Fertilization with microalgal biomass from wastewater impacts the soil and rhizosphere active microbiomes, greenhouse gas emissions and plant growth

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Abstract

Microalgae are highly effective in nutrient recovery and have strong potential as a sustainable wastewater treatment technology. For this experiment, we obtained microalgae cultivated in pilot-scale tubular photobioreactor (PBR) located in a glass greenhouse of the Netherlands Institute of Ecology (NIOO-KNAW), in Wageningen, the Netherlands. The culture medium was vacuum collected and anaerobically treated black water. Here we describe the experimental set up and methods to determine the effects of inorganic NPK and organic microalgae biomass as fertilizer on soil chemical composition, soil CO2 and N2O fluxes, active bacterial and protist communities in soil and rhizosphere in different stages of barley development, abundances of functional genes of nitrification and denitrification in soil with barley at different stages of development, barley productivity, grain yield and grain nutritional quality. Additionally, we provide the statistical methods and scripts used to analyse the data.

Keywords: N2O emission, Sustainable strategy, Organic fertilization, Bacterial community, Protist community, Black water

1. Method details

1.1. Experimental design and soil sampling

The soil was collected from The Soil Health Experiment (SHE) located at the experimental field station in Vredepeel, in the south east of the Netherlands (51 32’ 27.10” N and 5 51’14.86” E). The soil was homogenized and sieved through a 5-mm sieve to remove large stones, plant material, and macrofauna. The greenhouse pot experiment consisted of four completely randomized blocks containing three treatments with different N sources: 80 kg ha⁻¹ of N provided by the microalgae, 80 kg ha⁻¹ of N provided by ammonium nitrate (NPK) and a control with no N input. In treatments with inorganic N, P and K were applied as recommend rates for barley in the Netherlands, P as single...
Figure 1. (a) On-site wastewater treatment scheme at NIOO-KNAW used for the cultivation of microalgae biomass. (b) Schematic overview of the experiment and results of the impact of microalgae biomass on plant growth, quality and grain yield, greenhouse gases (GHG) emissions, biological processes linked to nitrous oxide (N₂O) emission and soil active bacterial and protozoan communities in bulk soil and rhizosphere of different stages of barley development.
superphosphate (45 kg P2O5 ha−1) and K as KCl (70 kg K2O ha−1). As we used the 0-10 cm layer of soil (1,000,000 kg ha−1 soil) to apply the organic and inorganic fertilizers, the N, P2O5 (P) and K2O (K) rates applied per kg of soil in each pot were 80, 45 (20) and 70 (58) mg kg−1 soil, respectively. Treatments with microalgae biomass had no mineral fertilizer supplementation, and the nutrients were applied in the concentration of 80, 16 and 116 mg kg−1 soil of N, P and K, respectively.

Microalgae Chlorella sorokiniana was cultivated in pilot-scale tubular photobioreactor (PBR) located in a glass greenhouse of the Netherlands Institute of Ecology (NIOO-KNAW), in Wageningen, the Netherlands (Figure 1a). The culture medium was vacuum collected and anaerobically treated black water originating from a full-scale Upflow Anaerobic Sludge Blanket (UASB) reactor operated at 35°C and 8.7 days hydraulic retention time. The total volume of the reactor is 893L, the diameter and height of the UASB are 0.66m and 2.75m respectively with 5 taps installed every 0.46m from the bottom. After collection, the AnBW was filtered to remove seeds and large solids, which could cause clogging of the PBR influent tubing. The AnBW was kept in a 200L container at ≤ 10°C, to prevent further digestion, for a maximum of 4 days, when new AnBW was added.

The PBR consists of two parallel systems that are connected to ensure homogeneous PBR content. Each system consisted of 25 meters of horizontal tubes, and two vertical air lift columns. At the top of the air lift columns there was a cylindrical box that ensured the complete mixing of the PBR's content. The inner diameter of the horizontal tubes was 5.6 cm and the inner diameter of the air lift columns was 12 cm. The volume of the horizontal tubes was 140L, the airlift columns 63L, and the top box 8L, resulting in a total volume of ± 211L. A pH and temperature sensor was placed in the cylindrical box. An air outlet tube connected the cylindrical box with outdoors and allowed the excess air to escape. Light sensors (LICOR QUANTUM) were located in three places; on both sides of the reactor and on top of the mixing box. Temperature of the liquid was not controlled, but the air temperature of the greenhouse was controlled to 24°C. The data from the three light sensors, pH, and liquid temperature were logged constantly with custom made NIOO logger V1.0 software. At the beginning of the experimental run, the PBR was filled with AnBW and then inoculated with precultured Chlorella sorokiniana containing 105 cells.mL−1. Following the inoculation, the PBR was operated in batch mode during 10 days to allow the microalga to adapt to the AnBW and environmental conditions. After the acclimation period (microalga count of 7.95 × 107 cells.mL−1), the operation was switched to continuous mode, with an hydraulic retention time of 5 days (inflow of fluid of 42.2 L.d−1 and dilution rate of 0.2 d−1). The PBR was operated under natural light conditions. The PBR content was mixed by 10% CO2 enriched excess air to escape. Light sensors (LICOR QUANTUM) were located in three places; on both sides of the reactor and on top of the mixing box.

1.2. Soil analyses

Bulk soil samples were collected at 0–10 cm depth to measure soil properties. Soil moisture was determined gravimetrically by drying the soil at 105°C for 24 h, expressed per gram of dry soil. Soil mineral N (NH4+ -N and NO3− -N) was measured with a continuous flow analytical system (FlA lab-2500 System) after extraction with 2 M KCl in 1:10 soil-to-solution ratio and soil pH was determined in CaCl2 solution.

1.3. Plant productivity and quality

After 120 days, at the time of barley harvest, shoots and grains fresh weights were determined. After rhizosphere sampling, the plant roots were gently washed and root fresh weight were measured. Shoots, roots and grains where then oven-dried at 40 °C until a constant weight was achieved for dry weight measurement. The dry shoots and grains samples were then ground to a fine powder using a pulverizing mill before measuring macro (N, P, K, Ca, S and Mg) and micronutrients (Fe, Mn, Zn, Cu, and B) determined according to Bataglia et al. (1983).

1.4. Greenhouse gas sampling

The assessment of CO2 and N2O fluxes were collected using the static chamber method (Lourenço et al., 2018a). We modified the gas chambers to polyvinyl chloride (PVC) pipes of 8 cm diameter and 18 cm length. The top lid was made of a PVC cap, and the lower end was trimmed to be inserted 6 cm into the soil from the top. The PVC cap was equipped with two openings, each fitted with a valve, one for gas sampling and the other for internal and external pressure equilibrium. Chambers were installed at the day of fertilization, and the chambers remained in place with the cap opened during the entire growing season. During sampling, after closing the chambers, 30 ml gas samples were collected after 1, 15, and 30 min using syringes. The samples were transferred and stored in pre-evacuated extainer vials (12 ml) and analyzed in a gas chromatograph (model GC-2014, Shimadzu Co.) with an electron capture detector for N2O determination and with flame ionization detector for CO2. The vials were evacuated to a pressure of ~172 kPa and prepared 1 day before sampling. Air and gases samples were obtained between 9 am and 11 am, starting at day 0 after the fertilizer and microalgal application. The gases were sampled three times per week during the experiment. The cumulative GHG emissions were calculated by linear interpolation of fluxes emission in two consecutive days, follow the trapezoidal rule (Soares et al., 2016, Lourenço...
The emission factors for \( \text{N}_2\text{O} \) were calculated according to Lourenço et al. (2018a).

\[ EF = \frac{N_2O - N_{\text{treat}} - N_2O - N_{\text{control}} \times 100}{N_{\text{applied}}(\text{Fert} + \text{Vinasse})} \]  

(1.1)

where \( EF \) is the \( \text{N}_2\text{O} \) emission factor (%), \( N_2O - N_{\text{treat}} \) and \( N_2O - N_{\text{control}} \) are the cumulative emissions (mg m\(^{-2}\) of \( \text{N}_2\text{O} \)) of the fertilized and unfertilized chambers, respectively, and \( N \) applied is the mass of \( N \) fertilizer added to the chamber with \( N \) mineral fertilizer or \( N \) from microalgae biomass (mg m\(^{-2}\) of fertilizer-\( N \)).

### 1.5. Molecular analysis – Nucleic acids extraction, quantification of \( N \) cycle genes and amplicon sequencing

Total RNA and DNA were simultaneously extracted from soil samples of three replicates of each treatment using the RNA PowerSoil® Total RNA Isolation Kit (Qiagen, Valencia) and DNA Elution Accessory Kit (Mo Bio), respectively. We also tried to extract RNA from microalgae samples, without being successful. The isolated RNA and DNA were quantified using a Nanodrop ND-1000 spectrophotometer (Thermo, Waltham, MA, USA), checked via electrophoresis gel and for fragment size in the 2100 Bioanalyzer (Agilent Technologies) using a High Sensitivity DNA kit (Agilent Technologies). Maxima First Strand cDNA Synthesis Kit and random hexamer primers (Fermentas, Thermo Fisher Scientific Inc., USA) were used for cDNA synthesis.

Quantifications of phylogenetic gene markers (16S rRNA Bacteria and Archaea) and functional genes (\( \text{amoA} - \) Bacteria and Archaea, \( \text{nirK} \) and \( \text{nirS} \)) using DNA representing the first 3 growing stages of barley (days 8, 14 and 30) were carried out by real-time PCR using the CFX96 TouchTM thermocycler (BIO-RAD, CA, USA) and gene-specific primers. Each sample was quantified in duplicates and the copy numbers of each gene were calculated from the standard copy numbers as per Lourenço et al. (2018a). The amplification specificity was checked by melt curve analysis determined by 95°C (10 sec), from 65°C to 95°C in increments of 0.5°C (5 min). The details on plasmid standards, gene-specific qPCR primers, reaction mixtures and thermal programs are shown in Table 1.

Bacterial/archaeal 16S rRNA partial genes and protozoa 18S rRNA partial gene were amplified from duplicate cDNA to access active community. PCR primers and conditions that target the region V4 (515F/806R) for bacteria and V4 (Euk1391F / EukBr) for downstream paired-end Illumina (Illumina, Inc., San Diego, CA, USA) barcoded sequencing (Caporaso et al., 2012) are found in Table S2. We pooled duplicate amplified samples and purified them with Agencourt AMPure XP purification kit (Beckman Coulter, Brea, CA, USA). The final concentration of amplicon products in each sample was determined by Fragment Analyzer (Advanced Analytical, IA, USA). Equal amounts of the amplicon products were pooled and subjected to MiSeq V3 library preparation for MiSeq sequencing. For more info please refer to the guidelines of Illumina MiSeq System.

### 1.6. Data analyses

The bacterial and protozoan amplicon raw sequences, obtained from the Illumina MiSeq platform, were quality filtered for expected errors >0.5 or a length shorter than 100bp eliminated. The paired-end reads of bacteria were assembled into a single read using VSEARCH (Rognes et al., 2016) while single-end sequences were used for protozoa. Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using the usearch global method implemented in VSEARCH and chimeric sequences were removed using UCHIME (Edgar et al., 2011). Representative OTUs were classified and assigned using BLAST against Silva database (version 128) for bacteria and PR2 database (Guillou et al., 2013) for protozoa using SINA (Pruesse et al., 2012). Sequences identified as singletons (only occurring once), or belonging to chloroplast or mitochondria were discarded for bacteria while for protozoa Streptophyta, Metazoa, fungal and unclassified Opisthokonta sequences were discarded. All trimmed sequence reads generated in this study are available in ENA European Read Archive under accession number PRJEB32941.

Downstream analyses were done with an OTU table normalized with Hellinger’s transformation. We tested for significant shifts in overall community composition or functional gene belonging to nitrogen cycle (\( \text{amoA} \) bacteria and archaea, \( \text{nirK} \) and \( \text{nirS} \)) across bulk soil and rhizosphere compartments, time points (plant development stages) and \( N \) source using permutational multivariate ANOVA (PERMANOVA) implemented in the “Adonis” function in the vegan package in R with 10,000 permutations (Oksanen et al., 2017). A discriminant analysis of principal components (DAPC) was used to assess the phylogenetic \( \beta \)-diversity based on the Bray–Curtis matrix of the 16S rRNA partial gene sequence data set implemented in the ‘adegenet’ R package (Jombart et al., 2010) (scripts provided in Additional Material 1). The rarefaction analysis and the richness and diversity indices were calculated with QIME software (Caporaso et al., 2012). Linear discriminant analysis (LDA) was applied on OTU table according to the method of Segata et al. (2011), to identify bacterial taxa discriminant among different treatments and specific in each treatment in each time-point (plant development stages).

The soil (pH, \( \text{NH}_4^+ - N \) and \( \text{NO}_3^- - N \)) and environmental parameters as, \( \text{N}_2\text{O} \) and \( \text{CO}_2 \) fluxes and cumulative emissions, \( N \)-cycling genes abundances and gene ratios data were initially tested to verify assumptions of normality (Kolmogorov-Smirnov test). In cases where these conditions were not satisfied, the data were transformed via natural logarithm or square-root. The differences between the treatments (Control, NPK and Microalgae) for all the parameters were calculated using one-way ANOVA’s. Fisher’s Least Significant Difference (LSD) post hoc test was used to check for significance (P ≤ 0.05). Statistical analyses were conducted using SPSS (Chicago, IL, USA). Correlations between gas emissions, soil parameters and gene abundances and ratios (days and treatments combined and separated) were calculated.

## Credit authorship contribution statement

Afnan Suleiman, Kesia Lourenco and Eiko Kuramae: conceived the idea and designed the research with contributions of Tania Fernandes, Gustavo Silva and Louise Vet. Gustavo Silva and Tania Fernandes: conducted the microalgae cultivation experiments. Ronildson Luz and Kesia Lourenco: conducted the greenhouse experiments and gas measurements. Afnan Suleiman and Callum Clark: conducted the molecular analyses. Heitor Cantarella: chemical analysis of the plants and grains. Afnan Suleiman and Kesia Lourenco: performed the DNA sequencing and statistical analyses. Afnan Suleiman, Kesia Lourenco, Callum Clark and Eiko Kuramae: wrote the paper. All authors reviewed the manuscript.

## Additional Material 1: The scripts used to run PERMANOVA and DAPC analysis are shown below

```r
#loadpackagessource("https://bioconductor.org/biocLite.R")
bicLite("phyloseq") library("phyloseq") library("vegan") library(mvpart) library(adegenet)

#permanova otu_table <- read.table("For_R.txt",sep = \"\t\",header=T) otu_table_Hel <- otu_table[,(-1:5)] abund_table2 <- decostand ((otu_table_Hel), method="hellinger")
Bacteria_Hel <- data.frame(otu_table[,c(1:5)], (abund_table2) - decomand
Bacteria_HelTreatment <- factor(Bacteria_HelTreatment) Bacteria_HelTime- factor(Bacteria_HelTime) Bacteria_HelScompartment <- factor(Bacteria_HelScompartment) Bacteria_Hel_P <- Bacteria_Hel [,c(1:4)] Bacteria_Hel_map <- Bacteria_Hel [,c(5:8775)] adonis
```
Table 1
Details of the qPCR and PCR runs. The targeted genes included: ammonia monooxygenase (amoA) for both bacteria (AOB) and archaea (AOA), bacterial nitrite reductase (nirK and nirS), 16S rRNA for total bacteria (TB) and total archaea (TA), 16S rRNA for bacteria sequencing and 18S rRNA for protozoan sequencing.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>PCR Mix (Total volume 12 μl)</th>
<th>Thermocycler Conditions</th>
<th>Plasmid</th>
<th>Average Efficiency</th>
<th>Average R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>amoA-AOB (Rotthauwe et al., 1997)</td>
<td>amoA1F 5'-GGGGTTC-TACTGGTGGT-3'</td>
<td>amoA2R 5'-CCCTCCKGSA-AAGCITCCTC-3'</td>
<td>6 μl Sybergreen Bioline</td>
<td>95°C (10 min), 40 × 95°C (10 sec), 65°C (25 sec)</td>
<td>Nitrosospira multiforme ATCC25196</td>
<td>78.8 %</td>
<td>0.964</td>
</tr>
<tr>
<td>amoA-AOA (Francis et al., 2005)</td>
<td>Arch-amoAF 5'-STAATGGTCT-GGCTTAGACG-3'</td>
<td>Arch-amoAR 5'-GCGGCCATCC-ATCTGTATGT-3'</td>
<td>6 μl Sybergreen Bioline</td>
<td>95°C (5 min), 46 × 95°C (10 sec), 64°C (10 sec)/72°C (20 sec)</td>
<td>Nitrosospira viennensis EN76</td>
<td>92.8 %</td>
<td>0.993</td>
</tr>
<tr>
<td>nirK (Henry et al., 2004)</td>
<td>NirK876 5'-ATYGGCGGV-AYGGCGA-3'</td>
<td>NirK1040 5'-GCTCCGATCA-GRTTRTGTTT-3'</td>
<td>6 μl Sybergreen Bioline</td>
<td>95°C (5 min), 41 × 95°C (15 sec), 62°C (15 sec), 72°C (20 sec)</td>
<td>Paracoccus denitrificans DSM 413</td>
<td>99.4 %</td>
<td>0.971</td>
</tr>
<tr>
<td>nirS (Throbäck et al., 2004)</td>
<td>nir5cDaF 5'-GASTGGGRT-GSCTCTGA-3'</td>
<td>EUB 518 5'-ATTACCGCG-GCTGCTGG-3'</td>
<td>6 μl Sybergreen Bioline</td>
<td>95°C (3 min), 40 × 95°C (10 sec), 59°C (35 sec), 72°C (20 sec)</td>
<td>Enterobacter cloacae 146</td>
<td>93.1 %</td>
<td>0.997</td>
</tr>
<tr>
<td>TB 16S (Klindworth et al., 2013)</td>
<td>EUB 338 5'-ACTCCTACGG-GAGGCAGCAG-3'</td>
<td>EUB 518 5'-ATTACCGCG-GCTGCTGG-3'</td>
<td>25 μL of PCR mixture consisted of 12.5 μL 5X KAPA HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems, Inc., Boston, MA, USA), 0.4 μl of each primer, and 100 ng of cDNA template</td>
<td>94°C (2 min), 25 × 94°C (45 s); 55°C (45 s) and 72°C (1 min); followed by 72°C (6 min)</td>
<td>pgemT</td>
<td>76.3 %</td>
<td>0.981</td>
</tr>
<tr>
<td>TA 16S (Klindworth et al., 2013)</td>
<td>Arch1017R 5'-AGGAATTGGC-GGGGGAGCAC-3'</td>
<td>GOG_2015 81.0 %</td>
<td>95°C (10 min), 40 × 95°C (10 sec), 64°C (10 sec), 72°C (20 sec)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>16S sequencing (Turner et al., 1999, Caporaso et al., 2012)</td>
<td>515F 5'-GGTCCAGCMG-CGGGGTAA-3'</td>
<td></td>
<td></td>
<td>95°C (3 min), 35 × 95°C (30 sec), 59°C (35 sec), 72°C (20 sec)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18S sequencing (Meddin et al., 1998, Stackebrandt &amp; Goodfellow, 1991)</td>
<td>Eub1r 5'-TGATCCTCCTGC-AGGTCACTC-3'</td>
<td></td>
<td></td>
<td>95°C (3 min), 35 × 95°C, (10 s); 55°C (20 s); 72°C (20 s); followed by 72°C (5 min)</td>
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</table>
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Supplementary materials


References


