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From toilet to agriculture: Fertilization with microalgal biomass from wastewater impacts the soil and rhizosphere active microbiomes, greenhouse gas emissions and plant growth

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Abstract

Human activities are pushing earth beyond its natural limits, so recycling nutrients is mandatory. Microalgae are highly effective in nutrient recovery and have strong potential as a sustainable wastewater treatment technology. Here, nutrients from black water (toilet wastewater) were recovered as microalgal biomass, which was dried and assessed as a fertilizer in pot experiments compared with inorganic fertilizer. We deciphered the effects of microalgal biomass as a biofertilizer on plant growth and quality and the biological processes linked to greenhouse gas (GHG) emissions. In addition, we elucidated the assembly of the active microbiome in bulk soil and rhizosphere during barley development. Microalgal biomass application and inorganic fertilizer (NPK) resulted in similar plant productivity (16.6 g pot^{-1}). Cumulative nitrous oxide (N_2O) emissions were 4.6-fold higher in the treatment amended with microalgal fertilizer (3.1 % of applied N) than that with inorganic fertilizer (0.5 % of applied N). Nitrification by bacteria was likely the main pathway responsible for N_2O emissions (R^2 = 0.7, $p \leq 0.001$). The application of nitrogen fertilizers affected the structures of both the active bacterial and protozoan communities, but these effects were less obvious than the strong plant effect, as the recruited microbiota varied among different plant developmental stages. Both treatments enriched similar bacterial and protozoan taxonomic orders but with different distributions through time across the plant developmental stages. Furthermore, the bacterial community showed a clear trend of resilience from the beginning of the experiment until harvest, which was not observed for protozoa. Our results indicate that the use of microalgal biomass as a fertilizer is a viable option for recycling nutrients from wastewater into plant production.

1. Introduction

Fertilization is a common agricultural practice in which organic and inorganic fertilizers are used to improve plant nutrition and yield. Animal manure, plant residues and biosolids are frequently used as biofertilizers in agriculture. As recovery of all P in human excreta could supply 22% of global P demand, microalgae cultivated in wastewater have recently been tested as fertilizers based on their high effectiveness in recovering nutrients (Mihelcic *et al.*, 2011). Microalgae cultivated in wastewater can assimilate essential nutrients such as N, P and K as well as other micro-nutrients such as magnesium (Mg), zinc (Zn) and cobalt (Co) (Mulbry *et al.*, 2008, Boelee *et al.*, 2012, Vasconcelos Fernandes *et al.*, 2015, Silva *et al.*, 2019). Microalgae assimilate nutrients at varying ratios depending on species and environmental and operation conditions. Overall, N:P assimilation ratios between 10 and 30 have been reported for microalgae and cyanobacteria (Geider & Roche, 2002, Fernandes *et al.*, 2017, Silva *et al.*, 2019). In particular, the green alga *Chlorella sorokiniana* can remove most of all N and P from nutrient-rich black water (toilet wastewater) with N:P ratios ranging from 15 to 26. However, the assimilation of N and P is imbalanced relative to the N:P stoichiometry of black water, resulting in rapid removal of P but slower removal of N (Fernandes *et al.*, 2017). The few assessments of microalgae cultivated in wastewater as fertilizer have reported positive effects on crops and successful replacement of inorganic fertilizers (Coppens *et al.*, 2016, Renuka *et al.*, 2017, Das *et al.*, 2019). Mulbry *et al.* (2005) showed that cucumber and corn plants in a potting mix containing microalgae assimilated 38% to 60% of the P applied with the microalgae and had comparable dry weight and nutrient content as plants in inorganic fertilizer-amended treatments. Similarly, Castro *et al.* (2017) reported that application of microalgae obtained from wastewater resulted in similar yields of millet dry biomass after 60 days compared with urea fertilization.

In addition to affecting crop yields, the type and amount of fertilizer amendment influence the physicochemical properties of the soil, which has a significant long-term impact on soil fertility, health and productive capacity. Organic matter in the form of organic amendments is considered a sustainable alternative to inorganic fertilizers (Thangarajan *et al.*, 2013). When applied to soil as biofertilizers, organic residues produced during industrial and agricultural processes provide nitrogen (N), phosphorus (P) and potassium (K) as well as other macro- and micro-nutrients to the plant. Other positive effects of organic amendments include increased soil organic matter (SOM), structure and moisture and stimulation of microbial growth and enzyme activities (Lupatini *et al.*, 2017, Rahman *et al.*, 2017, Sarma *et al.*, 2017), even in the short term (Dinesh *et al.*, 2010, Suleiman *et al.*, 2018). Besides, the high micronutrients content, such copper, in the organic residues can have a potential risk of soil pollution (Xiong *et al.*, 2010)

Compared with their positive effects on soil quality and microbiota, the influence of organic fertilizers on emissions of nitrous oxide (N_2O), a main greenhouse gas (GHG) produced in crop production, is less clear (Chen *et al.*, 2013, Zhao *et al.*, 2016). Comparisons of N_2O emissions for different types of organic and inorganic fertilizers have yielded conflicting results. Stalenga and Kawalec (2008) reported that the application of inorganic N fertilizer increased N_2O emissions nearly twofold

compared with animal manure application, whereas Castro *et al.* (2017) found a fivefold increase in N_2O emissions under microalgae compared to urea treatment. However, in general, the average emission factor (EF; percentage of N applied lost as N_2O) for organic amendments (Charles *et al.*, 2017) of 0.57±0.30% is lower than the default value assumed by IPCC of 1% for inorganic and organic fertilizers (IPCC, 2013).

The enhancement of microbial activity by organic management systems includes direct effects on microbes responsible for N_2O production through different pathways (Baggs, 2011, Hu *et al.*, 2015) as well as on bacterial, fungal and protist communities in the soil and rhizosphere (Lupatini *et al.*, 2019). However, the changes in soil microbiota due to organic fertilization can be temporary (Suleiman *et al.*, 2016, Lourenço *et al.*, 2018b) because of microbial community resilience. Bacteria and fungi are the primary decomposers, while protozoans are the main predators of bacteria and perform functions in the soil food web such as recycling nutrients, promoting plant growth, and transferring end energy to higher trophic levels (Trap *et al.*, 2016, Jousset, 2017). Protozoan are the central core of the soil microbiome and may regulate the effects of changes in specific members of the bacterial and fungal microbiomes on soil management (Xiong *et al.*, 2017). Overall, organic fertilization increases the complexity of primary decomposers and predators, including the bacterial and protozoan communities, thereby strengthening diverse microbe-eukaryote interactions such as survival, predation and cooperation strategies (Suleiman *et al.*, 2019).

In the present study, the impact of microalgae cultivated in wastewater as a biofertilizer on the environment was comprehensively investigated for the first time, revealing mutual effects on soil, plant and atmosphere. The goal was to understand the effects of biofertilization with microalgae both aboveground by assessing GHG emissions, plant productivity and grain quality and belowground by determining soil chemistry and microbial community composition in bulk soil and rhizosphere, including key functions of the nitrogen cycle. Specifically, the following questions were addressed: (1) Is microalgal fertilizer as efficient as NPK inorganic fertilizer in terms of plant productivity and quality? (2) Does microalgal fertilizer produce higher or lower GHG emissions than inorganic fertilizer? (3) What is the main biological process related to N_2O emissions? (4) What is the impact of microalgal fertilizer on the assembly of active bacterial and protozoan communities in the soil and rhizosphere of barley at different growth stages?

2. Material and methods

2.1. 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 superphosphate (45 kg P_2O_5 ha^{-1}) and K as KCl (70 kg K_2O 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, P_2O_5 (P) and K_2O (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, respectivelly.

Microalgae *Chlorella sorokiniana* was cultivated in a 211 L tubular photobioreactor (PBR) located in a temperature-controlled (25 °C) 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 originating from an Upflow Anaerobic Sludge Blanket (UASB) reactor operated at 35 °C and 8.7 days hydraulic retention time. The PBR was operated under natural light conditions and at a hydraulic retention time of 5 days. The PBR content was mixed by 10% CO_2 enriched air bubbling, and pH was automatically controlled at 6.7 ± 0.1. The microalgal biomass producted in the tubular photobioreactor was collected and settled at different operational times. The settled microalgal biomass was dried in trays at 60 °C for 72 h and ground into a powder in a leaf milling machine. The chemical properties of soil and of the microalgal fertilizer are described at Table 1.

Both N fertilizer sources were manually applied in the soil at the same day. Before sowing the pots, seeds of spring barley (*Hordeum vulgare* L.) were surface sterilized by rinsing with 70% ethanol for 1 min followed by washing with sterile demi water. The experimental period covered four replicates for time 0 (before experiment) and for the six plant growth stages, as follows: two emerging leaves (7 days), tillering (14 days), advanced tillering (30 days), heading (60 days), flowering (90 days) and maturity (120 days). For soil microbiome analyses, 3 soil replicates were used while for other analysis the 4 replicates were considered. Two different pot sizes, 27 kg and 4.5 kg were used to evaluate plant productivity, GHGs emissions and soil microbial changes (Figure 1). Pots containing 27 kg of soil were used to evaluate the GHGs emissions throughout the experiment, therefore gas chambers were installed in each pot for the 3 different treatments. Moreover, the same size pots were used to evaluate plant biomass for the last growth stage of barley and in the last sampling for soil microbial community (6^{th} sampling). The pots with 4.5 kg were implied to evaluate the microbial analysis and biomass of the first 5 time points. Immediately after fertilization, 12 and 6 seeds were sown (at 5 mm depth) in pots containing 27 and 4.5 kg of soil, respectively. After 8 days, three seedlings were left in each pot for the 5 first time points, while 6 seedlings were left (at 5 mm depth) in pots containing 27 kg of soil representing the last time point.The experiment was carried out in a greenhouse at 16/8 h light/dark at 21 °C/16 °C. The soil water-holding capacity of 60% (w/w) was maintained by watering three times weekly throughout the experiment period, in which pots were weighed and deionized water was added, after gas samplings. Cores of three spots from the 4.5 kg pots and five cores from the 27 kg pots were taken to represent bulk soil. Furthermore, the rhizosphere of three plants were collected and separately for each compartment, placed on ice and immediately transported to the laboratory. For the rhizosphere, after

shaking off the loosely adhering soil of the roots, the tightly adhering rhizosphere soil was collected with a sterile brush. Both samples, bulk and rhizosphere soil, were stored at -80 °C for molecular analysis.

2.2. 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 (NH_4^+-N and NO_3^--N) was measured with a continuous flow analytical system (FIAlab-2500 System) after extraction with 2 M KCl in 1:10 soil-to-solution ratio and soil pH was determined in $CaCl_2$ solution.

2.3. 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).

2.4. Greenhouse gas sampling

The assessment of CO_2 and N_2O 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 exetainer vials (12 ml) and analyzed in a gas chromatograph (model GC-2014, Shimadzu Co.) with an electron capture detector for N_2O determination and with flame ionization detector for CO_2. 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 microalgae 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 *et al.*, 2018a). Emission Factors (EF) were calculated according to Lourenço *et al.* (2018a).

2.5. 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 (QIAGEN, Valencia) and DNA Elution Accessory Kit (Mo Bio), respectively. We also tried to extract RNA from microalgae samples, but with no success. 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 (*amoA* - Bacteria and Archaea, *nirK* and *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 Tables S1.

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 (515 F/806 R) 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 S1. 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.

2.6. 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 (amoA bacteria and archaea, nirK and 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 β-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). The rarefaction analysis and the richness and diversity indices were calculated with QIIME software (Caporaso *et al.*, 2012). Linear discriminant analysis (LEfSe) 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, NH_4^+-N and NO_3^--N) and environmental parameters as, N_2O and 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 ($P \leq 0.05$). 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 by Spearman's correlation in SigmaPlot Version 13 (Systat Software, San Jose, CA).

3. Results

3.1. Plant productivity and nutrition

Overall, both microalgal and inorganic fertilization significantly affected plant productivity and enhanced the shoot, root, and grain biomass of barley compared with the control (Figure 2). The total biomass yield was 17.2 g pot^{-1} for microalgal fertilization and 16.1 g pot^{-1} for inorganic fertilizer. In addition, grain macronutrient content was higher in both the microalgal and inorganic fertilizer treatments than in the control (Table 2). The microalgal treatment was associated with higher Ca grain concentration but lower protein (N concentration) than the inorganic fertilizer treatment (Table 2). Additional information on the macro- and micronutrient content in the leaves is shown in Supplementary Figure 1.

3.2. Greenhouse gas emissions

Fluxes of N_2O emissions were significantly affected by the microalgal treatment, as shown in Figure 3a. Three different periods with high N_2O emissions were distinguished during the 120 days of the experiment: an initial peak phase until day 7; a second phase with constant N_2O emissions above the control treatment between days 7 and 30; and a third phase between days 30 and 120 in which N_2O emissions diminished to control levels until the end of the experimental period. On day 3, the flux of N_2O emissions was 245.9 mg N m^{-2} d^{-1} from the microalgal treatment, 18.8 mg N m^{-2} d^{-1} from the inorganic treatment and 5.8 mg N m^{-2} d^{-1} from the control ($P < 0.001$). After 120 days, the cumulative N_2O emissions from the microalgal treatment were equivalent to 750.7 mg N m^{-2}, a 4.6-fold increase compared with the inorganic treatment (162.4 mg N m^{-2}) ($P = 0.001$) and a 15-fold increase compared with the control (50.0 mg N m^{-2}) ($P < 0.001$) (Figure 3c). The flux of N_2O was positively correlated with NO_3^--N (Table 3; Table S2). The EF was 3.1% N for the microalgal treatment but 0.5% for the inorganic treatment.

The microalgal treatment significantly affected CO_2 emissions between days 0 and 14 ($P<0.05$), but this difference was no longer significant by day 30 (Figure 3b). The largest differences were between the microalgal and NPK treatments and between the microalgal and control treatments. However, after day 40, CO_2 emissions were higher in the inorganic and microalgal treatments than in the control. The cumulative CO_2 emissions of the microalgal, inorganic and control treatments were equivalent to 188.4, 161.2 and 130.4 g CO_2-C m^{-2}, respectively (Figure 3d).

3.4. Abundances of functional genes in the nitrogen cycle

The patterns of the gene abundances of the nitrification gene *amoA* from ammonia-oxidizing bacteria (AOB) and archaea (AOA) (referred to as *amoA*-AOB and *amoA*-AOA, respectively) were similar among the treatments (Figure 4a-b). In addition, the population size of the *amoA*-AOA gene was ~2-fold higher than that of the *amoA*-AOB gene for all treatments, with an average of 7.06×10^5 copies g^{-1} dry soil versus 3.28×10^5 copies g^{-1} dry soil, respectively (Figure S2a). Significant differences in the abundances of *amoA*-AOB and *amoA*-AOA among the treatments were observed on day 8. Most notably, the copy numbers of *amoA*-AOB and *amoA*-AOA were ~4- and ~3-fold higher in the inorganic-amended soil than in the control (Figure 4a-b).

With respect to denitrification, no significant changes in *nirS* and *nirK* copy numbers were observed in either fertilized treatment on any sampled day (Figure 4c-d), but *nirK* abundance was on average 3317-times higher than *nirS* abundance for all treatments (Figure S2b). Both *nirK* and *nirS* were more abundant than the nitrification genes, with averages of 2.92×10^9 copies g^{-1} dry soil and 9.04×10^5 copies g^{-1} dry soil, respectively. The abundance values of the bacterial and archaeal 16S rRNA genes were nearly stable in all treatments, with no differences between treatments (Figure 4e-f). The total abundance of the bacterial 16S rRNA gene was greater than that of the archaeal 16 rRNA gene by ~7 orders of magnitude in all treatments.

Concomitant with these changes in gene abundance were alterations of the dynamics of N_2O emissions (Figure S3). The *amoA*-AOA and *amoA*-AOB abundances were positively correlated with N_2O fluxes (0.39, p = 0.04 and 0.66, p = 0.00, respectively), with a higher correlation for AOB (Table 3), However, no correlation between the AOA/AOB ratio and N_2O that would indicate which group (bacteria or archaea) contributed most to N_2O emissions was found (Table 3). The *nirK* abundance was significantly correlated with N_2O emissions (0.33, p= 0.09), but no correlation between the *nirK*/*nirS* ratio and N_2O was detected (Table 3). Thus, neither the bacterial not the archaeal community was significantly correlated with N_2O emissions.

3.5. Bacterial community differences between N fertilizer sources

A total of 1,533,300 high-quality sequences were assigned to 7,982 OTUs, excluding singletons. The numbers of reads from the soil and rhizosphere samples ranged from 4,654 to 38,695 across all treatments. The range of Good's coverage values was 78–91% at a 97% similarity cutoff, indicating sufficient sequence reads to capture the bacterial diversity. The addition of different nitrogen sources did not induce significant ($p > 0.05$) changes in the alpha (Chao and Shannon) diversity of the bacterial community (Figure S4). The comparison of bacterial community composition showed significant effects of time and the interaction of time and treatment but not of compartment (bulk or rhizosphere) (Table 4, $p<0.05$), which is partly supported by DAPC analysis (Figure 5). The beta diversity of the active bacterial communities based on RNA varied among the three treatments (microalgae, NPK and control) without considering the distinction between the soil and rhizosphere compartments (Figure 5a). Differences in the rhizosphere community were observed among both the treatments and time points, indicating a strong plant developmental stage effect (Figure 5b-d). DAPC analysis revealed a visible differences between the bulk and rhizosphere samples. The addition of nitrogen sources caused greater sample dispersion of the bacterial community over time compared with the control, and for all treatments, a clear trend of decreasing dissimilarity of the bacterial community from the beginning of the experiment until harvest was observed. Because there were no differences in the bacterial community between the rhizosphere and bulk soil compartments by PERMANOVA and the soil and rhizosphere communities clearly differed according to plant development stage and not treatment, the rhizosphere and bulk soil compartments were not considered separately in further analyses. LEfSe analysis revealed 10 significantly active bacterial groups that were sensitive to N source type in soil ($p < 0.05$, LDA > 0.2; Figure 6a). The abundances of several bacterial groups decreased in response to the addition of microalgae and inorganic fertilizer, including seven taxa within the phyla Delta- and Gamma-proteobacteria, Bacteroidetes, Acidobacteria and Actinobacteria. The order *Subsection III* (phylum Cyanobacteria) was enriched in the inorganic treatment, while the orders *Cytophagales* (phylum Bacteroidetes) and *Bradymonadales* (phylum Delta-proteobacteria) were enriched in the microalgal treatment (Figure 6a). LEfSe analysis of the different time points of each treatment revealed 24, 22 and 36 significantly different bacterial groups in the control, inorganic and microalgal treatments, respectively (LDA>3; Figure S5a-S7a). Many orders were shared among the treatments, but their distributions during

plant growth differed. *Burkholderiales* and *Vamprivibrionales* increased in abundance on days 8 and 14, respectively, in the control and microalgal treatments but on days 14 and 30, respectively, in the inorganic treatment. Furthermore, some common orders highlighted on day 0 in both the inorganic and microalgal treatments (i.e, *Rhodospirillales*, *Nitrospirales*, *Gaiellales* and *NB1-j*) were not selected in the control. Other orders were unique to each treatment, including *Pseudomonadales* at day 8, *Rhizobiales* at day 60 and *Oligoflexales*, *Legionellales* and *Gammaproteobacteria-incertae sedis* at day 120 in the microalgal treatment and *Sphingobacteriales* and *Herpetosiphonales* at day 60 and *Myxococcales*, *Solibacterales* and *FCPU453* at day 120 in the inorganic treatment.

3.6. Protozoan community differences between N fertilizer sources

After quality filtering and exclusion of non-protist eukaryote sequences, the remaining 677,067 sequences were clustered into 2,012 protist OTUs for both the bulk and rhizosphere compartments. The numbers of reads in the soil samples ranged between 1,066 and 20,042 in the treatments. The Good's coverage values were in the range of 84-92% at a 97% similarity cutoff, indicating sufficient sequence reads to capture the protozoan diversity in these soils. Similar to the bacterial community, the alpha diversity of protozoan communities was not affected ($p > 0.05$) by the application of the different N sources (Figures S4c and S4d). However, stronger effects of treatment and the interaction of time and treatment than of the bulk or rhizosphere soil compartment were found (Table 4, $p<0.05$). The protozoan DAPC showed dissimilarity among the treatments (Figure 7a). For all treatments, the structure of the active protozoan communities according to RNA changed over time without recovering to the original active protozoan state (Figure 7b-d). The greatest differences in the protozoan community were observed in the control treatment in the first 8 and 30 days due to the plant effect; after this period, the changes in the community were randomly dispersed among the sampling days. However, after 120 days, the bulk soil protozoan community was similar to that on day 0. For the fertilized treatments, we could not track the dynamics of the protozoan community in either bulk soil or rhizosphere, and no recovery of the protozoan community to its original state was observed after 120 days. LEfSe analysis revealed 23 protozoan biomarkers (Figure 6b), but only 2 orders were sensitive to the type of N fertilizer treatment ($P < 0.05$, $LDA > 1.0$). The order *Leptomyxida* (phylum Amoebozoa) decreased with inorganic and microalgal fertilization, whereas the order *Nassophorean* (phylum Alveolata) increased in the microalgal treatment. LEfSe analysis at the different time points of each treatment identified 9, 2 and 3 significant biomarkers (LDA>3; Figure S5b-S7b) for the control, inorganic and microalgal treatments, respectively. The only shared order was *Euamoebida*, which was found only in the control and microalgal treatments at days 0 and 30, respectively. The unique orders selected by LEfSe analysis at day 8 were *Cryptophyceae* in the control, *Chrysophyceae_Synurophyceae* in the inorganic treatment and *Diplonemida* in the microalgal treatment.

4. Discussion

4.1. Plant productivity and nutritional status

Barley biomass and productivity measured at 120 days were stimulated and significantly enriched in the treatments with organic N from microalgae and inorganic N from NPK fertilizer. The increase in total yield was slightly higher in the microalgal than in the inorganic treatment, although this difference was not significant. Several studies (Faheed & Fattah, 2008, Hernández-Herrera *et al.*, 2014, Renuka *et al.*, 2016, Renuka *et al.*, 2017) have reported positive associations among nutrient uptake, biomass accumulation, and crop yields due to the use of microalgae as a biofertilizer. The increment of barley yield due to microalgal fertilizer indicates that the nutrients contained in the microalgae were mostly plant-available in the short term as also noted by Castro *et al.* (2017). Microalgal amendment was also able to supply micronutrients as organic amendments carry small amounts of iron, manganese, copper, and zinc, to soil (Tarakhovskaya *et al.*, 2007, Alobwede *et al.*, 2019, Yadav *et al.*, 2019). However, the total protein (N content) was lower in the algae than in the inorganic N treatment (Table 2), which can be explained as most of the N in the algae is in the organic form (Table 1), which must undergo mineralization in the soil as plants absorb N mainly in the inorganic form. In addition, as suggested by Tarakhovskaya *et al.* (2007), certain microalgal extracts have high levels of auxins, gibberellins, and cytokinins, regulators that are related to plant growth and thus could increase plant productivity. Overall, the results support the potential benefits of using microalgae from black water as a sustainable organic fertilizer in the form of increased crop yields and nutritional quality of barley comparable with inorganic fertilization.

Concerns have been raised about the presence in wastewater, including black water, of contaminants of emerging concern such as pharmaceuticals, which may accumulate in conventional wastewater treatment sewage sludge and also microalgal biomass. We previously reported that the concentrations of pharmaceuticals such as diclofenac and carbamazepine that accumulated in microalgal biomass cultivated on black water were low compared with other wastewater sources (de Wilt *et al.*, 2016). However, evaluation of the contamination level of biomass generated from wastewater will always be mandatory for soil applications.

4.2. Linking soil microbial community dynamics to N_2O emissions

In addition to increasing barley yield and quality, fertilization with microalgae increased emissions of N_2O and CO_2, gases that contribute to global warming. The highest N_2O fluxes occurred within the first days after fertilization due mineral N availability (Table 1). Inorganic fertilization also had a major impact on N_2O emission fluxes and cumulative emissions. On average, N_2O emissions were 4.6-fold and 15-fold higher in the microalgal treatment than the inorganic and control treatments, respectively. Different fertilizer types may dramatically affect N_2O emissions from agricultural soils (Bouwman *et al.*, 2002, Stehfest & Bouwman, 2006), and our findings are consistent with other studies showing that the addition of organic residues increases GHG emissions (Zhai *et al.*, 2011, Suleiman *et al.*, 2016, Castro *et al.*, 2017). Similar to our results, Castro *et al.* (2017) showed that the efficiency of microalgal biofilm as an organic fertilizer was similar to that of traditional chemical fertilizers in shoot production and N content

assimilated by the plant, but N_2O emissions were higher compared with inorganic fertilizer. These higher N_2O emissions may be due to the low C:N ratio of microalgae that favors mineralization rather than N immobilization (Gregorich *et al.*, 2005, Jiang *et al.*, 2018) after fertilization possibly causing a primer effect. Microalgae biomass application could increase the microbial activity with increment in the population of nitrifiers and heterotrophic microbes (Oosterkamp et al., 2016), creating anaerobic microsites and boosting N_2O production (Lourenço et al., 2018). The interaction between C and N are the main responsible for high N_2O and CO_2 emissions (Liang et al., 2015).

N_2O can be produced under both aerobic and anaerobic conditions (Hu *et al.*, 2015, Medinets *et al.*, 2015). Here, N_2O emissions were correlated with the abundances of nitrification genes (*amoA*) from bacteria (0.66, $p \leq 0.00$) and archaea (0.39, $p \leq 0.05$) and with the denitrification gene *nirK* from bacteria (0.33, $p \leq 0.10$), indicating that both processes likely occurred during our experiment. Nitrification apparently triggered denitrification, inducing a cascade of reactions in the soil resulting in high N_2O production. Lourenço *et al.* (2018a) found similar results in tropical soils when organic residue from ethanol production was applied as a biofertilizer. During nitrification, N_2O is produced in the presence of O_2 and/or low availability of degradable carbohydrates; during denitrification, N_2O is synthesized when O_2 is lacking and/or nitrate (or nitrite) accumulates. Faster C mineralization requires extra oxygen, resulting in the formation of anaerobic soil microsites that support the local creation of N_2O through denitrification (Paré & Bedard-Haughn, 2013). This interpretation is reinforced by Cui *et al.* (2016), who observed a rise in the percentage of potential N_2O emissions resulting from denitrification after the addition of organic matter. Supporting our hypothesis, the significant positive relation between the rates of N_2O and CO_2 emissions suggests that soil C degradation is a factor controlling N_2O production. This correlation implies that denitrification might also play a role in net N_2O production in our soil samples (Huang *et al.*, 2004). Our results revealed that microalgal application significantly increased soil CO_2 emissions by 17% and 44% compared with the inorganic and control treatments, respectively. Elevated CO_2 often indicates increased belowground allocation of plant photosynthates to roots, high soil labile C (Alberton *et al.*, 2005), microbial biomass and microbial activities (Hu *et al.*, 1999), including heterotrophic denitrifying microbes (Knohl & Veldkamp, 2011). Furthermore, higher emissions of CO_2 could be due to the release of C from the microalgae biomass, enhanced soil microbial activity (autotrophic and heterotrophs microbes), and possibly from root respiration. Treatments with organic or inorganic fertilizer showed higher root biomass than control. Therefore, it is expected that the amount CO_2 released to the atmosphere is higher when organic fertilizer is added to the soil. Moreover, plant growth was stimulated by fertilization and part of the photosynthates are released as exudates (carbon compounds) by the roots which are used by soil microbes consequently emitting CO_2 via their activity.

Despite the importance of denitrification in N_2O emissions, nitrification was the dominant process controlling N_2O production in the current study, especially in the microalgal treatment (0.80, $p = 0.01$, $n = 9$). The slightly positive correlation of N_2O emissions with *nirK* copy number and the negative correlation with the (*nirK*+*nirS*)/*amoA*-AOB ratio (-0.60, $p \leq .00$) showed that *amoA*-AOB was the most important gene related to N_2O emissions. Thus, despite the high copy numbers of *nirK* and *nirS* genes, *amoA*-AOB was relatively more important in producing elevated N_2O emissions, thus indicating that

nitrification by AOB dominates N_2O production. In soil with ≤ 60% water-filled pore space, similar to our experimental conditions, N_2O is mainly produced by organisms involved in the ammonium oxidation process (bacteria and archaea) (Bateman & Baggs, 2005, Baggs *et al.*, 2010). These results suggest that AOB actively grow under high NH_4^+-N concentrations, leading to microtoxic or anoxic conditions. These conditions in turn induce denitrification by heterotrophic denitrifiers, resulting in high N_2O emission fluxes.

4.4. Bacterial community differences between N fertilizer sources

The bacterial communities associated with the different fertilization treatments showed notable differences except in alpha diversity, in line with earlier studies (Yu *et al.*, 2013, Lourenço *et al.*, 2018b). The microalgal and inorganic fertilizers significantly enriched members of different orders: *Subsection III* (phylum Cyanobacteria) in the inorganic treatment and the orders *Cytophagales* (phylum Bacteroidetes) and *Bradymonadales* (phylum Delta-proteobacteria) in the microalgal treatment. Unexpectedly, an increase in Cyanobacteria was observed in the inorganic treatment. Although similar increases have been reported previously (Ai *et al.*, 2018, Kumar *et al.*, 2018) this remains an interesting result because Cyanobacteria are well-known obligate autotrophs and active N-fixing bacteria. High concentrations of macronutrients (N, P, K) are expected to favor processes such as nitrification and nitrogen mineralization, resulting in a reduction of N-fixing organisms. *Cytophagales* was also found in low concentrations in the microalgal treatment, and members of *Cytophagia* are capable of lysing a variety of microalgae (Rivas *et al.*, 2010), which may explain their increased abundance in this treatment. The order *Bradymonadales* contains rare microorganisms, and information on the ecology of this order in soils is still limited. The first genome from this order was sequenced only recently from an organism in a marine system that, like members from the order *Myxococcales*, preys on other bacteria (Wang *et al.*, 2015, Wang *et al.*, 2019).

Temporal changes in the microbial community were apparent in both the control and fertilized treatments, indicating a strong plant effect. Plants alter the chemical and physical properties of soil, thereby influencing the rhizosphere and bulk microbial community structure and, in turn, plant performance. We also observed differences between the bulk soil and the rhizosphere community composition in each treatment (Figure 5, 7). These differences were expected, since the microbial community structure in the rhizosphere is dynamic and changes over the course of plant development (Lundberg *et al.*, 2012), in part due to changes in the quantity and quality of root exudates (Chaparro *et al.*, 2014), (Baudoin *et al.*, 2002). Many studies have also demonstrated that rhizosphere microbes are significantly affected by plant developmental stage (Schlemper *et al.*, 2017, Schlemper *et al.*, 2018).

The addition of nitrogen sources caused greater sample dispersion of the microbial community over time compared with the control. However, there was a clear trend of decreasing dissimilarity at the final time point of 120 days, indicating recovery to the original active bacterial status at harvest for all treatments. Similarly, for *Arabidopsis*, potato and sorghum, the microbial communities in the early rhizosphere are distinct from those in bulk soil during early plant development, and these differences

decrease as the plant ages (İnceoğlu *et al.*, 2011, Schlemper *et al.*, 2017, Schlemper *et al.*, 2018). It is hypothesized that the roots of seedlings release sugars as substrates for a diverse range of microbes in the early stages of development, but as the plant ages, specific substrates and potentially antimicrobial compounds are released to select for particular microbial inhabitants of the rhizosphere (Badri *et al.*, 2013, Chaparro *et al.*, 2014). Interestingly, although distributed differently through time, the orders that changed during the growth period were consistent, independently of the addition of a N source. For example, the abundances of *Burkholderiales* (phylum Betaproteobacteria) and *Vamprivibrionales* (phylum Cyanobacteria) increased on days 8 and 14, respectively, in both the control and microalgal treatments, while both orders were enhanced in the inorganic treatment on days 14 and 30, respectively. For *Arabidopsis,* Chaparro *et al.* (2014) found that bacteria classified as Cyanobacteria were significantly more abundant in the soil at bolting than in the seedling and vegetative stages before decreasing in abundance at the flowering stage. By contrast, compared with the control, the abundance of Cyanobacteria was higher in the inorganic treatment on day 30 (advanced tillering) and in the microalgal treatment on day 14 (tillering). Furthermore, the relative abundance of *Burkholderiales*, endophytic plant growth-promoting bacteria (Santoyo *et al.*, 2016), increased in the seedling and flowering stages. *Burkholderiales* typically contribute to plant growth-promoting activity by producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase and indole-3-acetic acid (IAA). These results indicate that while soil fertilization appears to be a determinant of the rhizosphere and bulk community bacterial structure, plant and growth stage also have effects. Remarkably, some orders were unique and increased in each treatment, such as the higher abundance of *Legionellales* at day 120 in the microalgal treatment (Figure S7). Wastewater treatment plants have confirmed the presence of but underestimated sources of *Legionella*, which belongs to *Legionellales* and causes Legionnaires' disease (Caicedo *et al.*, 2019). Therefore it is vital to consider mitigation strategies such as ultraviolet light, which can inactivate *Legionella* and possibly other pathogens (Jjemba *et al.*, 2015), after microalgal harvesting.

4.5. Protozoan community differences between N fertilizer sources

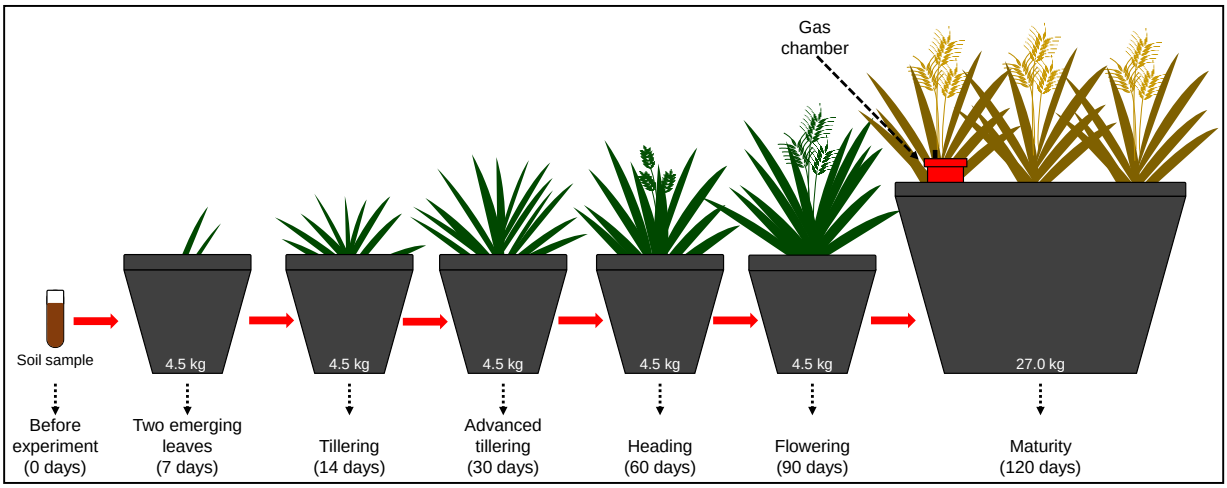
The application of organic and inorganic fertilizers affected the composition of the active protozoan community without affecting alpha diversity. The protozoan communities were notably distinct across different fertilization types. Furthermore, the protozoan communities did not recover within days to the pre-perturbation state in either the fertilized treatments or the control, revealing a strong plant growth stage effect, similar to the results for the bacterial community. For the fertilized treatments, the changes in the active protozoan community in the bulk and rhizosphere did not follow a defined pattern, and none showed recovery to the original state after 120 days, indicating not only a plant effect but also an additional nutritional boost from the fertilizers. In our study, the order Leptomyxida (phylum Amoebozoa) decreased with both N fertilizer sources, while the order Nassophorea (phylum Alveolata) increased solely with the addition of microalgae. Leptomyxida are characteristic of natural habitats such as forests, and thus their decreased abundance is likely due to perturbation caused by the addition of nitrogen in agricultural soil (Geisen *et al.*, 2015). Interestingly, nassophoreans have a basketlike structure that

allows them to consume typically filamentous cyanobacteria or blue-green algae (Lynn, 2008), suggesting that they could be consuming the cyanobacteria that increased in the microalgal treatment.

This study is the first to comprehensively analyze the variations of the dynamics of active protozoa in different plant development stages under nitrogen source application. Our results indicate that the protozoan community composition of barley, including in the bulk and rhizosphere, changed mainly in the first 8 days but did not vary significantly in other plant developmental stages, in contrast to bacteria. *Euamoebida* was the only shared order and was found only in the control and microalgal treatment at days 0 and 30, respectively. These organisms are considered parasites of the gut of invertebrates or vertebrates (Baker, 1994) suggesting the need for precautions, as they may remain active after collection from human wastewater, like bacteria. The unique orders selected as significant at day 8 according to DAPC were *Cryptophyceae* for the control, *Chrysophyceae-Synurophyceae* for the inorganic treatment and *Diplonemida* for the microalgal treatment. Interestingly, *Cryptophyceae* was previously found to be favored by reductions of nutrient loading in shallow lakes (Jeppesen *et al.*, 2007). Furthermore, *Diplonemida* are euglenids, a group of free-living, single-celled flagellates that thrive predominantly in aquatic environments. Flagellates from this order have remarkable adaptability to diverse environmental conditions and are able to metabolize various exogenous carbon sources (Tani & Tsumura, 1989). Remarkably, overall the results suggest that microalgal application creates specific niches favoring the growth of organisms that are also adapted to aquatic environments.

6. Conclusions

Microalgae are efficient at recovering nutrients from wastewater and represent a sustainable alternative to conventional wastewater treatment systems. Here, we focused on closing the nutrient cycles within wastewater treatment systems by providing a comprehensive understanding of the impact of biofertilization with microalgae cultivated on black water. We assessed GHG emissions, plant productivity and quality, and soil chemistry and microorganisms within the bulk soil and rhizosphere, including specific functions of the nitrogen cycle. We conclude that (1) microalgae from black water are an effective biofertilizer for plant growth; (2) microalgal biofertilizer increases N_2O and CO_2 emissions, (3) which apparently is due to different pathways, with nitrification as the main process; (4) different N sources affect the active bacterial and protozoan microbiomes, but the plant has a stronger effect in selecting for a subset of microbes at different stages of plant development. Overall, the use of microalgal biofertilizer in agriculture is a viable option to close the wastewater treatment and nutrient cycles. However, sustainable solutions such as management practices of microalgal biofertilizer, including inhibition of nitrification, are necessary to mitigate the N_2O emissions of microalgal biofertilizer. In addition, further studies are needed to clarify the presence and persistence of pathogens in such residues.



578

579 **Fig. 1.** Schematic view of the pot experiment and each barley plant development stage. Pots containing
580 4.5 kg of soil were used to evaluate the impact of fertilization with microalgal biomass on plant growth
581 and the soil microbial community. Pots containing 27.0 kg of soil were used to evaluate the last growth
582 stage of barley and GHG emissions. For both pot sizes, bulk and rhizosphere samples were collected
583 for each treatment.

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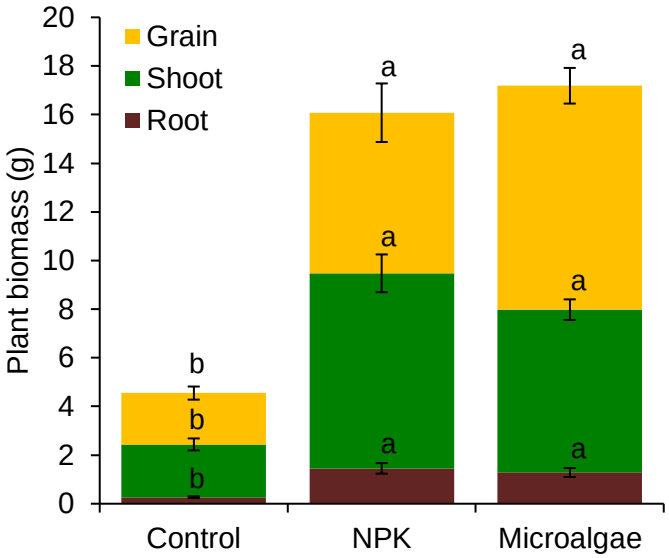

585

586 **Fig. 2.** Dry plant biomass partitioned at harvest into root, shoot and grain yields. Standard errors are
587 shown (n=4). Significant differences ($P < 0.05$) between partitioned yields for each treatment are
588 depicted by lowercase letters.

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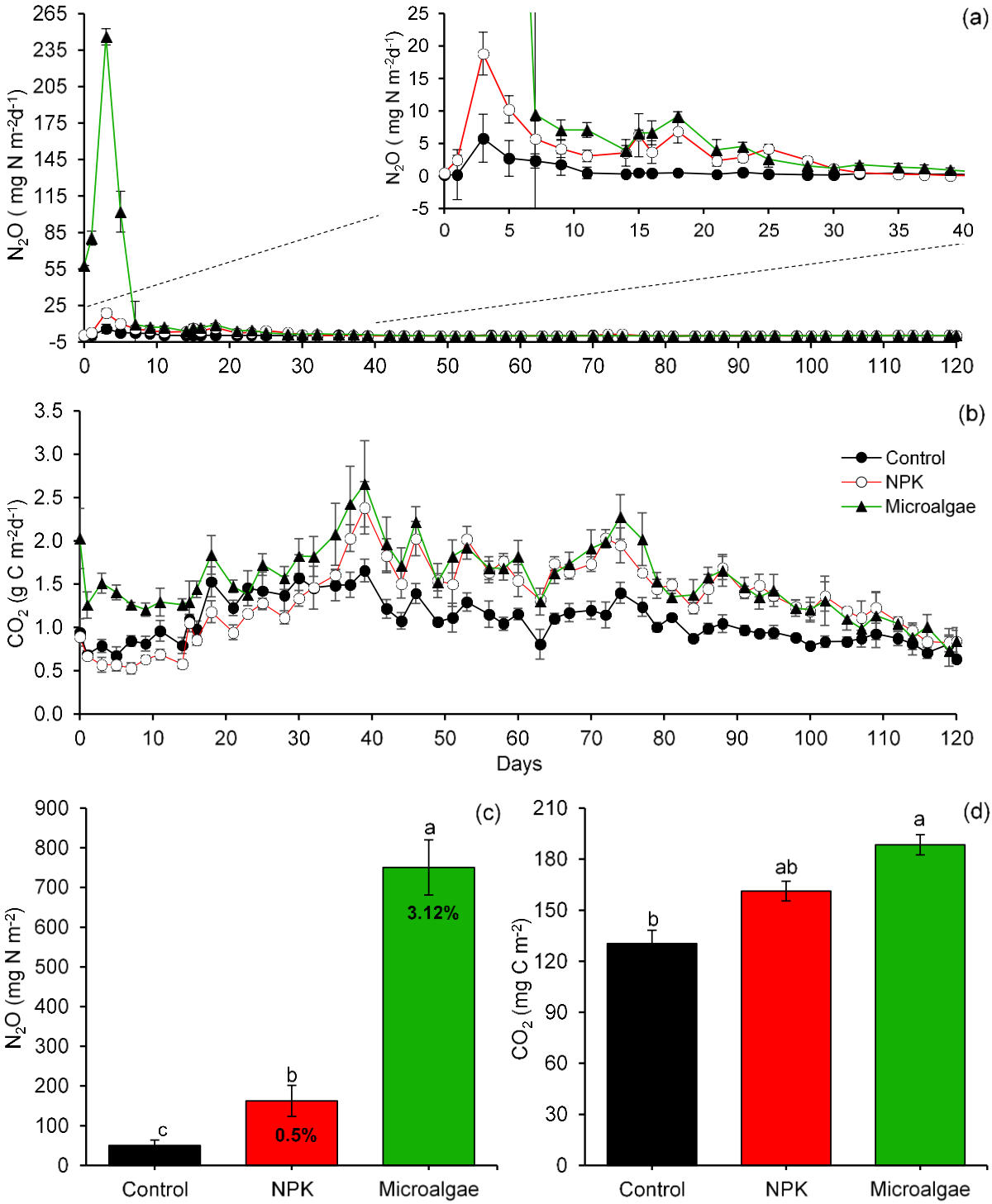


Fig. 3. Daily N_2O (a) and CO_2 (b) fluxes over the entire experiment (120 days) for all treatments. A close-up view of the N_2O fluxes over the first 40-day period is also provided. Cumulative N_2O (c) and CO_2 (d) emissions over the entire experiment (120 days) for all treatments and respective N_2O-N emission factors (within the bars, %). Standard errors are shown (n=4). Significant differences ($P < 0.05$) between treatments are depicted by lowercase letters.

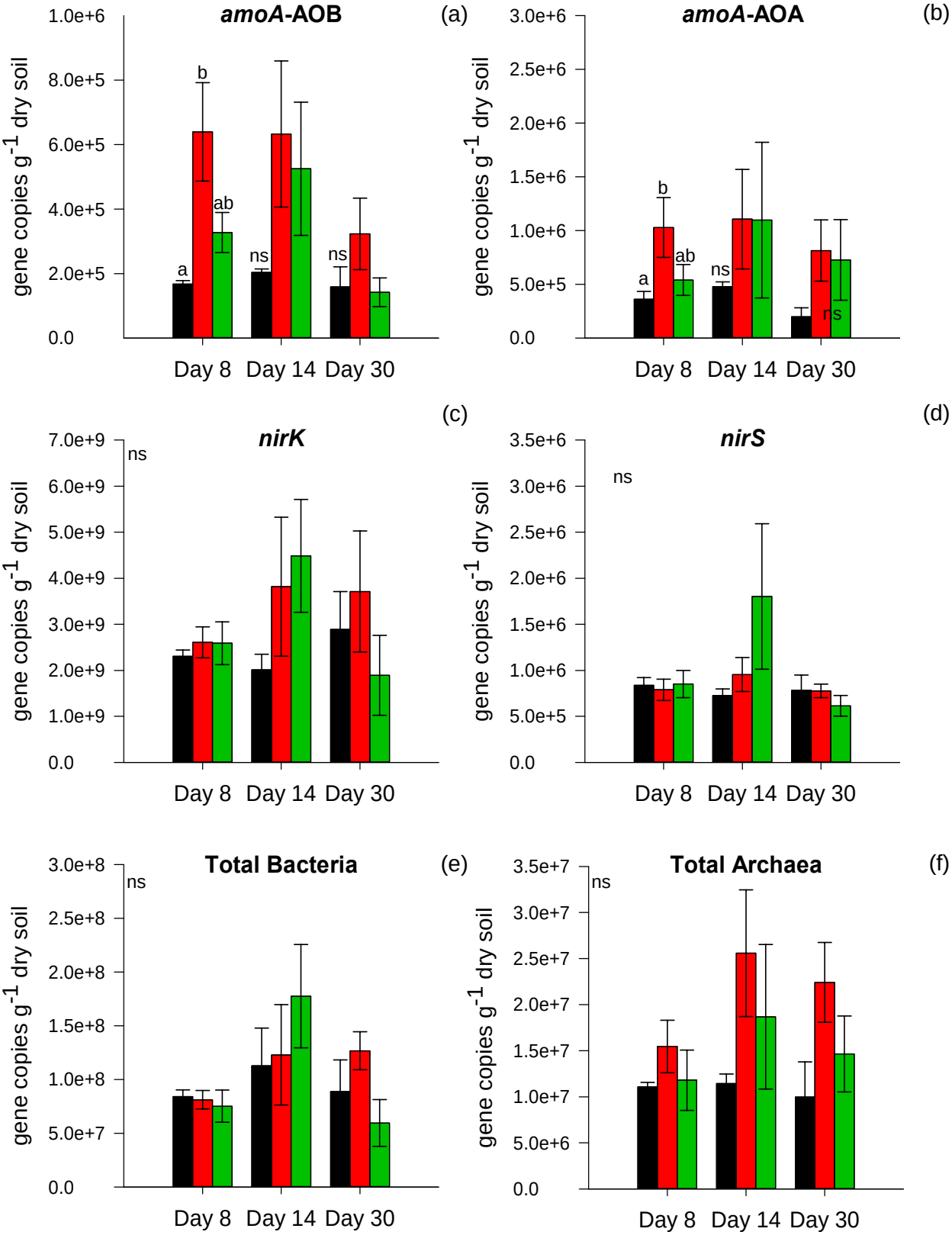
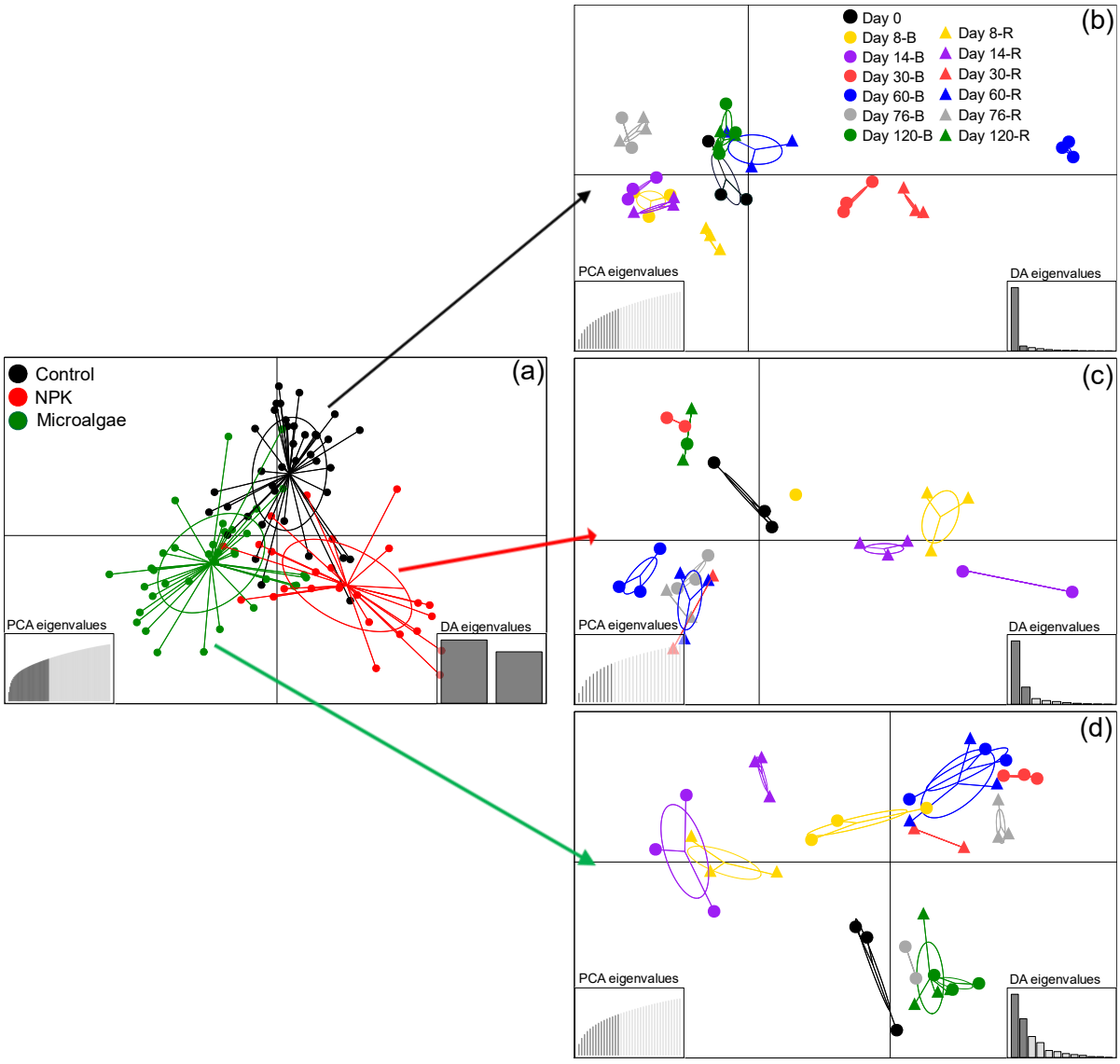


Fig. 4. Gene abundances of a) bacterial (AOB) and b) archaeal (AOA) ammonia monooxygenases, c) and d) nitrite reductases (*nirK* and *nirS*), e) total bacterial 16S rDNA and f) total archaeal 16S rDNA. Black bars are the control, red bars are inorganic fertilizer, and green bars are microalgal biofertilizer. Standard errors are shown (n = 3). Significant differences between treatments ($P < 0.05$) are depicted by lowercase letters; ns means nonsignificant. Sampling for soil conditions and gene abundances was conducted on Days 8, 14 and 30.



627

628 **Fig. 5.** Discriminant analysis of principal components (DAPC) plot at (a) the bacterial OTU level
629 revealing distinct clustering of the control, inorganic fertilizer (NPK), and organic fertilizer (microalgae)
630 treatments without considering distinctions between time points and compartments; (b) the bacterial
631 OTU level revealing distinct clustering of the control treatment, (c) inorganic treatment and (d) microalgal
632 treatment at different time points during the 120-day experimental period. Legend: "B" indicates bulk
633 soil and "R" the rhizosphere.

634

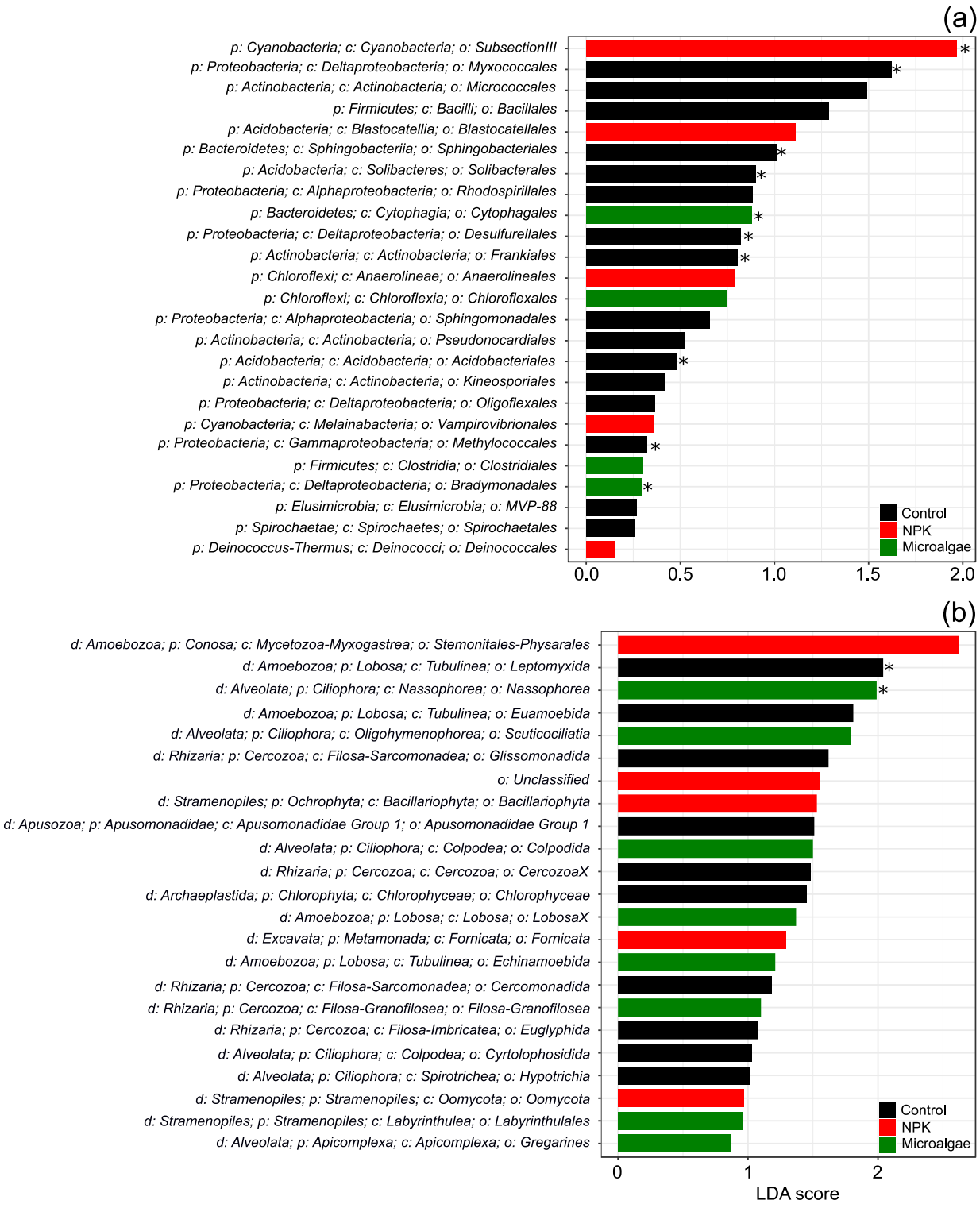


Fig. 6. Linear discriminant analysis Effect Size (LEfSe) of active (a) bacterial orders and (b) protozoan orders that most likely explain the differences between treatments.

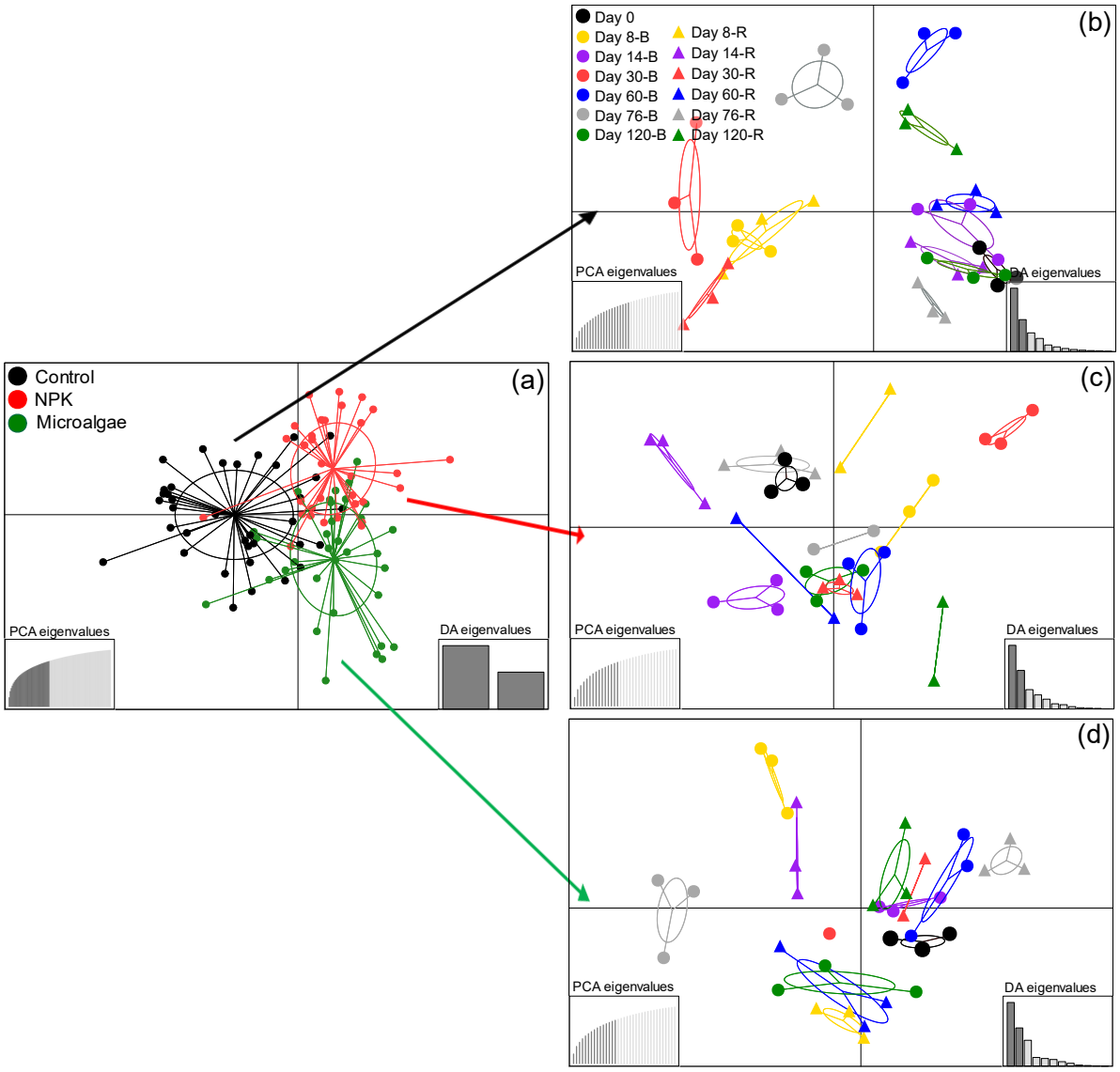


Fig. 7. Discriminant analysis of principal components (DAPC) plot at (a) the protozoan OTU level revealing distinct clustering of the control, inorganic fertilizer (NPK), and organic fertilizer (microalgae) treatments without considering distinctions between time points and compartments; (b) at the protozoan OTU level revealing distinct clustering of the control treatment, (c) inorganic treatment and (d) microalgae treatment at different time points during the 120-day experimental period. Legend: “B” indicates bulk soil and “R” the rhizosphere.

Tables

Table 1. Properties of the microalgae fertilizer and the soil used in the experiment.

Algae (*Chlorella sorokiniana*)		**Soil characteristics**	
C content	20.5 (%)	Organic matter	3.7
Total N content	60.9 (g kg^{-1})	N – total (mg kg^{-1})	980
C/N ratio	3.4	C/N ratio	22
Mineral nitrogen	14.0 (g kg^{-1})	pH	5.7
P	12.3 (g kg^{-1})	P- plant availability (mg kg^{-1})	1.3
K	88.7 (g kg^{-1})	K- plant availability (mg kg^{-1})	96.7
Ca	8.7 (g kg^{-1})	Ca - plant availability (mg kg^{-1})	169.7
Mg	3.4 (g kg^{-1})	Mg - plant availability (mg kg^{-1})	174.0
S	9.6 (g kg^{-1})	S - total (mg kg^{-1})	230.0
Fe	350.0 (mg kg^{-1})	Fe - plant availability (µg kg^{-1})	<2010
Mn	28.6 (mg kg^{-1})	Mn - plant availability (µg kg^{-1})	560.0
Cu	94.9 (mg kg^{-1})	Cu - plant availability (µg kg^{-1})	<21
Zn	154.6 (mg kg^{-1})	Zn - plant availability (µg kg^{-1})	506.7
B	44.4 (mg kg^{-1})	B - plant availability (µg kg^{-1})	277.3
		Na - plant availability (mg kg^{-1})	8.0
		Sand/Silt/Clay	90/6/1
		CEC ($mmol^{+}$ kg^{-1})	64.7
		Microbial biomass (mg N kg^{-1})	16

654 **Table 2.** Macro- and micronutrient content (± standard error) in the dry grain of the last development
655 stage of barley. Significant differences between treatments (Tukey test, P < 0.05) are depicted by
656 lowercase letters.
657

Treatment	Dry Grain	N	P	K	Ca	Mg	S	Fe	Mn	Cu	Zn	B
	g pl^{-1}	----------		g kg^{-1}		----------		----------		mg kg^{-1}	----------	
Control	2.1 ±0.3 b	26.7 ±2.2 a	3.5 ±0.1	5.8 ±0.5	0.2 ±0.0 b	1.1 ±0.1	1.3 ±0.1 a	27.6 ±2.6	3.5 ±0.6 a	4.8 ±0.5	44.1 ±4.1	1.3 ±0.1
NPK	6.6 ±0.7 a	26.2 ±0.8 a	3.8 ±0.2	5.0 ±0.4	0.2 ±0.0 b	1.3 ±0.1	0.8 ±0.0 b	27.1 ±2.5	3.3 ±0.5 a	4.8 ±0.2	47.2 ±3.4	1.3 ±0.2
Microalgae	9.2 ±1.2 a	17.1 ±1.1 b	3.7 ±0.5	5.2 ±0.8	0.3 ±0.0 a	1.3 ±0.2	1.1 ±0.1 ab	25.2 ±4.3	9.7 ±2.8 a	5.7 ±0.8	39.8 ±5.1	1.3 ±0.2

658

659

660 **Table 3.** Spearman's correlation coefficients between N_2O emission flux (mg m^{-2} day^{-1}) and the
661 abundances of ammonia-oxidizing bacteria (AOB) and archaea (AOA), nitrite reductase genes (*nir*K and
662 *nir*S), total bacteria and total archaea.
663

Parameter (*n* = *27*)	**N_2O-N**
*amo*A AOB	**0.661*** **
*amo*A AOA	**0.390***
AOA/AOB ratio	-0.177
nirK	**0.335'**
nirS	0.277
nirK/*nirS* ratio	-0.049
(*nirK*+*nirS*)/AOB	**-0.603*** **
(*nirK*+*nirS*)/AOA	-0.311
Total Bacteria	0.186
Total Archaea	0.281
pH	-0.202
NH_4^+-N	-0.253
NO_3^--N	**0.430****
CO_2-C	-0.012

664 Bold values indicate significant differences: 'p ≤ .10, *p ≤ .05, **p ≤ .01 and ***p ≤ .001.
665
666

667 **Table 4.** Results of PERMANOVA testing of the effects of fertilization (inorganic or microalgae) or the
668 control treatment on bacterial and protozoan communities in bulk soil and rhizosphere of barley, time
669 (for 120 days), and their interaction on active bacterial and protozoan soil communities.
670

Microbial group	Effect	Pseudo-F value	R^2	P value
Bacteria	**Treatment (Tr)**	**1.47432**	**0.02561**	**0.0800**
	Time (Ti)	**2.07354**	**0.10804**	**0.0002**
	Compartment (C)	0.81709	0.00710	0.5961
	Tr x Ti	**1.52980**	**0.13285**	**0.0024**
	Tr x C	1.01979	0.01771	0.3875
	Ti x C	1.05823	0.04595	0.3414
	Tr x Ti x C	**1.23173**	**0.10696**	**0.0678**
Protozoa	**Treatment (Tr)**	**1.28978**	**0.02477**	**0.0462**
	Time (Ti)	0.92894	0.05352	0.7898
	Compartment (C)	0.94030	0.00903	0.5478
	Tr x Ti	**1.14731**	**0.11017**	**0.0297**
	Tr x C	0.92823	0.01783	0.6604
	Ti x C	1.02683	0.04930	0.3494
	Tr x Ti x C	0.95815	0.09201	0.7207

671 Bold values indicate significant differences (p value ≤ 0.10).

672

CRediT authorship contribution statement

Afnan Suleiman, Kesia Lourenco and **Eiko Kuramae**: conceptualization, designed 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 experiment 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 bioinformatical and statistical analyses. **Afnan Suleiman, Kesia Lourenco, Callum Clark** and **Eiko Kuramae**: wrote the paper. All authors reviewed the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financialinterests or personal relationships that could have appeared to influ-ence the work reported in this paper.

Availability of data and materials

The raw sequences were submitted to the European Nucleotide Archive (ENA) under study accession number PRJEB32941.

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