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REGULAR ARTICLE

Management of nitrogen fertilizer application, rather than functional gene abundance, governs nitrous oxide fluxes in hydroponics with rockwool

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Abstract
Aims Nitrous oxide (N_2O) is a strong greenhouse effective gas (GHG); the primary human source of N_2O is agricultural activities. Excessive nitrogen (N) fertilization of agricultural land is now widely recognized as a major contributor. In soil, the microbial processes of nitrification and denitrification are the principal sources of N_2O. However, it remains poorly understood how conventional hydroponics influences GHG emission. Here, we compared GHG fluxes from soil and rockwool used for hydroponics under identical nutrient conditions.

Methods Tomato plants (*Solanum lycopersicum*, momotaro) were grown in soil or by hydroponics using rockwool. In situ emissions of CH_4, CO_2, and N_2O, and the abundance of genes involved in nitrification and denitrification were measured during cultivation.

Results Hydroponics with rockwool mitigated CO_2 emission by decreasing the microbial quantity in the rhizosphere. Dilution of the nutrient solution significantly decreased N_2O emission from rockwool. Although proliferation of nitrifiers or denitrifiers in the rhizosphere did not induce N_2O emission, reuse or long-term use of rockwool induced a 3.8-fold increase in N_2O emission.

Conclusions Our data suggest that hydroponics has a lower environmental impact and that adequate fertilizer application, rather than bacterial control, governs N_2O fluxes in hydroponic cultivation using rockwool.

Keywords Greenhouse gases (GHGs) · Nitrous oxide (N_2O) · Rhizobacteria · Rockwool · Tomato

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Introduction

In addition to carbon dioxide (CO_2), nitrous oxide (N_2O) is a very important greenhouse gas (GHG), which has, so far, contributed an estimated 4–5 % of global warming (IPCC 2001). Emission of N_2O is increasing at a rate of 0.2–0.3 % per year (IPCC 2007) and it is 310-fold more effective in heat trapping in the atmosphere than CO_2 for a 100-year timescale (IPCC 2001). Thus, even a small accumulation of N_2O may have destructive effects for

centuries (Robertson et al. 2000). The primary human source of N_2O is agriculture, which accounts for two thirds of worldwide N_2O emissions. Currently, efforts to predict and mitigate N_2O emission are quite important challenges for the establishment of sustainable agriculture (Kennedy et al. 2013). Excessive nitrogen (N) fertilization of agricultural land is now widely recognized as a major factor in the increase in N_2O emission, as indicated by an increase in the atmospheric concentration of N_2O. For example, microbial oxidation of ammonia (nitrification) and reduction of nitrate (denitrification) are known to be a predominant processes for N_2O emission in agricultural land (Hwang and Hanaki 2000; Inamori et al. 2008; Wang et al. 2008).

Nitrification is a bacterial respiratory process, involving two successive steps that are carried out by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Nitrification-provoking N_2O emission can be achieved by adjusting environmental conditions to be favorable for AOB, but not for NOB, resulting in nitrite accumulation; this is called partial nitrification (Ahn et al. 2010, 2011). Since nitrite can be reduced to N_2O by denitrification by nitrifiers (Kool et al. 2011), so the nitrite accumulation is known to be a triggering factor for N_2O emission (Ahn et al. 2010). On the other hand, denitrification consists of four enzymatic reductive steps as follows: NO_3^- reduction (NO_3^- to NO_2^-), NO_2^- reduction (NO_2^- to NO), NO reduction (NO to N_2O), and N_2O reduction (N_2O to N_2) (Kandeler et al. 2006). Genes responsible for these steps are widely used as molecular markers in the study of denitrifying bacterial communities. There is a large and diverse range of bacteria, archaea, and fungi involved in the nitrification/denitrification processes, and N_2O fluxes are usually determined by their abundance and composition, and by variable environmental factors in the rhizosphere, such as temperature, pH, moisture, oxygen level, organic content, and nitrogen availability (Bergsma et al. 2002; Hwang and Hanaki 2000; Inamori et al. 2008; Liu et al. 2013; Robertson et al. 2000; Tiedje 1988).

Compared to soil-based field agriculture, hydroponics is a promising technique for the cultivation of vegetable crops, using controlled local environmental conditions to maximize crop yields and minimize various environmental challenges (Ryu et al. 2012; Van't Oster et al. 2012). The recent contributions of hydroponics to protected horticulture are not limited to the above-mentioned fields, but also illustrate more sustainable production, using less resources and producing less waste, and therefore reducing environmental impact (Torrellas et al. 2012). Thus, so-called plant factories have drawn attention globally, as this holds the potential for mitigating the total GHG emission during protected horticultural crop production (Torrellas et al. 2013). However, the actual nutrient status of the hydroponic solution and rhizospheric microbial number and composition can gradually change during the natural course of cultivation, because the nutritional requirement is highly dependent on the growth and developmental stage, for example, flowering and ripening (Berendsen et al. 2012; Duineveld et al. 1998; Ibekwe et al. 2010). Importantly, when gaseous loss of nitrogen was investigated, it was found that more than 10 % of N input was released as N_2O in cucumber hydroponic culture (Daum and Schenk 1996). However, it remains poorly understood how conventional hydroponics influences GHG emission and whether it can result in a lowered environmental impact.

Understanding the precise environmental factors influencing N_2O flux is of particular importance for mitigating N_2O emission from future hydroponic agricultural systems. Our aim in this study was to compare GHG emission between conventional soil cultivation and hydroponic cultivation with rockwool. In addition, we assessed the impact of remaining N compounds on N_2O flux using once-used cultivation materials. We hypothesized that (a) conventional hydroponic cultivation with rockwool would have an advantage in mitigating CO_2 emissions compared to soil cultivation; and (b) reuse (or long-term use) of rockwool would increase not only crop yields, but also N_2O emission, by possibly increasing N substrate availability for both crop and rhizobacteria; and (c) nitrifier or denitrifier proliferation in the rhizosphere is necessary, but not sufficient, to increase N_2O emission.

Materials and methods

Plant material and growth conditions

Tomato (*Solanum lycopersicum*, cv. Momotaro-York) plants were grown either by soil cultivation or by hydroponic cultivation using rockwool in an air-conditioned greenhouse in Abiko, Chiba, Japan (N35.9°, E140.0°) from September 2010 to March 2011 (first-year cultivation), and from September 2011 to March 2012 (second-year cultivation). Average temperature was set at 23

±3 °C. Three-week-old tomato seedlings grown in black soil (purchased from a garden center; pH 6.3, total organic carbon content 26 %, bulk density 1.2 Mg m^{-3}, cation exchange capacity 13.2 $cmol_c$ kg^{-1}) or rockwool blocks made of mineral fibers generally used for hydroponics (Grotop Master, Gordan, Denmark) were transplanted to 1/2,000-a Wagner pots filled with the soil or the rockwool blocks that were sub-irrigated with a nutrient solution (Otsuka House Solution A, Otsuka Chemical Co. Ltd., Osaka, Japan). Nutrient solution was prepared with tap water at 2.3 dS m^{-1} EC (EC2.3) in the first year of cultivation and at 1.3 dS m^{-1} EC (EC1.3) in the second year of cultivation, i.e., there was a low concentration of nutrients in the second year. During cultivation, the nutrient solution was supplied using drip irrigation (100 mL h^{-1}) so that the total amount of supplied nutrients was equal to the nitrogen accumulation and was synchronized among all the pots. Fruits were harvested every 2 weeks until February, and the total fresh weight per pot (fruit yield) was measured. Plant height was measured at the end of cultivation. Old leaves and lateral shoots were removed on a weekly basis, dried, and weighed as inedible biomass.

Experimental set-up and gas sampling

We performed the first year of cultivation (exp. 1) using freshly prepared soil (FS) and freshly prepared rockwool (FR). In addition to FS and FR, both the soil and rockwool used for the first-year cultivation were reused for the second-year cultivation (exp. 2), denoted as used soil (US) or used rockwool (UR). Every experiment (FS and FR in the first year, and FS, US, FR, and UR in the second year) consisted of four pots. CO_2, CH_4, and N_2O emissions from the cultivation materials in the pots were captured using a closed-chamber technique (Rolston 1986). The gas sampler was designed as a lid for 1/2,000-a Wagner pots. The top of the lid apparatus had a gas sampling three-way valve connected to a syringe, while a Tedlar sampling bag and an air bag to control atmospheric pressure inside the chamber, as described before (Nkongolo et al. 2010). The apparatus could be opened and closed with a hinged lid, and flexible urethane foam was used to avoid injury to plant stem when the apparatus (lid) was closed and set on a Wagner pot to isolate air circulation with greenhouse air. The height and diameter of the chamber was 10 and 25 cm, respectively. Gas sampling started 60 days after seeding (week 1) and carried out every week until the tomato plants died (week 16). Weeks 1 to 4, 5 to 8, 9 to 12, and 13 to 16 samples, respectively, correspond to the vegetative growth phase, flowering phase, ripening phase, and senescence phase. At each sampling occasion, the chamber was placed onto the pot for 20 min between 14:00 and 15:00 on the sampling day. Then, a sample of the enclosed atmosphere inside the chamber was withdrawn using a syringe and transferred into the connected sampling bag. Samples of the ambient air in the greenhouse were also captured, to provide a background for analyze gas fluxes according to Nkongolo et al. (2010).

GHG flux measurement

A gas chromatography 7890A GC system equipped with a HaySepQ80/100 separation column (Agilent Tech. Inc., Santa Clara, CA, USA) was used to measure CO_2, CH_4, and N_2O. The CO_2 concentration was analyzed using 2 mL min^{-1} nitrogen as the carrier gas, and the temperature of the thermal conductivity detector and oven were set at 200 and 60 °C, respectively. The CH_4 concentration was analyzed using 2 mL min^{-1} He was the carrier gas, and the temperature of the flame ionization detector and oven were set at 250 and 60 °C, respectively. The N_2O concentration was analyzed using 2 mL min^{-1} argon-containing 5 % methane as the carrier gas, and the temperature of the electron capture detector and oven were set at 300 and 60 °C, respectively. The obtained value (gas flux per 20 min) was used as an average flux value during 1 week (from one sampling point to the next sampling point) and, based on this definition, the total gas emitted during the developmental phase was calculated.

DNA extraction and quantification of the gene abundance

Soil and rockwool samples for the quantification of gene abundance were taken from separate pots for sampling every 2 weeks and immediately used for DNA extraction. Taking into consideration that biofilm formation could occasionally be observed on the rockwool surface, visible biofilm-free surface (5–10-mm depth from surface) and interior (10–100-mm depth from surface) were separately sampled. DNA

was extracted from soil samples using an ISOIL DNA isolation kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. DNA extraction from rockwool samples were performed as described previously (Postma et al. 2005). In brief, 40 mL (2 g) of rockwool samples were mashed with a homogenizer (Retsch MM300, Haan, Germany), followed by phenol-chloroform extraction, and DNA purified using standard procedures. DNA quantity was determined using a Multiskan GO (Thermo Fisher Scientific K.K., Yokohama, Japan) and the extracted DNA was stored at −80 °C until required for use. To determine extraction efficiency, a known quantity of microbes was mixed with freshly prepared soil or rockwool before DNA extraction. The DNA recovery indexes were determined, in triplicate, to be approximately 0.82 in soil and 0.75 in rockwool. The determined index was used for the estimating the actual DNA quantity (gene abundance) included in each sample, as described below.

Quantification of expression of all genes related to nitrification and denitrification were performed using a Thermal Cycler Dice Real Time System TP850 (Takara bio, Japan) and SYBR Premix Ex-Taq (Takara bio, Otsu, Japan) with the following gene-specific primers: 16S rRNA (5′-actcctacgggaggcagcag-3′, 5′-attaccgcggctgctgg-3′), AOB-16S rRNA (5′-ggagraaagcaggggatcg-3′, 5′-ctagcyttgtagtttcaa-3′), *amoA* (5′-ggggtttcatactggtggt-3′, 5′-cccctckgsaaagccttcttcttc-3′), *nirS* (5′-gtsaacgtsaaggar-acsgg-3′, 5′-gasttcggttgsgtcttga-3′), *nirK* (5′-ggmathht-kccstggca-3′, 5′-gcctcgatcagrttrtggtt-3′) *norB* (5′-actgga-acaacgtggagacc-3′, 5′-gtcggcagatagggttgta-3′), and *nosZ* (5′-cgytgttcmtcgacagccag-3′, 5′-cgsaccttsttgccstygcg-3′) (Ahn et al. 2006; Braker et al. 1998; Degrange and Bardin 1995; Dionisi et al. 2002; Kowalchuk et al. 1997; Rotthauwe et al. 1997; Throback et al. 2004). Melting curves were routinely checked to confirm purity of the amplified products. Standard curves were obtained using 10-fold serial dilutions of plasmid DNA harboring *amoA*, *nirK*, *nirS*, *nosZ*, *norB*, and 16S rRNA with a linear response ranged from 10^2 to 10^7 copy number per microliter DNA; feasible PCR efficiencies (r^2>0.98) and low coefficient of variations <17 % were obtained with all primer sets. Variations against the actual copy number of the standard plasmid were below 30 %. The total microbial community was quantified using 16S rRNA as a molecular marker. Abundance of each gene per volume (milliliter soil or rockwool) was calculated from copy number and extraction efficiency.

Ammonium and nitrate measurement

Ammonium and nitrate were extracted from rockwool samples at the end of the tomato cultivation with a homogenizer (Retsch MM300), followed by distilled water extraction. Ammonium and nitrate contents were measured with an ICS-1500 ion chromatography system (Dionex Corp., Osaka, Japan). Accuracy was established using cation-mixed standard solution II and anion mixes standard IV (Kanto Chemical Co., Inc., Tokyo, Japan).

Statistical analysis

All statistical tests were performed using KyePlot 4.0 (KyensLab Inc., Tokyo, Japan). Two-way analysis of variance (ANOVA) with repeated measures was used to determine whether there was a significant difference in the interaction of sampling time. We analyzed the difference between cultivation methods by Student's *t* test. Differences were considered statistically significant at P<0.05.

Results

Plant growth and fruit yield

Soil culture showed slight, but non-significantly (p=0.17) higher fruit yield per plant (FS, 555.0±85.7 g) than did hydroponic culture using rockwool (FR, 433.3±39.7 g) in exp. 1. Plant heights at the end of cultivation were similar: FR, 2.5±0.3 m; FS, 2.3±0.2 m (Fig. 1), suggesting that tomato plants cultivated in either system would take up and utilize nearly equal amounts of nutrients. While FS and FR cultures showed the same number of fruit, fruit yield increased slightly in FR culture in exp. 2 (Fig. 1). UR markedly increased fruit yield (number and size) compared to FR. Interestingly, UR culture did not promote plant growth (height and biomass of inedible parts; Fig. 1c, d).

Characteristics of distinct GHG emissions in different cultivation systems

Flux data of exp. 1 and 2 showed substantial differences, resulting from annual environmental variations, for example solar radiation, which makes it difficult and unreliable to compare growth phase-dependent flux between exp. 1

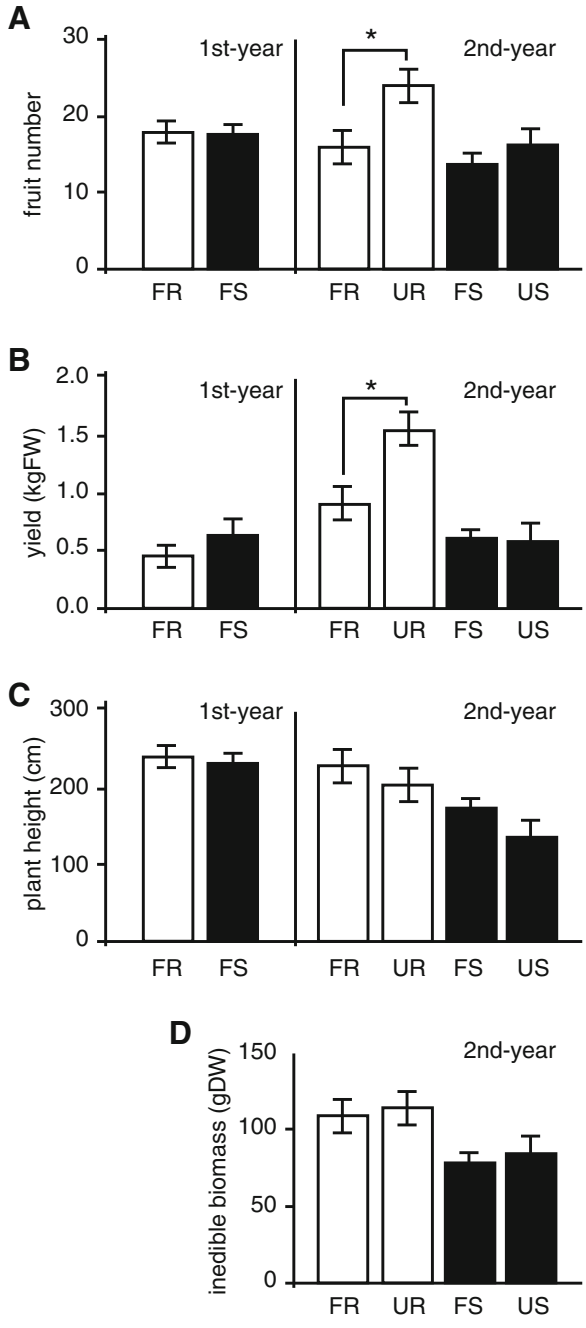

Fig. 1 Fruit yield and growth from different cultivation procedure. **a**. Total number of fruit harvested from a single tomato plant by February 8 on 2011 (first year) and 2012 (second year). **b**. Total fruit yields from a single tomato plant by February 8 on 2011 (first year) and 2012 (second year). **c**. Individual tomato plant height at the end of cultivation on March 10 (2011, first year) and on February 8 (2012, second year). **d**. Total weight of inedible plant materials from a single tomato plant, including the plant body, and the removed old leaves and lateral shoots, by the end of cultivation (February 8, 2012). *Black bar* indicates data obtained from soil cultivation and *white bar* indicates data obtained from hydroponics using rockwool. Mean values (± standard deviation) were calculated and significance from a *t* test analysis ($P<0.05$) is presented by an *asterisk* (*)

and 2 (Supplementary Fig. 1). Thus, below, we focused on comparative analysis of exp. 2 between FS and US, and FR and UR. The CH_4 fluxes fluctuated greatly during the cultivation period (Fig. 2a, d), but showed no tendency among experimental blocks or growth phases ($p=0.77$; Kruskal–Wallis). It is known that N fertilization increases soil uptake of CH_4 (Li et al. 2012) and inhibits CH_4 oxidation (Acton and Baggs 2011; Alam and Jia 2012), so that we cannot exclude the possibility that the manner and timing of nutrient supply in our protocol contributed to CH_4 flux variation.

In contrast, the fluxes of CO_2 were statistically and significantly different between FS and US ($P<0.05$; two-way ANOVA) and growth phase ($P<0.05$; two-way ANOVA). FS displayed a constant CO_2 emission (approximately 400 g m^{-2}). In contrast, the CO_2 emission from US was the highest, viz., approximately 750 g m^{-2}, at the beginning of cultivation (first 4 weeks after planting; phase I) and gradually decreased to the same level as in FS (Fig. 2b). On the other hand, CO_2 emissions from FR and UR were similar throughout the cultivation periods (Fig. 2e). Average CO_2 emissions of all rockwool cultivation never exceeded 400 g m^{-2} during any phase.

N_2O fluxes were statistically significantly different between FR and UR, and FS and US ($P<0.01$; two-way ANOVA), but showed marked differences from the CO_2 flux pattern. Both FS and FR maintained low levels of N_2O emissions from phase I to IV. Unlike CO_2, N_2O emission from US did not increase at the beginning of cultivation (Fig. 2b, c). However, the emission of N_2O was markedly enhanced during phases III and IV. N_2O emission from UR also increased during later growth phases, whereas those from FR did not (Fig. 2f).

Abundance of genes related to nitrification and denitrification during different modes of cultivation

In order to investigate why reused cultivation materials emitted more GHGs, we assessed the microbial dynamics

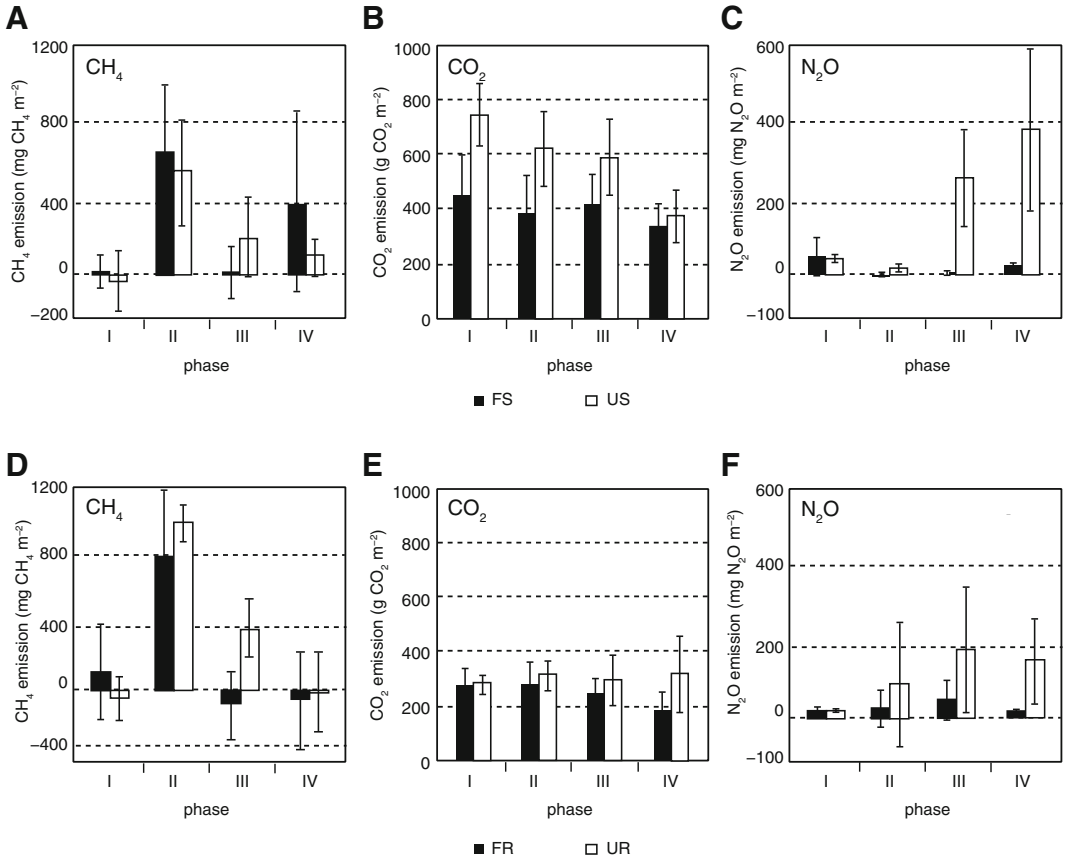

Fig. 2 Greenhouse gas fluxes from soil and rockwool during tomato cultivation. Greenhouse gas fluxes of soil cultivation (**a**–**c**) and hydroponics with rockwool (**d**–**f**) in exp. 2. The cultivation period was divided into four phases (I, vegetative growth; II, flowering; III, ripening; IV, senescence). Total fluxes of CH_4 (**a** and **d**), CO_2 (**b** and **e**), and N_2O (**c** and **f**) from individual pots during each phase are presented as mean values (± standard deviation). *Black bar* indicates data obtained from freshly prepared cultivation materials of second-year cultivation, and *white bar* indicates data obtained from reused cultivation materials of second year cultivation

inside the cultivation materials of exp. 2. The copy number of 16S rRNA genes was similar among the different sampling points within US, UR-surface, and UR-inside (Fig. 3a–c). As predicted, the 16S rRNA gene abundance observed in the early phases (I and II) of FS was markedly less than in the later phases (III and IV), and the gene abundance at phase IV was similar to that in US (Fig. 3a). A 10-fold lower 16S rRNA gene abundance was observed in rockwool samples (Fig. 3b, c). Unlike soil samples, only phase I samples of FR demonstrated a smaller 16S rRNA copy number than UR (Fig. 3b, c) suggesting that microbial proliferation reached a plateau before phase II.

Next, we focused on the microbial community responsible for N_2O emission, because the major difference between FS and US, or FR and UR, was N_2O emission during the later growth phase (Fig. 2c and f). The process by which N_2O is emitted by the rhizosphere is dependent on two types of nitrogen metabolism, viz., nitrification and denitrification. Therefore, we estimated the amounts of nitrifiers and denitrifiers by quantification of the abundance of functional genes. The abundance of all key genes involved in N cycling was higher under US than FS during phases I to III, but not during phase IV (Fig. 3d). This is because rhizospheric microbes proliferated during the first-year cultivation of tomato plants. However, the larger number of microbes in US is unlikely to have contributed to N_2O emission, since the highest N_2O emission was detected during phase IV, rather than phase II (Fig. 2c), while the highest CO_2 emission occurred during phase I of US (Fig. 2b). N_2O emission and functional gene abundance were not highly correlated

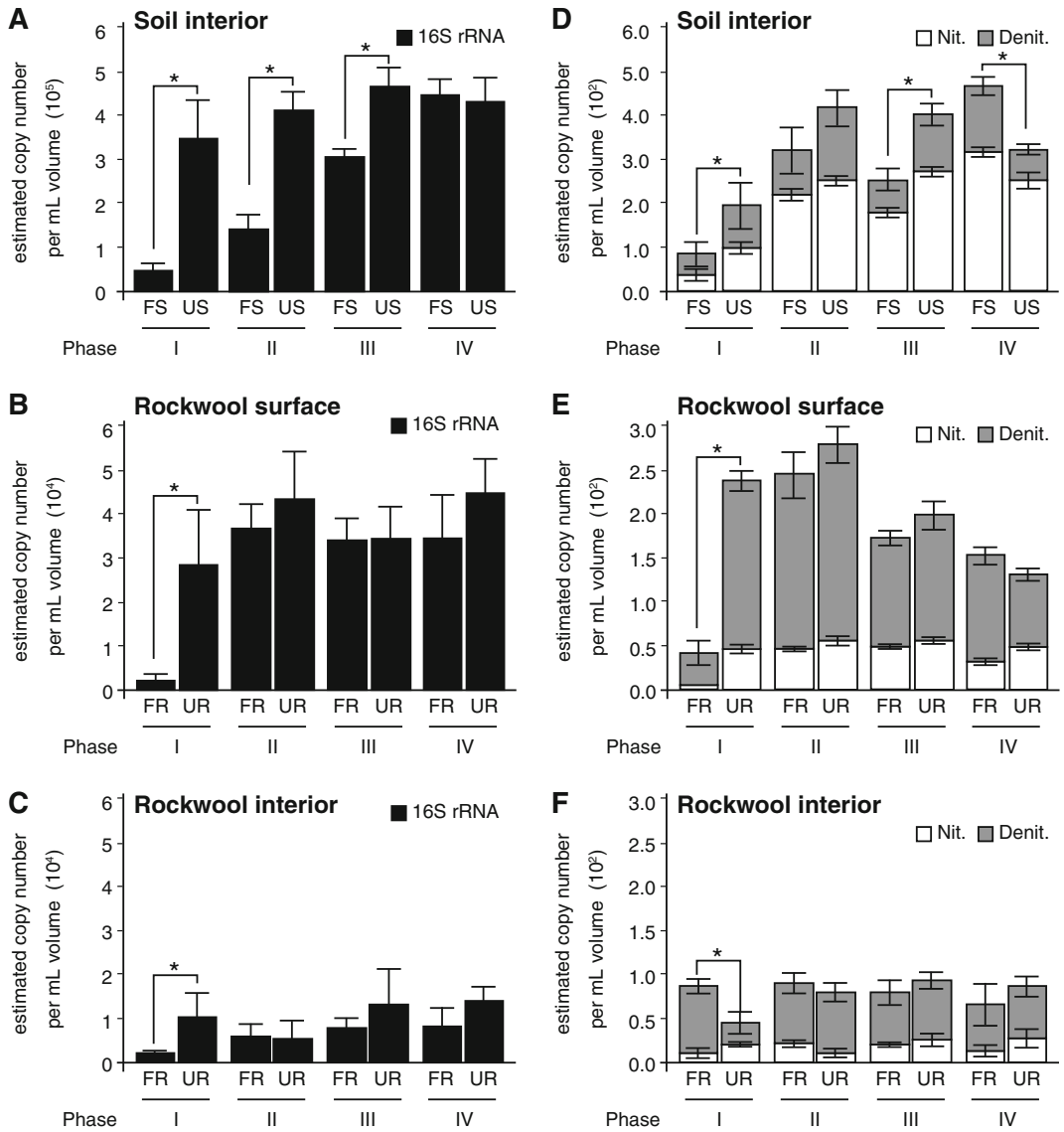

Fig. 3 Abundance of bacterial 16S rRNA gene and nitrogen-cycling associated genes in soil and rockwool. 16S rRNA gene, for quantifying the bacterial proliferation in soil and rockwool (**a**–**c**). Sum of the abundance of genes associated with nitrification (AOB, *amoA*, NSR, and FGPS) and denitrification (*nirK*, *nirS*, *norB*, and *nosZ*) (**d**–**f**). Gene abundance expressed as the number of gene copies per milliliter soil (**a** and **d**), rockwool surface (**b** and **e**), and rockwool interior (**c** and **f**). *Black*, *white*, and *gray bars* indicate 16S rRNA, nitrification (Nit.) and denitrification (Denit.), respectively. Mean values (± standard deviation) were calculated and significance from a *t* test analysis ($P<0.05$) is presented by the *asterisk* (*)

(AOB; r=0.268, *amoA*; r=0.182, *norB*; r=0.041, *nosZ*; r=0.331). The amount of nitrifiers and denitrifiers on the surface of rockwool was more abundant in UR than in FR during the early growth phase (Fig. 3e). In contrast, nitrifiers and denitrifiers inside the rockwool displayed little difference between FR and UR, and remained fewer than that on the surface of the rockwool (Fig. 3f). Therefore, the surface (<2-cm depth) rhizosphere-mediated nitrification or denitrification process may influence the emission of N_2O arising from hydroponics with rockwool, as observed in taro cultivation (Penton et al. 2013).

Based on the quantification results, it appeared that nitrification- or denitrification-associated genes were abundant when N_2O was vigorously released from the

cultivation material (see phases II–IV in Figs. 2f, 3e, f). However, N_2O was not consistently emitted, even when the numbers of nitrifiers and denitrifiers reached a plateau by phase II (see phase I in Figs. 2f, 3e, f). As in soil, functional gene abundance did not show any clear relationship to N_2O emission (AOB; r=0.312, *amoA*; r=0.266, *nirS*; r=−0.309, *nirK*; r=0.192, *norB*; r=0.275, *nosZ*; r=0.284).

Nitrogen nutrient status

In hydroponic culture, a substantial amount of inorganic nitrogen remains in cultivation materials when cultivating crops incompletely take up the supplied nitrogen fertilizers, such as N-NH_4 and N-NO_3. In our tomato hydroponic cultivation of exp. 2, the FR contained 1.6–9.8 mg/L N-NH_4 at the end of cultivation, while UR contained 1.2–15.5 mg/L N-NH_4 at this time-point (Fig. 4, NH_4). On the other hand, the level of N-NO_3 of UR was 3.8–655.9 mg/L at the end of cultivation, whereas FR contained 11.5–160.7 mg/L N-NO_3 (Fig. 4, NO_3). Based on these results, we estimated that, on average, more than 4.4 mg/L N-NH_4 and 85.0 mg/L N-NO_3 would be included in UR at the beginning of hydroponic cultivation of exp. 2 (dashed line in Fig. 4), because the nutrient solution at EC2.3 was used for exp. 1; thus, the tomato plants used in UR would have been exposed to a large amount of nitrogen fertilizer.

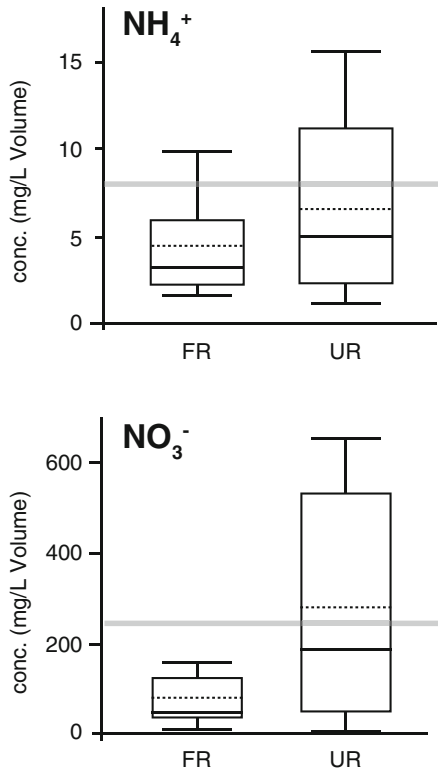

Fig. 4 Residual inorganic nitrogen in rockwool, supplied as nitrogen fertilizer at the end of cultivation. Ammonium and nitrate contents in rockwool at the end of cultivation were determined. Rockwool samples were randomly prepared from around the root zone and root debris removed before measurement. Data is shown as a 5-number summary (minimum, first quartile, median, third quartile, and maximum) for each line, with the mean denoted as a dashed line. Concentrations of ammonium and nitrate supplied in the second year cultivation are shown by the thick gray line

Discussion

Mitigation of GHG emission by hydroponics using rockwool

Currently, the plant industry and protected horticulture is aiming at stable production, quality improvement, and, in part, high yield. An increasing number of studies that focused on the environmental impact of horticulture have reported that GHG emissions from horticultural production are mainly due to the cultivation process, rather than the industrial production of cultivation materials: viz., electricity, fertilizer, biocides, and rockwool (Anton et al. 2005; Ingram and Thomas Fernandez 2012; Pluimers et al. 2000; Torrellas et al. 2012). Our study focused on the cultivation process and compared CO_2, CH_4, and N_2O emissions from conventional cultivation between soil and hydroponics using rockwool.

To summarize the GHG emissions during our tomato cultivation, CO_2 equivalents (CO_2-eq) of CH_4 (20-fold) and N_2O (310-fold) were estimated based on the heat-trapping effect per molecule (Fig. 5). The total GHG emissions from FR and FS, as CO_2-eq, averaged 853 g (exp. 1) and 999 g (exp. 2), and 1,303 g (exp. 1) and 1,509 g (exp. 2), respectively. FR (exp. 1) and FR (exp. 2) reproducibly showed lower total emissions than FS (exp. 1) and FS (exp. 2). Total CO_2-eq emission from UR and US amounted to 1,292 and 2,424 g, respectively. Thus, even if rockwool is repeatedly used for hydroponics, a lower environmental impact can be achieved, compared to that from conventional soil agriculture. The larger emission of CO_2-eq from soil than from rockwool resulted from CO_2 emission (Fig. 2b, e, and white bars in Fig. 5).

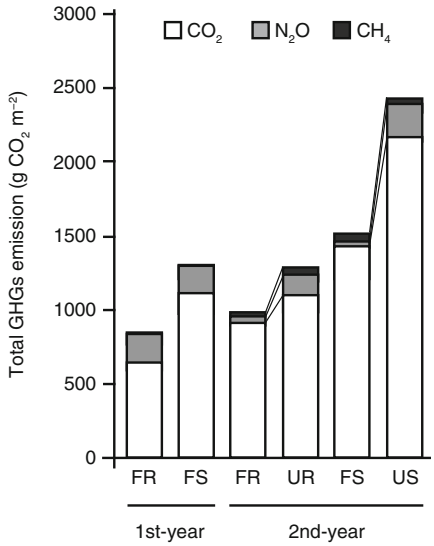

Fig. 5 Total CO_2 equivalent greenhouse gas emissions during tomato cultivation. Accumulating averaged emissions of CO_2 equivalents (CO_2-eq) of CH_4 (20-fold) and N_2O (310-fold) were calculated based on the heat-trapping effect per molecule (IPCC 2001). *White*, *gray*, and *black bars* indicate CO_2 emission, N_2O-CO_2-eq emission, and CH_4-CO_2-eq emission, respectively

Therefore, CO_2 emission was apparently reduced by hydroponic culture using rockwool; there was a 0.62-fold decrease using freshly prepared materials and 0.52-fold decrease utilizing used materials. Generally, soil contains a large pool of carbon compared to rockwool that is made from synthetic or natural minerals. In addition, 16S rRNA gene abundance in soil samples was 10-fold higher than rockwool samples (Fig. 3). Thus, a higher amount of CO_2 emission from soil can be simply explained by the large number of microbes and the resultant high microbial soil respiration rate (Wang et al. 2003). Moreover, a prominent difference between FS and US was the highest CO_2 emission of US at the beginning of cultivation (Fig. 2b). In fact, some root debris resulting from the first year culture undoubtedly remained, providing plenteous organic compounds (data not shown). Thus, even a single use of soil can influence CO_2 emission by increasing organic matter decomposition, and autotrophic and heterotrophic respiration. Noteworthy, re-use (long-term use) of rockwool demonstrated only slight increase in CO_2 emission (Fig. 5).

N_2O emission from hydroponics with rockwool

Compared to the N_2O proportion in the gaseous loss of N input in previous reports, which ranged from 0.001 to 6.8 % (Daum and Schenk 1996; Eichner 1990), reasonable proportions of the N input were estimated to be released from soil and rockwool, in the form of N_2O, in this study. The proportion of N_2O amounted to 4.9 % from FS (exp. 1), 3.8 % from FS (exp. 1), 4.1 % from FS (exp. 2), and 8.1 % from FR (exp. 2). The proportion of N_2O emitted from US and UR in exp. 2 amounted to 26.0 and 16.7 of the 2-year total N input. This estimation strongly suggest that some amounts of N input at EC2.3 of the first year cultivation remains inside the rockwool, and the remaining N compounds could be transformed to N_2O and be released into the atmosphere during the second year of cultivation. Therefore, we speculate that the dynamics of microbes in the rhizosphere, which converts the remaining N compounds to N_2O, may correlate with N_2O fluxes.

However, the relationships between N_2O emission and functional gene abundance were not significant. Neither the number of nitrifiers nor the number of denitrifiers directly contributed to increase in N_2O emission from hydroponics using rockwool. Thus, we concluded that microbial proliferation was necessary, but was not sufficient, for N_2O emission.

Interestingly, nitrifiers dominated in soil, whereas denitrifiers dominated in rockwool (Fig. 3d–f). These results suggested that the underlying process enabling N_2O emission from rockwool may differ from that of soil cultivation, so that distinct guidelines are required for reducing N_2O emission from hydroponic cultures. One of the important factors can be substrate availability, rather than functional gene abundance. The possibility was confirmed by our results that (a) hydroponic UR maximized yields (Figs. 1) and (b) hydroponic UR contained higher amount of N compounds (Fig. 4). Firstly, the increased growth and yield in UR strongly suggested increased N availability due to the remaining N compounds supplied from the growth media reused from exp. 1. Unlike UR, US culture was not different from FS in terms of fruit yield. This may be due to differences in the rhizosphere regions, such as labile N availability from soil particle, environmental differences from rockwool, microbial numbers, and community (Fig. 3). Secondly most tomatoes planted in FR (exp. 2) seemed to efficiently use the supplied nitrogen fertilizer at EC1.3, because the rockwool at the end of FR cultivation (exp. 2) contained much lower concentrations of N-NH_4 and N-NO_3 (4.4 and 85.0 mg/L, respectively) that that in the fertilizer solution (7.6 and 250 mg/L, respectively; thick gray line in Fig. 4).

Excess N-NH_4 and N-NO_3 accumulated in UR during the hydroponic cultivation period (6.7 and 280.7 mg/L, respectively). Thus, the residual N-NO_3 in UR consequently demonstrated an average of 3.8-fold and a median of 4.6-fold increase inside the rockwool beds by the end of cultivation. On the other hand, UR contained only 1.3-fold the N-NH_4 levels at the end of cultivation. Therefore, an explanation why UR increased N_2O emission could be the large amount of residual N-NO_3.

Consequently, UR released 3.8-fold higher N_2O-CO_2-eq than FR (gray bars in Fig. 5). This result indicates that long-term use of rockwool without optimal fertilization and cultivation management has the potential to accumulate excess amount of N-NO_3, which could be released as N_2O via microbial-denitrification, resulting in massive amounts of N_2O emission in future. In order to reduce GHG emission from tomato hydroponics using rockwool, management of N fertilizer application is a plausible further target for improvement aimed at mitigation of N_2O emission, so further lowering the environmental impacts of horticulture. An alternative way to achieve this is by enzymatic reduction of N_2O to N_2 by activation of N_2O reductase (N_2OR) (Itakura et al. 2008). This process does not promote consumption of N fertilizer by microbes, so that no negative effects on crop production would be expected. Inoculation of rockwool with a N_2OR-fortified rhizobium could mitigate N_2O emission by increasing N_2 emission from hydroponic cultures, as occurs in soil systems (Itakura et al. 2012).

Conclusions

Our findings demonstrated that hydroponics using rockwool is capable of producing substantial fruit yield, while lowering CO_2 emission by suppressing microbial proliferation in the rhizosphere. Dilution of the nutrient solution from EC2.3 to EC1.3 significantly decreased N_2O emission. Nitrification- and denitrification-associated bacterial proliferation in the rhizosphere of freshly prepared rockwool reached a plateau in the first 4 weeks, without marked N_2O emission. Re-use (long-term use) of rockwool resulted in increased fruit yields and markedly accelerated N_2O emission from hydroponic cultures by accumulating excessive ammonium and nitrate. We concluded that the rockwool environment, rather than the number of nitrifiers and denitrifiers governs N_2O fluxes. Further research is required to investigate the effect of the substrate used on N_2O production, its dynamics during hydroponic cultivation, and its relation to N_2O fluxes. Future studies may facilitate optimization of fertilizer application to minimize GHG emission from hydroponic culture.

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References

Acton SD, Baggs EM (2011) Interactions between N application rate, CH4 oxidation and N2O production in soil. Biogeochemistry 103:15–26

Ahn JH, Kim MC, Shin HC, Choi MK, Yoon SS, Kim T, Song HG, Lee GH, Ka JO (2006) Improvement of PCR amplification bias for community structure analysis of soil bacteria by denaturing gradient gel electrophoresis. J Microbiol Biotechnol 16:1561–1569

Ahn JH, Kim S, Park H, Rahm B, Pagilla K, Chandran K (2010) N2O emissions from activated sludge processes, 2008–2009: results of a national monitoring survey in the United States. Environ Sci Technol 44:4505–4511

Ahn JH, Kwan T, Chandran K (2011) Comparison of partial and full nitrification processes applied for treating high-strength nitrogen wastewaters: microbial ecology through nitrous oxide production. Environ Sci Technol 45:2734–2740

Alam MS, Jia Z (2012) Inhibition of methane oxidation by nitrougenous fertilizers in a paddy soil. Front Microbial 3:1–13

Anton A, Montero JI, Munoz P, Castells F (2005) LCA and tomato production in Mediterranean greenhouses. Int J Agr Res Gov Eco 4:102–112

Berendsen RL, Pieterse CM, Bakker PA (2012) The rhizosphere microbiome and plant health. Trends Plant Sci 17:478–486

Bergsma TT, Robertson GP, Ostrom NE (2002) Influence of soil moisture and land use history on denitrification end-products. J Environ Qual 31:711–717

Braker G, Fesefeldt A, Witzel KP (1998) Development of PCR primer systems for amplification of nitrite reductase genes (nirK and nirS) to detect denitrifying bacteria in environmental samples. Appl Environ Microbiol 64:3769–3775

Daum D, Schenk MK (1996) Gaseous nitrogen losses from a soilless culture system in the greenhouse. Plant soil 183:69–78

Degrange V, Bardin R (1995) Detection and counting of *Nitrobacter* populations in soil by PCR. Appl Environ Microbiol 61:2093–2098

Dionisi HM, Layton AC, Harms G, Gregory IR, Robinson KG, Sayler GS (2002) Quantification of *Nitrosomonas oligotropha*-like ammonia-oxidizing bacteria and *Nitrospira* spp. from full-scale wastewater treatment plants by competitive PCR. Appl Environ Microbiol 68:245–253

Duineveld BM, Rosado AS, Van Elsas JD, Van Veen JA (1998) Analysis of the dynamics of bacterial communities in the

rhizosphere of the *Chrysanthemum* via denaturing gradient gel electrophoresis and substrate utilization patterns. Appl Environ Microbiol 64:4950–4957

Eichner MJ (1990) Nitrous oxide emissions from fertilized soils: summary of available data. J Environ Quality 19:272–280

Hwang S, Hanaki K (2000) Effects of oxygen concentration and moisture content of refuse on nitrification, denitrification, and nitrous oxide production. Bioresour Technol 71:159–169

Ibekwe AM, Poss JA, Grattan SR, Grieve CM, Suarez D (2010) Bacterial diversity in cucumber (*Cucumis sativus*) rhizosphere in response to salinity, soil pH, and boron. Soil Biol Biochem 42:567–575

Inamori R, Wang Y, Yamamoto T, Zhang J, Kong H, Xu K, Inamori Y (2008) Seasonal effect on N2O formation in nitrification in constructed wetlands. Chemosphere 73:1071–1077

Ingram DL, Thomas Fernandez R (2012) Life cycle assessment: a tool for determining the environmental impact of horticultural crop production. Hort Technology 22:275–279

IPCC (2001) The supplementary report to the IPCC scientific assessment. Cambridge University Press, Cambridge

IPCC (2007) Climate change. Cambridge University Press, Cambridge, UK

Itakura M, Tabata K, Eda S, Mitsui H, Murakami K, Yasuda J, Minamisawa K (2008) Generation of *Bradyrhizobium japonicum* mutants with increased N2O reductase activity by selection after introduction of a mutated dnaQ gene. Appl Environ Microbiol 74:7258–7264

Itakura M, Uchida Y, Akiyama H, Takada-Hoshino Y, Shimomura Y, Morimoto S, Tago K, Wang Y, Hayakawa C, Uetake Y, Sanchez C, Eda S, Hayatsu M, Minamisawa K (2012) Mitigation of nitrous oxide emissions from soils by *Bradyrhizobium japonicum* inoculation. Nature clinate change 11:1–5

Kandeler E, Deiglmayr K, Tscherko D, Bru D, Philippot L (2006) Abundance of narG, nirS, nirK, and nosZ genes of denitrifying bacteria during primary successions of a glacier foreland. Appl Environ Microbiol 72:5957–5962

Kennedy TL, Suddick EC, Six J (2013) Reduced nitrous oxide emissions and increased yields in California tomato cropping systems under drip irrigation and fertigation. Agric Ecosyst Environ 170:16–27

Kool DM, Dolfing J, Wrage N, Van Groenigen JW (2011) Nitrifier denitrification as a distinct and significant source of nitrous oxide from soil. Soil Biol Biochem 43:174–178

Kowalchuk GA, Stephen JR, de Boer W, Prosser JI, Embley TM, Woldendorp JW (1997) Analyses of ammonia-oxidizing bacteria of the β subdivision of the class Proteobacteria in coastal sand dunes bydenaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl Environ Microbial 63:1489–1497

Li K, Gong Y, Song W, He G, Hu Y, Tian C, Liu X (2012) Responses of CH(4), CO(2) and N(2)O fluxes to increasing nitrogen deposition in alpine grassland of the Tianshan Mountains. Chemosphere 88:140–143

Liu X, Chen CR, Wang WJ, Hughes JM, Lewis T, Hou EQ, Shen J (2013) Soil environmental factors rather than denitrification gene abundance control N2O fluxes in a wet scerophyll forest with different burning frequency. Soil Biol Biochem 57:292–300

Nkongolo NV, Hatano R, Kakembo V (2010) Diffusivity models and greenhouse gases fluzes from a forest, pasture, grasslans and corn field in northern Hokkaido, Japan. Pedosphere 20:747–760

Penton CR, Deenik JL, Popp BN, Bruland GL, Engstrom P, Louis DS, Tiedje J (2013) Importance of sub-surface rhizosphere-mediated coupled nitrification-denitrification in a flooded agroecosystem in Hawaoo. Soil Biol Biochem 57:362–373

Pluimers JC, Kroeze C, Bakker EJ, Challa H, Hordijk L (2000) Quantifying the environmental impact of production in agriculture and horticulture in The Netherlands: which emissions do we need to consider? Agric Syst 66:167–189

Postma J, Geraats BPJ, Pastoor R, van Elsas JD (2005) Characterization of the microbial community involved in the suppression of *Pythium aphanidermatum* in cucumber grown on rockwool. Phytophathology 95:808–818

Robertson GP, Paul EA, Harwood RR (2000) Greenhouse gases in intensive agriculture: contributions of individual gases to the radiative forcing of the atmosphere. Science 289:1922–1925

Rolston DE (1986) Method of Soil Analysis, Part 1, Physical and Mineralogical Methods, 2nd ed., Agronomy Monograph. pp. 1103–1119

Rotthauwe JH, Witzel KP, Liesack W (1997) The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microbiol 63:4704–4712

Ryu DK, Chung SO, Park SU, Kim SJ, Lim YP (2012) Construction and operatinoal test of a plant factory. In American Society of Agricultural and Biological Engineers Annual International Meeting 2012:5497–5505

Throback IN, Enwall K, Jarvis A, Hallin S (2004) Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol Ecol 49:401–417

Tiedje JM (1988) Ecology of denitrification and dissimilatory nitrate reduction to ammonium. John Wiley & Sons, New York

Torrellas M, Anton A, Lopez JC, Baeza EJ, Parra JP, Munoz P, Montero JI (2012) LCA of a tomato crop in a multi-tunnel greenhouse in Almeria. Int J Life Cycle Assess 17:863–875

Torrellas M, Anton A, Montero JI (2013) An environmental impact calculator for greenhouse production systems. J Environ Manage 118:186–195

Van't Oster A, Van Ieperen W, Kalaitzoglou P (2012) Model study on applicability of a semi closed greenhouse concept in Almeria: effects on greenhouse climate, crop yield and resource use efficiency. Acta Horticulture 927:51–58

Wang WJ, Dalal RC, Moody PW, Smith CJ (2003) Relationship of soil respiration and microbial biomass, substrate availability and clay content. Soil Biol Biochem 35:273–284

Wang Y, Inamori R, Kong H, Xu K, Inamori Y, Kondo T, Zhang J (2008) Nitrous oxide emission from polyculture constructed wetlands: effect of plant species. Environ Pollut 152:351–360