, N_2O can also be released as an intermediate product of microbial nitrification (Anderson et al. 2010; Fagerstone et al. 2011). This process requires NH_4^+ or NO_2^- as a substrate, both of which were not detected in the system. However, if these substrates are gradually formed, an immediate consumption by nitrifiers is possible, thereby leaving them undetected. NH_4^+ can be

produced in the bacterial decomposition of amino acids, while the supply of gaseous NO to the media can lead to the formation of NO_2^-. Therefore, a moderate contribution of nitrification to the observed N_2O emissions cannot be excluded, but is likely not the main source of this gas. According to previous studies, both steps of nitrification are suppressed at high irradiance levels, typically present in PBRs and high salinities as the presently applied 1 M NaCl (Lingling et al. 2009). The same reasoning holds for the process of heterotrophic nitrification (Robertson et al. 1989; Kuenen and Robertson 1994).

In the lab scale experiment, N_2O emissions were up to 23.78±12.36 μg N_2O L^{-1} day^{-1}, in the Gas-N treatment (Table 5). Compared to the field scale trial (4261 μg N_2O L^{-1} day^{-1}), these lower values are in line with a lower microbial activity and less anaerobic microsites for denitrification, due to previous autoclaving and a shorter culture period (9 instead of 18 days). As shown by Fagerstone et al. (2011), the presence of NO_3^- as a substrate for denitrification is crucial for the emission of N_2O in algae cultures. Therefore, it is conspicuous that in the lab scale experiment, significantly more N_2O was emitted by treatment Gas-N, while most NO_3^- was present in the positive control. It appears that the form in which N is supplied to the system influenced the emission of N_2O. NO is an obligate intermediate in the respiratory reduction of NO_2^- to N_2O in most denitrifiers (Ye et al. 1994). However, the accumulation of NO can inhibit all steps of denitrification due to its toxicity (Rodionov et al. 2005; Ye et al. 1994). Therefore, NO levels are well regulated in denitrifiers (Goretski et al. 1990), suggesting that the reduction of NO does not only serve as electron acceptor, but most immediately is a detoxification process (Ye et al. 1994). Since NO can diffuse through the cell membrane of denitrifiers (Goretski and Hollocher 1988; Zafiriou et al. 1989), supplying it to the media in high concentrations might trigger an immediate detoxification reaction, leading to an increased emission of N_2O during denitrification, yet this remains to be substantiated in dedicated experiments. Furthermore, the presence of NO_2^- as a substrate for denitrification can yield larger N_2O emissions compared to NO_3^- (Körner and Zumft 1989; Schulthess von et al. 1995; Schreiber et al. 2009). NO_2^- can be formed upon solution of NO in the media and was likely present to some extent. For future experiments it is suggested to use ^{15}N-labelled NO gas as N-source for algae cultures, in order to unravel the complex kinetics of NO consumption by the algae and the production of N_2O.

Low emissions of CH_4 were recorded during both the field scale experiment (13.03 μg CH_4 L^{-1} day^{-1}) and lab scale experiment (0.34±0.08 μg CH_4 L^{-1} day^{-1}). Natural production of CH_4 is usually linked to processes of anaerobic microbial decomposition by Archaea (Conrad 1989; Kiene 1991; Cicerone and Oremland 1988). In the lab scale experiment, CH_4 production was two orders of magnitude lower than in the field scale trial. Since bottles were previously sterilized, this trend substantiates the involvement of microbial activity in CH_4 production. The production of N_2O and CH_4 requires completely different windows of redox potentials. Significant CH_4 production takes place under strong reducing conditions, typically below −150 mV (Cicerone and Oremland 1988; Yu et al. 1997, 2001), while the production of N_2O is regulated within a narrow range of +120–250 mV (Yu et al. 2001). If produced in the same anaerobic microsites, this could explain why N_2O was produced at a higher rate compared to CH_4.

Results for the GHG balance in the field scale experiment suggest that the emissions of CH_4 and N_2O outweighed the moderate retention of CO_2. In fact, during phases I and II, 5.9 and 4.3 times more CDE were discharged into the air by treating the flue gas (Table 2). It is expected that by using sterile cultures, the emission of GHG can be reduced, and in the lab scale trial a net retention of CDE took place (Table 5). A supplementation of algae cultures with NO_x as a source of N significantly increased the production of N_2O, compared to treatments with NO_3^- as N-source.

In conclusion, the present study showed that algae cells can use C and N for growth, when supplied with biodiesel flue gas. A low sequestration rate for CO_2 and emissions of CH_4 and N_2O led to a negative GHG balance for the used system. Results suggest that N_2O and CH_4 productions in algae cultures are mainly the outcome of anaerobic microbial processes. Although higher productivities and sequestration rates for CO_2 can certainly be achieved, which would mitigate the negative outcome of this study, emissions of CH_4 and N_2O will always need to be reviewed critically. In light of these results, the suitability of algae cultures for the biotreatment of flue gases remains questionable. Currently, the most economic systems for flue gas treatment are open ponds, which are generally characterized by poor mixing, non-sterility and anaerobic conditions in sediment layers; therefore, their GHG emissions could be even higher. Further research in the area is required, not only to assess the environmental sanity of algae cultures for flue gas treatment but also to yield further insight into those mechanisms that lead to the formation of GHG.

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