UTILIZATION OF AZOLLA IN RICE CULTIVATION FOR CLIMATE CHANGE ADAPTATION AND MITIGATION

By

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Declaration of Originality

I, Samuel Munyaka KIMANI hereby declare that this dissertation entitled “Utilization of Azolla in Rice Cultivation for Climate Change Adaptation and Mitigation” is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not currently submitted in candidature of any other degree. Any contribution made to the research by others with whom I have worked with, is explicitly acknowledged in the dissertation.

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Abstract

Rice (*Oryza sativa L.*) is one of the most important crops in the world. And with a predicted population increase in the near future, an increase in rice production to meet its demands is inevitable. This projected rise in production however faces several threats occasioned by climate change. These threats mainly include; (1) irrigation water shortages as a result of competition for water from other uses as well as the threat of droughts, (2) soil fertility and organic matter loss as a consequence of chemical fertilizer overuse and low manure amendments, and (3) increasing global warming as a result of anthropogenic greenhouse gas (GHGs) emissions. However, rice is not just a victim of climate change but also a major contributor, as paddy fields are considered the most important sources of anthropogenic GHGs; methane (CH_4) and nitrous oxide (N_2O) emissions. Therefore, adaptation and mitigation strategies are needed to cope with the effects of climate change on rice production as well as reduce water loss and GHGs emissions from rice fields.. Azolla is a genus of small aquatic ferns that is naturally found in temperate and tropical regions worldwide, particularly in constantly flooded paddy ecosystems. Through its symbiosis with the cyanobacterium *Anabaena azollae*, Azolla is capable of fixing atmospheric nitrogen (N) and has thus been successfully utilized as green manure in lowland rice fields. However, the mitigation efficiency of Azolla on GHGs emissions from rice paddies remains contradictory. Furthermore, literature on its influence on water loss remains scarce. Within this context, this research study aimed to determine the potential of Azolla application not only as a promising alternative to chemical fertilizers but also as a possible water saver and a likely management practice to decrease the CH_4 and N_2O emissions from flooded paddy ecosystems.

First, to determine the influence of the rapidly growing Azolla cover on evapotranspiration (ET), two independent pot experiments were conducted in an incubation chamber (experiment 1) and greenhouse (experiment 2). The results showed that Azolla cover significantly decreased ET losses on average by 17.3% (experiment 1) and 20.0% (experiment 2) compared with open water surfaces and both open water surfaces and green polyester covered mats (analogous to plant cover), respectively. The ET reduction potential by Azolla in both experiments was attributed to, but not limited to, its anatomy, horizontal placement of its leaves, and smaller leaf area, which possibly restricted simultaneous evaporation-transpiration losses by shielding much of the water surface.

Second, to investigate the effect of Azolla cover on simultaneous CH_4 and N_2O emissions from the constantly flooded rice paddies, an outdoor pot experiment was setup in a single rice-growing season in 2016. Two treatments, control (rice plant only) and Azolla cover (rice plus Azolla covering on the flooding water) were established in four replications. The bulk alluvial soil used in this experiment was collected from a rice field at the University Farm. Results showed that dual cropping of Azolla with rice significantly suppressed CH_4 emissions by 34.7% compared with

the control, likely due to an increase in dissolved oxygen concentration and redox potential at the soil-water interface between the flooding water and soil surface. However, the Azolla cover did not significantly affect N_2O emissions from both treatments. This implied that Azolla cover did not affect extra N_2O flux from dual Azolla and rice cropping ecosystems.

Third, to investigate the influence of Azolla incorporation as green manure and its subsequent growth as a dual crop in conjunction with chemical fertilizers, on CH_4 and N_2O emissions from flooded paddy soil planted with rice, an outdoor pot experiment was setup in a single rice-growing season in 2017 with three treatments, chemical fertilizer (NPK) as control, incorporation of Azolla as green manure (AGM), and AGM plus basal chemical fertilizer (NPK + AGM). Results showed that AGM and NPK + AGM treatments significantly increased seasonal CH_4 emissions by 31.5% and 43.5%, and decreased seasonal N_2O emissions 3.4 and 4.6 folds compared to NPK, respectively. Both the CH_4 increase and N_2O decrease were attributed to the effect of the incorporated Azolla particularly at the early rice growth stages. Significantly higher grain yields were observed under AGM (32.5%) and NPK+ AGM (36.3%) compared to NPK. There were no significant differences in the CH_4 emissions per grain yield among treatments, however, compared to NPK, AGM and NPK+ AGM treatments significantly reduced N_2O emissions per grain yield by 78.7% and 84.1%, respectively.

Fourth, in the same batch of experiments as highlighted in the third section above, we investigated the effects of poultry-litter biochar (hereinafter biochar) amendment and its co-application with NPK and AGM (i.e., NPK + biochar and AGM + biochar) on the simultaneous CH_4 and N_2O emissions. The main objective was to determine the influence of AGM (incorporated as green manure and its successive growth as a cover) co-applied with biochar in lowland rice paddies on simultaneous CH_4 and N_2O emissions. Results showed that compared with AGM and NPK + biochar treatments, AGM + biochar did not significantly influence cumulative CH_4 emission during the whole rice growth period. Conversely, AGM + biochar significantly reduced N_2O emissions by 76.4%-95.9% compared with the other treatments, with a significantly high interaction ($P < 0.01$) between biochar and fertilizer amendments. Additionally, compared with all other treatments, AGM + biochar significantly increased rice grain yield by 27.3%–75.0%, and consequently, decreased both yield equivalent CH_4 emissions by 24.7%–25.0% and N_2O emissions by 81.8%–97.7%. These results suggest that the co-application of Azolla and biochar offers a novel approach to increase yield while mitigating CH_4 and N_2O emissions.

Fifth, to determine the effect of biochar application and its co-treatment with NPK and/or Azolla as green manure on rice yield, N uptake, and N use efficiency, eight treatments were compared; no amendment (control), NPK, AGM, NPK+ AGM, without and with biochar amendment. Biochar was the main factor, with fertilizer N sources (NPK and AGM) as the sub-

factors. Results showed biochar amendment significantly increased grain yield (32.4%), grain N uptake (23.9%), apparent N recovery efficiency (28.1%), agronomic N efficiency (50.0%), and internal N utilization efficiency (35.9%), and decreased the soil N dependence rate (-15.2%) compared with the without biochar amended treatments. No significant synergistic interactions between biochar and the fertilizer N sources were observed on all determined parameters in this study setup.

In conclusion, the utilization of Azolla in rice cultivation has the potential to reduce chemical fertilizer application needs and irrigation water, increase rice yield, and reduce and/or mitigate CH_4 and N_2O emissions. However, these results were based on pot experiment setups in the laboratory and glasshouse, and on in situ outdoor setups during single rice cropping systems. Thus, long-term field studies should be carried out to validate the findings.

要旨和訳

イネは世界で最も重要な作物の 1 つであり、人口増加に伴うコメの増産は避けられない。しかし、コメの増産は、気候変動から幾つかの脅威に直面している。それらの脅威は主に次の 3 つが挙げられる。（1）他の用途からの水との競争および干ばつや降水パターンらが齎した灌漑用水の不足、（2）過剰な化学肥料と少ない有機物資材の施用による土壌肥沃度の低下、（3）増加し続ける温室効果ガスの人為的な排出による地球温暖化。また、水田は温室効果ガスのメタン（CH_4）と一酸化二窒素（N_2O）の重要な発生源であるため、稲作は気候変動の被害者だけではなく、気候変動の加害者でもある。従って、稲作における気候変動の適応と緩和戦略として、節水栽培と温室効果ガスの削減は必要である。アゾラは、温帯および熱帯地域に分布する水生シダ植物で、藍藻の *Anabaena azollae* との共生関係を持ち、大気中の窒素を固定できるため、昔から水田の緑肥として利用されてきた。しかし、今までの研究で、水田からの温室効果ガス放出量に及ぼすアゾラの影響は相違があり、またアゾラ施用の節水効果に関する研究はまだ報告されていない。上述の研究背景を踏まえ、本研究では、稲作生産における化学肥料の代わりになる有機質肥料だけでなく、節水栽培と温室効果ガス CH_4 と N_2O 排出量を削減するために、アゾラ応用の可能性を明らかにすることを目的とした。

第一に、水面からの蒸発散量（ET）に及ぼすアゾラの影響を調べるために、2 つのポット実験を人工培養器（実験 1）と温室（実験 2）で行った。アゾラに覆われているポットからの ET は、覆われていないポットとグリーンポリエステルマット（植物カバーの類似物）に覆われているポットからの ET より、実験 1 で 17.3％、実験 2 で 20.0％有意に減少したことを示した。アゾラに覆われている両方のポット実験における ET の減少は、アゾラ葉の水平配置および小さい葉面積など解剖学的構造の特徴により、アゾラに覆われている水面からの蒸発と蒸散は同時に制限されることを示唆した。

第二に、常時湛水の水田土壌からの CH_4 と N_2O の放出量に及ぼすアゾラ被覆の影響を調査するために、対照（稲のみ）とアゾラ被覆の 2 つの処理区を設け、4 反復の室外ポット実験を 2016 年に行った。実験用土壌は山形大学農場から採集した沖積土であった。その結果、アゾラに覆われている被覆区の水田土壌からの CH_4 排出量は、対照区と比べると 34.7％で有意に抑制された。これは、アゾラ被覆区の表水層と土壌表面の間に溶存酸素濃度と酸化還元電位がアゾラ被覆によって上昇され、CH_4 の酸化が促進されたと考えられる。一方、両処理区からの N_2O 放出量は大きな差がなく、アゾラの被覆は N_2O 放出量に与える影響がなかったことを示唆した。

第三に、アゾラを緑肥として水田土壌にすき込んだ後、引き続きアゾラに覆われている水田土壌からの CH_4 と N_2O の放出量を調査した。対照の化学肥料（NPK）、アゾラをすき込み（AGM）、AGM と化学肥料両方施用（NPK+AGM）の 3 つの処理区も設け、2017 年に室外ポット実験を行った。NPK と AGM 区の窒素施肥は、同量であった。その結果は、イネ生育期間中に、AGM および NPK+AGM 処理区から CH_4 放出量は、NPK と比べると、31.5％および 43.5％有意に増加したが、逆に N_2O 放出量は、それぞれ 3.4 倍および 4.6 倍で有意に減少した。CH_4 の増加と N_2O の減少は、主にイネの初期成長段階で生じたもの

で、すき込んだアゾラに影響されたと考えられる。しかし、NPK と比べると、AGM と NPK+AGM 区の籾収量は、それぞれ 32.5％と 36.3％で、有意に高かったため、処理間で籾収量あたりの CH_4 放出量は、3 処理区の間に有意差がなかった。一方、籾収量あたりの N_2O 放出量は、AGM と NPK+AGM 処理区が、NPK よりそれぞれ 78.7％と 84.1％で有意に削減された。

第四に、アゾラと家禽厩肥からできたバイオチャー（以下にバイオチャと省略）の同時施用は、どのように水田土壌からの CH_4 と N_2O の放出量に与える影響を明らかにするため、上記の第三実験の NPK 区と AGM 区と合わせて、バイオチャー同時施用区（NPK ＋バイオチャー区と AGM ＋バイオチャー区）を加えて、第三実験と同様な調査を行った。その結果は、NPK+バイオチャー区と AGM と比較して、生育期間中に AGM+バイオチャー区からの CH_4 放出量は、有意な差がなかった。逆に、AGM ＋バイオチャー区は、他の処理と比較して N_2O 排出量が 76.4％～95.9％で有意に削減された。バイオチャーとアゾラ施用の相互作用も有意であった（$P < 0.01$）。さらに AGM ＋バイオチャー区の籾収量は、他処理区より 27.3％～75.0％で有意に増加し、次第に籾収量あたりの CH_4 放出量は 24.7％～25.0％で、籾収量あたりの N_2O 排出量も 81.8％～97.7％で減少した。これらの結果は、アゾラとバイオチャーの同時施用は、イネの収量の増加や CH_4 と N_2O 放出量の削減に効果的なアプローチであることを示唆した。

第五に、バイオチャーとアゾラ緑肥の同時施用は、どのようにイネの収量、窒素吸収量、窒素利用率に及ぼす影響を明らかにするため、8 つの処理のポット実験を用いて、２つ因子の統計解析を行った。処理区には、無施肥（対照）、化学肥料施用（NPK）、アゾラ緑肥施用（AGM）、AGM と化学肥料両方施用（NPK+AGM）の４つの処理区に、バイオチャーを施用した４つの処理区を加えた。バイオチャーが主因子とし、その他が副因子とした。その結果は、バイオチャーの施用により、籾収量（32.4％）、籾窒素吸収量（23.9％）、窒素の見かけの回収率（28.1％）、実用施肥窒素効率（50.0％）、内部窒素利用効率（35.9％）らは、有意に増加した。一方、土壌 N 依存率（-15.2％）が減少した。なお、バイオチャーと他肥料因子の間に有意な相互作用は認められなかった。

以上のことから、稲作におけるアゾラの利用は、CH_4 と N_2O の放出量を軽減または緩和しながら、化学肥料の施用量と用水量を減らし、米の収量を増やすのは、可能であることが示唆された。ただし、これらの結果は、それぞれ単年度の室内外のポット実験に基づいたもので、実際の水田圃場での連続的な長年調査はさらに必要である。

Abbreviations

Abbreviation	Full name
GHGs	Greenhouse gases
CH_4	Methane
CO_2	Carbon (IV) oxide
N_2O	Nitrous (IV) oxide
CO_2eq	Carbon (IV) oxide equivalent
GWP	Global warming potential
IPCC	Intergovernmental Panel on Climate Change
NH_4^+-N	Ammonium nitrogen
NO_3^--N	Nitrate nitrogen
TN	Total nitrogen
SOC	Soil organic carbon
EC	Electrical conductivity
RGR	Relative growth rate
T_d	Biomass doubling time
NHI	Nitrogen harvest index
ARE_N	Apparent recovery efficiency
SNDR	Soil nitrogen dependent rate
AE_N	Agronomic efficiency
PE_N	Physiological efficiency
IUE_N	Internal utilization efficiency
PFP_N	Partial factor productivity

CHAPTER I

1. General Introduction

1.1. Rice Production and Global Climate Challenges

In recent years, research emphasis on global climate change and the relationship and potential influence of these changes on rice farming has gained momentum. Rice (*Oryza sativa* L.) is a major source of dietary protein for over half of the world's population and is the second most important cereal crop in the world, after maize alone. With a predicted population growth to nearly 9 billion over the next 20 to 30 years, demand for rice production will have to increase by 25% by 2050 to meet the anticipated increase in population. Climate change, however, is projected to have a major effect on rice production (Chauhan et al., 2017).

According to the Intergovernmental Panel on Climate Change (IPCC), the accumulation of greenhouse gases (GHGs) in the atmosphere and particularly methane and nitrous oxide gases has caused changes in the global climate. Methane (CH_4) has a relative global warming potential (GWP) of 34 times that of carbon dioxide (CO_2) over 100 years and is emitted during the production and transport of coal, natural gas, and oil, as well as from livestock, natural wetlands, anthropogenic activities, and from biomass burning. On other hand, the release of nitrous oxide (N_2O) with a relative GWP of 298 times that of CO_2 at a period of 100 years is mainly due to; (1) agricultural practices such as the inputs of synthetic nitrogen fertilizers and animal manure application, and (2) industrial activities related to the combustion of fossil fuels. Overall, CH_4 and N_2O emissions contribute approximately 20% and 7%, respectively, to the global radiative forcing. Hence, the continued release and accumulation of these two gases in the atmosphere could lead to substantial modifications in both land and water resources for rice production as a result of; (1) increases in global temperature, (2) rising sea levels and, (3) changes in rainfall patterns and distribution in different regions globally. Additionally, the rising sea levels and temperatures are expected to exacerbate the threat of water scarcity on rice agriculture by increasing the rates of evaporation (E) and evapotranspiration (ET) from open water sources and vegetation, respectively, as a result of (1) less rainfall, (2) lack of cloud cover, and, (3) low humidity levels. On the contrary, rice agriculture is not only a victim of global climate change but also a leading cause.

Lowland rice fields make up to about 55% of the global harvested area and are notably one of the largest anthropogenic sources of atmospheric CH_4 and N_2O emissions, with an estimated global CH_4 emission rate of 25-60 Tg yr-1 and an annual global N_2O contribution of 13-24% (IPCC 2013). As reviewed by (Malyan et al., 2016) CH_4 production is the terminal step of bacterial degradation of complex organic matter under anaerobic soil conditions (**Fig. 1.1**). This process is known as methanogenesis and the bacteria and/or archaea involved are referred to as methanogens. Total CH_4 emissions from constantly flooded rice paddies are determined by CH_4 production, oxidation, and transport. These processes are dependent on factors like; (1) soil organic matter content, (2) soil pH,

(3) soil texture, (4) redox potential of soil, (5) fertilizers application, and (6) soil temperature, (7) rice cultivar, and (8) water management. CH_4 made in the soil is transported and emitted to the atmosphere through three possible mechanisms; (1) ebullition (bubble), (2) diffusion through the paddy water column, or (3) through the rice plants (aerenchyma).

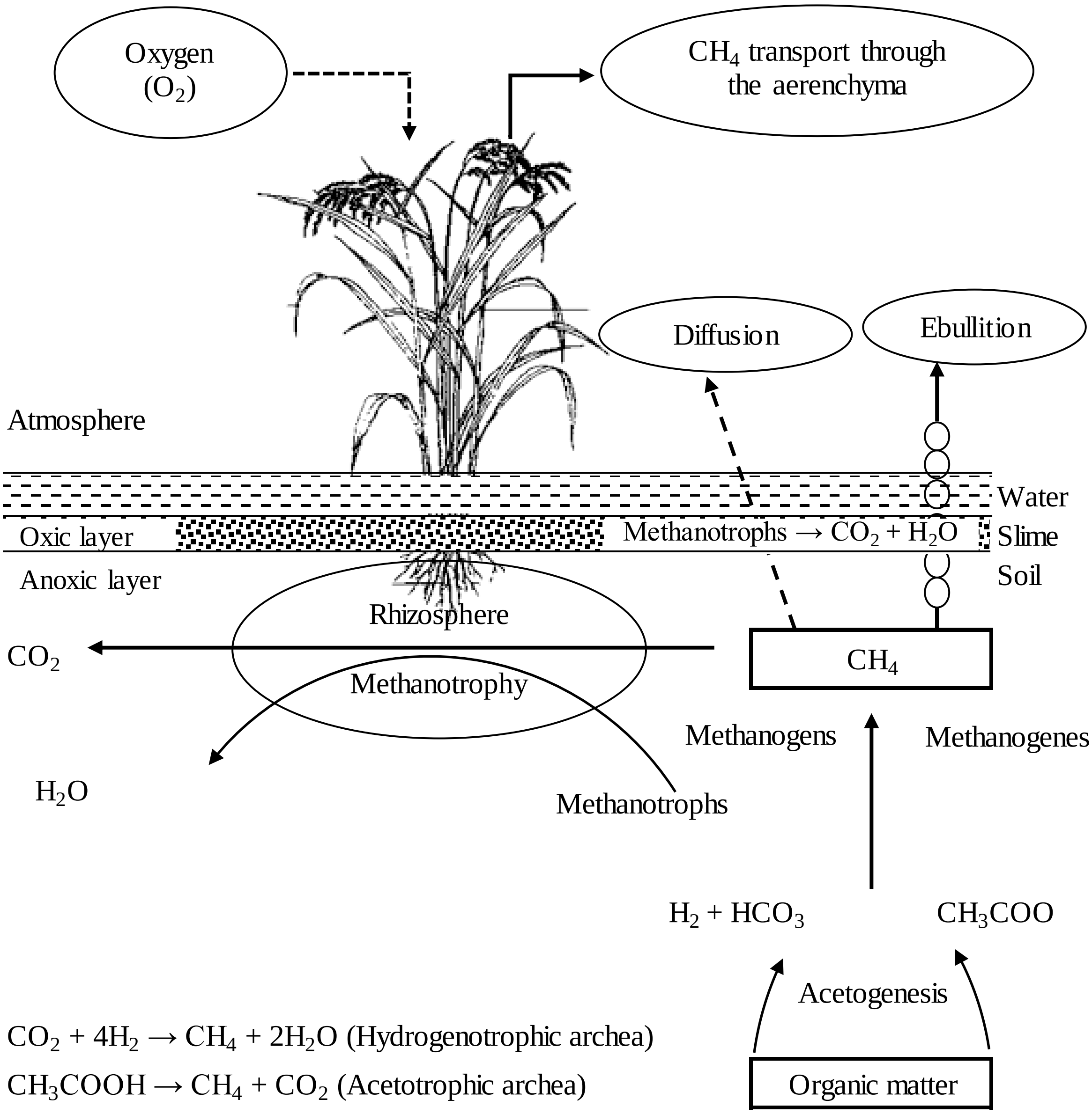

Fig. 1.1. Conceptual schematic diagram of methane production, oxidation, and emission from paddy field (Source: Dubey, 2005).

Nitrous oxide (N_2O) production is primarily a byproduct of (1) nitrification -the aerobic microbial oxidation ammonium (NH_4^+) to nitrate (NO_3^-)- and, (2) an intermediate product of denitrification -the anaerobic microbial reduction of NO_3^- to nitrogen gas (N_2) (**Fig. 1.2**)- (Signor and Cerri, 2013). Although upland fields are recognized as the major sources of N_2O emission, continuously flooded paddy soils are considered among the important sources of N_2O emission resulting from high amounts of N fertilization and during the mid-season drainage and dry-wet episodes (Cai et al., 1997; Chen et al., 1997). Therefore, the projected rice production increase will predictably be accompanied by an increase in CH_4 emissions as well as a proportionate increase in N_2O emissions following among other factors the increased chemical fertilizer use.

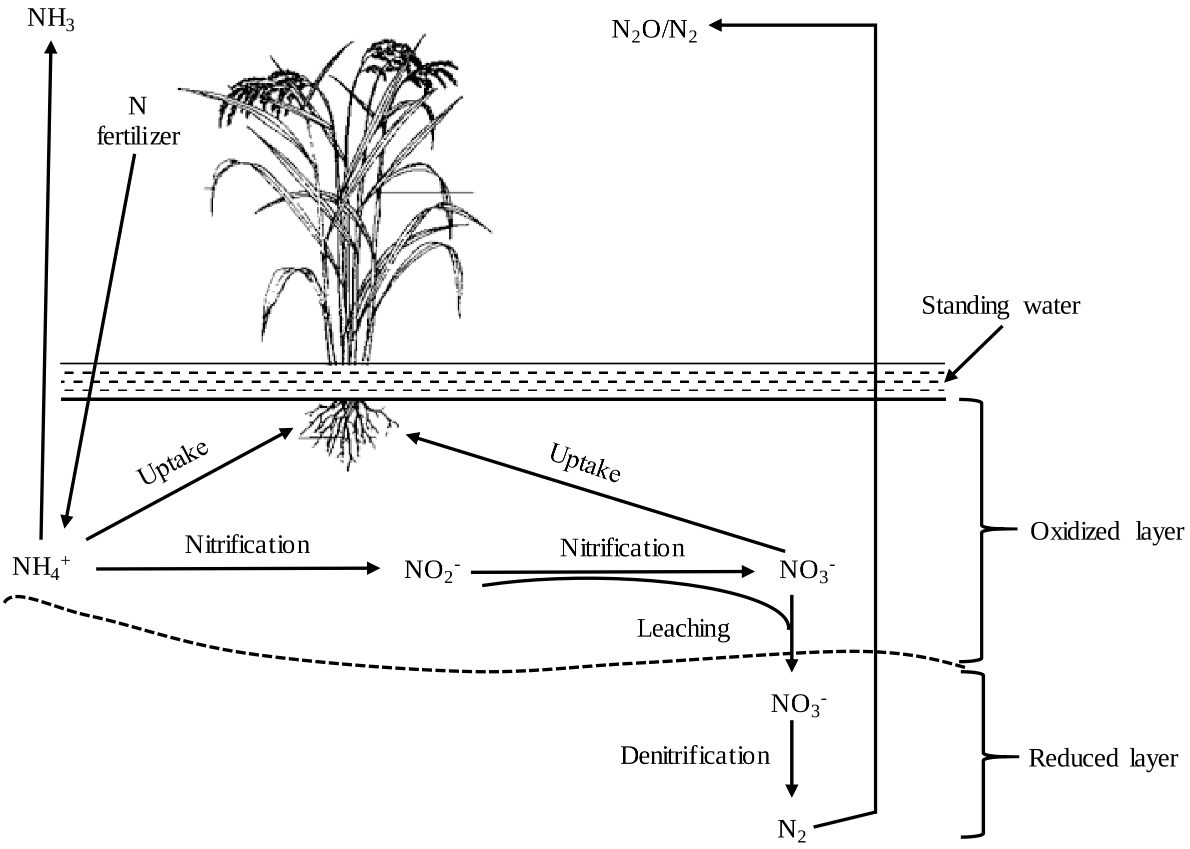

Fig. 1.2. Schematic representation of nitrogen (N) transformation in lowland rice soil.

Lowland rice fields account for about 75% of global rice production annually and are thus among the major consumers of global freshwater utilizing about 24-30% of the total world’s accessible freshwater. With the predicted “physical” and “economic” irrigation water scarcity in the near future in most rice-producing countries, the increase in rice production is expected to require high efficiency of water use (Tuong and Bouman, 2003). Currently, no proportional increase in water productivity is observed relative to rice productivity. Additionally, as rice is mostly grown under ponded conditions, its consumptive water use (herein ET) per unit area is higher compared to maize and wheat -two other most important world cereal crops (Bouman et al., 2007). Moreover, due to its ponded nature, the paddy field has a water-balance different from that of dryland crops. This water balance consists of inflows by irrigation, rainfall, and capillary rise, and outflows by seepage and percolation, as well as transpiration (T) and evaporation (E)-herein taken as evapotranspiration (ET), since E and T water losses are difficult to measure separately in the field (**Fig. 1.3**) (Djaman et al., 2017). Thus, with an estimated increase in rice production to meet the demand of the growing population, the demand for irrigation water will inevitably increase and subsequently lead to an increase in ET losses.

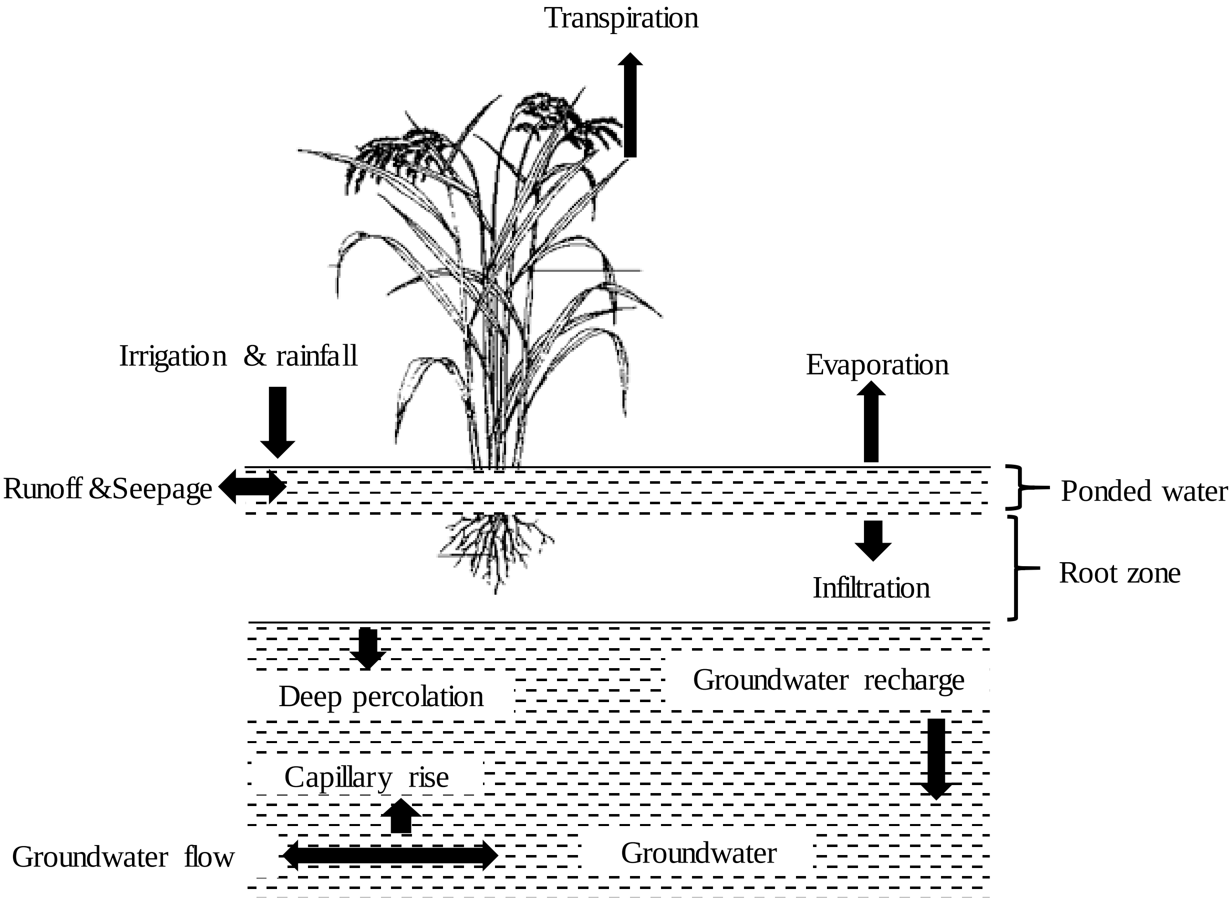

Fig. 1.3. Schematic representation of the water balance of a flooded rice field.

Previously, several water-saving strategies aimed at minimizing water requirements and improving the efficiency of its use in rice production ecosystems have been developed and proposed. These include but are not limited to direct wet-seeding and intermittent irrigation and drainage practices. However, these techniques primarily address water losses that are largely controlled by edaphic factors such as topography and soil characteristics and that are highly site-specific (Tuong et al., 2005). The techniques are therefore largely restricted to water losses caused by percolation, seepage, and surface run-off. Evapotranspiration, on the other hand, is linked to meteorological factors (Feng et al., 2020). Given this, steps to fix losses from; (1) paddy soil, (2) open water, (3) intercepted rainfall, as well as transpiration losses from paddy vegetation, which account for around 30-40% of the total water losses to the atmosphere (Tomar and O'Toole, 1980), are still scarce in the literature, and this is of interest to study.

1.2. Azolla Utilization in Rice Agriculture

Azolla species are aquatic ferns native to Asia, Africa, and the Americas. Reportedly, these species are the smallest but most economically important macrophytes in the world that float on the water surface. The genus *Azolla* Lam. (established by Lamarck in 1783) is classified into two subgenera as stated by Wagner (1997), i.e., *Euazolla* and *Rhizosperma*. The subgenera of *Euazolla* is characterized by three megaspore floats and consists of four species, namely: (i) *A. caroliniana* Willd., (ii) *A. filiculoides* Lam., (iii) *A. mexicana* Presl., and (iv) *A. microphylla* Kaulf. The subgenera of *Rhizosperma* is characterized by nine megaspore floats and consists of two species, namely: (i) *A. pinnata* R. Br., and (ii) *A. nilotica* Decne. The early rationalization of the Azolla species and the nature of taxonomic description is summarized in **Fig. 1.4.**

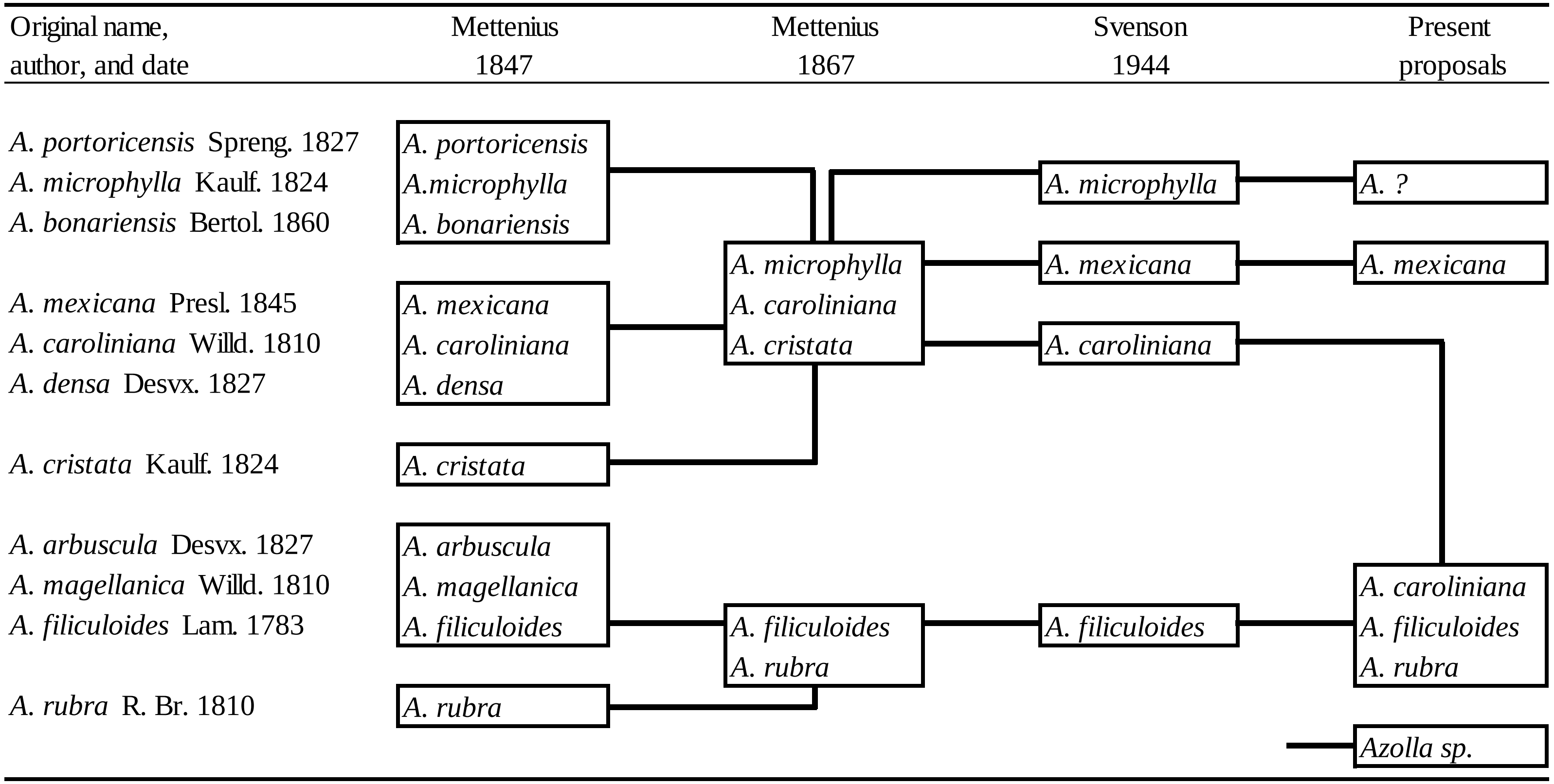

Fig. 1.4. Taxonomy in section Azolla: previous recognition of taxa and current proposals. (Source: Dunham and Fowler (1987).

For many decades, Azolla has been used as green manure for rice cultivation in China and Vietnam, and more recently in Africa due to its ability to fix atmospheric nitrogen (N) through its unique symbiotic relationship with cyanobacterium *Anabaena azollae* contained within its leaf cavities (**Fig. 1.5**).

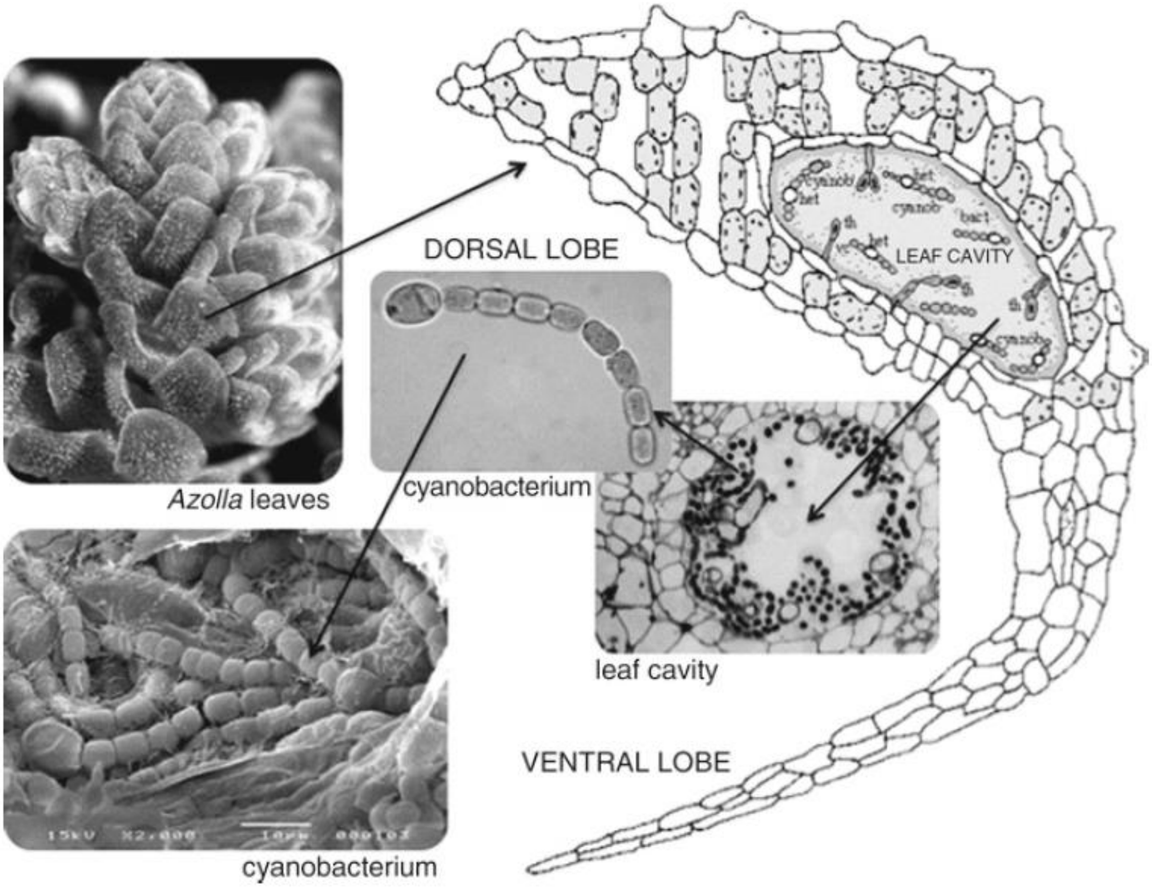

Fig. 1.5. Location of the leaf cavity in Azolla filiculoides Lam. and the cyanobacterium Anabaena azollae (Source: Carrapiço, 2010).

All known Azolla species and strains contain the N_2-fixing blue-green alga *Anabaena azollae* and have the potential to fix atmospheric N at high rates of up to 1000 kg N per acre per year. Nitrogen (N) is the most restrictive factor in agricultural production and substantially high quantities of synthetic N are added to maintain production. However, poor N recovery by rice cause major economic losses for farmers and negatively affects the climate (Mosier et al., 2013). As a result of the increasing and highly justified concern about environmental conservation and protection, improvement of agricultural soil productivity, and the need to adopt renewable and sustainable resources in agriculture, Azolla use as a biofertilizer in agricultural production has been widely recorded as a promising alternative strategy (Carrapiço, 2010; Moore, 1969; Wagner, 1997; Yao et al., 2018). While the potential of Azolla or soil and agricultural management in lowland rice ecosystems has been documented in numerous studies, there is still a lack of consensus on its potential for mitigating climate change in rice-based cropping systems.

1.3. Biochar for Climate Change Mitigation

Biochar is a carbon (C) rich product derived from the pyrolysis of organic biomass in an oxygen-depleted environment under high temperatures; i.e., above 250 ºC but not greater than 700 ºC. And although it is similar to charcoal, biochar is defined by its intended application (e.g. as a soil amendment or growth medium) to the soil for environmental functions. Thus, biochar may be produced from different biogenic feedstock materials that deliver specific functions based on the properties of the soils and the desired environmental response after biochar amendment (Lehmann and Joseph, 2012).

Biochar offers the potential to sequester recently fixed atmospheric C in addition to a range of environmental services and benefits including but not limited to; (1) promotion of plant growth, (2) improvement of soil water-holding capacity, (3) reducing soil CH_4 and N_2O emissions, and (4) reducing of nutrient leaching loss which in turn reduces fertilizer needs (Biederman and Harpole, 2013; Sohi et al., 2010). These derived benefits are majorly attributed to the biochar’s porous structure, high surface area, and affinity for charged particles, and the subsequent interaction with physical and biological components of soil (Lehmann and Joseph, 2012).

Although biochar has been proposed as a ‘win-win-win’ solution to meeting the global environmental challenges as a result of its high potential to contribute to C sequestration while simultaneously increasing yield and reducing fertilizer use; firstly, its application in nitrogen (N) deficient soils may lead to N immobilization and subsequent decrease in crop yields as biochar does not comprise appreciable quantities of N, and secondly, biochar can affect GHG emissions directly following its application to soils, and indirectly by adding carbonized instead of non-carbonized residue which usually has higher emissions following application (Clough et al., 2013). Furthermore, the addition of biochar with mineral fertilizers or different organic wastes to soil has been reported to improve crop productivity and increase, decrease or effect no change in soil GHG fluxes (Jeffery et al., 2011, 2015; Xie et al., 2013). Surprisingly, despite the rise in green manure adoption in lowland rice paddies, in particular, Azolla green manure, reports on biochar and Azolla co-treatment and the ensuing synergistic effects on rice yield and/or simultaneous CH_4 and N_2O emissions remain scarce. Due to their short-lived duration once incorporated as a result of their accelerated decomposition rates, green manure biofertilizers require occasional applications (Partey et al., 2014). Biochar has, however, been postulated to offer a remedy to this limitation in addition to providing additional soil management options (**Fig. 1.6**) (Lehmann et al., 2006).

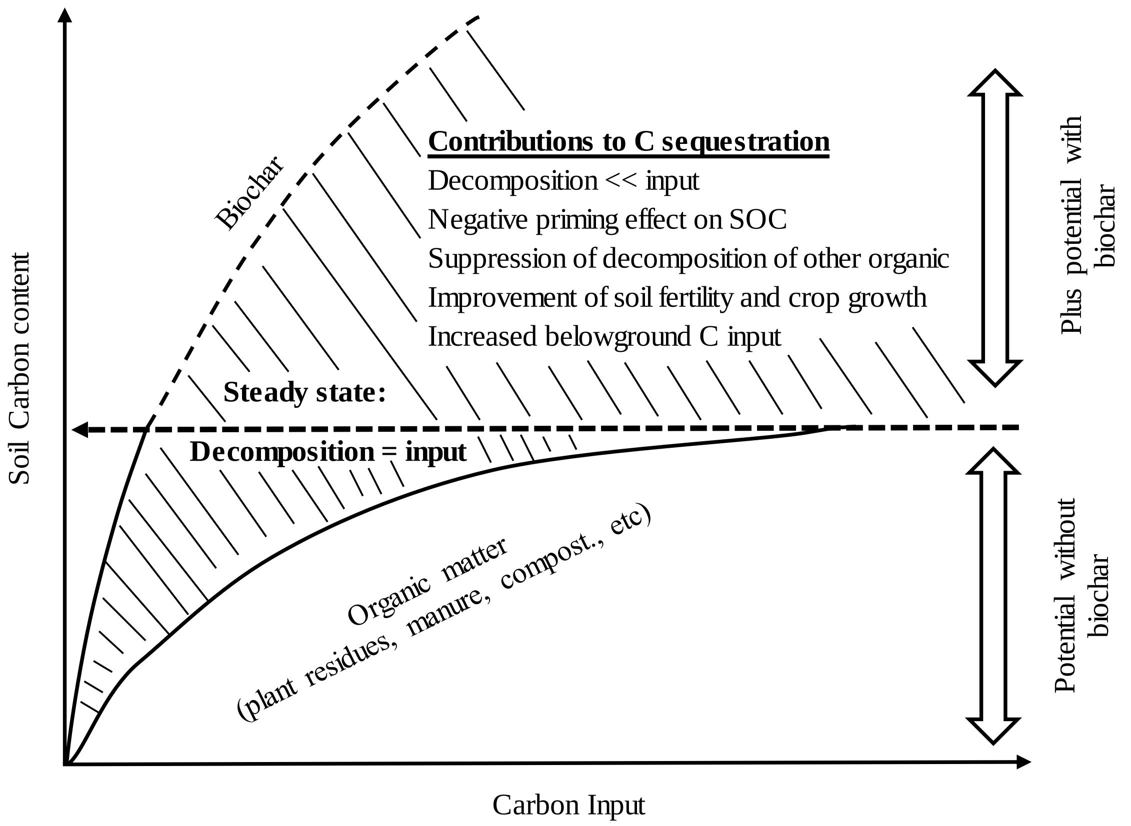

Fig. 1.6. Increased carbon (C) sequestration following biochar application. (Source: Wang, Xiong, et al., 2016).

1.4. Research Justification and Objectives

In recent decades, there has been a consistent increase in cover crop adoption in constantly flooded rice paddies and this has necessitated the need for research to understand their relationship with climate change (Kaye and Quemada, 2017). Although the ability of cover crops to improve soil quality, fix atmospheric nitrogen (N), minimize erosion and N leaching has been widely documented, the effects of cover crops, in particular, Azolla on ET and the simultaneous CH_4 and N_2O emissions from continuously flooded lowland ecosystems remain scarce and/or contradictory. Therefore, this thesis presents the recent finds on; (**1**) the relative reduction in the ET efficiency of Azolla from flooded water surfaces under different flooding water depths (**Chapter II**); (**2**) the significant CH_4 reduction potential of Azolla cover (applied as dual cropping with rice) from flooding rice paddy to the atmosphere (**Chapter III**); (**3**) the significant N_2O reduction potential of Azolla incorporated as green manure and dual cropping from a flooded paddy ecosystem (**Chapter IV**); (**4**) the synergistic effects on simultaneous CH_4 and N_2O emissions from rice paddy soil following the co-application of biochar (a carbon-rich material) and Azolla as green manure and dual cropping (**Chapter V**); and, (**5**) the potential of Azolla as green manure co-applied with biochar as a feasible fertilizer management practice to increase rice production and improve nitrogen (N) use efficiency (**Chapter VI**). In addition, the research findings as presented in **Chapter III** and **IV** are currently under verification in the field. The results of a single rice-growth season are briefly introduced (as the study is still in progress) in the last section of this thesis (**Chapter VII**).

CHAPTER II

2. Floating *Azolla* Cover Influences Evapotranspiration from Flooded Water Surfaces

2.1. Abstract

Floating vegetation is a common sight in flooded environments and plays a key role in regulating the global water balance, particularly through evapotranspiration (ET). *Azolla* is a dominant floating fern in lowland paddy fields, and its biological characteristics contribute mainly to nitrogen (N) fertilization. Here, to determine the potential of the rapidly growing *Azolla* as an alternative N source and its role in water conservation in flooded paddy ecosystems, we investigated its productivity and the resulting reduction in ET in two separate pot experiments. *Azolla* cover significantly decreased ET losses under high and low flooding depths compared with open water surfaces and green polyester covered mats (analogous to plant cover). Additionally, *Azolla* showed significantly higher biomass productivity, carbon assimilation, and N accumulation in presence of phosphorous (P) than in its absence under both high and low flooding depths. Overall, our results indicate that the efficiency of *Azolla* as an N alternative in agricultural ecosystems can be realized in presence of P, regardless of the flooding water depth. Additionally, we predict a relative reduction in the ET efficiency of *Azolla* in rice paddies. Field studies are, however, necessary to confirm these findings.

2.2. Introduction

Vegetation plays a key role in regulating the global water balance by influencing the transfer of liquid water to the atmosphere through evapotranspiration (ET) (Drexler et al 2004; Djaman et al 2017). In addition, ET reportedly affects water depth, temperature, salinity, and the areal extent of water coverage and inundation duration (Drexler et al 2004). Flooded paddy ecosystems are the largest man-made wetlands, with a water balance between water inflow due to irrigation, rainfall, and capillary rise and outflow due to seepage, percolation, and ET (Djaman et al 2017). In recent decades, there has been a consistent increase in cover crop adoption in constantly flooded rice paddies because of the heightened awareness of climate change (Kaye and Quemada 2017); however, studies on the influence of these cover crops on ET remain scarce.

Azolla, a genus of small aquatic ferns, is naturally found in temperate and tropical regions worldwide, especially in flooded paddy ecosystems. Recently, *Azolla* has gathered significant scientific interest because of its potential ability to modify the physical, chemical, and biological properties of soil and soil–water interface in paddy ecosystems (Cheng et al 2015a; 2015b; Wagner 1997; Sadeghi et al 2014; Kollah et al 2016; Xu et al 2017; Brouwer et al 2018; Kimani et al 2018). Although research on the link between *Azolla* and climate change is increasing, the influence of *Azolla* on ET from flooded paddy fields remains minimally explored, with perhaps the exception of two studies, Diara and Van Hove (1984) and Liu and Zheng (1992), which reported relative reduction in ET due to floating *Azolla* cover.

According to Mohamed et al (2012), the outcomes of climate change, particularly rising sea levels and temperatures, in most regions are expected to increase the rate of evaporation (E) and ET from open water sources and vegetation, respectively, as a result of less rainfall, lack of cloud cover, and low humidity. Although past studies have compared ET from floating vegetation to that from open water sources from clearings (Snyder and Boyd 1987; Rao 1988), agricultural drains (Rashed 2014), and below forest canopy (Allen et al 2016), no studies have been conducted on the effects of extensive floating *Azolla* mats on ET from flooded ecosystems. By contrast, extensive literature is available on both the ecological and socio-economic impacts of other floating and/or submerged aquatic macrophytes and wetland covers (Kirzhner and Zimmels 2006; Villamagna and Murphy 2010; Jiménez-Rodríguez et al 2019). Therefore, the objectives of this study were to investigate the influence of *Azolla* on ET under different depths of flooding water, and under no variation in flooding water depth.

The future of flooded paddy ecosystems is threatened by the increasing cases of water scarcity and losses occasioned not only by the increasing intensities of extreme weather events and competition from other sectors but also by the vegetation-mediated water fluxes within the soil–vegetation–atmosphere continuum (Katul et al 2012; Wang and Dickson 2012). Consequently, viable water conservation techniques in paddy ecosystems have been explored that reduce the amount of water supplied but maintain flooding at levels not considered threatening to the overall paddy production (Bouman et al 2007). On the other hand, water depth is reportedly a major factor influencing the optimal growth and biomass production of *Azolla*, as its efficiency as an alternative N source is highly dependent on its growth rate, minimum biomass, and N-fixing potential (Arora and Singh 2003; Biswas et al 2005; Sadeghi et al 2014). Moreover, it is challenging to avoid the deficiency and loss of phosphorus (P) from paddy fields (Zhang et al 2003), as it is the most critical nutrient for *Azolla* proliferation and its subsequent N-fixation through its symbiotic relationship with cyanobacteria *Nostoc azollae* (Watanabe et al 1980; Brouwer et al 2018).

Thus, the objectives of this study are twofold: (1) to investigate the influence of *Azolla* on ET in an environmentally-controlled chamber under different flooding water depths (experiment 1) and in a greenhouse without variations in flooding water depth (experiment 2); (2) to further our understanding of the effects of flooding water depth on the growth and N accumulation potential of *Azolla* (experiment 1) in the absence and presence of P since N accumulation potential is the major factor affecting its efficiency as an alternative N source in agricultural ecosystems (Arora and Singh 2003).

2.3. Material and Methods

2.3.1. Experiment Design and Management

To investigate the influence of *Azolla* on ET, two separate pot experiments were conducted (experiment 1 in a laboratory growth chamber and experiment 2 in a greenhouse) over a 28- and 5-day observation period, respectively, during the summer of 2016 at the Experimental Farm belonging to the Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata Prefecture located in northeastern Japan (38°44′N, 139°50′E). Subsequently, to further understand the effects of flooding water depth on *Azolla* growth and N accumulation potential in the absence and presence of P, pots only in experiment 1 were investigated at weekly intervals. Although *Azolla* grows in this region during the summer rice-growing season, no native *Azolla* was available because of harsh winter weather. Therefore, an introduced *Azolla* species (IRRI code F1.1001), namely *Azolla filiculoides* Lam., was adopted for this study, as in previous studies (Cheng et al 2010; Kimani et al 2018). Pots used in both experiments were of similar dimensions (16 cm inside diameter, 13.2 cm height, and 10.9 cm bottom diameter). Each pot was filled with 0.75 kg of gray sandy soil collected from the plow layer (approximately 15-cm top layer) of a typical rice field in Kujukuri, Chiba Prefecture, Japan. Chemical properties of the paddy soil are listed in **Table 2.1**. Additional details on the experimental design and treatments per experiment are as described below.

Table 2.1. Chemical properties of the experimental paddy soil

Organic C (g kg^{-1} dw)	6.20
Total N (g kg^{-1} dw)	0.80
C/N	7.75
pH (1:5, soil to H_2O ratio)	6.74
EC (dS m^{-1})	0.12
Available P (mg P_2O_5 kg^{-1})	63.6

2.3.2. Experiment 1: Influence of Flooding Water Depth and P on the Growth of *A. filiculoides*

To investigate the influence of different flooding water depths and the absence or presence of P on *Azolla* growth, carbon (C) assimilation, and N accumulation, *A. filiculoides* was grown in 12 plastic pots in an environmentally-controlled plant growth chamber (Model LH-240N) at 30°C day/25°C night temperature and 75% day/60% night humidity to mimic summer conditions in the local area. The soil in six pots was enriched with superphosphate of lime at 1 g P_2O_5 per pot (+P), three at high flooding depth (5 cm; H + P) and three at low flooding depth (2 cm; L + P) and compared against six P-deprived (−P) pots at similar depths (H − P and L − P). On April 1, 2016 (a day after soil was mixed with P), a removable cross-shaped plastic bar was floated on the water in each pot to divide the pot into four equal quadrants. Each quadrant (equivalent to a replicate) was inoculated with 5 g of *A. filiculoides* (**Fig. 2.1**).

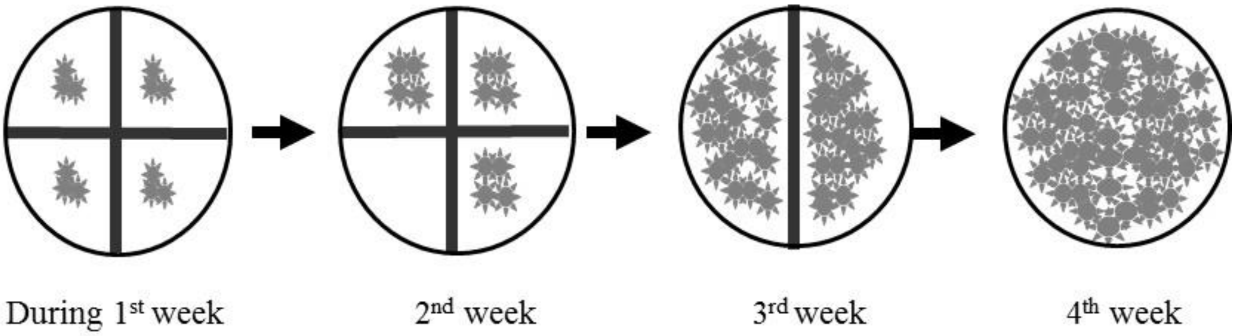

Fig. 2.1. Diagram showing the experimental sampling schedule and method in experiment 1. A removable cross was used to divide 4 times plant sampling. See detail in the text.

The amount of P fertilizer applied was similar to that described by Cheng et al (2010). Thus, the following four treatments were conducted:

1) "H + P" high flooding water depth (5 cm) with P + *Azolla* cover
2) "H − P" high flooding water depth (5 cm) without P + *Azolla* cover
3) "L + P" low flooding water depth (2 cm) with P + *Azolla* cover
4) "L − P" high flooding water depth (2 cm) without P + *Azolla* cover

Samples of the floating *Azolla* mat from each block in all pots were collected at 7, 14, 21, and 28 days after inoculation (**Fig. 2.1**) using a stainless steel mesh. Samples were then washed with tap water, oven-dried at 70°C for 48 h, and weighed. Dried ferns for the final samples were ground, and the C and N contents of tissues were determined using an automatic highly sensitive Nitrogen-Carbon analyzer (Sumigraph NC-220F, Japan).

Exponential relationships for changes in *Azolla* biomass over the whole growth period were determined according to Eq. (1) (Cheng et al 2010), while the relative growth rate (RGR) and biomass doubling time (T_d) were calculated using Eq. (2) and Eq. (3) (Jackson 1980):

$$y = A \times exp(kx) \qquad (1)$$

Where y is the *Azolla* biomass (mg) on x day; A is the *Azolla* biomass (mg) at the beginning of the experiment, and k is the growth rate constant (day^{-1}) for the exponential model.

$$RGR = \ln(DW_2) - \ln(DW_1)/(t_2 - t_1) \qquad (2)$$

Where DW_1 and DW_2 are dry weights (mg) measured on the first day (t_1) and last day (t_2) of the experiment, respectively.

$$T_d = \ln(2)/RGR \qquad (3)$$

2.3.3. Experiments 1 and 2: Influence of *A. filiculoides* on ET

In experiment 1, 4 pots with open water surfaces (2 H and 2 L) were set up adjacent to the 12 pots described above to investigate the influence of *Azolla* on ET under different depths of flooding water. These control treatments were designated as described below:

5) "H ctr" high flooding water depth (5 cm) without *Azolla*.
6) "L ctr" low flooding water depth (2 cm) without *Azolla*.

The second experiment in the greenhouse (experiment 2) was set up as a consequence of the ET results from experiment 1 to include possible field conditions and other environmental factors beyond our control. Here, five plastic pots inoculated with *A. filiculoides* and fertilized with P (as described above) but not divided into blocks (as in experiment 1) were compared with five green polyester mat covered pots (analogous to canopy cover) and five control pots (open water surface). The experiment was set up from May 15–20, 2016, approximately two weeks after the end of experiment 1. In both experimental setups, water loss was measured gravimetrically, based on the decrease in the weight of the pots every 2 days (in experiment 1) or every 24 h (in experiment 2) compared with the initial pot weight. After every measurement, water loss was compensated by the addition of deionized and tap water in experiments 1 and 2, respectively, to maintain the initial pot weights.

In experiment 2, the diurnal water temperature cycle was measured in all pots at 30-s intervals using the Thermo recorder TR-71U (T&D Corp., Tokyo, Japan) and plotted against the diurnal variations in air temperature and sunshine time. The average estimates of daily hourly air temperature and sunshine time data were downloaded from the Japan Meteorological Agency database for the Tsuruoka Meteorological Observatory (http://www.data.jma.go.jp/obd/stats/etm/index.php).

2.3.4. Data Analysis

Statistical analysis of all parameters measured at the last sampling, including P, flooding water depth, and P x flooding water interactions in experiment 1 and temperature effects in experiment 2, was conducted by the ANOVA. Significant differences in parameters were analyzed by the least significant difference test at $P < 0.05$. All statistical analyses were carried out using the SPSS 20 statistical package (SPSS Inc., Chicago, IL, USA).

2.4. Results

2.4.1. Experiment 1: Effects of P and Flooding Water Depth on *A. filiculoides* Growth

In experiment 1, changes in *Azolla* biomass over the 28-day growth period showed significant exponential relationships under the four combinations of P and flooding water depths (**Fig. 2.2a**). The initial dry weight (DW) was 19.07 mg in each treatment. The growth rate constant *k* increased under high and low flooding water depths with added P by 22.7% and 17.5%, respectively. At the final sampling, total *Azolla* biomass was significantly increased by P application and flooding water depth of 5 cm ($P < 0.01$) and 2 cm ($P < 0.05$) (**Table 2.2**). On average, the application of P increased *Azolla* biomass by 2.4-fold. The C concentration of *Azolla* tissues at the final sampling was similar (approximately 40%) among the four treatments, so the rate of C assimilation by *A. filiculoides* followed the same pattern as that of dry weight (**Table 2.2**).

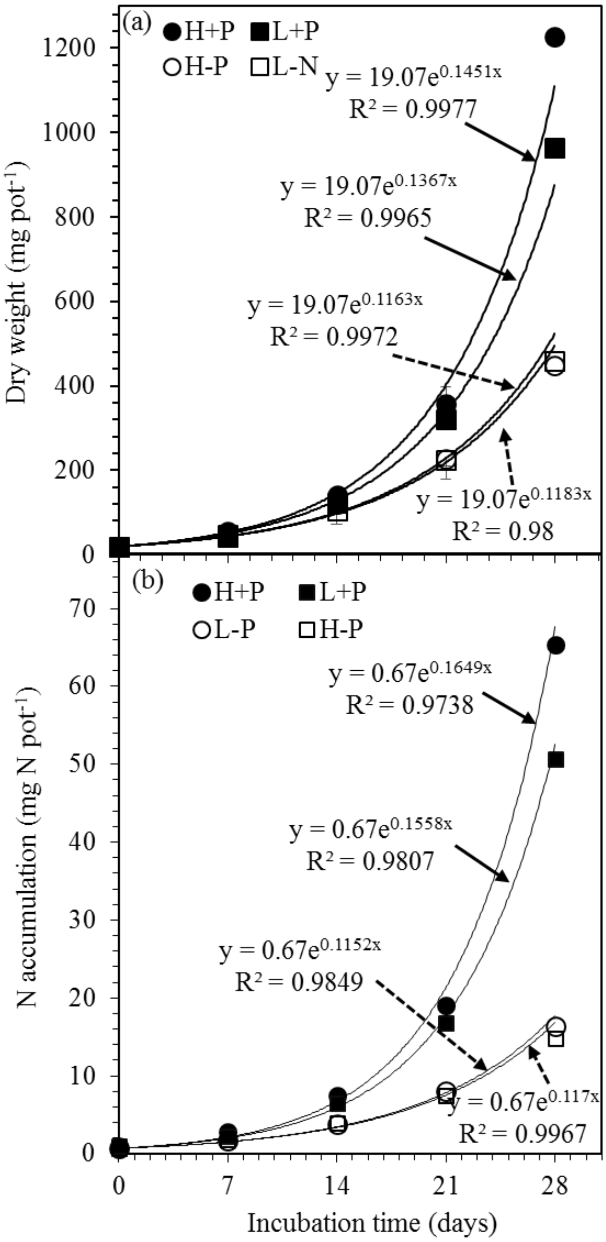

Fig. 2.2. Changes in dry weight (a) and the amounts of accumulated nitrogen (b) of *A. filiculoides* in pots with (+P) and without (-P) phosphorus application under both high (5 cm) and low (2 cm) flooding water depths during 28-day incubation in a controlled-environment incubator (experiment 1).

The initial N concentration of *A. filiculoides* was 0.67 mg pot^{-1} in each of the four treatments in experiment 1. The rate constant *k* increased under high and low flooding water depths with added P by 40.9% and 35.2%, respectively (**Fig. 2.2b**). Percent N concentration of *A. filiculoides* tissues was significantly higher with P than without P under both high and low flooding water depths ($P < 0.01$), with significant influence of flooding water depth and no interaction between P and flooding water depth. The accumulation of N in *A. filiculoides* tissues was significantly greater (on average 3.8-fold) following P application under both high and low flooding water depths and was significantly affected by flooding water depth and the interaction between P and flooding water depth ($P < 0.05$) (**Table 2.2**).

Table 2.2. Effects of flooding water depth and phosphorus on *A. filiculoides* Lam. growth in experiment 1

Flooding water depth (cm)	Phosphorus (P) With P (+P) Without P (-P)	Dry weight (mg/pot)	C concentration (%)	N concentration (%)	C/N (wt/wt)	C assimilation (mg C/pot)	N accumulation (mg N/pot)
High (5cm)	H+P	1227.61	44.47	5.32	8.36	545.95	65.28
	H-P	447.87	37.47	3.29	11.39	167.81	14.74
	% change by +P	174.10	18.69	61.63	-26.57	225.33	343.02
Low (2cm)	L+P	964.22	41.61	5.25	7.92	401.23	50.64
	L-P	459.41	38.72	3.57	10.85	177.88	16.39
	% change by +P	109.88	7.47	47.23	-27.01	125.56	209.01
ANOVA results							
Phosphorous		**	ns	**	**	**	**
Flooding water depth		*	ns	ns	*	**	*
Phosphorus x Flooding water depth		**	ns	ns	ns	**	*

ns: not significant, *: $P < 0.05$, **: $P < 0.01$

Values of RGR and T_d followed a logistic curve in the +P treatments compared with the −P treatments (**Fig. 2.3a, b**). The RGR for all treatments ranged between 0.288 and 0.374 mg DW pot^{-1} d^{-1}, while the T_d values ranged between 7.41 to 9.62 days.

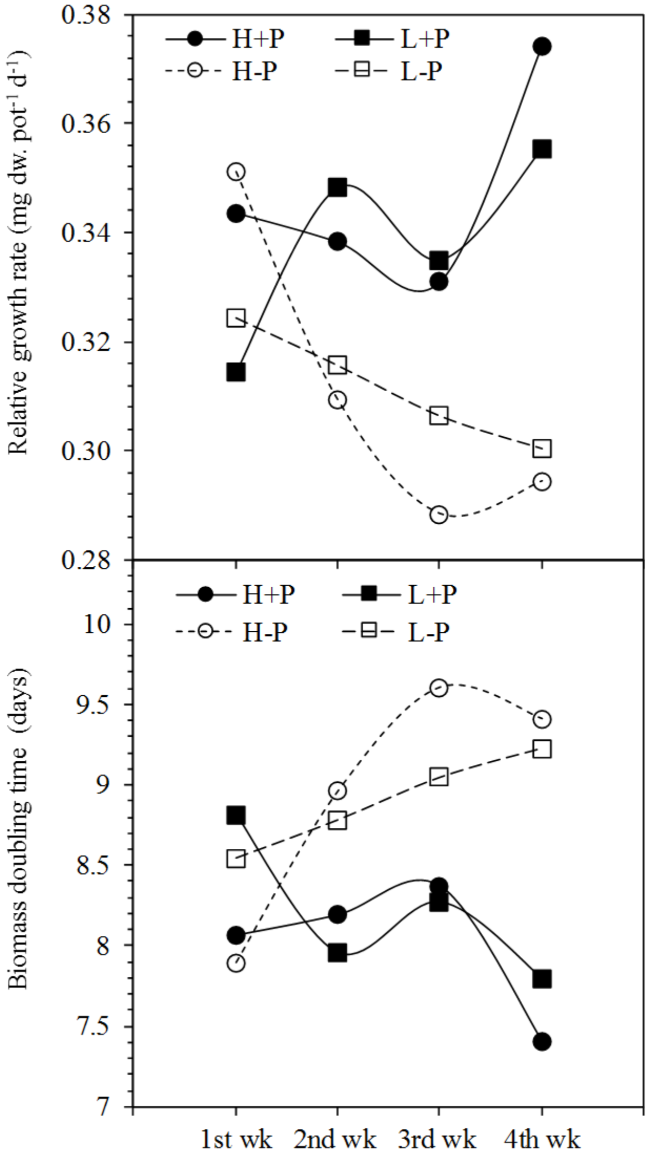

Fig. 2.3.Increase of Azolla biomass expressed as relative growth rate (rgr = Ln (DW_2)-Ln (DW_1) / (t_2-t_1)) (a), and biomass doubling time (t_d=Ln (2)/rgr) calculated every week from April 8th to April 29th 2016 (b) (experiment 1).

2.4.2. Experiment 1: Influence of *A. filiculoides* Cover on ET

Under controlled growth chamber conditions (**Fig. 2.4**), the rates of ET in *Azolla* inoculated treatments under high flooding water depth with or without P showed no significant differences over the study period, with average ET values of 1503.3 and 1525.0 mg pot^{-1} in H + P and H − P treatments, respectively. Similarly, no significant differences were detected in ET values under low flooding water depth with or without P, with average rates of at 1208.3 and 1181.7 mg pot^{-1} in L +

P and L − P treatments, respectively. However, significant differences in ET were detected between treatments and the control treatments in both cases, with H ctr values at 1887.5 mg pot^{-1} and L ctr at 1390.0 mg pot^{-1} (**Fig. 2.4**).

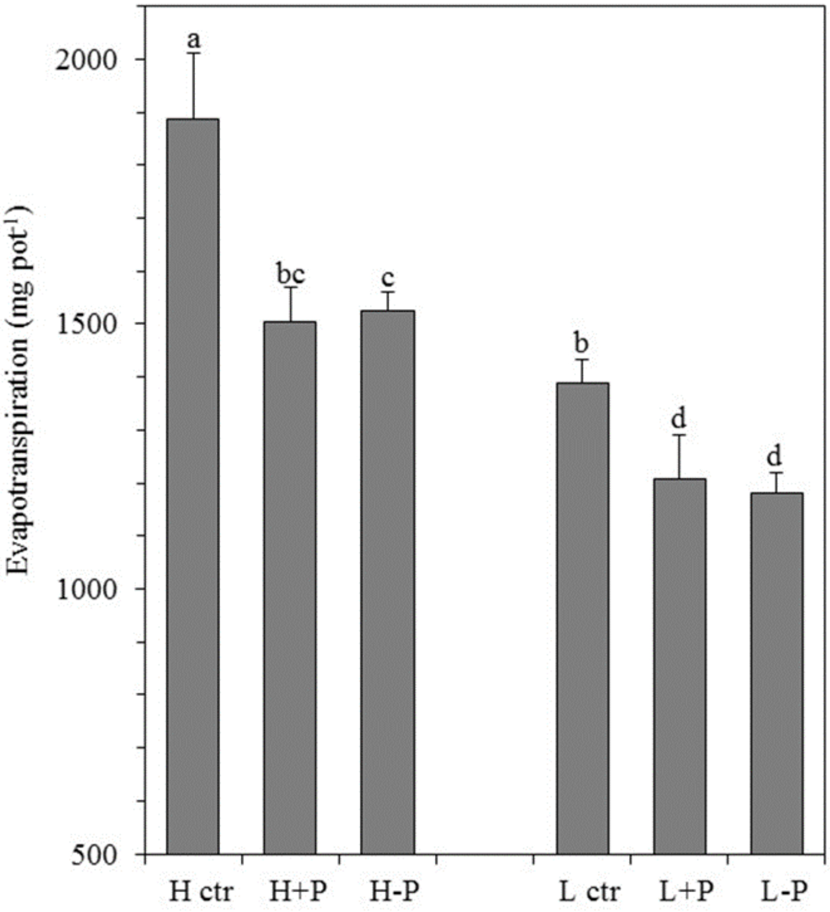

Fig. 2.4. Estimated evapotranspiration losses between treatments in the controlled-environment incubator (experiment 1) in 28 days. High and Low flooding water depth without Azolla (H ctr and L ctr, respectively); High and Low flooding water depth with Azolla plus phosphorus (P) (H+P and L+P, respectively); and High and Low flooding water depth with Azolla minus phosphorus (P) (H-P and L-P, respectively). Bars indicate standard deviation (n=3). Different letters above bars indicate significant differences (Fisher's LSD)

2.4.3. Experiment 2: Influence of *A. filiculoides* Cover on ET

In the greenhouse experiment, values of daily ET and E losses were significantly low from May 15–20, 2016, under *Azolla* cover than under the green polyester mat (analogous to plant canopy) and control (open water) treatments, respectively (**Fig. 2.5a**).

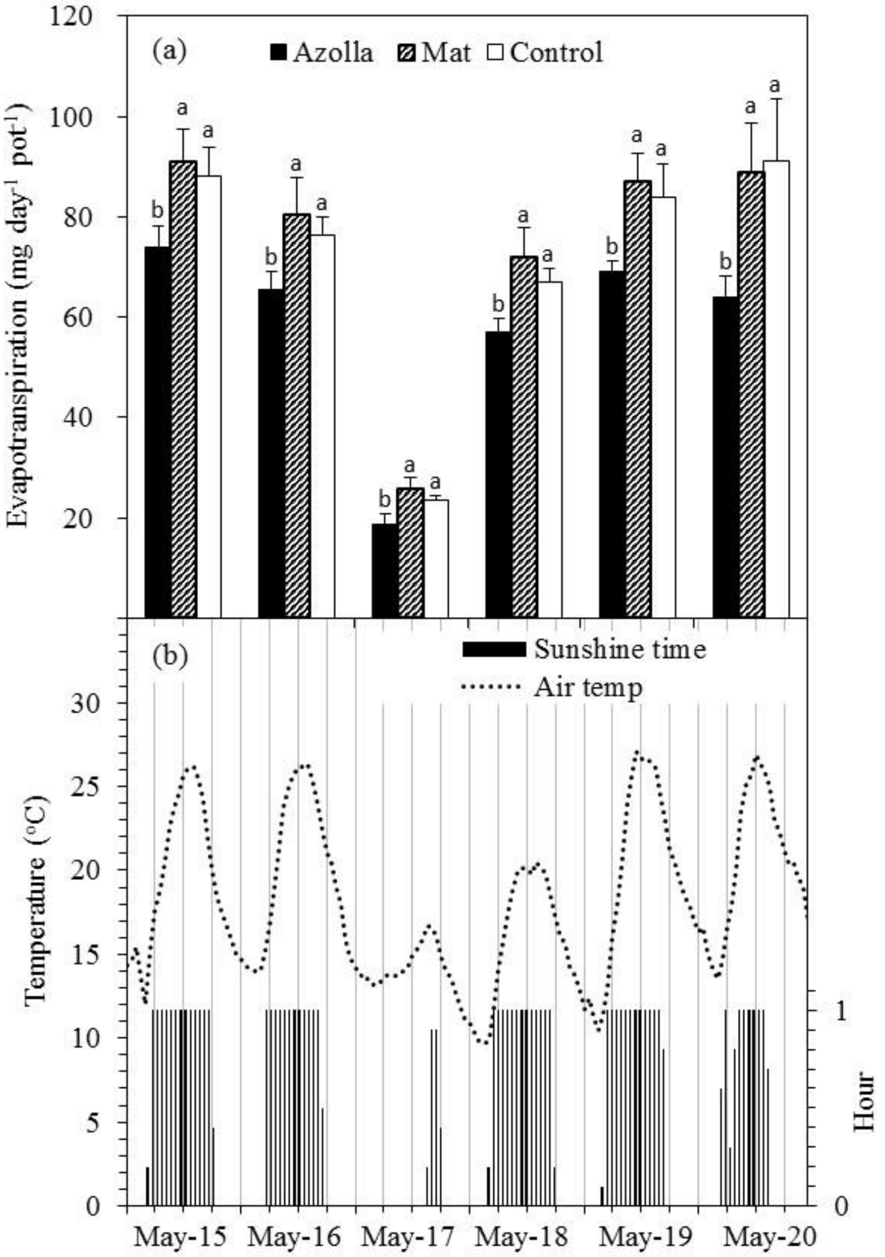

Fig. 2.5. Estimated daily evapotranspiration losses among treatments in the greenhouse (experiment 2) recorded every 24h between 15th and 20th May 2016 (a), and the recorded daily hourly air temperature and sunshine time during the experiment period (b). Bars indicate standard deviation (n=5). Different letters above bars indicate significant differences (Fisher's LSD).

The observed daily ET and E variations were in accordance with the recorded sunshine time and air temperature throughout the experiment (**Fig. 2.5b**). Higher water losses (ET and E) were observed on days with higher sunshine time and air temperature (May 15, 16, 18, 19, and 20) in three treatments, while the lowest values were recorded on May 17 as a result of cloudy weather conditions leading to low sunshine time and air temperature. Lower water losses recorded on May 18 compared with the remaining days, except May 17, were consistent with low air temperature on this day. Despite the variations in daily hourly air temperature and sunshine time (**Fig. 2.6**), floating *Azolla* cover maintained significantly low ET values compared with the mat and control treatments.

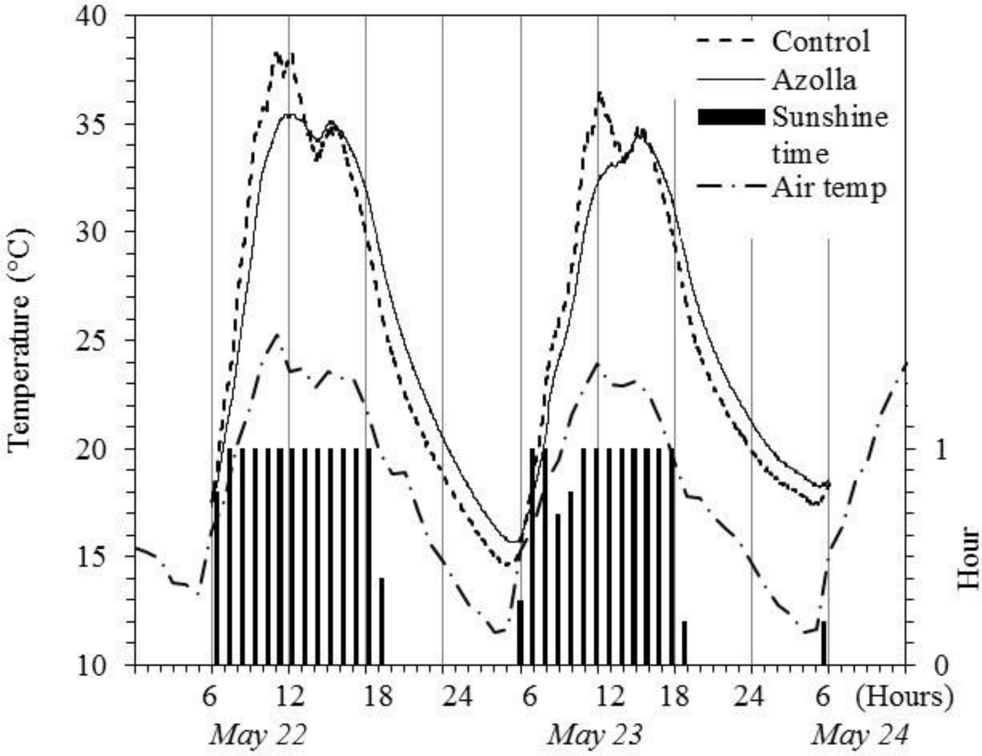

Fig. 2.6. Diurnal variations of air temperature (black dashed line), sunshine time (black bars), water temperature under Azolla (blacked solid line), and open water surface (Control, thin black dotted line) over 54h (recorded every 30 seconds) between 22nd and 24th May 2016 in the greenhouse (experiment 2).

2.5. Discussion

2.5.1. Experiment 1: Influence of P and Flooding Water Depth on *A. filiculoides* Biomass, C Assimilation, and N Accumulation

In this study, under both high and low water depths, P addition significantly increased *A. filiculoides* dry matter (H + P, 174.10 %; L + P, 109.88%), C assimilation (H + P, 225.33 %; L + P, 125.56%), and N accumulation (H + P, 343.02%; L + P, 209.01%) compared with the −P treatments (**Fig. 2.2a,b**; **Table 2.2**). Significantly high interactions were detected between P and flooding water depth. The present study demonstrated a positive relationship between *A. filiculoides* growth, P, and flooding water depth, consistent with previous studies, which showed that P addition and optimum flooding water depth positively influence the growth of *Azolla* (Subudhi and Watanabe 1981; Wagner 1997; Sadeghi et al 2013).

Previously, 3–5 cm of flooding water depth both in laboratory and field conditions has been recommended for optimal growth of *Azolla* (Pabby et al 2003). In addition, P rather than N is the principal limiting nutrient for *Azolla* growth, since the N requirement for *Azolla* is satisfied by cyanobacteria (Arora and Singh 2003; Biswas et al 2005; Cheng et al 2010). In this study, we demonstrate that a reduction in flooding water depth (below the optimum but above the soil surface; in this case, 2 cm) in the presence of P (i.e., L + P) significantly increased biomass production, C assimilation, and N accumulation compared with the optimal depth in absence of P (i.e., H − P) as well as in L-P (**Fig. 2.2a, b; Table 2.2**). Therefore, while the overall optimal performance of *Azolla* might be closely related to water depth (as clearly observed under H + P treatments), considerably more benefits of *Azolla* can still be realized under favorable flooding water depth in the presence of P, especially in water-scarce environments.

2.5.2. Experiment 1 and 2: Influence of *A. filiculoides* on ET

In experiment 1, over a 28-days period, *Azolla* cover significantly reduced ET losses from pots by 17.3% under different flooding depths both in the absence and presence of P (L ± P, 14.0%; H ± P, 19.8%) compared with the open water pots (**Fig. 2.4**). In experiment 2, over a 5-day period, *Azolla* covered pots significantly reduced ET losses by 20.0% compared with both open water and green polyester mat covered pots (**Fig. 2.5a**). No significant differences were observed between the open water and polyester mat covered pots during this period. These observed reductions, though relatively low, were in agreement with those previously reported by Diara and Van Hove (1984), who showed more than 20.3% reduction in ET by *A. filiculoides* grown in plastic pots in a partially-controlled and well-ventilated greenhouse in the Sahelian countries.

Previously, macrophyte stand density and height have been reported as the principal physiological characteristics effecting significant ET losses (Pauliukonis and Schneider 2001; Rashed 2014). However, in contrast to the vertical growth of many aquatic macrophytes and land plants, *Azolla*, like other floating macrophytes, expands horizontally on the surface, avoiding competition for light as long as the surface is not fully covered (Brouwer et al 2018); this is fully reflected in the high RGRs obtained in this study. Our results in experiment 1 showed that *A. filiculoides* maintained high RGR throughout the experimental period (P = 0.054; **Fig. 2.3a**), which were within the reported range of 0.12–0.50 d^{-1} (Cary and Weerts 1992; Maejima et al 2001). Additionally, *Azolla* showed significantly different T_d values among treatments ($P < 0.05$; **Fig. 2.3b**), which was within the reported range of 2–10 days (Becking 1979). Thus, the ET reduction potential by *Azolla* in both experiments may be attributed to, but not limited to, its anatomy, horizontal placement of its leaves, and smaller leaf area, which possibly restricted simultaneous E-transpiration losses by shielding much of the water surface. Plant physiological traits have been previously reported as key components influencing E, transpiration, and interception losses (Hussey and Odum 1992; Moore and Owens 2012).

Furthermore, studies have previously shown a strong connection between water temperature and ET, with losses significantly increasing in tandem with the water temperature (Kadlec 2006; Finch and Calver 2008; Papaevangelou et al 2012). In the current study, despite the significantly higher water temperatures (above 5°C) in *Azolla* covered pots relative to the surrounding air temperature during the day (**Fig. 2.6**), these pots maintained significantly higher ET reduction rates compared with the control pots, which were only a degree higher during the daytime hours (**Fig. 2.6, Fig. 2.5a**). Floating vegetation has been reported to modify microclimate conditions around the ecological system compared with ecosystems without vegetation, producing an increase and associated decrease in the net radiation and evaporation losses (Carrington et al 2001; Wang et al 2001; 2002). Hence, significantly higher ET reduction rates under *Azolla* cover, in spite of the significantly high water temperature, may be attributed to a greater total reflectance of the incoming solar radiation and enhanced modification of the surrounding microclimate by the dense mat of the floating *A. filiculoides*.

Daily variations in ET and E losses in experiment 2 throughout the study period (**Fig. 2.5a**) were partly attributed to 1) the influence of *Azolla* anatomy (as discussed above) and 2) the variation in sunshine time and air temperature (**Fig. 2.5b**). The lowest ET and E losses recorded on May 17, 2016, were thought to be a result of cloudy weather conditions during the day, while the subsequent lower losses on May 18, 2016, compared with the remaining days (except May 17), were mostly due to lower air temperature (about 20°C). Variations in weather conditions have previously been reported to influence water losses (Peters et al 2011; Guo et al 2017).

In conclusion, the success of *Azolla* under low flooding water depth in the presence of P to maintain sufficient N accumulation is a conviction that shallow flooding is not only a useful technical improvement over high flooding water depth cultivation but also a new and useful development in growing *Azolla* for agricultural use, especially in adverse water conditions and under the newly proposed water conservation techniques in rice production (Tuong et al 2005; Sujono et al 2011; Darzi-Naftchali and Ritzema 2018). Although this study did not take into account the micrometeorological parameters, the practicality of maintaining a permanent floating *Azolla* cover, or the interference of rice itself with ET, the amount of water lost by E is often much higher under natural conditions than under our experimental setup. Thus, on the scale of this study, we predict a relative ET reduction efficiency of *A. filiculoides* in rice paddy fields. Further experiments under field conditions are needed to validate these findings.

CHAPTER III

3. Azolla Cover Significantly Decreased CH_4 but not N_2O Emissions from Flooding Rice Paddy to Atmosphere

3.1. Abstract

Azolla (*Azolla filiculoides*) is a common aquatic fern that has been used successfully as a dual crop with lowland rice. It grows rapidly and has the ability to fix N_2 for rice paddy. However, its ecological significance especially on greenhouse gases emissions remains unclear. To investigate the effect of Azolla cover on methane (CH_4) and nitrous oxide (N_2O) emissions from the rice paddy, a pot experiment with two treatments, control (rice plant only) and Azolla cover (rice plus Azolla covering on the flooding water) was carried out in Tsuruoka, Yamagata, Japan, in 2016. The results showed that the rice growth parameters, like shoot height, maximum and productive tiller numbers, and plant biomass were not significantly different between the two treatments. Dual cropping of Azolla with rice significantly suppressed CH_4 emissions, likely due to an increase in dissolved oxygen concentration and redox potential at the soil-water interface between flooding water and soil surface. There were significant ($P<0.05$) positive correlations between CH_4 flux and night respiration (CO_2 emissions) between the two treatments. The cumulated CH_4 emission during the growth period until 106 days after transplanting (DAT) was significantly lower at 36.2 g C m^{-2} from Azolla cover treatment than that from the control treatment pot at 55.4 g C m^{-2}. A prolonged non-significant N_2O emission under the Azolla cover treatment after the initial highest peak at 15 DAT was recorded due to denitrification of the nitrate in the initial soil. No further N_2O emissions were recorded thereafter from both treatments. Azolla cover did not affect N_2O emissions from both treatments.

3.2. Introduction

Atmospheric concentrations of greenhouse gases (GHGs), such as carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) have significantly increased as a result of anthropogenic activities. CH_4 accounts for 15-20% of the radiative forcing with a global warming potential (GWP) of 25 times greater than carbon dioxide (CO_2) on a mass basis, while N_2O is 298 times more potent in heat-trapping as compared to CO_2 and accounts for 6-8% of the current global warming (IPCC 2007). According to IPCC (2007), agriculture is estimated to account for 10-12% of anthropogenic emissions of GHGs worldwide, including 50% global CH_4 and 60% of global N_2O emissions. Rice paddies are considered as one of the most important sources of anthropogenic CH_4, but they also emit N_2O and the intensity of emissions is related to the nitrogen (N) fertilizer application rate (Akiyama *et al.* 2006). CH_4 and N_2O gases are simultaneously emitted from rice paddy soils depending on rice cultivation system, soil organic matter, soil moisture level, microbial activity, redox status, exogenous application of nitrogenous fertilizer and organic materials (Akiyama *et al.* 2006; Snyder *et al.* 2009; Nishimura *et al.* 2011). The trade-off relationship between CH_4 and N_2O emissions in rice paddy fields is a strong indication that possible techniques to reduce these emissions simultaneously should be developed (Li 2007).

Azolla (*Azolla filiculoides*) is a floating aquatic fern growing in tropical and temperate freshwater ecosystems. As a result of its symbiosis with N_2 fixation cyanobacteria, *Anabaena azollae*, within its leaf cavities, it has been cultivated in rice paddy for improving rice N nutrient in southern China and northern Vietnam as green manure for many centuries (Watanabe and Liu 1992; Wagner 1997). Even though chemical N fertilizers have substituted the role of Azolla as green manure, Azolla is still cultivated by organic farmers, especially in rice-fish-Azolla or rice-duck-Azolla multiple eco-production systems in China and Japan (Cheng *et al*. 2015a, b). Additionally, Azolla is recognized to modify the physical, chemical, and biological properties of soil and the soil-water interface between flooding water and soil surface in rice fields for mobilizing fixed phosphates, retarding NH_3 volatilization which accompanies the application of chemical N fertilizer, suppressing aquatic weeds in flooding rice field and reducing evapotranspiration losses for rice production (Mandal *et al.* 1999; Cissé and Vlek 2003; Cheng *et al*. 2015a; Kimani *et al.* 2016; Kollah *et al*. 2016). Recently, Azolla has been highlighted again for biofertilizer and biodiesel production (Bocchi and Malgioglio 2010; Jumadi *et al*. 2014; Brouwer *et al.* 2016; Kollah *et al.* 2016).

Previous studies have shown that Azolla application in the paddies could increase (Chen *et al.* 1997; Ying et *al.* 2000) or decrease (Bharati *et al*. 2000; Ali *et al.* 2015; Singh and Strong 2016) CH_4 emissions from rice soils. The discrepancy between these studies may be partly due to different experimental conditions, including different rice cultivars and soil types. N_2O is a byproduct or an intermediate product of microbial nitrification and denitrification on the N cycling in soil-plant ecosystems (Cheng *et al*. 2004a, b). There are many studies showing N_2O emissions from symbiotic N_2 fixation plants, such as leguminous crops and acacia trees as being larger than non-symbiotic N_2 fixation plants (Arai *et al*. 2008; Mori *et al*. 2010, Uchida and Akiyama 2013; Zhang *et al*. 2014). The N_2O emission from soybean ecosystems could be mitigated by inoculation of high N_2O reductase N-fixing rhizobium (Akiyama *et al*. 2016). As a symbiotic N_2 fixing plant, how Azolla affects N_2O emission from rice paddy ecosystems as dual crops (rice and Azolla covering on flooding water) is not well understood.

Although numerous studies on the exchange of CH_4 and N_2O between the rice paddies and atmosphere have been conducted from the 1980s, most of those studies place their focus on either of the two gases. To understand how Azolla cover on the flooding water affect CH_4 and N_2O emissions simultaneously from dual cropping (rice and Azolla covering on flooding water) rice paddy systems, we conducted a pot experiment in the 2016 summer season in Tsuruoka, Yamagata, Japan.

3.3. Material and Methods

3.3.1. Experiment Site, Design, and Management

This research was conducted in 2016 at the Experiment Farm, Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata Prefecture, located in northeastern Japan (38°44'N, 139°50'E, 16 m elevation). According to the Japan Meteorological Agency database for Tsuruoka Meteorological Observatory (http://www.data.jma.go.jp/obd/stats/etrn/index.php), which is located inside of the Experiment Farm, Faculty of Agriculture, Yamagata University, the climate condition during the rice growth season (31st May to 15th September) in 2016 was hotter and sunnier than the historic average of 1981-2010 (**Fig. 3.1**). The daily average air temperature and sunshine times were 23.71 °C and 6.16 hours than those of the historic average of 1981-2010 at 22.60 °C and 5.75 hours, respectively.

In situ pots were used with two treatments in four replications, (1) control (rice plant only, single cropping), and (2) Azolla cover (rice plus Azolla covering on the flooding water, dual cropping). Although Azolla grows in this region during summer, rice-growing season, no native Azolla was available because of the harsh winter conditions. An introduced species (IRRI code FI 1001) was adopted and grown in a glasshouse for this experiment (**see details in Chapter II)**. Rice cultivar was Haenuki, which is a popular edible rice variety grown widely in the local area, Yamagata Prefecture.

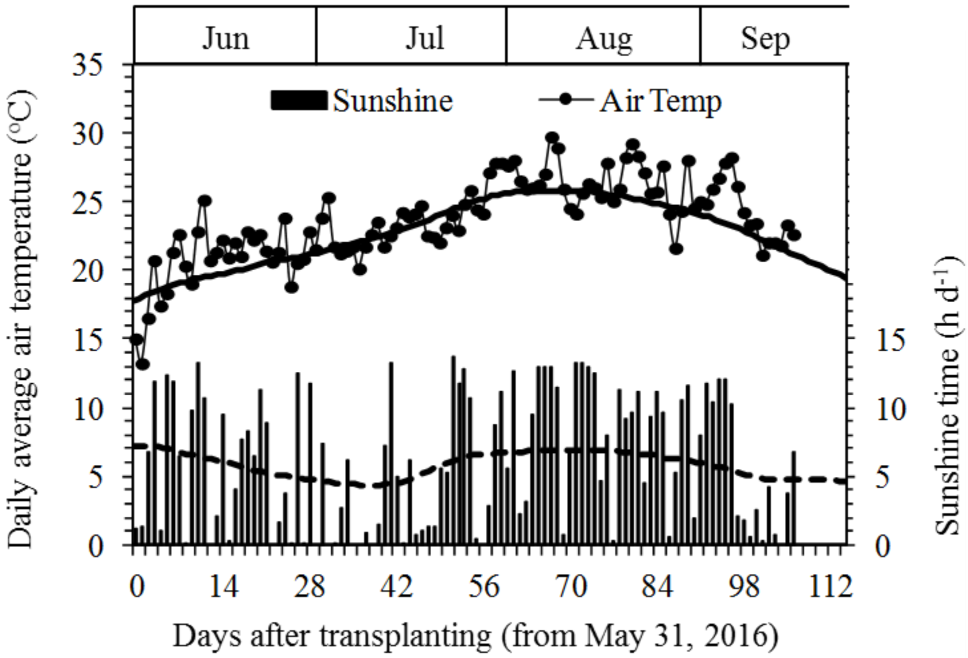

Fig. 3.1. Daily sunshine time (■) and average air temperature (●) during the experiment period from 31st May to 15th September 2016 in Tsuruoka, Japan. The bold line and dish line crossed Air Temp and Sunshine time are the average values for 1981-2010 (Av. 30 years). Data were from the Japan Meteorological Agency.

The bulk soil used in this experiment was collected from a rice field at the University Farm and classified as an alluvial (**Table 3.1**). The soil was air-dried, sieved (5-mm mesh size), and mixed well before use. On 26th April 2016, germinated seeds were sown in a seedling tray (three seeds per cell). Three weeks after sowing, on May 31st, 2016, the seedlings were transplanted to 8 plastic pots (19.5 cm inside diameter, 27 cm height, and 0.2 cm thickness). Before transplanting, 6.0 kg (4.5 kg dry soil equivalent, water content was 25% in w/t) of the alluvial soil was mixed with 0.87g of KH_2PO_4 and 0.87g $CO(NH_2)_2$ and filled to each pot. The amounts of nitrogen, phosphorous, and potassium of the basal fertilizers were 0.60, 0.20, and 0.25 g pot^{-1}, respectively. At 49 days after transplanting (DAT), we top-dressed with 0.20 g N, 0.10 g P, and 0.13 g K pot^{-1} by 0.43 g $CO(NH_2)_2$ and 0.43 g KH_2PO_4. At the end of the experiment, the total amounts of applied N, P, and K fertilizers at field level were, 183.84, 89.73, and 112.88 kg per ha, respectively. The flooding water was maintained at about 5 cm depth throughout the experimental period by adding tap water. Five grams fresh weight of Azolla was inoculated into the water of each of the Azolla cover treatment pots after rice transplanting and its dry final dry weight determined before rice harvesting.

Table 3.1. *Major properties of experimental paddy soil.*

Organic C (g kg^{-1} DW)	28.5
Total N (g kg^{-1} DW)	2.90
C/N	9.83
pH (H_2O)	6.25
EC (μS cm^{-1})	570
NH_4^+ (mg N kg^{-1} DW)	4.79
NO_3^- (mg N kg^{-1} DW)	79.7

3.3.2. Measurements of Plants Growth

Rice height and tiller number of each plant were measured once a week beginning at 8 days after transplanting (DAT). At that time, top rice leaf greenness (SPAD value, indicating the amount of chlorophyll present) was measured using a SPAD-502 Plus chlorophyll meter (Konica Minolta Inc., Tokyo, Japan). The data from four hills were averaged for each container during all rice growth periods. Above-ground parts of rice plants were harvested at 107 DAT and the roots were thoroughly washed with water. The samples were promptly oven-dried at 80ºC for 48 h for dry weight calculations. The dry weights of each part for all the plants used in the experiment were also determined. Much of the ears were lost due to the field mouse invasion before harvest, so the dry weight of ears was not shown in this paper. Before harvest, Azolla in the flooding water was also collected and oven-dried at 70ºC for 48 h for dry weight calculations.

3.3.3. Measurements of CH_4 and N_2O fluxes, and Plants Dark Respiration (CO_2 flux)

Seasonal variations in CH_4 and N_2O fluxes, and plants dark respiration (CO_2 flux) from the rice pots placed in outdoor water tanks (two pots per tank) (65 cm length × 46 cm width × 32 cm depth) filled with water, were collected with a cylindrical, transparent acrylic, closed-top chamber (20.5 cm inside diameter, 100 cm height, and 0.3 cm thickness). During the sampling time of 30 min, each pot was covered with the chamber to capture gas exchanged between the pots and the atmosphere. At 0, 15, and 30 min after the chamber was placed, a gas sample of about 30 mL was drawn with a 30mL plastic syringe through a capillary tube at the top of the chamber and injected into a 19mL vacuum bottle with a rubber stopper and screw cap. The bottles were sent to the Institute for Agro-Environmental Sciences, NARO, where CH_4, N_2O, and CO_2 concentrations in the bottles were analyzed using an automated analysis system for three gases of CO_2, CH_4, and N_2O. This system consists of two gas chromatographs (GC-14B, Shimadzu, Kyoto, Japan), of which one has both a thermal conductivity detector (TCD) and a flame ionization detector (FID), and the other has an electron capture detector (ECD). This system can analyze 40 samples consecutively with a modified automated headspace sampler (HSS-2B, Shimadzu, Kyoto, Japan) (Sudo 2006). The fluxes were calculated from the increase in the gas concentration inside the chamber per square meter per hour (Cheng *et al.* 2006, 2008). We measured CH_4, N_2O, and CO_2 fluxes at night time about 20:00~22:00, because the average CH_4 flux from rice paddy was similar to the flux at day time between 8:00~10:00 and night time 20:00~22:00 (Cheng *et al.* 2008; Minamikawa *et al.* 2015), and the increase in CO_2 concentration in the chamber in the evening samples represented plants night respiration. The three gases fluxes were measured once every 2 weeks for the first 56 DATs. Later, gas measurements were carried out after every week and terminated after 106 DATs, a day before the final rice plant harvesting.

3.3.4. Measurements of Dissolved CO_2 and CH_4, and NO_3^--N in Soil Solution

Soil solution in each pot was sampled with a Rhizon soil-solution sampler (10 Rhizon SMS-MOM; Eijkelk-amp Agrisearch Equipment, Giesbeek, the Netherlands). The sampler consists of a microporous polymer tube 10 cm long (2.5 mm outside diameter × 1.5 mm inside diameter) and a PVC tube (50 cm length × 2.7 mm outside diameter × 1.0 mm inside diameter). The microporous polymer tube was inserted vertically into the soil between the rice plant and pot edge at a depth of 10-15 cm in each pot 1 day after rice transplanting. About 8 mL of solution was sucked out of the polymer tube with a 10 mL vacutainer (Terumo Ltd, Tokyo, Japan) to remove impurities from the tube before the main sampling. Then a 19 mL semi-vacuum bottle (filled with pure N_2 gas at 0.5

atm) fitted with a rubber stopper and a screw cap was connected to the sampler tube. About 9.5 mL of soil solution was sucked into the bottle by the time the pressure in the bottle reached 1 atm. The amount of solution collected and headspace volume were determined by weighing the bottle before and after sampling. CO_2 and CH_4 were measured in the laboratory with a gas chromatograph (Shimadzu GC-7A) with TCD and FID detectors, respectively. Concentrations of CO_2 and CH_4 dissolved in the soil solution were calculated by Henry's law according to the concentrations of CO_2 and CH_4 in the headspace (Cheng *et al.* 2005, 2006). The NO_3^--N concentration in soil solution was analyzed by colorimetric techniques at 450 nm by using a UV-1200V spectrophotometer (Shimadzu, Japan).

3.3.5. Statistical Analysis

Two paired-sample t-test was applied for each parameter for which significant differences were found ($P<0.05$) between control and Azolla cover treatments. The analysis was done with SPSS 20 (SPSS Inc., Chicago, IL USA) statistical package.

3.4. Results

3.4.1. Changes in Rice Plant Height, Tiller Number, and SPAD Value during the Entire Experimental Period

Rice growth period from transplanting to harvesting was about 107 DAT for both treatments (control and Azolla cover). Plant height increased steadily in both setups until the grain-filling stage about 85 DAT (**Fig. 3.2a**). At harvest, the shoot height was 78.0 and 80.0 for control and Azolla cover setups, respectively. There was no significant difference in rice plant height between the two treatments (**Table 3.2**).

Rice tiller numbers reached a maximum at 43 DAT for both setups (**Fig. 3.2b**). The maximum tiller numbers per hill were 50.8 for control treatment and 48.5 for the Azolla cover treatment. At harvest, the productive tiller numbers per hill were 39.5 and 40.0 for control and Azolla cover setups, respectively (**Table 3.2**).

Top rice leaf greenness (SPAD value) increased to maximum value until 22-29 DAT, 44.5, and 45.3, respectively, then declined slowly for both setups. Later, the leaf greenness was improved by first added fertilization on 49 DAT (**Fig. 3.2c**). During the entire rice growth period, the SPAD value of the Azolla cover treatment was mostly darker than that of the control. There was a significant difference in SPAD value between the two treatments between 92-106 DAT (**Fig. 3.2c**), with SPAD values between 28.6 - 17.4 for the control and 30.9 - 21.8 for Azolla cover treatment, respectively.

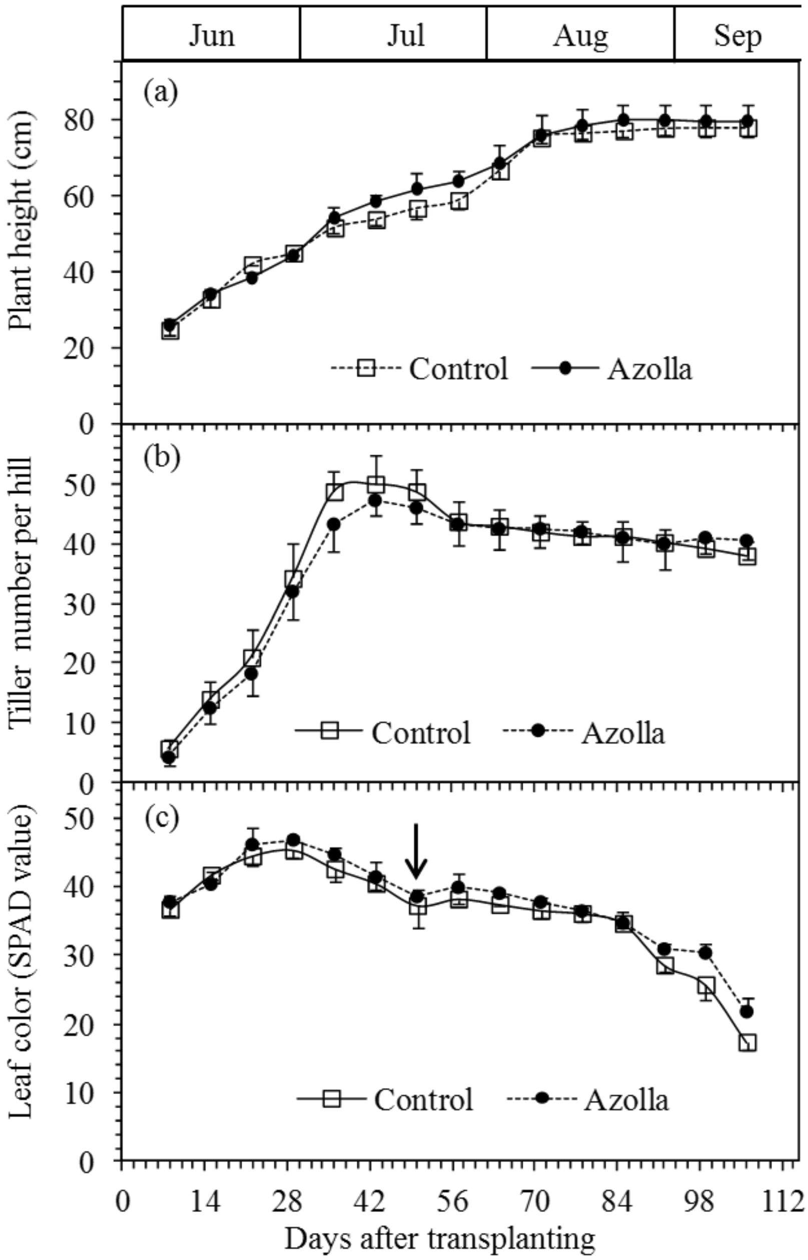

Fig. 3.2. Changes in plant heights (a), tiller numbers of rice plants (b), and leaf color in SPAD values (c) between the rice growth on the two treatments of absence (Control) and presence of *A. filiculoides* (Azolla) throughout the experiment. Bars indicate standard deviation (n=4). Arrow indicates the day of fertilizer addition.

Table 3.2. The main rice growth parameters, dry weight of rice plants and Azolla at harvest, and the cumulated CH_4 and N_2O emissions during the experimental period between two treatments of absence (Control) and presence of *A. filiculoides* (Azolla).

Treatments	Maximum tiller	Productive tiller	Shoot height at harvest	Dry weight of rice at harvest¶			Dry weight of Azolla at harvest	Total CH_4 emission	Total N_2O emission
				Leaf	Stem	Root			
	(No. hill^{-1})		(cm)	(g hill^{-1})			(g pot^{-1})	(g C m^{-2})	(mg N m^{-2})
Control	50.8a*	39.5a	78.0a	9.36a	57.11a	9.75a	–	55.4a	2.72a
Azolla	48.5a	40.0a	80.0a	9.93a	56.97a	10.11a	13.60	36.2b	2.59a

*Values within each column followed by the different letters indicated a significant difference between 2 treatments (P<0.05). ¶*The ear dry weight of rice plants was lost due to the damage by field mouse.

3.4.2. Dry Weight of Rice Plants and Azolla at Harvest

Dry weights of leaf, stem, and roots for the two treatments were not significantly different. Leaf biomass per hill was 9.36 and 9.93 for control and Azolla cover treatments, respectively. The stem biomass per hill was 57.11 for control and 56.97 for Azolla cover treatments, while the roots biomass per hill was 9.75 and 10.11 for control and Azolla cover treatments, respectively (**Table 3.2**). At harvest, the dry biomass of flooding Azolla in the Azolla cover pots was 13.60 g pot^{-1}.

3.4.3. Changes in CH_4 Fluxes and Accumulated Emissions

The pattern/intensity of CH_4 emissions from the pot experiment varied with the stage of rice growth (**Fig. 3.3a**). The emission rates for the control and Azolla cover treatments were relatively low and similar (0.00-7.60 mg C m^{-2} h^{-1}) during the initial stage (15-57 DAT) and increased as the crop matured. The rates peaked on 71 DAT, (70.73 mg C m^{-2} h^{-1}) and (52.51 mg C m^{-2} h^{-1}) for control and Azolla cover treatments, respectively, and decreased following ripening of the rice crop (46.38-27.71 mg C m^{-2} h^{-1}) for the control and (24.69-17.25 mg C m^{-2} h^{-1}) for the Azolla cover treatments. While the Azolla cover treatment recorded a continuous CH_4 emission decrease, a second peak for the control treatment was recorded at 85 DAT. Total seasonal average CH_4 emission rates were (55.4 and 36.2 g C m^{-2}) for control and Azolla cover treatments, respectively (**Table 3.2**). Variations of CH_4 emissions from the two treatments in the pot experiment differed significantly between the heading (71 DAT) and maturity (106 DAT) stages.

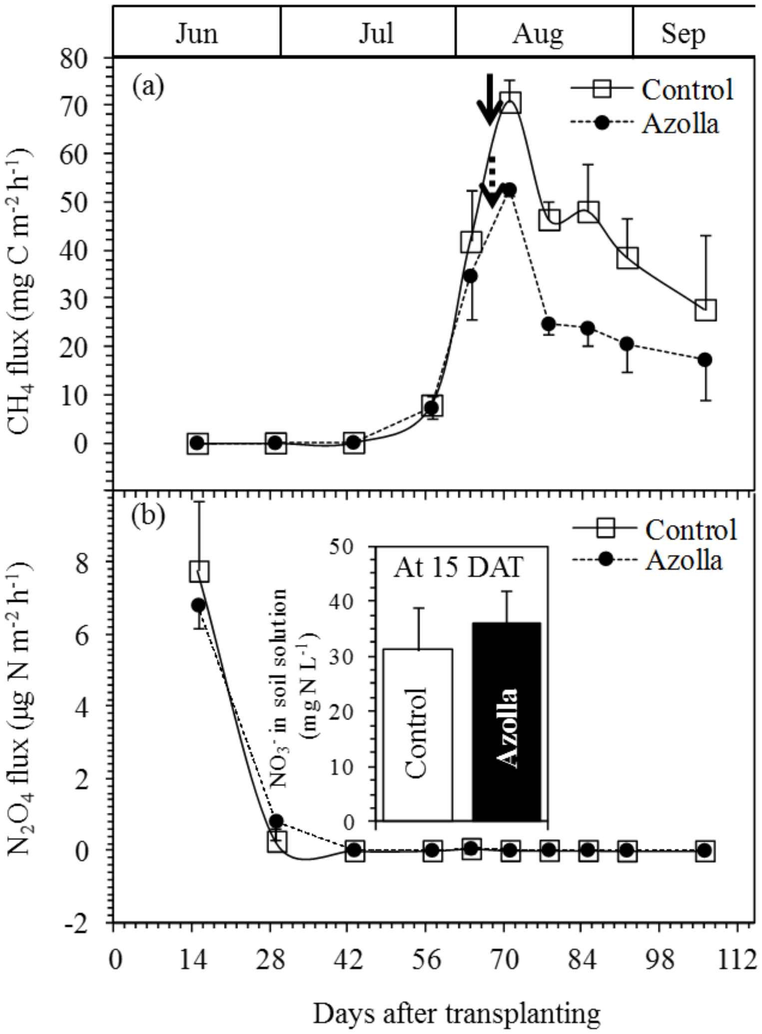

Fig. 3.3. Changes in the daily CH_4 (a) and N_2O (b) fluxes from the pots between the rice growth on the two treatments of absence (Control) and presence of *A. filiculoides* (Azolla) throughout the experiment. Bars indicate standard deviation (n=4). Insert in (b) shows the concentration of NO_3^--N dissolved in soil solution at the first gas sampling day (15 DAT). **Bold arrows** in (a) indicate the heading days for each treatment.

3.4.4. Changes in N_2O Fluxes and NO_3^--N Concentrations in Soil Solution

The pattern/intensity of N_2O emissions from the pot experiment recorded varied emission rates during the initial stage (15-29 DAT) and decreased rapidly following the development of anaerobic conditions in the soil. The emissions peaked on 15 DAT (7.77 μg N m^{-2} h^{-1}) and (6.81 μg N m^{-2} h^{-1}) for control and Azolla cover treatments, respectively (**Fig 3.3b**). Variations of N_2O emissions from the two treatments during 15-106 DAT were not significant. However, the Azolla cover treatment showed slightly prolonged N_2O emission during 15-29 DAT, 0.27 μg N m^{-2} h^{-1} for control, and 0.81 μg N m^{-2} h^{-1} for the Azolla cover treatment on 29 DAT. The NO_3^--N concentration in the solution was only detected on 15 DAT and was 36.15 and 31.12 mg N L^{-1} for control and Azolla cover treatments, respectively (**insert Fig. 3.3b**).

3.4.5. Changes in CO_2 Night Respiration

Night respiration (the CO_2 mostly emitted from rice plant and floating Azolla) ranged from 90.4 to 863.9 mg C m^{-2} h^{-1} during the entire experimental period, and this parameter varied with the growth stage. The highest peak of nighttime CO_2 respiration was found at 10 weeks after rice transplanting for the two treatments (**Fig. 3.4a**). The small peaks before and after the highest one were as a result of daily sunshine time (**Fig. 3.4b**). Nighttime CO_2 was significantly higher at 15 DAT and from the 64 and 106 DAT ($P<0.05$). The average values of CO_2 respiration during the entire experimental period were 412.6 and 491.67 mg C m^{-2} h^{-1} from control and Azolla cover treatments, respectively.

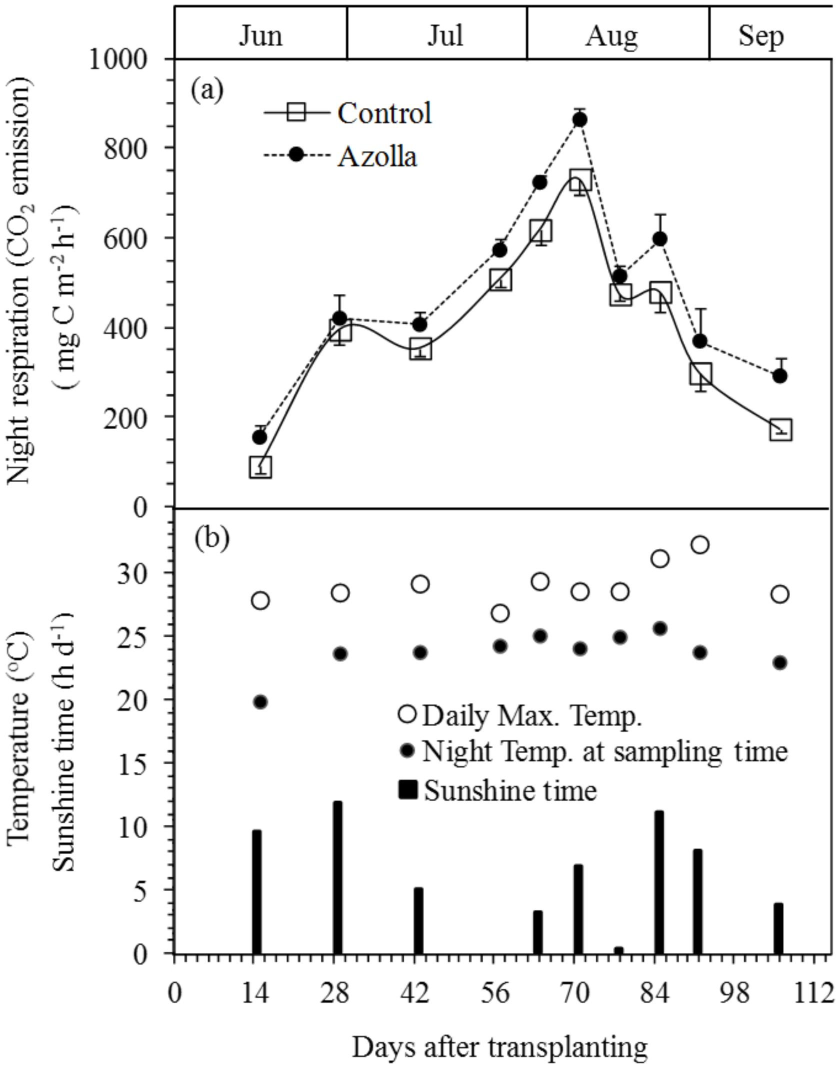

Fig. 3.4. Changes in night respiration (CO_2 emission) of rice plants grown in the pots between treatments of absence (Control and presence of *A. filiculoides* (Azolla) throughout the experiment period (a). Bars indicate standard deviation (n=4). The daily maximum temperature and the temperature at sampling time (21:00), and sunshine time on the day of gases sampling were shown in (b).

3.4.6. Changes in CO_2 and CH_4 Concentrations in Soil Solution

Variations of soil solution CO_2 concentrations between the two treatments during 15-57 DAT were not significantly different (**Fig. 3.5a**).

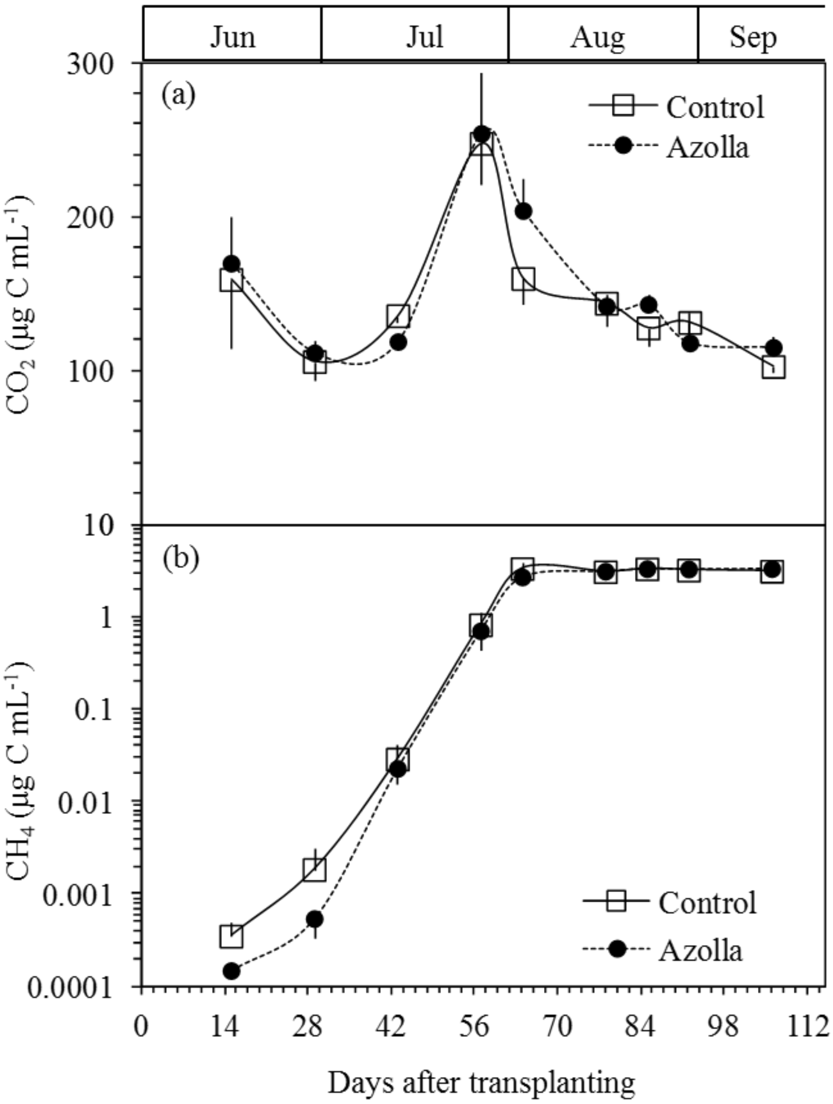

Fig. 3.5. Changes in the concentration of CO_2 (a) and CH_4 (b) dissolved in the soil solution in the pots between the treatments of absence (Control), presence of *A. filiculoides* (Azolla) throughout the experiment period. Bars indicate standard deviation (n=4).

After the highest peak at 57 DAT for both treatments, at 64 DAT a significant soil concentration level ($P<0.05$), was recorded with no further significant observations thereafter. Dissolved CH_4 in the soil solution recorded non-significant variations between the two treatments throughout the growth period, 15-106 DAT (**Fig. 3.5b**). However, Azolla cover treatment showed lower CH_4 soil solution concentrations in the first 15-43 DAT compared to control. The dissolved soil concentrations of CH_4 for both treatments increased uniformly with the highest peak at 64 DAT and later stabilized. The average values of soil dissolved CO_2 concentration during the experimental period were 146.0 and 153.0 µg C mL^{-1} for control and Azolla cover treatments, respectively. While those of soil dissolved CH_4 concentrations were 1.89 µg C mL^{-1} and 1.85 µg C mL^{-1} for control and Azolla cover treatments, respectively.

3.5. Discussion

3.5.1. Effects of Azolla Cover on Rice Growth

Though the tiller number of the control treatment during the early rice growth stage and the maximum tiller number were higher than those of Azolla cover treatment (**Fig. 3.2b**), the productive tiller number at harvest was similar (**Table 3.2**). The SPAD values of the control treatment were slightly higher than those of the Azolla cover treatment, but there were not significant in most cases, except for the last 2 times (**Fig. 3.2c**). The overall performance of the rice under control and Azolla cover in respect to above and below ground dry weight biomasses was statistically similar (**Table 3.2**). Although we lost much of the aboveground biomass due to field mouse invasion, the results indicated that Azolla cover during the single rice season did not affect rice growth. The reasons can be explained as follows. Firstly, covering Azolla was not plowed into the soil during the rice growth season as biofertilizer, thus, the Azolla cover treatment could not provide extra N for increasing rice growth. Secondly, covering Azolla absorbed the nutrition elements from flooding water, but it did not compete against rice to absorb the nutrition elements from the soil. Nonetheless, covering Azolla as dual cropping did not compete for sunshine with rice and hence no decrease in rice growth. Related observations have been reported by Mujiyo *et al.* (2016) for organic rice systems in Indonesia.

3.5.2. Effect of Azolla Cover on CH_4 Emission

The earliest research regarding the effect of Azolla application on CH_4 emission from rice soil ecosystems was started in the 1990s. Chen *et al.* (1997) and Ying et *al.* (2000) reported that Azolla application increased CH_4 emission, likely due to the exudation of Azolla root and decomposition of dead Azolla, but not due to Azolla transportation of CH_4 in the soil as rice plant did. However, the most recent reports showed that Azolla cover decreased CH_4 emission from rice soil ecosystems, likely due to the increase in redox potential in the root region and dissolved oxygen concentration at the soil-water interface (Ali *et al.* 2015; Singh and Strong 2016; Liu *et al.* 2017). In this study, we had not obviously observed the dead Azolla until harvest, and even if the exudation of Azolla roots contributed to the organic matter in the soil surface, the contribution could have become an insignificant source of CH_4 since the surface layer of submerged rice paddy always maintained oxic conditions. Hence, the possibilities of the Azolla covering in our experiment becoming a source for CH_4 production and emission in the dual cropping Azolla-rice soil ecosystem was highly unlikely. Our results showed that CH_4 fluxes occurred 8 weeks after rice transplanting at 7.91 and 7.28 mg C m^{-2} h^{-1} for control and Azolla cover treatments, respectively. After that, CH_4 fluxes from the control treatment were always higher than those from the Azolla cover treatment (**Fig. 3.3a**). The cumulated CH_4 emissions during the growth period until 106 DAT (most during the periods after heading) was significantly lower at 36.2 g C m^{-2} from Azolla cover treatment than that from control treatment pot at 55.4 g C m^{-2}. Azolla cover decreased the seasonal CH_4 emission at 34.7% compared to the control treatment without Azolla cover (**Table 3.2**). This result was consistent with previous recent studies (Bharati *et al.* 2000; Ma *et al.* 2012; Ali *et al.* 2015; Liu *et al.* 2017). The cumulated CH_4 emission from the control treatment pot at 55.4 g C m^{-2} was similar to the value gathered in a field experiment in Yamagata (Itoh *et al*. 2011). Not only Azolla but also common duckweed (*Lemna minor*), the other floating macrophyte, significantly reduced the mean CH_4 emission from flooded rice paddies in China by 20.4% (Wang *et al.* 2015). It is well known that CH_4 emission from flooded rice paddy soils occurs through plant-mediated transport, ebullition, and diffusion and that plant-mediated transport is the dominant pathway (Nouchi *et al.* 1990). Net CH_4 emission is determined by the balance between CH_4 production and CH_4 oxidation in rice plant–paddy soil ecosystems (Inubushi *et al.* 2003). Redox potential, which is an indirect indicator of the CH_4 flux pattern from rice ecosystem (Wang et al. 1993), is directly related to dissolved oxygen concentration, and soils with higher redox potential could inhibit CH_4 production and contribute to CH_4 oxidation and hence lower CH_4 emission rate (Malyan et al. 2016). Although we did not measure the dissolved oxygen content in the standing water and redox potential in this study, (Xu et al. 2017) reported 30.0-42.8 and 24.1-44.8% markedly enhanced dissolved oxygen at the soil-water interface and enhanced soil redox potential by 14.5-19.8 and 12.7-19.4% during the early and late rice growing seasons, respectively, of rice + Azolla without N fertilizer treatment compared with the conventional rice without N fertilizer. Thus, for our study, the moderating effect of floating plants on CH_4 emission from dual Azolla-rice soil ecosystem could be reconsidered due to two main reasons. Firstly, photosynthetically released oxygen by the floating plants into the flooding water, could directly stimulate CH_4 oxidation at the soil-water interface and rhizosphere of the surface layer, indirectly leading to a decrease in CH_4 emission from plant-mediated transport (through the aerenchyma tissues). Secondly, the moderating effect on CH_4 emission from dual Azolla–rice soil could be attributed to the large masses of floating plants covering the flooding water surface of rice soil which could serve as a physical barrier obstructing the diffusion of CH_4 from anaerobic soil to the atmosphere, which provides the other pathways for CH_4 emitting to the atmosphere as ebullition and diffusion (van der Steen et al. 2003). Our results showed no significant differences in rice growth parameters and dry biomass at harvest (**Fig. 3.2** and **Table 3.2**), and the soluble CH_4 and CO_2 concentrations in soil solutions (**Fig. 3.5**) between control and Azolla cover treatments, implying that Azolla cover did not affect the CH_4 production in the submerged dual cropping Azolla-rice soil ecosystem. This confirmed that the decreased CH_4 emission by Azolla cover was due to stimulated CH_4 oxidation (Bharati et al. 2000; Ali et al. 2015; Liu et al. 2017).

The sources of CH_4 production are from both older matter (e.g., native soil organic matter, incorporated organic material such as straw and manure (Nakajima *et al.* 2016) and new matter from plant growth (e.g., root exudates and plant debris). In this pot experiment, we used light air-dried soil (at 25% w/t moisture) without any visible plant residues for cultivating single cropping rice and dual cropping Azolla and rice. CH_4 fluxes were not detected or were relatively low during the early rice growth period until 43 DAT (**Fig. 3.3a**), even though the amount of CH_4 dissolved in the soil solution increased following the rice growth until the rice heading stage (**Fig. 3.5b**). It implied that the native soil organic matter was not the main source of CH_4 production. The CH_4 flux increased shapely after 43 DAT and reached the highest peaks at the heading stage for both treatments (**Fig. 3.3a**). During the same period, the plant night respirations also recorded the highest peaks for both treatments (**Fig. 3.4a**). Also, significant positive correlations were found between CH_4 flux and night respiration for both the control ($P<0.05$) and Azolla cover ($P<0.01$) treatments throughout after 9 weeks of rice transplanting (**Fig. 3.6**). These results implied that the sources of CH_4 productions from both treatments were mostly from new matter through root exudates and plant debris, not from older matter, such as native soil organic matter and incorporated organic material, which is consistent with our previous results (Cheng *et al.* 2006, 2008; Lou *et al.* 2008).

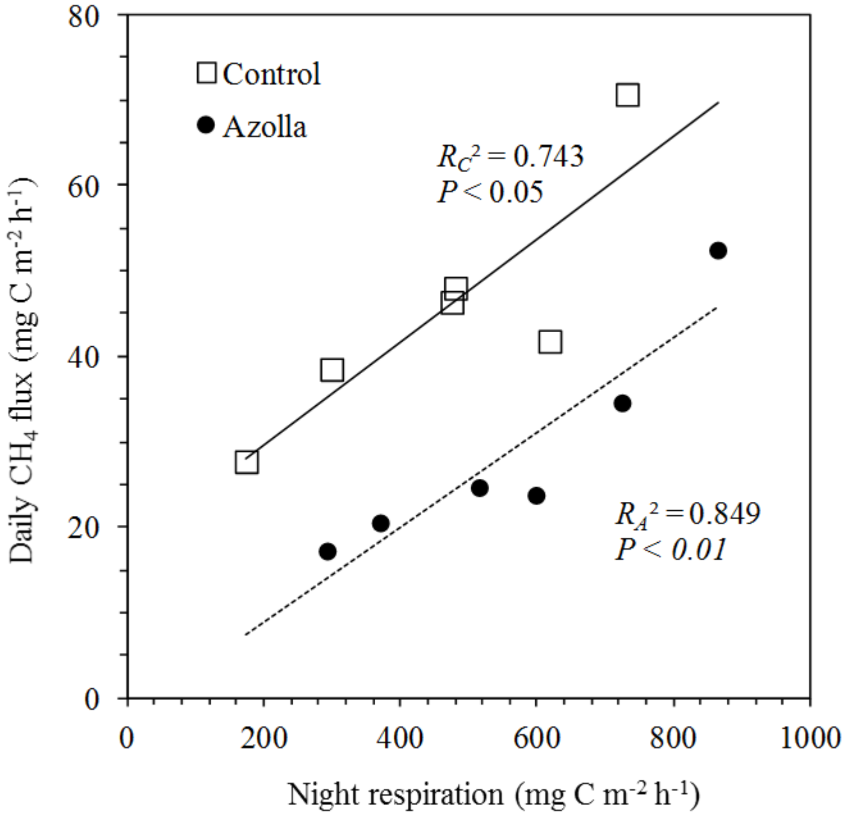

Fig. 3.6. Relationship between daily CH_4 flux and night respiration (CO_2 emission) between treatments of absence (Control) and presence of *A. filiculoides* (Azolla) throughout after 9 weeks of rice transplanting.

Also, the night respirations of Azolla cover treatments were always higher than those of control treatments due to CO_2 respired from both the Azolla cover as dual cropping and rice (**Fig. 3.4a**). It should be noted that the higher CO_2 respiration from Azolla cover treatment than control did not directly relate to different CH_4 emissions between both treatments. Night CH_4 emission and respired CO_2 could be affected by many environmental factors, such as temperature, and sunshine time. In this experiment, the temperature at sampling time (around 21:00) and daily maximum temperature at day time did not affect both night CH_4 emission and respired CO_2 during the entire rice growth period (**Fig. 3.3a** and **Fig. 3.4a, b**). However, the daily sunshine time likely affected night CO_2 respiration (**Fig. 3.4a, b**).

3.5.3. Effect of Azolla Cover on N_2O Emission

To our knowledge, N_2O emission from rice paddy affected by Azolla cover was only reported by Chen *et al.* (1997) and Ma *et al.* (2012). Their results showed that Azolla cover increased N_2O emission from rice paddies due to N-fixation by Azolla providing a source for N_2O production through nitrification and denitrification, especially when the Azolla died. The reason is highly similar to that of N_2O emissions from the other symbiotic N_2 fixation plants ecosystems, such as leguminous crops and acacia trees ecosystems, which emitted more N_2O to the atmosphere than non-symbiotic N_2 fixation plant ecosystems (Arai *et al.* 2008; Mori *et al.* 2010, Uchida and Akiyama 2013; Zhang *et al.* 2014). However, in this experiment, N_2O flux was not detected or was relatively low (<0.01μg N m^{-2} h^{-1}) after 4 weeks after rice transplanting from both control and Azolla cover treatments (**Fig. 3.3b**). It implied that Azolla cover did not bring extra N_2O flux from dual Azolla and rice cropping ecosystems. The reason can be explained as discussed in the last section, as we had not observed the dead Azolla until harvest. Though relatively high N_2O fluxes were detected at 15 DAT (first gas sampling) from both treatments at 7.77 and 6.81 μg N m^{-2} h^{-1} for control and Azolla cover treatments, respectively (**Fig. 3.3b**), the source for the high N_2O can be attributed to the NO_3^--N in the initial soil before flooding. The NO_3^--N in the initial soil was 79.7 mg N kg^{-1} DW (**Table 3.1**). Even at 15 DAT, the NO_3^--N concentrations were 31.1 and 36.2 mg N L^{-1} in the soil solutions of control and Azolla cover treatments, respectively (**insert Fig. 3.3b**). High NO_3^--N in the initial soil and high NO_3^--N dissolved in soil solution, and no statistical significance of NO_3^--N amounts and N_2O fluxes between control and Azolla cover treatments confirmed that the N_2O flux from the early rice growth period was from NO_3^--N denitrification in the initial soil. This result was consistent with our previous experiment (Cheng *et al.* 2006). Though there were no significant differences in N_2O emissions between the control and Azolla cover treatments in our study. Later decomposition and/or incorporation of the covering Azolla, if not removed from the pots, may increase N_2O emission during the subsequent rice-growing season.

3.6. Conclusions

To determine whether Azolla cover affects CH_4 and N_2O emissions from dual Azolla and rice cropping paddy soil, a pot experiment was carried out in Tsuruoka, Yamagata, Japan in 2016. The results showed that Azolla cover did not affect rice growth, because Azolla did not compete against rice to absorb the nutrition from soil and sunshine from rice canopy. Azolla cover did not affect CH_4 production in the submerged rice soil, but dual cropping of Azolla with rice significantly suppressed CH_4 emissions, likely due to an increase in dissolved oxygen concentration and redox potential at the soil-water interface, thereby stimulating CH_4 oxidation. The cumulated CH_4 emissions during the growth period until 106 DAT (most during the periods after heading) was significantly lower at 36.2 g C m^{-2} from Azolla cover treatments than that from control treatments pot at 55.4 g C m^{-2}. Azolla cover decreased the seasonal CH_4 emission at 34.7% compared to the control treatment without Azolla cover. A prolonged non-significant N_2O emission under the Azolla cover treatment after the initial highest peak at 15 days after transplanting (DAT) was recorded due to denitrification of the nitrate in the initial soil. No further N_2O emissions were recorded thereafter from both treatments. Azolla cover did not affect N_2O emissions from both treatments. It should be noted that our results are based on a pot experiment, field studies should be carried out in the future.

CHAPTER IV

4. Azolla Incorporation and Dual Cropping Influences CH_4 and N_2O Emissions from Flooded Paddy Ecosystems

4.1. Abstract

To investigate the influence of Azolla (*A. filiculoides* Lam.) incorporated as green manure and its subsequent growth as a dual crop with rice on simultaneous methane (CH_4) and nitrous oxide (N_2O) emissions from a flooded alluvial soil planted with rice, a pot experiment with three treatments, chemical fertilizers (NPK) as the control, incorporation of Azolla as green manure (AGM), and AGM plus basal chemical fertilizers (NPK+AGM) was conducted in Tsuruoka, Yamagata, Japan in 2017. AGM and NPK+AGM treatments significantly increased CH_4 emissions at early rice growth stages before 63 days after transplanting (DAT) by 123.0% and 176.7% compared to NPK, respectively. At late rice growth stages (after 63 DAT), only the NPK+AGM treatment significantly increased CH_4 emission by 22.1% compared to NPK. However, percentage of CH_4 emitted after 63 DAT relative to the seasonal CH_4 emission followed the order of NPK (86.2%) > AGM (76.5%) > NPK+AGM (73.3%). Higher CH_4 emissions from AGM and NPK+AGM before 63 DAT were attributed to the incorporated Azolla, while the higher emissions after 63 DAT in all treatment groups were ascribed to rice photosynthesis. AGM and NPK+AGM treatments significantly decreased N_2O emissions by 71.6% and 81.1% compared to NPK, respectively, at early rice growth stages. Azolla incorporation may have restricted N_2O emission from initial soil nitrate before 63 DAT and not have contributed to N_2O emissions after 63 DAT. Significantly higher grain yields were observed under the AGM (32.5%) and NPK+AGM (36.3%) compared to NPK. Together, AGM and NPK+AGM treatments significantly increased seasonal CH_4 emissions by 31.5% and 43.5%, and decreased seasonal N_2O emissions 3.4- and 4.6- fold compared to NPK, respectively. There were no significant differences in the CH_4 emissions per grain yield among the treatments; however, compared to NPK, AGM and NPK+AGM treatments significantly reduced N_2O emissions per grain yield by 78.7% and 84.1%, respectively.

4.2. Introduction

Methane (CH_4) has a global warming potential (GWP) by mass that is 34 times higher than carbon dioxide (CO_2) over 100-years and is estimated to account for 15-20% of atmospheric radiative forcing; while nitrous oxide (N_2O) is 298 times more potent as a heat trapper than CO_2 and may account for 6-8% of radiative forcing in the atmosphere (IPCC 2013). As the two most important trace greenhouse gases (GHG) contributing to global warming, and with the continued increase in global temperatures, CH_4 and N_2O gas emissions have necessitated more research, particularly in paddy ecosystems (Akiyama, Yan, and Yagi 2006; Yan et al. 2009; Yao et al. 2012; Gao et al. 2016; Kimani et al. 2018).

Rice paddy fields are one of the most important sources of atmospheric CH_4 and N_2O emissions, with an estimated global CH_4 emission rate of 25-60 Tg yr^{-1} (Reay, Smith, and Amstel 2010), and an annual global N_2O contribution of 13-24% (Mosier et al. 1998; Olivier et al. 1998). Methane is produced through anaerobic degradation of organic matter by archaea and oxidized by methanotrophic bacteria, while N_2O originates naturally in soils through microbial processes of nitrification and denitrification (Bouwman 1998; Inubushi et al. 2003; Cheng et al. 2004a, 2004b). Although the extent of N_2O emissions from rice paddies is much lower than that of CH_4, agricultural practices that add nitrogen (N) to soil, such as increased organic and inorganic fertilizers, are major drivers of agricultural soil N_2O emissions (Zou et al. 2005). Additionally, CH_4 and N_2O gases are simultaneously emitted from paddy soils depending on the farming system, soil organic matter, soil moisture levels, soil microbial activities, reduction-oxidation status, and fertilizer management practices (i.e. exogenous application of N fertilizers and/or organic materials) (Snyder et al. 2009; Nishimura et al. 2011). With an estimated demand for increased rice production that must reach 828 million tons by 2025 to satisfy the needs of the rapidly growing population (Kubo and Purevdorj 2004), an accompanying increase in CH_4 emissions, as well as a proportionate increase in N_2O emissions following increased N fertilizer use, is likely (Bouwman 1991; Zou et al. 2009). Agricultural GHG fluxes are complex and heterogeneous, and active management strategies to mitigate the combined (or total) CH_4 and N_2O emissions from paddy fields are needed (Li 2007).

Azolla (*A. filiculoides*) is a free-floating aquatic fern common in lowland rice fields that has been used effectively in paddy fields in Vietnam and southern China as a biofertilizer due to its unique symbiotic relationship with the N-fixing cyanobacteria *Anabaena azollae*. Azolla is still cultivated in organically managed paddy fields, especially in rice-fish-Azolla or rice-duck-Azolla multi-eco-production systems in Asia (Cagauan, Branckaert, and van Hove 2000; Lu and Li 2006; Cheng et al. 2015). Additionally, Azolla has recently gained considerable importance for its multifaceted uses (Yadav et al. 2014; Kollah, Patra, and Mohanty 2016; Shukla et al. 2018; Chakraborty et al. 2019). The use of Azolla as green manure can either be through its incorporation into paddy soil at the beginning of land preparation before rice transplanting or grown as a dual crop along with rice plants (Xu et al. 2017). Dual cropping of Azolla with rice was reported to either increase (Chen et al. 1997; Ying et al. 2000) or decrease (Bharati et al. 2000; Kimani et al. 2018) CH_4 emission fluxes from flooded rice paddies with varying observations of N_2O emissions reported as well (Chen et al. 1995; Zou et al. 2005; Kimani et al. 2018). The lack of a consensus between all these studies is partly attributed to differences in experimental conditions, including different rice cultivars and soil properties. Recent studies have reported the influence of leguminous crop incorporation (e.g. Chinese milk vetch) on CH_4 and N_2O emissions in paddy fields (Zhu et al. 2012; Tang et al. 2015); however, the influence of Azolla on simultaneous CH_4 and N_2O emissions from a flooded paddy ecosystem when incorporated as green manure or as a dual crop with rice in conjunction with and without chemical fertilizers, is not well understood, perhaps only with the exception of Bharati et al. (2000), who reported cumulative CH_4 flux in the order of urea > Azolla (incorporated) + urea > Azolla (incorporated + dual crop) > no N control > urea + Azolla (dual crop).

Results from our previous study showed that when Azolla is as a cover crop, CH_4 production from submerged rice soil increased but CH_4 emissions were significantly suppressed, likely due to increased dissolved oxygen concentration and redox potential at the soil-water interface, which stimulated CH_4 oxidation (Kimani et al. 2018). Additionally, Azolla cover did not affect N_2O emissions due to denitrification of the initial soil (Kimani et al. 2018). Here, to investigate the influence of Azolla incorporation as green manure and its subsequent growth as a dual crop in conjunction with chemical fertilizers at basal and rice booting stages, on simultaneous CH_4 and N_2O emissions from a flooded paddy ecosystem, we conducted a pot experiment in the summer of 2017 in Tsuruoka, Yamagata, Japan.

4.3. Material and Methods

4.3.1. Experiment Site, Design, and Management

This research was conducted in 2017 at the Experiment Farm, Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata Prefecture located in northeastern Japan (38º44´N, 139º50´E, 16 m elevation). According to the Japan Meteorological Agency database for Tsuruoka Meteorological Observatory (**see details in Chapter III)**, the climatic condition during the rice growth season in 2017 (7 June to 20 September) was 0.1°C hotter and sunnier than the historic average 1981-2010 (**Fig. 3.1 in Chapter III**). The daily average air temperature and sunshine time during the rice growth season were 22.70°C and 6.38 h, while the historic averages of 1981-2010 were 22.60°C and 5.75 h, respectively.

In situ pots were used with three treatments in four replications (**Table 4.1**). *Azolla filiculoides* Lam. was grown in multiplication tanks and incorporated as fresh green manure at 12.2 g Azolla dry weight pot^{-1} (equivalent to 0.40 g N pot^{-1}) to experimental pots in the AGM and NPK+AGM treatment groups one day before rice transplanting. One week after Azolla incorporation, the Azolla started growing and formed a cover (i.e. Azolla dual crop) on the soil surface in the AGM and NPK+AGM treatments. Incorporated Azolla had dry weight concentrations of 339.9 g kg^{-1} organic C and 33.8 g kg^{-1} total N. Although Azolla grows in this region during the summer rice-growing season, no native Azolla was available because of the harsh winter conditions; therefore, an introduced species (IRRI code FI 1001) was adopted and grown in the greenhouse for this experiment (Cheng et al. 2010; Kimani et al. 2018). Haenuki is a popular edible rice cultivar widely grown in local areas of the Yamagata Prefecture and was used for this experiment.

Azolla filiculoides Lam., grown in multiplication tanks, was incorporated as fresh green manure at 12.2 g Azolla dry weight pot^{-1} (equivalent to 0.40 g N pot^{-1}) to experimental pots of second (AGM) and third (NPK+AGM) treatments a day before rice transplanting. One week after Azolla incorporation, the incorporated Azolla started growing and formed a cover (Azolla dual crop) on the soil surface of the second and third treatments. Incorporated Azolla had 339.9 and 33.8 organic C and total N at g kg^{-1} dry weight, respectively. Although Azolla grows in this region during the summer rice-growing season, no native Azolla was available because of the harsh winter conditions. An introduced species (IRRI code FI 1001) was adopted and grown in the greenhouse for this experiment (Cheng et al. 2015; Kimani et al. 2018). Haenuki cv., a popular edible rice cultivar grown widely in the local area, Yamagata Prefecture, was used for this experiment.

Table 4.1. Summary of the experimental treatments on fertilizers and Azolla application at the Experimental Farm, Tsuruoka, Japan.

Treatment code (in details)	Amendments		Total N application (g pot^{-1})
	Azolla application	Fertilizer amendment	
NPK (Chemical fertilizer)	-	0.40 g N, 0.20 g P, and 0.25 g K per pot were applied by KH_2PO_4 and $CO(NH_2)_2$ as basal fertilizer before transplanting. Half of the basal amounts were applied as top dressing at 49 DAT.	0.60
AGM (Azolla as green manure)	243 g fresh Azolla (95% water content, 12.2 g dry weight) incorporated as green manure at transplanting to provide 0.40 g N pot^{-1} eqv. (Azolla cover grew following Azolla incorporation).	Top dressing was applied at 49 DAT by KH_2PO_4 and $CO(NH_2)_2$ at 0.20 g N, 0.10 g P, and 0.13 g K per pot.	0.60
NPK + AGM (Chemical fertilizer plus Azolla as green manure)	243 g fresh Azolla (95%water content, 12.2 g dry weight) incorporated as green manure at transplanting to provide 0.40 g N pot^{-1} eqv. (Azolla cover grew following Azolla incorporation).	0.40 g N, 0.20 g P, and 0.25 g K per pot were applied by KH_2PO_4 and $CO(NH_2)_2$ as basal fertilizer before transplanting. No top dressing.	0.80

.

The pot experimental soil was collected from a rice field at the University Farm and classified as an alluvial (**Table 4.2**).

Table 4.2. Major properties of experimental paddy soil.

Organic C (g kg^{-1} DW)	14.50
Total N (g kg^{-1} DW)	1.40
C/N	10.36
pH (H_2O)	5.24
EC (µS cm^{-1})	170.0
Available P (mg P_2O_5 kg^{-1})	70.0
NH_4^+ (mg N kg^{-1} DW)	24.8
NO_3^- (mg N kg^{-1} DW)	101.6

The soil was air-dried, sieved (5-mm mesh size), and mixed well before use. On 26th April 2017, germinated rice seeds were sown in a seedling tray (three seeds per cell) and at five weeks after sowing, on 31st May 2017, the seedlings were transplanted (three seedlings per pot) to twelve plastic pots (19.5 cm inside diameter, 27 cm height, and 0.2 cm thickness). Before transplanting, 7.0 kg (4.9 kg dry soil equivalent, water content was 30% in water per total weight of soil) of alluvial soil was mixed with 0.87 g of KH_2PO_4 and 0.87 g $CO(NH_2)_2$ and filled to each pot of the first (NPK) and third treatments.

The amounts of nitrogen, phosphorus, and potassium of the basal fertilizers were 0.40, 0.20, and 0.25 g pot^{-1}, respectively. At 49 days after transplanting (DAT), the first and second treatments only were top-dressed with 0.20 g N, 0.10 g P, and 0.13 g K pot^{-1} by 0.43 g $CO(NH_2)_2$ and 0.43 g KH_2PO_4. At the end of the experiment, the total amounts of applied N, P, and K at field level were, 200.9-, 100.4, and 127.2 kg per ha, respectively. The flooding water was maintained at about 5 cm depth throughout the experiment period by adding tap water. Azolla cover (as a dual crop with rice) for the AGM and NPK+AGM treatments (herein Azolla amended treatments), was maintained throughout the rice growth period and its final dry weight was determined before rice harvesting.

4.3.2. Measurements of CH_4 and N_2O Fluxes, and Plants Night Respiration (CO_2 flux)

Whole rice growth period variations in CH_4 and N_2O fluxes, and plant nighttime respiration (CO_2 flux) from the rice pots (NPK and Azolla amended treatments), were collected and analyzed similarly to our previous pot experiment (Kimani et al. 2018). Briefly, a sample gas of about 30 mL at 0, 15, 30 min for each treatment replication was drawn with a 30 mL plastic syringe through a capillary tube at the top of 1m closed-top chamber and injected in a 19 mL pre-evacuated vial fitted with a rubber stopper and screw cap. All samples were analyzed at the Institute for Agro-Environmental Sciences, NARO, and the fluxes were calculated from the increase in gas concentration inside the chamber per square meter per hour (Sudo 2006; Cheng et al. 2008; Kimani et al. 2018). Fluxes of the three gases were measured once per week for the first 84 DATs. Later, the measurements were carried out every two weeks and terminated after 112 DATs, a day before the final rice harvesting (113 DATs).

4.3.3. Measurements of Dissolved CO_2 and CH_4, and NO_3^--N in Soil Solution

For the NPK and Azolla amended treatments, soil solutions in each of the treatment replications for dissolved CO_2, CH_4, and NO_3^--N analysis, were sampled, analyzed, and calculated as reported in our previous research (Kimani et al. 2018). Briefly, about 9.5 mL of soil solution was sucked into a 19 mL semi-vacuum bottle (filled with pure N_2 gas at 0.5 atm) fitted with a rubber stopper and a screw cap, through a 10 cm long microporous polymer tube (2.5 mm outside diameter x 1.5 mm inside diameter) fitted to a PVC tube (50 cm length x 2.7 mm outside diameter x 1.0 mm inside diameter) and inserted vertically into the soil between the rice plant and pot edge at a depth of 10-15 cm in each pot one day after rice transplanting. CO_2 and CH_4 were measured in the laboratory with a gas chromatograph (Shimadzu GC-7A) with TCD and FID detectors, respectively and their concentrations were calculated by Henry’s law according to the concentrations of CO_2 and CH_4 in the headspace (Cheng et al. 2005, 2006). The NO_3^--N concentration in soil solution was analyzed by colorimetric techniques at 450 nm using a UV-1200V spectrophotometer (Shimadzu, Japan).

4.3.4. Measurements of Plants Growth, Grain Yield, and Soil C Content

Data on rice height and tiller numbers of each plant per treatment were collected once a week beginning at eight DAT. At that time, top rice leaf greenness (SPAD value, indicating the amount of chlorophyll present) was measured using a SPAD-502 Plus chlorophyll meter (Konica Minolta Inc., Tokyo, Japan). The data from four hills per treatment were averaged during the rice growth period. Above-ground parts of rice plants were harvested at 113 DAT for all NPK and Azolla amended treatments. Before harvest, floating Azolla cover was also collected, oven-dried at 70 °C for 2 days for dry weight calculations. After harvest, the soil in the pots was divided into two equal parts from the center. One part for roots sampling (Kimani et al. 2018), and the other part for soil properties measurements, such as soil pH, EC, SOC, and TN contents which were measured by the air-dry soil samples procedures (JSSSPN 1986). Total dry weight biomass of rice plant included above-ground parts and root together. Total biomass, grain yield, and harvest index for all treatments were determined at harvest as described by Amanullah and Inamullah (2016). To measure grain yield, rice ears were air-dried for 1 month and grain was carefully threshed.

4.3.5. Evaluation of the Net GHG as Influenced by Azolla Green Manure Incorporation

Effects of fresh Azolla incorporation in flooded paddy soil on global warming potential (GWP), soil C sequestration, and the net GHG balance compared to the NPK only amendment, were evaluated. We derived the GWP in g CO_2 equivalent per square meter by combining the cumulative CH_4 and N_2O flux at harvest. In these calculations, the GWP values for CH_4 and N_2O were considered to be 34 and 298, respectively over a hundred-year time frame (IPCC 2013). Global warming potential (GWP) and Soil C sequestration was calculated using the following equations, respectively:

$$GWP_{CH_4\ to\ CO2eq} = \mathrm{CH_4\text{-}C\ emission} \times 16/12 \times 34 \tag{1}$$

$$\mathrm{GWP}_{\mathrm{N_2O\ to\ CO2eq}} = \mathrm{N_2O\text{-}N\ emission} \times 44/28 \times 298 \tag{2}$$

$$Soil\ C\ sequestration\ (g\ CO_{2eqv}\ m^{-2}) = \left(C_{tre} - C_{bef}\right) \times \left(\frac{S_{dw}}{P_{area}}\right) \times 44/12 \tag{3}$$

where C_{tre} is soil C content of the treatment after rice cultivation (g kg^{-1} dry soil), C_{bef} is the soil C content before the experiment (14.50 g kg^{-1} dry soil), S_{dw} is the amount of soil in the pot at the start of the experiment (4.9 kg dry weight), P_{area} is the pot area. Multiplied by a ratio of molecular weight of CO_2 to C (44/12) to calculate the C sequestration in CO_2 equivalent. Changes in the net GHG balance following the application of Azolla as green manure and its subsequent growth as a dual crop with rice was calculated relative to the NPK treatment.

4.3.6. Statistical Analysis

Data were submitted to an analysis of variance and means were compared based on Tukey-test to determine significant differences between the treatments for the measured plant parameters, the emissions of CH_4, N_2O, night respiration (CO_2 flux), and the dissolved CO_2, CH_4, and NO_3^--N in soil.

4.4. Results

4.4.1. Changes in Plant Growth Parameters, Grain Yield, and Azolla Cover Dry Weight

Rice growth period from transplanting to harvesting was about 112 DAT for all treatment groups (NPK, AGM, and NPK+AGM). Plant height increased steadily in all setups until the grain-filling stage 77-84 DAT (**Fig. 4.1a**). At harvest, the shoot height was 81.8, 88.5, and 84.7 cm for NPK, AGM, and NPK+AGM treatments, respectively. There was no significant difference in rice plant height among NPK and Azolla amended treatments.

Rice tiller numbers reached a maximum at 42, 91, and 49 DAT for NPK, AGM, and NPK+AGM treatments, respectively, (**Fig. 4.1b**). The maximum tiller numbers per hill were 46.0, 30.3, and 38.8 for, NPK, AGM, and NPK+AGM treatments, respectively. At harvest, the productive tiller numbers per hill were 32.0, 28.3, and, 32.3 for the NPK, AGM, and NPK+AGM treatments, respectively.

Top rice leaf greenness (i.e. SPAD value) reached its maximum value at 35 DAT, which was 48.6, 45.2, and 45.9 for NPK, AGM, and NPK+AGM, respectively, and then decreased sharply in all three treatments. The leaf greenness in both NPK and AGM treatments increased after top-dressing at 49 DAT (**Fig. 4.1c**), while NPK+AGM treatments were not top-dressed and the SPAD value decreased steadily until harvest. The AGM only treatment maintained significantly higher SPAD values (at $P < 0.05$) compared to NPK and NPK+AGM treatments between 56-70 DAT (**Fig. 4.1c**).

The total biomasses were 2.55, 2.65, and 2.85 kg m^{-2} in the NPK, AGM, and NPK+AGM treatment groups, respectively, and were not significantly different from one another. However, Azolla amended treatments maintained significantly higher grain yields of 1.06 kg m^{-2} for AGM and 1.09 kg m^{-2} for NPK+AGM, with harvest indexes of 40.27% and 38.30%, respectively, compared to 0.80 kg m^{-2} and 31.53% for the NPK treatment (**Table 4.3**). The dry biomasses of the floating Azolla cover in AGM and NPK+AGM treatments at harvest were 0.37 and 0.34 kg m^{-2}, respectively.

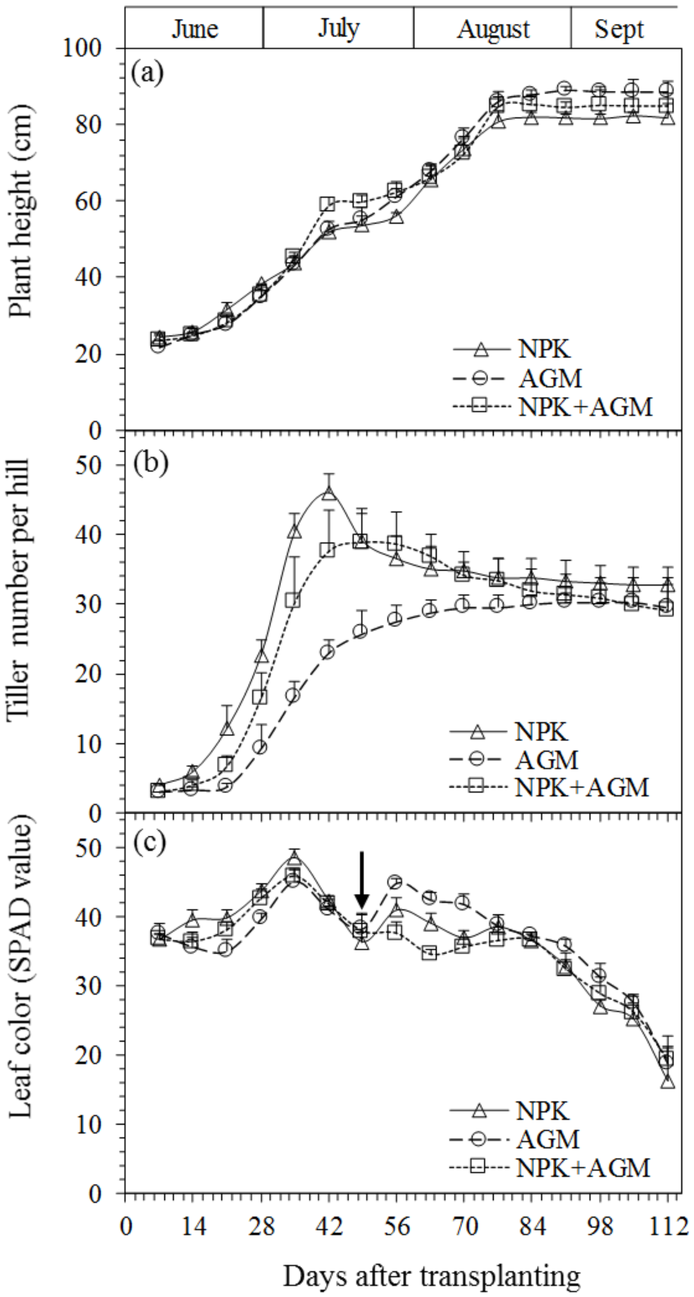

Fig. 4.1. Changes in plant heights (a), tiller numbers (b), and leaf color in SPAD values (c) of the rice plants, throughout the experiment period. Bars indicate standard deviation (n=4). The arrow indicates the day of fertilizer addition.

Table 4.3. Total biomass, grain yield, harvest index, cumulative CH_4 and N_2O emissions during early (before 63 DAT) and late (after 63 DAT) rice growth stages, and total CH_4 and N_2O emissions per grain yield equivalent among three treatments.

	Total biomass	Grain yield	Harvest index	Cumulative CH_4 emission			Cumulative N_2O emission			CH_4 emission per grain yield	N_2O emission per grain yield
				Early	Late	Total	Early	Late	Total		
Treatments	($kg\ m^{-2}$)		(%)	($g\ C\ m^{-2}$)			($\mu g\ N\ m^{-2}$)			($g\ C\ kg^{-1}$)	($\mu g\ N\ kg^{-1}$)
NPK	2.55 ± 0.25a	0.80 ± 0.08b	31.53 ± 3.64b	4.21 ± 0.47c	26.25 ± 2.44b	30.46 ± 2.85b	907.44 ± 137.58a	4.01 ± 3.62a	911.45 ± 140.25a	38.23 ± 4.45a	1156.15 ± 264.23a
AGM	2.65 ± 0.22a	1.06 ± 0.09a	40.27 ± 3.27a	9.40 ± 1.03b	30.65 ± 3.00ab	40.05 ± 3.58a	257.52 ± 0.00b	7.96 ± 5.83a	265.48 ± 113.8b	38.08 ± 7.19a	246.63 ± 94.80b
NPK+AGM	2.85 ± 0.09a	1.09 ± 0.07a	38.30 ± 1.69a	11.65 ± 0.94a	32.06 ± 2.01a	43.71 ± 2.36a	191.09 ± 0.00b	7.99 ± 2.90a	199.08 ± 33.24b	40.07 ± 2.59a	182.70 ± 31.99b

Values are means ± standard deviation (n=4). Values within each column followed by different letters refer to significant differences by Tukey's HSD test ($P < 0.05$).

4.4.2. Changes in CH_4 and N_2O Fluxes, and Accumulated Emissions

The pattern and intensity of CH_4 fluxes from the pot experiment varied with the stage of rice growth (**Fig. 4.2a**). The flux rates were relatively low and similar in all treatment groups (0.01-1.46 mg C m^{-2} h^{-1}) during the initial growth stage (15-35 DAT) and increased with the rice growth. Compared to the NPK treatment, AGM and NPK+AGM treatments had significantly higher fluxes at 42-56 DAT. The NPK and NPK+AGM treatments had single CH_4 flux peaks at 77 DAT (42.1 mg C m^{-2} h^{-1}) and 84 DAT (49.3 mg C m^{-2} h^{-1}), respectively, while the AGM treatment had two flux peaks, one at 63 DAT (32.0 mg C m^{-2} h^{-1}) that was slightly higher but not significantly different from the other two treatments (at P=0.06), and a second peak at 84 DAT (38 .0 mg C m^{-2} h^{-1}) that was different from the NPK and NPK+AGM treatment groups. Therefore, variations of CH_4 emissions from the three treatments in the pot experiment differed significantly during the early stage of rice growth (before 63 DAT) and from heading to maturity stages (after 63 DAT).

The pattern of seasonal variation in N_2O fluxes from rice soil differed from that of CH_4 fluxes (**Fig. 4.2b**). Fluxes at 7 DAT in the three treatments were not significantly different from each other, with values of 1151.8, 1473.4, and 860.2 µg N m^{-2} h^{-1}, for NPK, AGM, and NPK+AGM treatments, respectively. After the N_2O flux peaks at 7 DAT for AGM and NPK+AGM treatments, N_2O fluxes decreased rapidly following the development of anaerobic soil conditions. The NPK treatment that only included inorganic fertilizer had significantly higher N_2O flux rates during the initial growth stages (14-28 DAT, 1885.4-496.3 µg N m^{-2} h^{-1}), compared to AGM (32.1-1.15 µg N m^{-2} h^{-1}) and NPK+AGM (25.5-47.0 µg N m^{-2} h^{-1}) treatments. After 42 DAT, the N_2O fluxes were very low until rice harvest for all treatments.

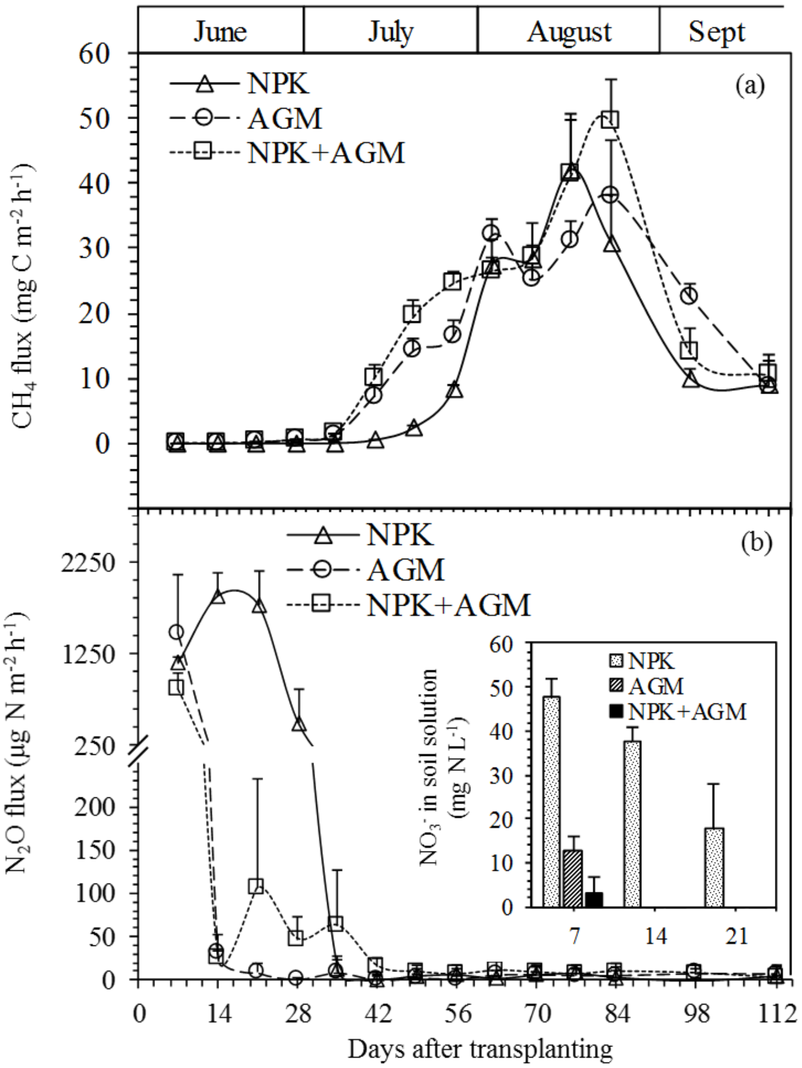

Fig. 4.2. Changes in CH_4 (a), and N_2O (b) fluxes from the pots among the three treatments throughout the experiment period. Bars indicate standard deviation (n=4). Insert in (b) shows the concentration of NO_3^--N dissolved in soil solution during the first three weeks at the day of gas sampling.

The total seasonal average CH_4 and N_2O emission rates in all treatments were divided into early (before 63 DAT) and late (after 63 DAT) rice growth periods (**Table 4.3**). Cumulative CH_4 emissions varied significantly among the three treatments during the early rice growth stages (4.21, 9.40, and 11.65 g C m^{-2} for NPK, AGM, and NPK+AGM, respectively), while cumulative CH_4 emissions were not significantly different between NPK and AGM, or AGM and NPK+AGM during the late rice growth stages (26.25 g C m^{-2} for NPK, 30.65 g C m^{-2} for AGM and 32.06 g C m^{-2} for NPK+AGM). Cumulative N_2O emissions during the early growth period were significantly higher for NPK treatment (907.44 mg N m^{-2}) compared to AGM (257.52 mg N m^{-2}), and NPK+AGM (191.09) treatments. No significant N_2O emission differences were recorded during the late growth period among the three treatments (**Table 4.3**).

Over the entire rice growth period, AGM and NPK+AGM recorded significantly higher total cumulative CH_4 emissions at 40.05, and 43.71 g C m^{-2} relative to NPK at 30.46 g C m^{-2}. While NPK recorded significantly higher cumulative N_2O emissions at 911.45 mg N m^{-2} compared to 265.48, and 199.08 mg N m^{-2}, for AGM and NPK+AGM treatments, respectively, (**Table 4.3**).

Total CH_4 emissions per grain yield were not significantly different among the treatments (P=0.832); however, compared with the NPK treatment, Azolla amended treatments significantly reduced N_2O emissions per grain yield from the rice soil ($P < 0.001$) (**Table 4.3**).

4.4.3. Changes in CO_2 Night Respiration

Night respiration was composed mostly of CO_2 emitted from rice plants in the NPK treatment and both rice plants and floating Azolla masses in the Azolla amended treatments, which ranged from 18.0 - 577.9 mg C m^{-2} h^{-1} during the experimental period and varied with the rice growth stage. The highest peak of nighttime CO_2 respiration was observed 6 weeks after rice transplanting in all treatments (**Fig. 4.3a**).

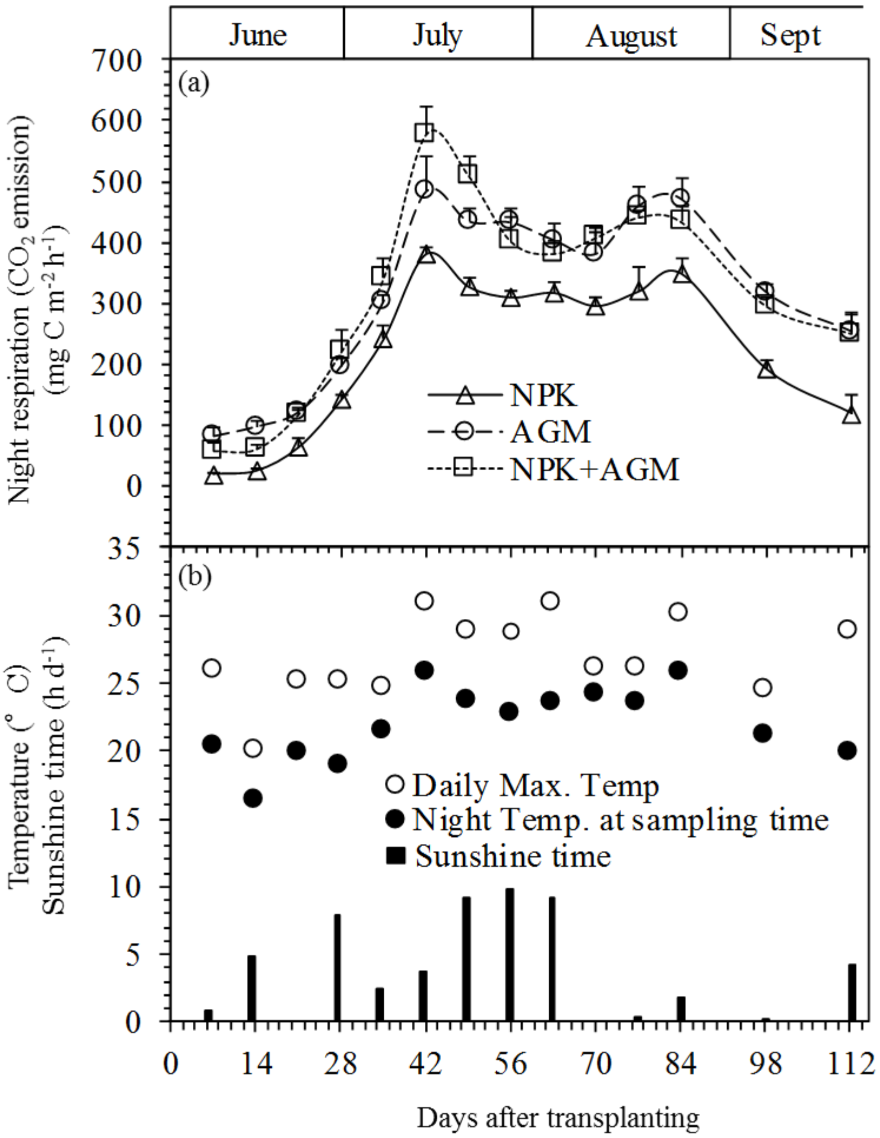

Fig. 4.3. Changes in night respiration (CO_2 emission) of rice plants grown in the pots among the treatments throughout the experiment period (a). Bars indicate standard deviation (n=4). The daily maximum temperature and temperature at sampling time (2100hrs), and sunshine time on the day of gas sampling are shown in (b).

The consecutive small peaks at 84 DAT were attributed to the daytime and high night temperature at sampling (**Fig. 4.3b**). Throughout the experiment, AGM and NPK+AGM treatments maintained a higher nighttime CO_2 respiration compared with the NPK treatment, significantly higher at 42-112 DAT ($P < 0.05$). The average values of CO_2 respiration during the entire experiment period were 220.8, 316.7, and 321.4 mg C m^{-2} h^{-1} from NPK, AGM, and NPK+AGM treatments, respectively.

4.4.4. Changes in CO_2, CH_4, and NO_3^--N Concentrations in Soil Solution

Dissolved CO_2 in soil solutions of the AGM and NPK+AGM treatments were significantly higher than that in the NPK treatment group at 7-42 DAT ($P < 0.01$) (**Fig. 4.4a**). At 28 DAT, significantly higher peaks of dissolved CO_2 were observed for the Azolla amended treatments compared with NPK treatment (222.87, 184.99, and 106.49 µg C mL^{-1} for AGM, NPK+AGM, and NPK, respectively). Dissolved CO_2 in soil solutions of all treatments converged at 56 DAT and no further significant observations were made thereafter.

Dissolved CH_4 in soil solutions were recorded for AGM and NPK+AGM treatments throughout the rice growth period (7-112 DAT) but were not significantly different. However, significantly lower soil dissolved CH_4 concentrations were observed in the NPK treatment compared to the Azolla amended treatments at 7-49 DAT (**Fig. 4.4b**). Dissolved CH_4 in soil solutions for all treatments increased uniformly with the highest peaks recorded at 112 DAT, which was the last sampling.

The average values of dissolved CO_2 concentrations in soil solution during the experimental period were 116.79, 148.92, and 147.39 µg C mL^{-1}, for NPK, AGM, and NPK+AGM treatments, respectively. Additionally, those of soil dissolved CH_4 concentrations were 3.81, 3.66, and 4.58 µg C mL^{-1} for NPK, AGM, and NPK+AGM treatments, respectively.

The NO_3^--N concentrations in the soil solutions were only detected on 7 DAT in the AGM (12.69 mg N L^{-1}) and NPK+AGM (3.28 mg N L^{-1}) treatments, while the concentrations in the NPK treatment were detected for 3 weeks and were 47.78, 37.49, and 17.98 mg N L^{-1} for 7, 14, and 21 DAT, respectively, (**insert Fig. 4.2b**).

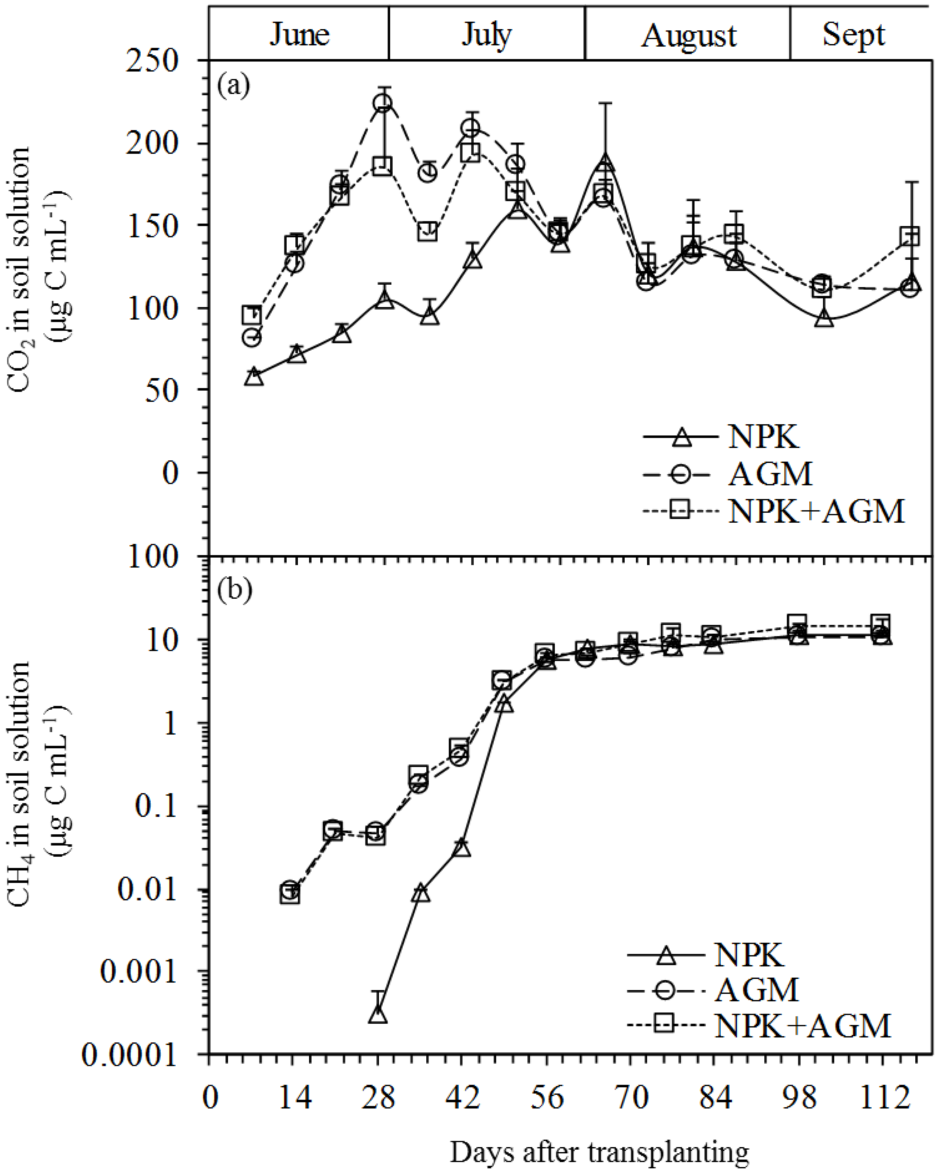

Fig. 4.4. Changes in the concentration of CO_2 (a) and CH_4 (b) dissolved in the soil solution in the pots among the three treatments throughout the experiment period. Bars indicate standard deviation (n=4).

4.4.5. Global Warming Potential, Soil C Sequestration, and Net Greenhouse Gas Balance

Regardless of the treatment setup and management, CH_4 was the most influential GHG that contributed to the bulk of the total combined GWP in early and late rice growth stage emissions (**Table 4.4**). Azolla amended treatments showed significantly higher combined GWP by the CH_4 and N_2O emissions values relative to NPK treatment (1807.97, 1939.90, and 2074.81 g CO_2 equivalent m^{-2}, for NPK, AGM, and NPK+AGM, respectively). However, the soil C sequestered in the Azolla amended treatments was significantly higher (1821.17 and 1955.92 g CO_2 equivalent m^{-2} for AGM and NPK+AGM treatments, respectively) compared to NPK treatment (1.63 g CO_2 equivalent m^{-2}). Subsequently, the Azolla amended treatments showed significantly lower net GHG balance when evaluating the difference between combined GWP by the CH_4 and N_2O emissions and amount of soil C sequestered (118.73 and 118.89 g CO_2 equivalent m^{-2} for AGM and NPK+AGM treatments, respectively) relative to the NPK treatment (1806.34 g CO_2 equivalent m^{-2}) (**Table 4.4.**). There were no significant differences between the Azolla amended treatments for the combined GWP values that were measured by the CH_4 and N_2O emissions, amount of soil C sequestered, and the net GHG balance.

Table 4.4. The net CO_2-equivalent greenhouse gas emissions balance from CH_4 and N_2O emissions (positive), and soil C sequestration (negative) among three treatments.

Treatment	Total CH_4 emission	Total N_2O emission	Soil C sequestration	Net GHG balance
NPK	1381.15 ± 129.24b	426.82 ± 65.68b	-1.63 ± 128.23b	1806.34 ± 29.63a
AGM	1815.58 ± 162.22a	124.32 ± 53.29a	-1821.17 ± 422.13a	118.73 ± 372.88b
NPK+AGM	1981.58 ± 107.15a	93.23 ± 15.56a	-1955.92 ± 130.16a	118.89 ± 217.83b

Total CH_4 and N_2O positive emissions were calculated from the GWP of CO_2:CH_4:N_2O = 1:34:298 (IPCC 2013). Soil C negative sequestrations were calculated from the difference of C stock before and after one rice growth season.

4.5. Discussion

4.5.1. Effects of Azolla Incorporation on CH_4 Emissions

In this experiment, incorporation of Azolla as green manure plus its subsequent growth as a cover crop in conjunction with chemical fertilizers (NPK) either at basal or top-dressing, significantly stimulated the total cumulative CH_4 emissions throughout the rice growth period (112 DAT) by 31.5% and 43.5% with AGM and NPK+AGM treatments, compared to the NPK treatment, respectively, (**Table 4.3**). Contrary to our previous research, where Azolla cover without incorporation significantly suppressed CH_4 emission from flooded rice soil (Kimani et al. 2018), the emergence of Azolla as a cover following incorporation of Azolla as green manure in this study did not suppress CH_4 emissions from AGM and NPK+AGM treatments, either during the early (before 63 DAT) or late (after 63 DAT) rice growth stages and the subsequent total cumulative CH_4 emissions (**Fig. 4.2a, Table 4.3**). These stimulating effects were consistent with previous findings that Azolla, either incorporated or as a dual crop with rice, increased CH_4 emissions from rice paddy soil pathways (Chen et al. 1997; Adhya et al. 2000; Ying et al. 2000), which was most likely due to the decomposition of the organic amendments by incorporated Azolla.

During the early rice growth stages before 63 DAT, no significant differences in rice growth parameters among all treatments were observed (**Fig. 4.1a, b**); however, dissolved CO_2 until 49 DAT and CH_4 concentrations in soil solutions until 63 DAT were significantly higher in the Azolla amended treatments compared to NPK, with no significant differences between the two Azolla amended treatments (**Fig. 4.4a, b**). During the early growth period, cumulative CH_4 emissions significantly increased in the Azolla amended treatments 123.3% (AGM) and 176.7% (NPK+AGM) compared to NPK (**Table 4.3**). These results were consistent with previous reports of increased CH_4 emissions of approximately 60% within 40 DAT from milk vetch and/or rye amended plots relative to NPK only (Kim et al. 2013). Therefore, CH_4 emissions in Azolla amended treatments before 63 DAT are likely attributed to readily available carbon substrates following incorporation of Azolla as green manure.

During the late rice growth stages after 63 DAT, the cumulative CH_4 emissions from all treatments were largely higher than those from early rice growth stages before 63 DAT, indicating probable changes of CH_4 carbon sources from initial soil organic matter and incorporated Azolla, to the photosynthetic products of the rice plants (Minoda, Kimura, and Wada 1996; Inubushi et al. 2003). As for the cumulative CH_4 emission, although the ratio to the total emission was highest in the NPK treatment (86.2%), the amount in the NPK treatment (26.25 g C m-2) was lower than those in the Azolla amended treatments (**Table 4.3**). Huang, Sass, and Fisher (1997) reported that more than 75% of the total seasonal CH_4 in a permanent flooding rice ecosystem was released during the rice reproductive and ripening stages without any organic matter incorporation. These results also showed that the carbon ratio of CH_4 emission to net photosynthetic production was about 8% during ripening periods, which was higher than that measured during the vegetative periods (about 1~2%). Moreover, the ratio was strongly dependent on plant biomass among the different rice cultivars tested. The highly significant and positive relationship explored in this and other studies between night CH_4 emission fluxes and night rice plant respiration (CO_2 emission) after 63 DAT in all tested treatments (**Fig. 4.5**) also suggests that rice photosynthesis supplies carbon substrates for methanogens in the rice soil (Aulakh et al. 2001b; Sass and Cicerone 2002).

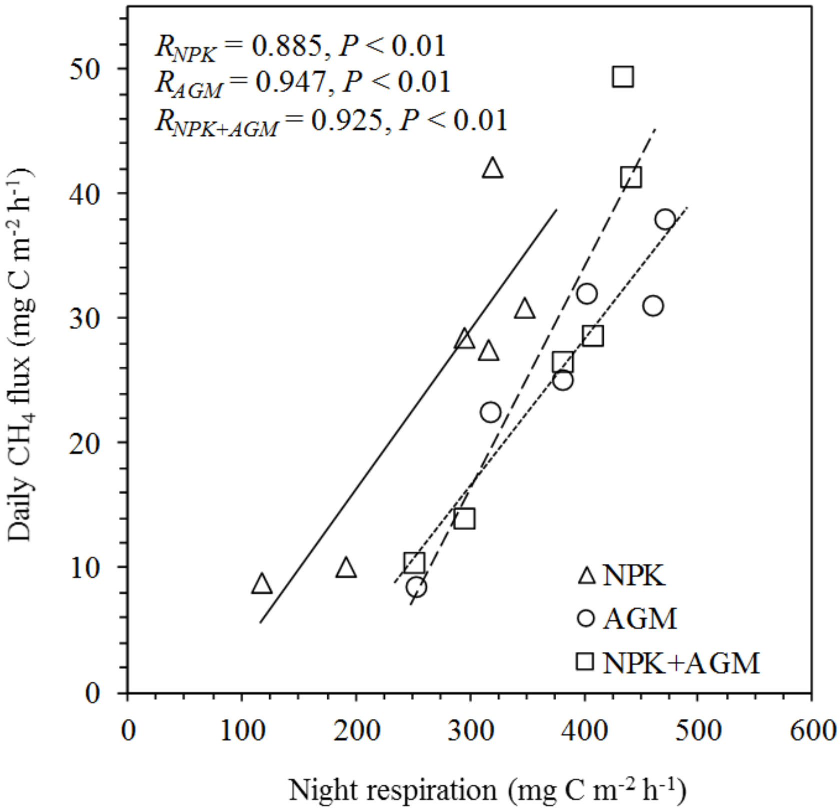

Fig. 4.5. Relationship between daily CH_4 flux and night respiration (CO_2 emission) among three treatments throughout after 9 weeks of rice transplanting (n=6).

In the current study, cumulative CH_4 emission after 63 DAT in the AGM treatment was not significantly different from the NPK treatment (**Table 4.3**), which suggests that incorporated Azolla as green manure at the beginning of the experiment, its subsequent growth as a cover, and the consequent observation of masses of dead Azolla cover (not quantified) at harvest neither promoted nor restricted CH_4 emission at the late rice growth stages. These observations are consistent with those by Zhu et al. (2012).

The carbon sources for CH_4 production in rice paddies can be native soil organic matter, incorporated organic materials such as rice straw and manure, and new carbon substrate from plant growth such as plant debris and root exudates (Nakajima et al. 2016; Wang et al. 2016). In this experiment, except for the incorporated Azolla in the Azolla amended treatments, the soil had no initial visible plant residues. Although the amount of CH_4 dissolved in the soil solution in the Azolla amended treatments significantly increased with increasing rice growth until 35 DAT, showing significantly lower soil solution dissolved CH_4 in the NPK treatment (**Fig. 4.4b**), aboveground CH_4 fluxes before 35 DAT were not detected or were relatively low during the early rice growth period (**Fig. 4.2a**). This result implies that the native soil organic matter was not the main source of CH_4 production.

Methane fluxes increased significantly between 42 and 56 DAT for the Azolla amended treatments relative to the NPK treatment (**Fig. 4.2a**). During the same period, the highest peaks of plant night respiration in the treatments were recorded (**Fig. 4.6a**). Similarly, the highest CH_4 emission peaks for all treatments occurred at 77 DAT for NPK and 84 DAT for both Azolla amended treatments, while additional smaller peaks of plant night respiration were recorded (**Fig. 4.2a, and 4.5a**). These results indicate that the sources of CH_4 production from the three treatments were less from the old matter from the native soil organic and incorporated Azolla green manure, and mostly from new carbon through rice plant root exudates and rice plant debris in all three treatments groups, which significantly contributed to cumulative CH_4 emissions after 63 DAT. These observations were consistent with what was reported previously (Chen et al. 1997; Ying et al. 2000; Cheng et al. 2006, 2008; Lou et al. 2008).

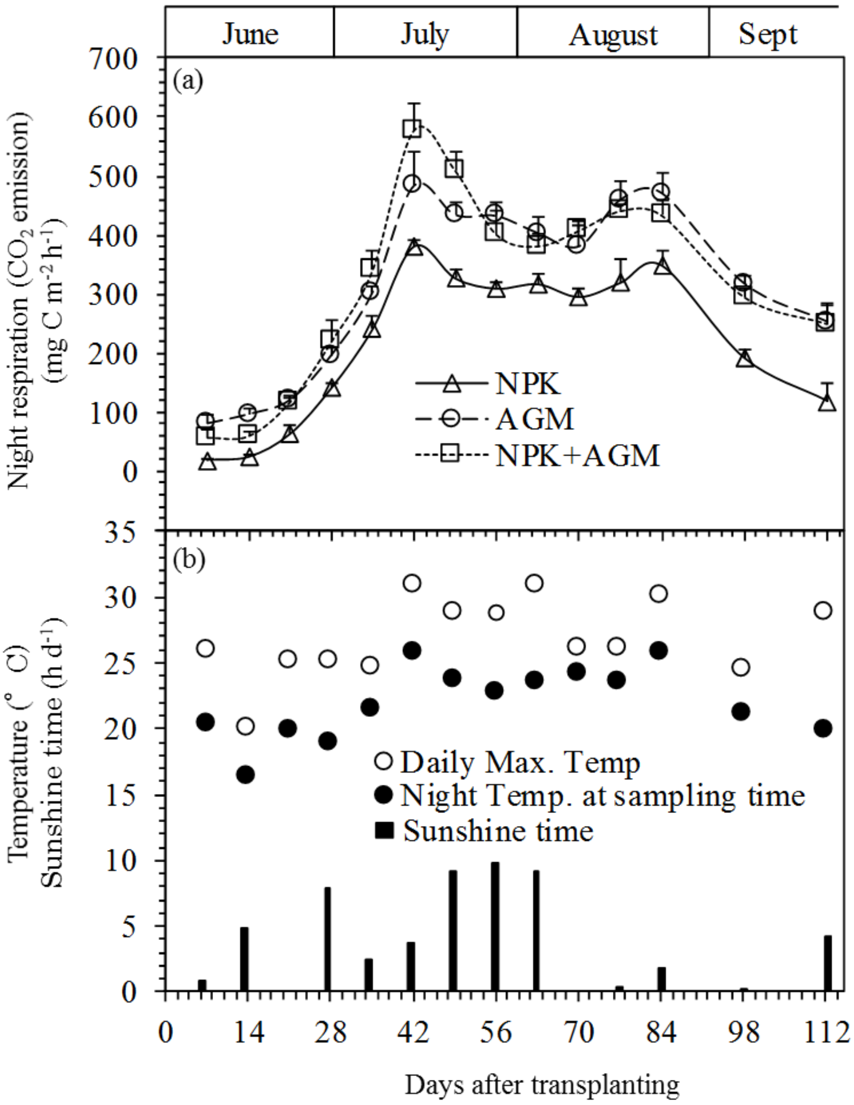

Fig. 4.6. Changes in night respiration (CO_2 emission) of rice plants grown in the pots among the treatments throughout the experiment period (a). Bars indicate standard deviation (n=4). The daily maximum temperature and temperature at sampling time (2100hrs), and sunshine time on the day of gas sampling are shown in (b).

On average, Azolla amended treatments in conjunction with chemical fertilizers used either as basal or top-dressing (both AGM and NPK+AGM) significantly increased rice grain yield by 34.4% and harvest index by 24.8% relative to NPK treatment (**Table 4.3**). Generally, reducing CH_4 emission per yield equivalent is a major challenge worldwide (Gao et al. 2015). In the current study, the values of CH_4 emission per grain yield in Azolla amended treatments were 38.08 and 40.07 g C kg^{-1} for AGM and NPK+AGM, respectively, which were not significantly different from 38.23 g C kg^{-1} measured in NPK. Since incorporated Azolla can decrease chemical fertilizer application in rice production, no change in CH_4 emission per yield equivalent in this study implies that Azolla incorporation as green manure is an alternative technique for sustainable rice production.

4.5.2. Effects of Azolla Incorporation on N_2O Emissions

Nitrous oxide emission is a microbial-mediated soil process in agricultural soils that acts through the nitrification-denitrification pathway (Butterbach-Bahl et al. 2013). Application of inorganic N fertilizers increases N_2O efflux by increasing substrate availability for nitrification and denitrification (Yao et al. 2012). Additionally, adequate amounts of NO_3^--N and availability of carbon (C) that are susceptible to mineralization either from native organic matter or newly incorporated crop residues are major factors in controlling denitrification, especially under limited oxygen conditions (Aulakh et al. 1991a, 2001a).

Our results showed that combined fresh Azolla incorporation and dual cropping significantly inhibited N_2O emission from flooded paddy soil compared with NPK treatment (**Fig. 4.2b, Table 4.3**). Despite the significant differences in the dissolved soil NO_3^--N concentrations among the treatments at 7 DAT (**insert Fig. 4.2b**), N_2O fluxes did not differ at 7 DAT among the treatments, (at P=0.137). Following the initial N_2O flux peaks for all treatments at 7 DAT, NPK treatment had prolonged and significantly higher N_2O fluxes 14, 21, and 28 DAT compared to Azolla amended treatments, whose fluxes dropped rapidly to relatively low emission values (< 106.53 µg N m^{-2} h^{-1}). Moreover, significantly higher dissolved soil NO_3^--N concentrations were recorded for the NPK treatment at 14, 21, and 28 DAT compared to the Azolla amended treatments (**Fig. 4.2b, insert Fig. 4.2b**). The cumulated total N_2O emissions during the entire rice growth period (112 DAT) were significantly lower in the Azolla amended treatments compared to NPK treatments (**Table 4.3**). Cumulatively high N_2O emissions were recorded during the early rice growth stages (before 63 DAT) in all treatments and on average, at the early rice growth stages, NPK treatment emitted 99.5% N_2O of all growth stages compared to the Azolla amended treatments (97.0% AGM and 96.0% NPK+AGM). There were no significant N_2O emission differences during the late rice growth stages among the three treatments. During the entire rice growth period, AGM and NPK+AGM treatments decreased seasonal N_2O emissions by 3.4 (70.9%), and 4.6 (78.2%) fold relative to NPK treatment, respectively, (**Table 4.3**).

Although we may have omitted the initial high N_2O emission peaks (e.g. at 2 - 5 days after soil flooding) from Azolla amended treatments and/or NPK treatments, lower N_2O emission fluxes during the early rice growth stages from Azolla amended treatments compared to NPK may have been due to a more effective reduction of N_2O to N_2 during denitrification as a result of the availability of readily decomposable organic matter, in this case, the incorporated Azolla. Availability of organic C has previously been reported to be a critical factor for denitrification (Aulakh et al. 1991b; Baruah and Baruah 2015).

Unlike in the NPK treatment where high N_2O fluxes (2-4 weeks after transplanting) were in tandem with the decrease in soil dissolved NO_3^--N concentrations, Azolla amended treatments maintained significantly low N_2O fluxes and dissolved NO_3^--N concentrations (**Fig. 4.2b, insert Fig. 4.2b**). This suggests that N_2O emissions at the early rice growth stage for all treatments were probably a result of high initial soil NO_3^--N levels. The experimental soil was stored outside for one year before the start of the experiment and a substantial amount of decomposable organic C and N may have already been converted into CO_2 and NO_3^--N, respectively. The amount of NO_3^--N in the initial soil was 101.6 mg N kg^{-1} in 4.9 kg of dry soil (**Table 4.2**). These observations were consistent with those by Cheng et al. (2006) and Kimani et al. (2018). These results favor the conclusion that incorporation of Azolla green manure as an N source may decrease N_2O emissions in flooded paddy soils. The combination of green manure and urea has been reported to mitigate urea N loss through N_2O flux (Zhu et al. 2012).

4.5.3. Effects of Azolla Incorporation on GWP, Soil C Sequestration and Net GHG Balance

According to Johnson et al. (2007), global warming potential (GWP) represents the expected effect on the radiation balance of the earth due to the addition of a unit of gas as a result of the specific gas mean lifetime and total quantity emitted. The major gases contributing to positive radiative forcing in the atmosphere are CO_2, CH_4, and N_2O (IPCC 2013). Compared to the NPK treatment, incorporation of Azolla as green manure plus its subsequent growth as a dual crop with rice significantly increased the combined GWP (CH_4 and N_2O emissions) in AGM (7.3%) and NPK+AGM (14.8%) treatments, respectively, (**Table 4.4**). This result was mainly attributed to the significantly higher seasonal CH_4 emissions from the Azolla amended treatments compared to the NPK treatment (**Table 4.3**). Methane emissions have been reported to be a key factor in determining the combined GWP during rice cultivation (Hwang et al. 2017; Setyanto et al. 2018; Tirol-Padre et al. 2018). As the contribution of N_2O to the combined GWP was very small in the flooded paddy soil condition irrespective of the treatment management, effective control of CH_4 emissions could be a useful mitigation approach to reducing total GHG emissions and hence, the subsequent combined GWP (Kim et al. 2013). Despite the high combined GWP from Azolla amended treatments, incorporated Azolla as green manure significantly increased soil organic carbon (SOC) content during the rice growth period by 23.9% on average for AGM and NPK+AGM treatments compared to NPK treatment (**Table 4.5**). The soil C sequestration values in the AGM and NPK+AGM treatments were significantly higher than those measured in the NPK only treatment, and reduced net GHG balance values in the Azolla amended treatments compared to the NPK only treatment (**Table 4.4**). Management practices that increase organic inputs (e.g. biomass and manure) have been reported to enhance microbial functions and SOC sequestration (Jarecki and Lal 2003).

Table 4.5. Soil properties at harvest among three treatments application of Azolla as green manure.

Treatments	pH (H_2O) (1:2.5)	EC (μS cm^{-1})	Total N (g kg^{-1} dry soil)	Soil Organic C (g kg^{-1} dry soil)	C/N
NPK	5.11a	80.13b	1.45b	14.50b	9.98a
AGM	5.18a	105.03ab	1.78a	17.84a	10.02a
NPK+AGM	5.13a	134.98a	1.81a	18.09a	10.04a

While the incorporation of Azolla as green manure and its subsequent growth as a dual crop in the flooded paddy soil with rice in this study increased the soil C sequestration relative to NPK treatment, the significantly high combined GWP from the Azolla amended treatments (**Table 4.4**) overshadowed the benefits of its SOC content increase. Despite this, significant increases in grain yield, and decreases in N2O and no significant CH4 emissions per grain yield at harvest in the Azolla amended treatments compared to NPK treatment (**Table 4.3**) is a positive result. Also, the cultivation and incorporation of Azolla is a process involving C accumulation from the atmosphere to the soil, while the production of synthetic N fertilizer consumes fossil fuels that release C and contribute to GHG emissions. Thus, in an effort to mitigate climate change, especially in organic farming practices, further long-term studies on field conditions are needed to not only quantify and recommend the best approaches for Azolla incorporation as green manure and cover in conjunction with or without chemical fertilizers but also to quantitatively evaluate changes in soil C storage related to organic matter application.

4.6. Conclusion

To determine whether Azolla incorporation as green manure (AGM) and its subsequent growth as a dual crop in conjunction with chemical fertilizers (NPK) either as basal or top-dressing affects both CH_4 and N_2O emissions from flooded paddy ecosystems, a pot experiment was carried out in Tsuruoka, Yamagata, Japan in 2017. Significantly higher CH_4 emissions from Azolla amended treatments (AGM and NPK+AGM) were observed in the early rice growth stage and attributed to the incorporated Azolla, while those at the late growth stage were a result of plant photosynthesis due to increased rice biomass. On average, AGM amendment increased rice yield by 34.4% and seasonal CH_4 emission by 37.5% but decreased N_2O emission by 74.5% relative to the NPK only treatment. Azolla amended treatments significantly increased total grain yield, global warming potential, soil organic content in the soil, and decreased the net GHG balance. It should be noted that our results are based on a pot experiment and field studies should be carried out in the future to confirm its application to farming conditions.

CHAPTER V

5. Co-application of Poultry-litter Biochar with Azolla has Synergistic Effects on CH_4 and N_2O Emissions from Rice Paddy Soils

5.1. Abstract

Poultry-litter biochar and Azolla as green manure amendments are reported to enhance paddy soil fertility and rice yields. However, whether their co-application in lowland rice paddies has synergistic effects and whether those benefits are accompanied by greenhouse gas (GHG) emissions remains unknown. The objective of this study was to determine the effects of poultry-litter biochar (hereafter: biochar) and its co-application with Azolla as green manure (hereafter: Azolla), on the simultaneous methane (CH_4) and nitrous oxide (N_2O) emissions from a lowland paddy soil planted with rice during a single rice-growing season in Tsuruoka, Yamagata, Japan. Biochar and Azolla amendments were applied once before rice was transplanted at a density of 20 t ha^{-1} and 133.9 kg N ha^{-1}, respectively. Compared with NPK, NPK + biochar, and Azolla only treatments, Azolla and biochar co-application (i.e., Azolla + biochar) significantly increased CH_4 emissions by 33% - 197.6% in the early stages of rice growth (before 63 days after transplanting, DAT), but did not significantly influence CH_4 emissions at both late rice growth stages (after 63 DAT,) and whole rice growth period (112 DAT). Conversely, Azolla + biochar significantly reduced N_2O emissions by 83.0% - 97.1% before 63 DAT, and by 76.4% - 95.9% during the whole rice growth period at 112 DAT, with significantly high interaction between biochar and fertilizer amendments. There were no significant N_2O emission differences among all treatments after 63 DAT. Additionally, Azolla + biochar significantly increased rice grain yield by 27.3%- 75.0%, and consequently, decreased both yield-equivalent CH_4 emissions by 24.7% - 25.0% and N_2O emissions by 81.8% - 97.7%. Our findings suggest that the co-application of poultry-litter biochar and Azolla as green manure offers a novel approach to increase rice yield while reducing the emissions of non-carbon dioxide greenhouse gases.

5.2. Introduction

Flooded rice fields are a significant anthropogenic source of greenhouse gases (GHG), with estimated global methane (CH_4) emission rate of 25–60 Tg yr^{-1} (Reay et al., 2010) and an annual global nitrous oxide (N_2O) contribution of 13%–24% (Saikawa et al., 2014). The global warming potential (GWP) by mass of CH_4 is 34 times while that of N_2O is 298 times that of carbon dioxide (CO_2) over 100 years (IPCC, 2013). According to Scialabba and Müller-Lindenlauf (2010) and Snyder et al. (2009), the exogenous application of inorganic and/or organic fertilizers to rice paddies exacerbates CH_4 and N_2O emissions. Accordingly, with the projected increase in rice demand by over 20% in the next 10 to 20 years, an increase in CH_4 emissions and a comparable increase in N_2O emissions resulting from increased fertilizer use is almost inevitable (Van Nguyen and Ferrero, 2006; Zou et al., 2009). Thus, it is important to find cultivation practices suitable to mitigate GHG emissions from constantly flooded rice paddies.

Despite the high overall contribution of chemical fertilizers to the carbon footprint of rice agriculture, their use is unavoidable to maintain rice growth and yield (Xu et al., 2013). However, given the current energy crisis, higher prices of inorganic fertilizers, and concerns about the detriments of climate changes, research interest in green manure use, especially in lowland rice, has been renewed (Brenzinger et al., 2018; Scialabba and Müller-Lindenlauf, 2010). Azolla, an aquatic fern often found in flooded rice fields, has long been used successfully as green manure to improve the N balance in lowland paddies in Vietnam and southern China, due to its symbiotic relationship with nitrogen (N)-fixing cyanobacteria *Anabaena azollae* (Cheng et al., 2015; Lu and Li, 2006). Nonetheless, the effects of green manure application on GHG emissions from lowland paddy fields remain contradictory. Bharati et al. (2000) demonstrated that incorporation of Azolla plus dual cropping significantly decreased CH_4 emissions by increasing the soil redox potential due to higher levels of dissolved oxygen (DO) concentration in the standing water effected by the floating Azolla cover. In contrast, Linquist et al. (2012) reported a significant increase in CH_4 emissions by 192% with the addition of green manure *Sesbania* compared to inorganic N fertilizers, mainly attributed to the amount of substrate available for methanogens. Meanwhile, Chen et al. (1997) reported substantial CH_4 and N_2O emissions from a rice field grown with Azolla as a cover, likely due to the exudation of Azolla root and decomposition of dead Azolla. Conversely, Kimani et al. (2018) reported that Azolla as a cover significantly decreased CH_4 emission by 34%, likely due to increased levels of DO concentrations and redox potential (Xu et al., 2017), and no significant influences on N_2O from a paddy soil planted with rice, attributed to no interferences by the Azolla cover (Cheng et al., 2006). The discrepancies in these results suggest, therefore, that the interactions between soil native and/or newly added N availability, management practices, and other site-specific factors influence CH_4 and N_2O emissions from lowland rice ecosystems (Linquist et al., 2012). Additionally, due to the accelerated decomposition rates of organic materials, a number of these benefits are short-lived and multiple applications per cropping season are required (Partey et al., 2014).

Biochar is the carbon-rich material obtained through the pyrolysis of biomass. Its application to agricultural soils leads to an increase in carbon sequestration and a corresponding decrease in GHG emissions subject to its high structural composition stability (chemically and biologically), characteristics that are of particular importance to the mitigation of climate change (Lehmann et al., 2006). Globally, biochar is readily produced from various sources of biomass under different pyrolysis conditions, resulting in products of varying properties, and consequently different soil amendment values. Accordingly, the use of biochar particularly in rice paddy ecosystems to decrease GHG emissions, though a promising option, remains contradictory (Kammann et al., 2017). For example, Singh et al. (2010) reported cumulatively higher N_2O emissions from poultry manure biochar amended soils by 32% compared to the control as a result of higher labile native N contents of the biochar. In contrast, van Zwieten et al. (2010) reported reduced N_2O emissions to 4.0% of the applied and available N by poultry litter biochar compared to control soil, mainly due to an increase in NO_3^- adsorption. Similarly, contradictory observations on CH_4 emissions have been reported (Jeffery et al. 2016). Liu et al. (2011) found that bamboo chips and rice straw-derived biochars amendments decreased methanogenic activities in the paddy soil, thereby significantly decreasing CH_4 emissions by 51.1% and 91.2%, respectively. Conversely, Zhang et al. (2012) revealed that amendment with wheat straw biochar at 40 t ha^{-1} significantly increased soil CH_4-C emissions by 34-41% probably due to increased substrate supply and the development of a conducive environment for methanogens, particularly in the early stages of rice growth (Jeffery et al., 2016). Meanwhile, some studies have reported no significant influences on CH_4 emissions, and a varying degree of N_2O emissions depending on the feedstock source (Clough et al., 2013; Xie et al., 2013). These contrasting results may be due to differing soil conditions, biochar feedstock, pyrolysis methods, biochar application rate and intervals, as well as experimental duration and management practices (Saarnio, 2015; Song et al., 2016).

Given the shortcomings of either inorganic and/or organic fertilizers and biochar use in lowland rice paddies as highlighted above, co-applications of inorganic and/or organic fertilizers and biochar amendments, though with differing effects, has been proposed as a suitable practice to achieve sustainable soil health, yield production, and GHG emissions mitigation (Rahman et al., 2020). For example, Abagandura et al. (2019) revealed a reduction in cumulative N_2O fluxes from a sandy loam soil amended with plant-based biochar plus dairy manure, attributed to improved aeration and a subsequent reduction in denitrification, and no significant effects on the cumulative.CH_4 fluxes, partly due to similar soil water contents among treatments. Similarly, Wu et al. (2019) also reported a significant decrease in cumulative N_2O emissions from a paddy soil co-treated with vermicompost and wheat straw-derived biochar, attributed to suppression of carbon and nitrogen mobilization. In contrast, Lin et al. (2017) found a significant increase in N_2O emissions by 256% with wheat-straw biochar co-applied with N fertilizer, mainly due to increased soil pH and its influence on the ammonia-oxidizing bacteria abundance. Additionally, Zhang et al. (2010) found a significant increase in total CH_4 emissions by 41% with wheat straw biochar application in N fertilized soils, partly due to increased substrate for methanogens in the early stages of rice development. As highlighted here, there are multiple studies on the effects of plant biomass-derived biochar co-applied with inorganic and/or organic fertilizers on agricultural GHG emissions. However, there are still few reports on the effects of animal manure derived biochar, and particularly poultry-litter biochar as a viable option to mitigate GHG emissions. With perhaps, the exception of Subedi et al. (2016) who observed significant increases in N_2O emissions by 0.65-3.41% from a soil amended with poultry-litter derived biochar in a laboratory study, attributed to greater availability of volatile compounds which may have acted as a potential substrate for the denitrifiers as well as increased availability of mineral N from the biochar itself (Cayuela et al., 2014). Furthermore, there are no studies on the combined effects of poultry-litter biochar and Azolla as green manure (herein Azolla) on both CH_4 and N_2O emissions in paddy soils.

Based on the previous findings (as highlighted above), we hypothesized that while biochar, inorganic fertilizers, and organic amendments show contrasting effects when applied independently, their co-application may have synergistic effects, resulting in simultaneous positive effects on CH_4 and N_2O emissions. Previously, in the same batch of the experiment, we reported a significant increase in seasonal CH_4 emission by 31.5% and a 3.4 fold N_2O emission decrease in Azolla amended paddy soil compared to NPK only treatment, mainly attributed to increased substrates availability favoring methanogens as well as accelerating denitrification (Kimani et al., 2020). Therefore, in the current study, we investigated the effects of poultry-litter biochar amendment and its co-application with NPK and Azolla (i.e., NPK + biochar and Azolla + biochar) on the simultaneous CH_4 and N_2O emissions. The main objective of this study was to determine the effects of poultry-litter biochar amendment and its co-application with Azolla (incorporated as green manure and its successive growth as a cover crop), on the simultaneous CH_4 and N_2O emissions from a flooded rice paddy soil planted with rice in single rice-growing season.

5.3. Material and Methods

5.3.1. Experiment Site, Design, and Management

The pot experiment was carried out on the ground at the Experimental Farm of Yamagata University (38º44´N, 139º50´E, 16 m a.s.l.) in 2017. Average daily air temperature in the rice growth season (7th June to 20th September 2017) was 0.1 ºC above the historic average for 1981–2010, coupled with a daily average air temperature of 22.7 ºC and 6.4 h sunshine time (**Fig. 3.1 in Chapter III**).

In situ pots were used with four treatments each replicated four times: chemical fertilizer (NPK) and Azolla (as green manure) without and with 20 tons per hectare biochar (**Table 5.1**). The experimental soil was classified and treated as explained in Chapter 3. The basic soil properties were determined using the air-dried soil sample procedures. Soil pH (1:5 soil-in–water ratio mixture) and electrical conductivity (EC) were determined with a handheld pH meter (D-51, Horiba, Kyoto, Japan) and an EC meter (DS-51 conductivity meter, Horiba), respectively. Soil organic carbon (SOC) and total nitrogen (TN) were analyzed by dry combustion using a Sumigraph NC 220F Analyzer (Sumika Chemical Analysis Service, Ltd., Osaka, Japan). The NH_4^+-N and NO_3^--N concentrations were determined by the nitroprusside and hydrazine reduction methods, respectively (JSSSPN 1986), and measured using Hitachi U-2900 Spectrophotometer (Hitachi High-Tech Science Corporation, Tokyo, Japan).

Table 5.1. Summary of the experimental treatments with chemical fertilizers, Azolla, and poultry-litter biochar application at the Experimental Farm, Tsuruoka, Japan.

Treatment code	Amendments				Total N application
(in details)	Basal fertilizer application	Azolla incorporation	Biochar incorporation	Additional fertilizer application	(g pot^{-1})
NPK (Chemical fertilizer)	0.40 g N, 0.20 g P, and 0.25 g K per pot were applied by KH_2PO_4 and $CO(NH_2)_2$ as basal fertilizer before transplanting.	-	-	Top dressing was applied at 49 DAT by KH_2PO_4 and $CO(NH_2)_2$ at 0.20 g N, 0.10 g P, and 0.13 g K per pot.	0.60
NPK + biochar	Basal chemical fertilizer applied as above.	-	66 g/pot dry wt. biochar (20 tons/ha eqv.) mixed with soil before transplanting.	Topdressing fertilizer applied as above.	0.60
Azolla (As green manure)	-	243 g fresh Azolla (95% water content, 12.2 g dry weight) incorporated as green manure at transplanting to provide 0.40 g N pot^{-1} eqv. [Azolla cover grew following Azolla incorporation].	-	Topdressing fertilizer applied as above.	0.60
Azolla + biochar	-	Fresh Azolla applied as above.	66 g/pot dry wt. biochar (20 tons/ha eqv.) mixed with soil before transplanting.	Topdressing fertilizer applied as above.	0.60

The poultry-litter biochar (a composition of poultry excreta and bedding materials sourced from commercial poultry farms in Kanazawa) used in this study was produced using commercial pyrolysis equipment under oxygen-limited conditions at 450 °C–500 °C (Meiwa Co., Ltd., Kanazawa, Ishikawa, Japan). The Azolla (*A. filiculoides* Lam.) species IRRI code FI 1001 (Cheng et al. 2015; Kimani et al., 2018, 2020) was used in this study. The primary properties of soil, biochar, and Azolla, determined as describe above, are as shown in **Table 5.2.** We also used Haenuki, a popular rice cultivar widely grown in Yamagata Prefecture, Japan.

Table 5.2. Characteristics of the experimental paddy soil, poultry-litter biochar, and Azolla (A. filiculoides Lam.).

	Soil	Biochar	Azolla
Organic C (g kg^{-1} DW)	14.50	284.50	339.90
Total N (g kg^{-1} DW)	1.40	26.70	33.80
C:N	10.36	10.66	10.06
pH (H_2O)	5.24	10.0	-
EC (µS cm^{-1})	170.0	2790.0	-
Available P (mg P_2O_5 kg^{-1})	70.0	2470.0	-
NH_4^+ (mg N kg^{-1} DW)	24.8	18.4	-
NO_3^- (mg N kg^{-1} DW)	101.6	550.8	-

One day before transplanting, 7 kg of soil (4.9 kg oven-dried soil equivalent, 30% water content per total weight of soil per pot) were mixed with 66 g pot^{-1} biochar (equivalent to 20 t ha^{-1}, an amount within a range of rates shown to have significant effects on plant growth (Biederman and Harpole, 2013)) for the with biochar treatments only (NPK + biochar and Azolla + biochar), fresh green manure at 12.2 g Azolla dry weight pot^{-1} (equivalent to 0.40 g N pot^{-1}) in the Azolla and Azolla + biochar treatments only, and 0.87 g of KH_2PO_4 and 0.87 g $CO(NH_2)_2$ for the NPK without and with biochar treatments only. Germinated rice seeds were grown in a seedling tray (three seeds per cell), then transplanted (three seedlings per pot) five weeks after sowing into 16 plastic pots (19.5-cm diameter, 27-cm height, and 0.2-cm thickness). Next, 49 days after transplanting (DAT), all treatments were top-dressed with 0.43 g of KH_2PO_4 and 0.43 g $CO(NH_2)_2$ (**Table 5.1**). The total amount of N application was the same at 200.9 kg ha^{-1} between NPK and Azolla treatments. The flooding water depth was maintained at about 5 cm above the surface of soil throughout the experiment period by continuously topping up with tap water. The surface cover of growing Azolla in the Azolla treatments without and with biochar was maintained throughout the rice growth period.

5.3.2. Quantification of CH_4 and N_2O Fluxes, and Night Respiration (CO_2 flux)

Emissions of CH_4 and N_2O, as well as nighttime respiration (CO_2 flux) rates from rice pots placed in outdoor water tanks (two pots per tank) (65-cm length, 46-cm width, and 32-cm depth) filled with water, were measured using a static closed-top chamber (height, 100 cm; inside diameter, 20.5 cm; thickness, 0.3 cm) as described previously (Kimani et al., 2018). After closure, a small fan was used to mix the gas in the chamber, and a 30-mL gas sample from the chamber headspace of each experimental pot was collected at 0, 15, and 30 min with a syringe and transferred into a 19-mL pre-evacuated vial. As detailed previously (Kimani et al., 2018), gas sampling was conducted between 20:00~23:00 once a week in the first 84 DAT. After this date, sampling was done every two weeks until 122 DAT, a day before rice harvesting (113 DAT). All gas samples were analyzed at the Institute for Agro-Environmental Sciences, NARO using an automated analysis system for three gases of CO_2, CH_4, and N_2O (Kimani et al., 2018; Sudo 2006). The GHG fluxes were calculated from the linear increase in gas concentrations inside the chamber per square meter per hour along with atmospheric pressure and temperature (Cheng et al., 2008; Kimani et al., 2018, 2020; Sudo, 2006).

5.3.3. Quantification of Dissolved CO_2, CH_4, and Nitrate in Soil Solution

For understanding the CH_4 and N_2O emissions with the C and N dynamics in the soils, the concentrations of dissolved CO_2 and CH_4 and nitrate (NO_3^--N) in soil solutions were sampled using a 10 cm long microporous polymer tube (outside diameter, 2.5 mm; inside diameter, 1.5 mm) fitted to a PVC tube (length, 50 cm; outside diameter, 2.7 mm; inside diameter, 1.0 mm) and inserted vertically into the soil between the rice plant and pot edge at a depth of 10–15 cm one day after rice transplanting, as previously described (Kimani et al., 2018). The 9.5-mL soil solution sample was aspirated into a 19-mL semi-vacuum bottle fitted with a rubber stopper and a screw cap and filled with pure N_2 gas at 0.5 atm (Kimani et al., 2018). The concentrations of CO_2 and CH_4 in the headspace volume were measured in the laboratory using a gas chromatograph (GC-7A, Shimadzu, Kyoto, Japan), fitted with a thermal conductivity detector (TCD), and a flame ionization detector (FID), respectively. The CO_2 and CH_4 concentrations were calculated with Henry’s law according to their respective concentrations in the headspace (Cheng et al., 2005, 2006). The NO_3^--N concentration in soil solution was analyzed using colorimetric techniques at 450 nm by a spectrophotometer (UV-1200V, Shimadzu, Japan). The soil solution samples were collected on the same day after gas measurements.

5.3.4. Effects of Poultry-litter Biochar and Azolla Co-application on Net GHG Emissions

Global warming potential (GWP), soil C sequestration, and the net GHG balance in g CO_2-equivalent (CO_2-eq) per square meter were calculated for all treatments. GWP was derived by combining cumulative CH_4 and N_2O emission fluxes. In these calculations, the GWP values for CH_4 and N_2O were considered to be 34 and 298, respectively, (IPCC, 2013). The GWP and soil C sequestration calculations were as below (Toma et al., 2019):

$$GWP_{CH_4\ to\ CO2eq} = \mathrm{CH_4\text{-}C\ emission} \times 16/12 \times 34 \tag{1}$$

$$\mathrm{GWP_{N_2O\ to\ CO2eq}} = \mathrm{N_2O\text{-}N\ emission} \times 44/28 \times 298 \tag{2}$$

$$Soil\ C\ sequestration\ (g\ CO_{2eqv}\ m^{-2}) = \left(C_{tre} - C_{bef}\right) \times \left(\frac{S_{dw}}{P_{area}}\right) \times 44/12 \tag{3}$$

where C_{tre} is the soil C content in each treatment after rice cultivation (g kg^{-1} dry soil), C_{bef} is the soil C content before the experiment (14.50 g kg^{-1} dry soil), S_{dw} is the amount of soil in the pot at the start of the experiment (4.9 kg dry weight), P_{area} is the pot area (m^{-2}), multiplied by a ratio of molecular weight of CO_2 to C (44/12) to calculate C sequestration in CO_2 equivalent. The ratios of 16/12 and 44/28 were used to convert CH_4-C to CH_4 and N_2O-N to N_2O, respectively. Changes in the net GHG balance following the co-application of poultry-litter biochar and Azolla were calculated relative to the other treatments.

5.3.5. Investigation of Plants Growth, Grain Yield, and Soil Analysis

The rice height and tiller number data per treatment were collected once a week beginning on 8 DAT. At that time, top rice leaf greenness (SPAD value) was measured using a SPAD-502 Plus chlorophyll meter (Konica Minolta Inc., Tokyo, Japan). Data from four hills per treatment were averaged during the rice growth period. At maturity (113 DAT), rice was harvested and separated into grains and straw, then air-dried for one month and weighed to determine total yield (Cheng et al., 2009). After harvest, soil in the pots was divided into two equal parts from the center. One part was used for roots sampling (Kimani et al., 2018) and the other part was air-dried for soil characteristics measurements, such as soil pH, EC, C, and N contents (JSSSPN, 1986).

5.3.6. Statistical Analysis

A two-way analysis of variance (ANOVA) to examine the direct and interaction effects of poultry-litter biochar and Azolla on soil properties, rice yield, cumulative CH_4 and N_2O emissions, night respiration (CO_2 flux), and the concentrations of soil solution dissolved CO_2, CH_4 and NO_3^--N. Significant differences among means were compared using Tukey's HSD test at $P < 0.05$ (unless stated otherwise). All data were analyzed using SPSS 20 software (SPSS Inc., Chicago, IL, USA).

5.4. Results

5.4.1. Changes in CH_4 and N_2O Fluxes and their Cumulative Emissions

The pattern and intensity of CH_4 and N_2O fluxes and their cumulative emissions during the rice growth period are shown in **Fig. 5.1a** and **b** and **Table 5.3,** respectively. The interaction effect of poultry-litter biochar and fertilizer amendments on the total cumulative CH_4 emissions for the whole rice growth period was not significant (P = 0.199; **Table 5.3**).

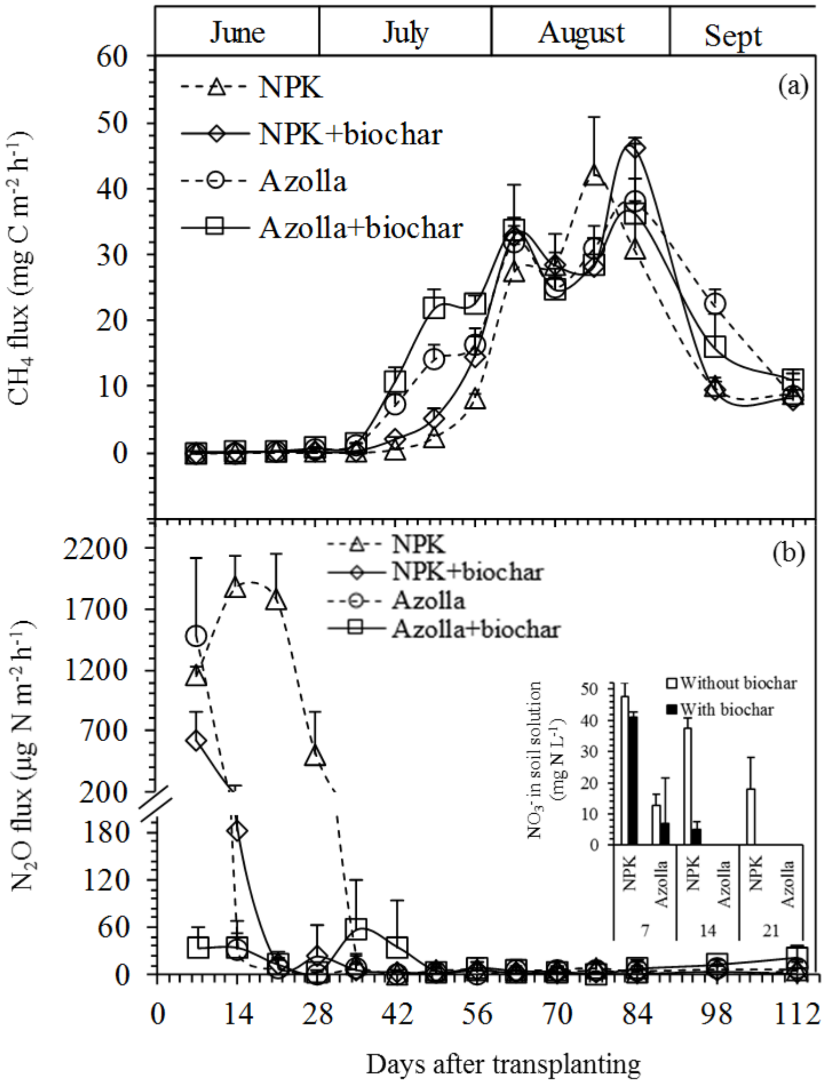

Fig. 5.1. Changes in CH_4 (a) and N_2O (b) fluxes from pots treated with NPK and Azolla (as green manure) with and without biochar throughout the experimental period. Bars indicate the standard deviation (n = 4). The insert in (b) shows the concentration of NO_3^--N dissolved in the soil solution during the first three weeks on the day of gas sampling. The data for the no biochar amendment treatments were obtained from Kimani et al. (2020).

During the early rice growth stages (i.e., before heading; before 63 DAT), the co-application of biochar and Azolla significantly increased cumulative CH_4 emissions by 197.6, 95.3, and 33.0% compared to the NPK, NPK + biochar, and Azolla treatments, respectively ($P < 0.01$; **Table 5.3**). The bulk of the CH_4 was emitted from 35 DAT after the soils changed to reduced condition. Furthermore, Azolla + biochar (10.86–22.60 mg C m^{-2} h^{-1}) had significantly higher CH_4 fluxes compared with NPK (0.54–8.20 mg C m^{-2} h^{-1}), NPK + biochar (1.91–14.54 mg C m^{-2} h^{-1}), and Azolla (7.22–16.39 mg C m^{-2} h^{-1}) between 42–56 DAT, (**Fig. 5.1a**). Amendment with biochar did not influence cumulative CH_4 emission during the late rice growth stages (i.e., heading to maturity; after 63 DAT) ($P = 0.682$; **Table 5.3**).

Throughout the rice growth period, Azolla + biochar significantly decreased total cumulative N_2O emission by 95.9, 76.4, and 86.1% compared to the NPK, NPK + biochar, and Azolla treatments, respectively, with significantly high interaction between the biochar and fertilizer amendments ($P < 0.01$; **Table 5.3**). Additionally, Azolla + biochar treatment significantly reduced N_2O emission before 63 DAT by 97.1, 83.0, and 89.9% compared to the NPK, NPK + biochar, and Azolla treatments, respectively, with a significantly higher interaction between biochar and fertilizer amendments ($P < 0.01$; **Table 5.3**). Furthermore, the co-application of biochar and Azolla significantly decreased N_2O fluxes within the first 28 DAT (Azolla + biochar: 33.6–2.87 μg N m^{-2} h^{-1}; NPK: 1151.8–496.3 μg N m^{-2} h^{-1}; NPK + biochar: 626.8–24.4 μg N m^{-2} h^{-1}; Azolla: 1473.4–1.2 μg N m^{-2} h^{-1}; **Fig. 5.1b**). The addition of biochar did not influence the cumulative N_2O emission during the late rice growth stages (after 63 DAT) ($P = 0.505$; **Table 5.3**)

Table 5.3. Cumulative CH_4 and N_2O emissions during the early (before 63 DAT) and late (after 63 DAT) rice growth stages, and total CH_4 and N_2O emissions per grain yield equivalent between four treatments.

Treatment		Cumulative CH_4 emission			Cumulative N_2O emission			CH_4 emission per grain yield	N_2O emission per grain yield
		Early	Late	Total	Early	Late	Total		
Fertilizer	Biochar	(g C m^{-2})			(μg N m^{-2})			(g C kg^{-1})	(μg N kg^{-1})
NPK (Chemical fertilizer)	Without biochar	4.2 ± 0.5d	26.3 ± 2.4a	30.5 ± 2.9b	907.4 ± 137.6a	4.0 ± 3.6a	911.5 ± 140.3a	38.2 ± 4.5a	1156.2 ± 264.2a
	With biochar	6.4 ± 1.3c	27.9 ± 2.8a	34.4 ± 4.1ab	152.6 ± 39.4b	4.0 ± 3.3a	156.5 ± 41.3b	32.0 ± 5.3ab	143.9 ± 29.6b
	% change by plus biochar	52.4	-	-	-83.2	-	-82.8	-	-87.6
Azolla (As green manure)	Without biochar	9.4 ± 1.0b	30.7 ± 3.0a	40.1 ± 3.6a	257.5 ± 110.8b	8.0 ± 5.8a	265.5 ± 113.8b	38.1 ± 7.2a	246.6 ± 94.8b
	With biochar	12.5 ± 0.6a	27.9 ± 1.5a	40.5 ± 1.4a	26.0 ± 10.1c	11.0 ± 4.2a	37.0 ± 13.9c	28.7 ± 2.4b	26.1 ± 10.1c
	% change by plus biochar	33.0	-	-	-89.9		-86.1	-24.7	-89.4
ANOVA results									
Fertilizer		**	ns	**	**	*	**	ns	**
Biochar		**	ns	ns	**	ns	**	*	**
Fertilizer x Biochar		ns	ns	ns	**	ns	**	ns	**

Values are means ± standard deviation (n=4). Different letters following values within the same column indicate significant differences among treatments (Tukey's HSD test [ns; not significant; *; $P < 0.05$; **; $P < 0.01$]). The data for the no biochar amendment treatments were obtained from Kimani et al. (2020).

The amendment of poultry-litter biochar significantly influenced the total CH_4 and N_2O emissions per grain yield equivalent (at $P < 0.05$; **Table 5.3**). Azolla + biochar significantly decreased CH_4 emissions per grain yield equivalent compared with NPK (24.9%) and Azolla (24.7%) treatments, but not NPK + biochar treatment, and total N_2O emissions per grain yield equivalent by 97.7% (NPK), 81.9% (NPK + biochar), and 89.4% (Azolla), with a significantly high fertilizer × biochar interaction ($P < 0.01$; **Table 5.3**).

5.4.2. Changes in CO_2 Night Respiration

Nighttime CO_2 respiration fluxes, composed mainly of CO_2 emitted from rice plants in the NPK and NPK + biochar treatments, and both rice plants and floating Azolla masses in the Azolla and Azolla + biochar treatments, are shown in **Fig. 5.2a**. Transient significant variations were observed between 8 and 35 DAT between the Azolla + biochar, NPK, and NPK + biochar treatments. However, between 42 and 112 DAT the Azolla + biochar treatment significantly increased nighttime CO_2 emissions compared to the NPK and NPK + biochar treatments, with no significant differences compared to Azolla throughout the rice growth period (**Fig. 5.2a**). The highest CO_2 respiration peak was observed at 42 DAT with significantly high emissions in the Azolla + biochar treatment (559.7 mg CO_2-C m^{-2} h^{-1}) compared to NPK (381.3 mg CO_2-C m^{-2} h^{-1}) and NPK + biochar (464.5 mg CO_2-C m^{-2} h^{-1}), but not Azolla (484.0 mg CO_2-C m^{-2} h^{-1}). Consecutive smaller peaks at 84 DAT observed in all treatments were attributed to the high daytime and night temperature at sampling (**Fig. 5.2a, b**). The average CO_2 respiration rates throughout the rice growth period were 220.8, 247.5, 316.7, and 369.9 mg CO_2-C m^{-2} h^{-1} for the NPK, NPK + biochar, Azolla, and Azolla + biochar treatments, respectively.

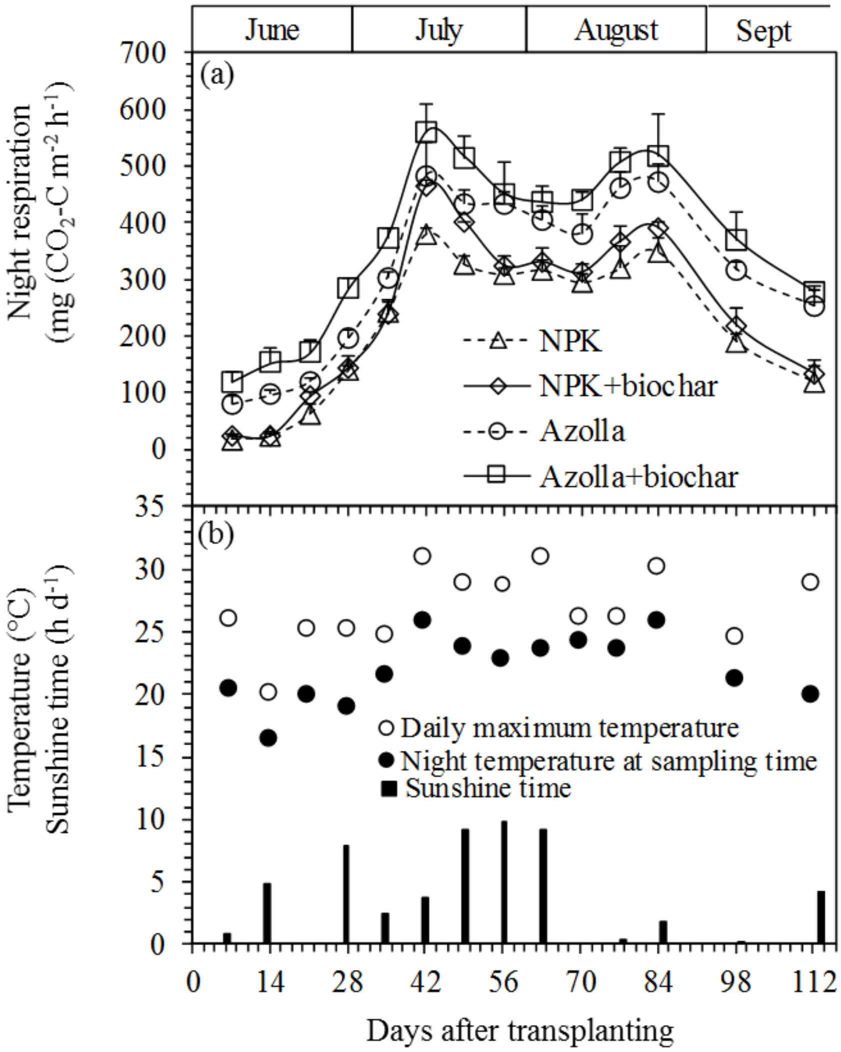

Fig. 5.2. Changes in night respiration (CO_2 emissions) of rice plants from pots treated with NPK and Azolla without and with biochar throughout the experimental period (a). Bars indicate standard deviation (n=4). The daily maximum temperature and temperature at sampling time (21:00) and sunshine time on the day of gas sampling are shown in (b). The data for the no biochar amendment treatments were obtained from Kimani et al. (2020).

5.4.3. Changes in Dissolved CO_2, CH_4, and NO_3^--N Concentrations in Soil Solution

The concentration of CO_2 dissolved in the soil solution increased significantly in the biochar and/or Azolla amended treatments (**Fig. 5.3a**). In the presence of Azolla, amendment with biochar (i.e., Azolla + biochar) significantly increased dissolved soil CO_2 concentration compared to NPK, NPK + biochar, and Azolla treatments between 7 and 42 DAT ($P < 0.01$; **Fig. 5.3a**). Between 7and 42 DAT, the dissolved CO_2 concentration in the Azolla + biochar treatment was between 202.4 and 368.7 µg C mL^{-1} compared with NPK (59.4–129.0 µg C mL^{-1}), NPK + biochar (184.5–206.5 µg C mL^{-1}), and Azolla (80.0–206.4 µg C mL^{-1}). The concentration of CO_2 dissolved in the soil solution for all treatments converged at 56 DAT and no significant differences occurred thereafter.

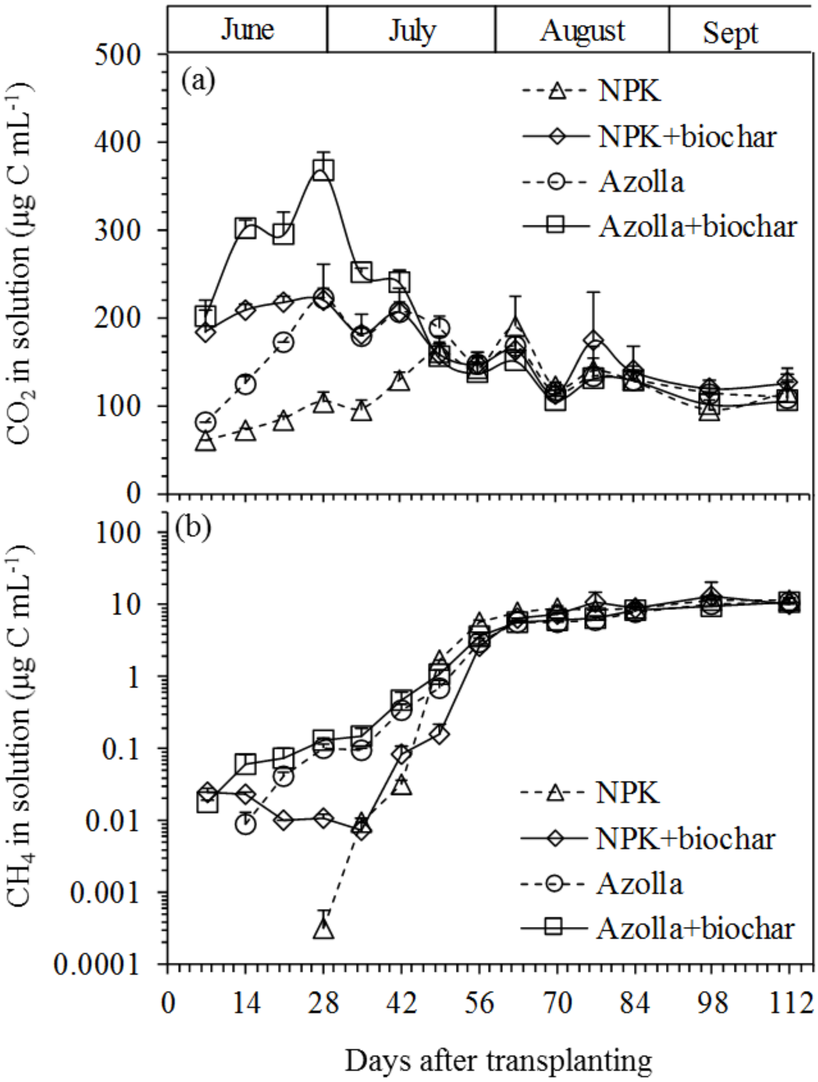

Fig. 5.3. Changes in the concentration of CO_2 (a) and CH_4 (b) dissolved in the soil solution in pots treated with NPK and Azolla with and without biochar throughout the experimental period. Bars indicate the standard deviation (n=4). The data for the no biochar amendment treatments were obtained from Kimani et al. (2020).

The concentration of CH_4 dissolved in the soil solution was significantly higher in the biochar and/or Azolla amended treatments compared with NPK treatment (**Fig. 5.3b**), and the effect of biochar and Azolla co-application on dissolved CH_4 was significantly higher than in the NPK and NPK + biochar, but not in the Azolla treatment between 7–49 DAT ($P < 0.01$; **Fig. 5.3b**). Between 7–49 DAT, the dissolved CH_4 concentration in the Azolla + biochar treatment was between 0.02 and 1.08 µg C mL^{-1}, while those of the NPK and NPK + biochar treatments were between 0.0–0.16 µg C mL^{-1} and 0.0–0.71 µg C mL^{-1} for the Azolla treatment. The concentration of CH_4 dissolved in the soil solution for all treatments increased uniformly with the highest levels recorded at 112 DAT (the last sampling day).

The concentrations of NO_3^--N in soil solution were significantly lower in the Azolla + biochar treatment compared to the NPK, NPK + biochar, and Azolla treatments ($P < 0.01$; insert **Fig. 5.1b**). Over three weeks, the concentration of NO_3^--N in the soil solution was significantly lower in Azolla + biochar treatment (0.0–7.12 mg N L^{-1}) while those of the NPK, NPK + biochar, and Azolla treatments ranged between 0.0–47.78 mg N L^{-1}. Nitrate-N was not detectable in the soil solutions after the three weeks for all treatments.

5.4.4. Rice Yield and Biomass

The addition of poultry-litter biochar and/or Azolla significantly influenced rice plant shoot height, total biomass, grain yield, and harvest index at harvest ($P < 0.05$, **Table 5.4**), with no significant interactions between the biochar and fertilizer amendments for all rice plant growth parameters. Poultry-litter biochar and Azolla co-application (i.e., Azolla + biochar) significantly increased rice grain yield by 75.0% (NPK), and 27.3% compared with both NPK + biochar and Azolla only treatments. Azolla (30.3) and Azolla + biochar (36.3) treatments recorded significantly lower maximum tiller numbers compared with NPK (46.0) and NPK + biochar (47.3) treatments. However, there were no significant differences in the productive tillers among treatments (**Table 5.4**). The dry biomasses of floating Azolla cover in Azolla and Azolla +biochar treatments at harvest were 15.6 and 14.9 g pot^{-1}, respectively.

Table 5.4. Synergistic effects of poultry-litter biochar and Azolla on maximum and productive tiller number, shoot dry weight at harvest, total biomass, grain yield, and harvest index.

Treatment		Maximum tiller	Productive tiller	Shoot height at harvest	Total biomass	Grain yield	Harvest index
Fertilizer	Biochar	(No. hill^{-1})		(cm)	(kg m^{-2})		(%)
NPK (Chemical fertilizer as control)	Without biochar	46.0 ± 2.7a	32.3 ± 2.9a	81.8 ± 3.7c	2.6 ± 0.3b	0.8 ± 0.1c	31.5 ± 3.6c
	With biochar	47.3 ± 2.6a	32.5 ± 1.3a	86.3 ± 1.4bc	3.0 ± 0.2a	1.1 ± 0.1b	36.0 ± 1.0bc
	% change by plus biochar	-	-	-	17.6	35.1	-
Azolla (As green manure)	Without biochar	30.3 ± 1.3b	28.3 ± 2.9a	88.5 ± 2.8ab	2.7 ± 0.2b	1.1 ± 0.1b	40.3 ± 3.3ab
	With biochar	36.3 ± 1.3b	32.8 ± 2.1a	90.4 ± 0.6a	3.2 ± 0.2a	1.4 ± 0.1a	43.7 ± 1.8a
	% change by plus biochar	-	-	-	22.4	33.2	-
ANOVA results							
Fertilizer		**	ns	**	ns	**	**
Biochar		ns	ns	*	**	**	*
Fertilizer x Biochar		ns	ns	ns	ns	ns	ns

Values are means _ standard deviation (n ¼ 4). Different letters following values within the same column indicate significant differences among treatments (Tukey's HSD test [ns; not significant; *; $P < 0.05$; **; $P < 0.01$]). The data for the no biochar amendment treatments were obtained from Kimani et al. (2020).

5.4.5. Changes in Soil Chemical Properties at Harvest

The chemical properties of soil at harvest are shown in **Table 5.5**. The co-application of biochar and Azolla significantly and positively influenced the soil pH and EC values with a significant synergistic interaction between biochar and fertilizer amendments ($P < 0.05$; **Table 5.5**). Additionally, biochar and Azolla amended treatments significantly increased the soil organic C and total N ($P < 0.01$). In the presence of Azolla, the poultry-litter biochar application significantly increased soil organic C by 44.1% (NPK), 25.9% (NPK + biochar), and 17.4% (Azolla), and total N by 33.3% (NPK), 17.6% (NPK + biochar), and 11.1% (Azolla). There were no significant differences in the C/N ratios among treatments (**Table 5.5**).

Table 5.5. Soil properties at harvest among four treatments without and with the amendment of poultry-litter biochar.

Treatment		pH (H_2O)	EC	Total N	Soil Organic C	C/N
Fertilizer	Biochar	(1:2.5)	(µS cm^{-1})	(g kg^{-1} dry soil)		
NPK	Without biochar	5.1 ± 0.1b	80.1 ± 14.4d	1.5 ± 0.0c	14.5 ± 0.2d	10.0 ± 0.1a
(Chemical fertilizer	With biochar	6.8 ± 0.1a	222.7 ± 15.3a	1.7 ± 0.1b	16.6 ± 0.6c	10.0 ± 0.2a
as control)	% change by plus biochar	33.6	177.9	14.4	14.1	-
Azolla	Without biochar	5.2 ± 0.0b	118.7 ± 12.0c	1.8 ± 0.1b	17.8 ± 0.8b	10.0 ± 0.1a
(As green manure)	With biochar	6.7 ± 0.1a	184.6 ± 24.3b	2.0 ± 0.1a	20.9 ± 1.0a	10.2 ± 0.3a
	% change by plus biochar	29.6	75.7	14.6	17.2	-
ANOVA results						
Fertilizer		ns	ns	**	**	ns
Biochar		**	**	**	**	ns
Fertilizer x Biochar		*	*	ns	ns	ns

Values are means _ standard deviation (n ¼ 4). Different letters following values within the same column indicate significant differences among treatments (Tukey's HSD test [ns; not significant; *; P < 0.05; **; P < 0.01]). The data for the no biochar amendment treatments were obtained from Kimani et al. (2020).

5.4.6. Global Warming Potential, Soil C Sequestration, and Net Greenhouse Gas Balance

The GWP of CH_4 and N_2O made up 93.6%–99.1% and 0.9%–6.4%, respectively, of the combined GWP (CH_4 plus N_2O) in the Azolla and Azolla + biochar treatments, as well as 76.4%–95.5% for CH_4 and 4.5%–23.6% for N_2O in the NPK and NPK + biochar treatments (**Table 5.6**). The application of Azolla significantly increased total CH_4 emissions g CO_2-eq m^{-2} (at $P < 0.01$) and combined GWP (at $P = 0.029$, **Table 5.6**). However, the co-application of poultry-litter biochar and Azolla had no significant influence on total CH_4 emissions g CO_2-eq m^{-2} but significantly decreased total N_2O emissions g CO_2-eq m^{-2}, with significantly high interaction between fertilizer and biochar amendments ($P < 0.01$, **Table 5.6**). Subsequently, in the presence of Azolla, the application of biochar did not significantly influence the combined GWP ($P = 0.086$) and no significant differences were observed in the combined GWP between treatments ($P = 0.056$). Application of biochar and/or Azolla significantly influenced soil C sequestration at harvest ($P < 0.01$) and the net GHG balance ($P < 0.01$) compared with NPK only treatment, with no significant interaction between biochar and fertilizer amendments (**Table 5.6**).

Table 5.6. The net CO_2-equivalent greenhouse gas emissions, soil C sequestration, and net GHG balance among four treatments during the whole rice growth period.

Treatment		Total CH_4 emission	Total N_2O emission	Soil C sequestration	Net GHG balance
Fertilizer	Biochar	(g CO_2eq m^{-2})			
NPK (Chemical fertilizer as control)	Without biochar	1381.2 ± 129.2b	426.8 ± 65.7a	-1.6 ± 128.2c	1806.3 ± 29.6c
	With biochar	1557.4 ± 187.2ab	73.3 ± 19.3b	-1731.3 ± 444.4b	-100.7 ± 451.2b
Azolla (As green manure)	Without biochar	1815.6 ± 162.2a	124.3 ± 53.3b	-1821.2 ± 422.1b	118.7 ± 372.9b
	With biochar	1834.1 ± 65.2a	17.3 ± 6.5c	-3485.8 ± 541.4a	-1634.4 ± 511.2a
ANOVA results					
Fertilizer		**	**	**	**
Biochar		ns	**	**	**
Fertilizer x Biochar		ns	**	ns	ns

Values are means ± standard deviation (n=4). Different letters following values within the same column indicate significant differences among treatments at P < 0.05. ns; not significant; *; $P < 0.05$; **; $P < 0.01$. (No biochar amendment treatments data referred from Kimani et al. (2020)).

5.5. Discussion

5.5.1. Effect of Poultry-litter Biochar and Azolla on CH_4 Emissions

Previously, Kimani et al. (2020) reported that incorporation of Azolla as green manure significantly increased CH_4 emissions during the early rice growth stages (i.e., before 63 DAT) by 123.3% and total cumulative CH_4 emissions by 31.5% compared to the NPK treatment (**Table 5.3**). This was largely attributed to the decomposition of the organic amendments by incorporated Azolla (Ying et al., 2000). Similarly, in this study, amendment with biochar in the presence of Azolla (i.e., Azolla + biochar), significantly increased CH_4 emissions both in the early rice growth stages before 63 DAT by 197.6% (NPK), 95.3% (NPK + biochar), and 33.0% (Azolla), and total cumulative CH_4 emissions (at 112 DAT) by 32.8% compared with NPK only treatment, with no significant emission differences relative to NPK + biochar or Azolla (**Table 5.3**). Furthermore, biochar amendment with chemical fertilizer (i.e., NPK + biochar) significantly increased cumulative CH_4 emissions before 63 DAT by 52.5% compared with NPK but reduced the emissions by 46.7% relative to Azolla treatment. No significant cumulative CH_4 emissions were observed among treatments at the late rice growth stages (i.e., after 63 DAT) (**Table 5.3**).

In our observations, the significant increase in cumulative CH_4 emissions before 63 DAT following the addition of biochar (at $P < 0.01$, **Table 5.3**), are consistent with Knoblauch et al. (2011) and Zhang et al. (2012) who revealed a 26% - 68% CH_4 emission increase in paddy soils after biochar applications. Similarly, Kim et al. (2013) reported increased CH_4 emissions of approximately 60% within 40 DAT from green manure amended plots relative to NPK only plots. During the early rice growth stages before 63 DAT, no significant differences in rice growth parameters among all treatments were observed (**Fig. 5.4a, b**); however, biochar and Azolla amendments significantly increased the concentrations of CO_2 and CH_4 dissolved in the soil solutions compared to NPK (**Fig. 5.3a, b**). This was likely as a result of increased microbial biomass and microbial activity after the application of biochar, which may have amplified the decomposition of both the newly-added (in this case Azolla) and native soil organic matter (SOM), as well as the decomposition of labile C pools derived from biochar (Steinbeiss et al., 2009). Therefore, the effect of biochar and Azolla applications on CH_4 emissions before 63 DAT is mostly attributed to the increased availability of carbon substrates following application of Azolla as green manure and/or biochar and their co-application (Jeffery et al., 2016).

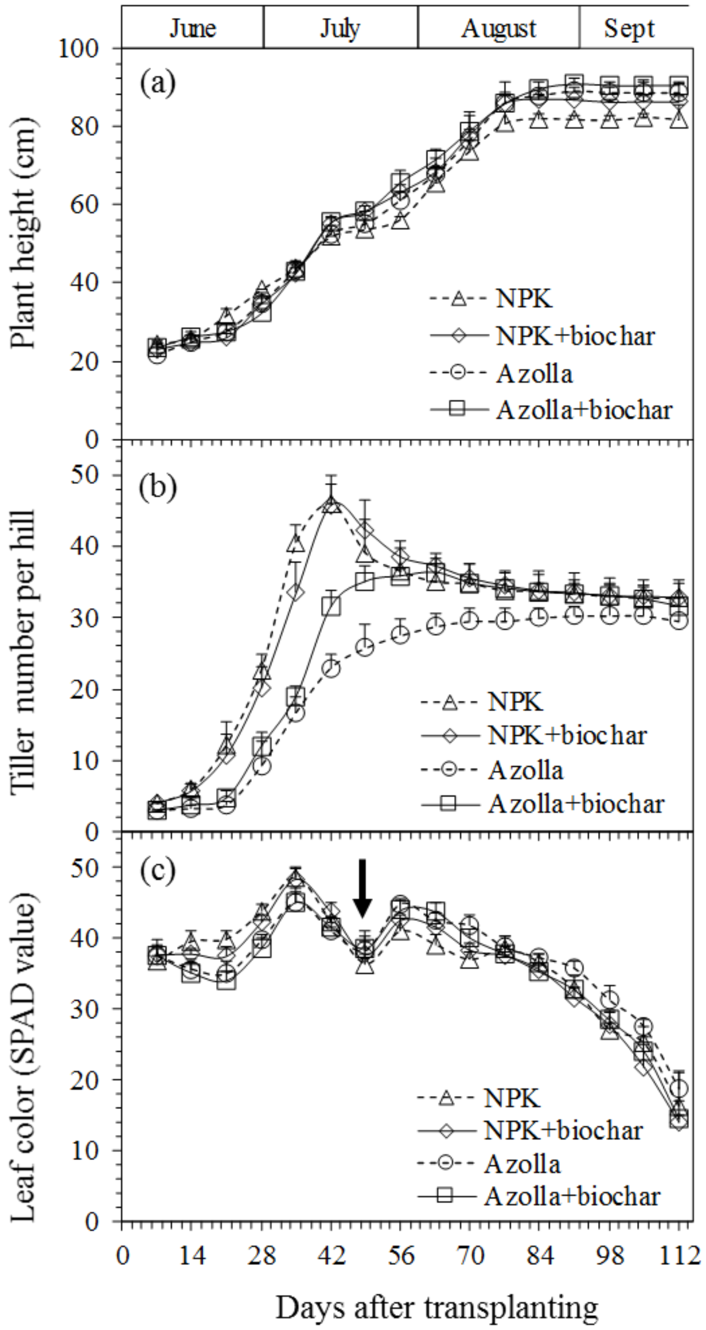

Fig. 5.4. Plant height (a), tiller number (b), and leaf color measured in SPAD values (c) of rice plants from pots treated with NPK and Azolla without and with biochar throughout the experimental period. Bars indicate standard deviation (n=4). The arrow indicates the day fertilizer was added to the pots. The data for the no biochar amendment treatments were obtained from Kimani et al. (2020).

Unlike in the early growth stages of rice, application of biochar did not significantly influence cumulative CH_4 emissions during the later stages (after 63 DAT; **Fig. 5.1a**, **Table 5.3**). In the same batch of treatments, Kimani et al. (2020) found that the percentage of CH_4 emitted after 63 DAT relative to the seasonal CH_4 emission following incorporation of Azolla as green manure (76.5%), was lower compared with NPK (86.2%) (**Table 5.3**). CH_4 emissions in flooded paddy soils are particularly affected by C availability (Wang et al., 2017). However, the highly stable nature of biochar is said to cause no significant changes in C availability (Jones et al., 2011). Moreover, the positive priming of soil organic matter and other organic matter inputs by biochar has been observed to persist for the short-term, due to the relatively small amounts of an easily-mineralizable fraction of biochar (Zimmerman et al., 2011). According to Partey et al. (2014) and Saarnio (2015), the labile C pools resulting from root exudates and root litters are thought to be significantly more compared to organic matter and/or biochar labile fractions. Considering this, our results could partly be ascribed to low soil C availability and supply after 63 DAT following the application of biochar and Azolla as suggested by the minor changes in dissolved CO_2 concentrations in the soil solution after 42 DAT vis a vis the initial (before 63 DAT) concentrations (**Fig. 5.3a**). Additionally, different to the correlation observations in the early growth stages of rice between the daily CH_4 flux and night respiration (CO_2 emissions) where only the Azolla and Azolla + biochar amended treatments showed positive effects ($P < 0.05$, **Fig. 5.5a**), the significantly high and positive correlations observed from all treatments during the late rice growth stages (at $P < 0.01$; **Fig. 5.5b**), suggest likely changes in carbon sources for methanogens, from either the initial SOM, incorporated Azolla as green manure, or biochar addition, to the photosynthetic products of rice plants (Aulakh et al., 2001; Minoda et al., 1996; Sass and Cicerone, 2002).

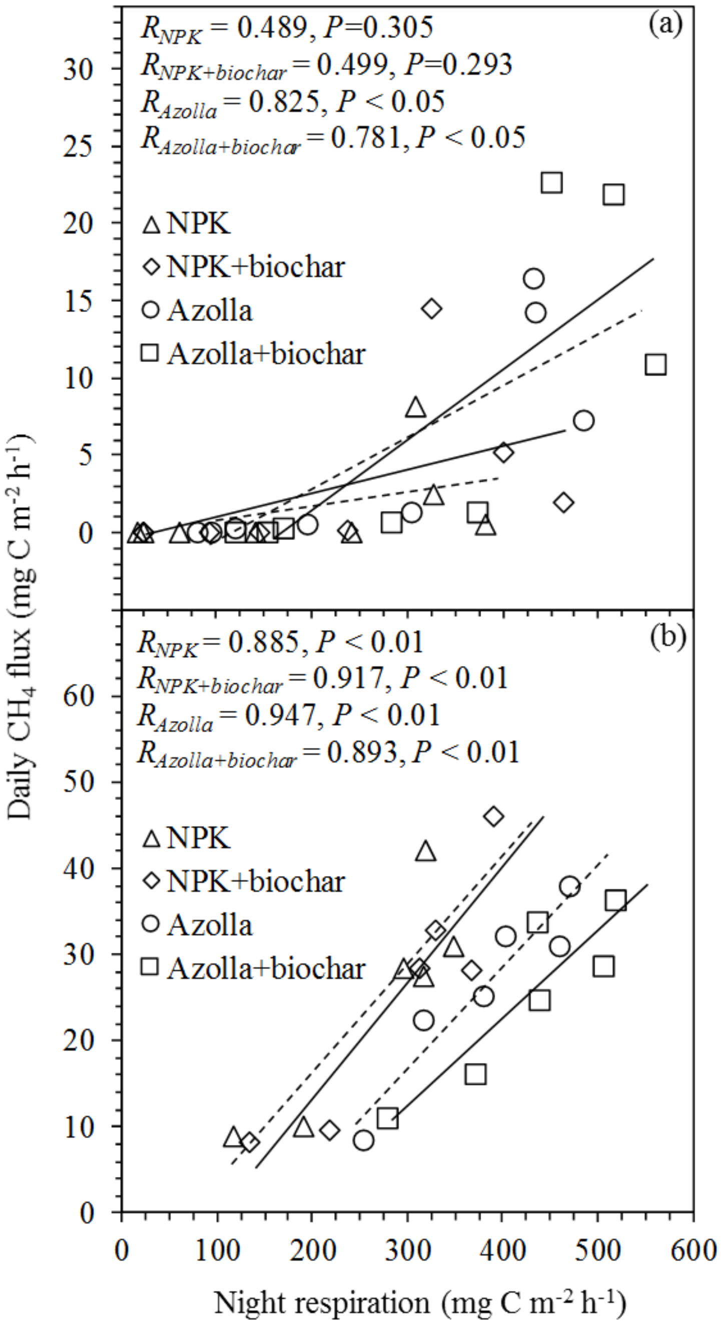

Fig. 5.5. Relationship between daily CH_4 flux and night respiration (CO_2 emissions) between treatments during the early (before 63 DAT) (a) and late (after 63 DAT) (b) rice growth stages (n=6). The data for the no biochar amendment treatments were obtained from Kimani et al. (2020).

According to Feng et al. (2013), decreasing yield equivalent agricultural CH_4 emissions remains a major global test. Cheng et al. (2018) reported a strong relationship between CH_4 emission and rice biomass. Similarly, (Sriphirom et al., 2020) found a significant reduction in yield-scaled CH_4 emissions by 15.2% - 25.5%, and higher rice biomass from biochar amended treatments compared with control under conventional or water management practices. In this study, compared with NPK and Azolla treatments, Azolla + biochar treatment significantly increased rice yield

(**Table 5.4**) and reduced yield-scaled CH_4 emissions (**Table 5.3**). The stimulatory effect of biochar on rice yield productivity is attributed to enhanced nutrient retention and addition, as well as improved nutrient turnover (Biederman and Harpole, 2013). As a result, the co-application of poultry-litter biochar and Azolla as green manure may be an alternative and feasible farming management practice for sustainable rice production.

5.5.2. Effect of Poultry-litter Biochar and Azolla on N_2O Emissions

The effects of biochar on N_2O emissions remain conflicting, ranging from stimulation (Lin et al., 2017), and reduction (Abagandura et al., 2019). In our study, Azolla + biochar significantly reduced the cumulative N_2O emission before 63 DAT compared with NPK (97.1%), NPK + biochar (83.0%), and Azolla (86.1%) (**Fig. 5.1a, Table 5.3**). Similarly, NPK + biochar significantly reduced cumulative N_2O emissions before 63 DAT by 82.8% relative to NPK treatment, with significantly high interaction between biochar and fertilizer (at $P < 0.01$; **Table 5.3**). There were no significant cumulative N_2O emissions observed after 63 DAT among the four treatments. During the entire rice growth period, Azolla + biochar significantly reduced the total cumulative N_2O emissions at 112 DAT by 95.9% (NPK), 76.4% (NPK + biochar), and 86.1% (Azolla), with a significant interaction between biochar and fertilizer (at $P < 0.01$; **Table 5.3**).

Nitrification and denitrification have been identified as the predominant pathways for N_2O production (Charles et al., 2017). According to Miller et al. (2008) availability of easily decomposable organic C and/or NO_3^- stimulates microbial metabolic activity, leading to increased oxygen consumption in the soil, and hence favoring denitrification. In our study, however, application of Azolla as green manure and/or biochar, and their co-application, did not result in additional N_2O production even though the microbial metabolisms, as seen by concentrations of CO_2 dissolved in the soil solutions in the early stages of rice growth, were significantly greater in NPK + biochar, Azolla, and Azolla + biochar treatments relative to NPK only treatment (**Fig. 5.3a**). In the same set of experiments, Kimani et al. (2020) reported that on average, incorporation of Azolla as green manure (AGM) significantly decreased both early (before 63 DAT) and seasonal N_2O emissions by 71.3% (**Table 5.3**). Similarly, Song et al. (2016) reported significant reductions in N_2O emissions in the first 30 days or after 90 days, with no significant differences in the 30-90 day period after biochar amendment. The significantly higher effects of Azolla and/or biochar, and their co-application, on N_2O emissions before 63 DAT would be explained by the effective reduction of N_2O to N_2 during denitrification due to increased availability of easily decomposable carbon from both Azolla and/or biochar (Cayuela et al., 2014; Miller et al., 2008).

Biochar application to soils has been reported to mitigate N_2O emissions through nitrification by probably altering the soil's physical, chemical, and biological properties (Kammann et al., 2017). Additionally, the inhibition of microbial pathways as a result of biochar toxicity, immobilization and adsorption of NH_4^+/NO_3^-, and aeration regulation, are believed to inhibit nitrification and subsequent N_2O emissions (Clough et al., 2013). Furthermore, amendment with biochar pyrolyzed at 400 ºC and 600 ºC has been reported to significantly decrease soil inorganic N by increasing the NH_4^+ adsorption by 62-81% (Zhang et al., 2015). In our study, the biochar was produced between 450 ºC - 500 ºC. Additionally, the application of biochar significantly reduced the NO_3^--N in the soil solution (**insert Fig 5.1b**), in addition to possible suppression of carbon (C) and of nitrogen (N) mobilization from both the native and freshly-added organic matter sources. Application of biochar amendments has been reported to induce a negative priming effect, inhibiting the decomposition of native soil organic carbon (SOC) and the stimulation effect of inorganic N on SOC degradation (Saarnio, 2015; Zimmermann et al., 2011).

Soil pH is a key variable affecting both N_2O production and consumption, as well as the N_2O/N_2 ratio of emissions, with the effect of biochar on the denitrification of N_2O suggested to mostly depend on its pH and the C/N ratios (Cayuela et al., 2014, 2015; Clough et al., 2013). According to van Zwieten et al. (2010), an increase in soil pH under flooded soil conditions possibly enhances the final stage of denitrification (i.e., reduction of N_2O to N_2). In the current study, application of biochar significantly increased the soil pH by 33.3% (NPK + biochar, 6.8 pH unit) and 28.8% (Azolla + biochar, 6.7 pH unit) compared with NPK (5.1 pH unit) and Azolla (5.2 pH unit) treatments, respectively, (**Table 5.5**). Similarly, Clough et al. (2004) reported lower cumulative N_2O fluxes from the soil at field capacity with pH values ≥ 5.9. On the other hand, the lack of significant influence on N_2O emissions after 63 DAT following biochar application in our observations (**Table 5.3**), might be a result of a decrease in the liming effect of biochar (Cayuela et al., 2014; van Zwieten et al., 2010).

In this study, the co-application of poultry-litter biochar and Azolla as green manure simultaneously decreased N_2O emissions and increased grain yield, thereby decreasing yield-scaled N_2O emissions during the rice growth period. Thus, the co-application of biochar and Azolla is an optimal practice for mitigating N_2O emissions and increasing rice yield.

5.5.3. Effect of Poultry-litter Biochar and Azolla on GWP, Soil C Sequestration and Net GHG Balance

In our study, the combined GWP (CH_4 plus N_2O) ranged from 1630.7 to 1939.9 g CO_2eq m^{-2} in all treatments. Biochar and Azolla amendments had no significant effects on the combined GWP (**Table 5.6**). In the same batch of the experiment, the incorporation of Azolla as green manure plus its subsequent growth as a dual crop significantly increased the combined GWP by 7.3% compared with NPK treatment (Kimani et al., 2020), and this was attributed to the significantly higher seasonal CH_4 emissions. The contribution of CH_4 emissions to the combined GWP is considered higher than that of N_2O emissions (Tirol-Padre et al., 2018). In the current observations, however, compared to the NPK treatment, biochar and/or Azolla amendments did not significantly influence the net CH_4 emissions, but significantly reduced net N_2O emissions with significant interactions between biochar and fertilizer (**Table 5.3 and 5.6**). Additionally, the application of biochar and/or Azolla amendments significantly increased the amounts of carbon sequestered in the soil, and subsequently significantly decreased net GHG (**Table 5.3 and 5.6**). According to Lehmann et al. (2006) application of biochar, independently or in combination with other amendments, is seen as a practical tool to mitigate GWP by enhancing soil C sequestration. Additionally, although the contribution of N_2O emissions to the combined GWP during rice cultivation is considered lower than that of CH_4 emissions (Tirol-Padre et al., 2018), our observations suggest that the co-application of biochar and Azolla in lowland rice fields could be a suitable management approach to reduce agricultural N_2O emissions without increasing CH_4 emissions and the subsequent GWP. However, the evaluation of appropriate years of long-term application is considered important in the future.

Nevertheless, the would-be role of biochar in climate change mitigation requires a comprehensive assessment of the energy consumption and carbon release from fossil fuels resulting from its production (Kammann et al., 2017), as well as the actual effect of biochar amendments on GWP. In other words, it is imperative to consider the balance of GHG gases from the production of biochar and the sinks its use may create (Mukherjee et al., 2014; Oomori et al., 2016). Considering this, future research should focus on evaluating the long-term effects of poultry-litter biochar and Azolla co-application to rice paddy fields and the resulting combined GWP and soil C sequestration. Moreover, future studies should also aim to provide a quantifiable basis for management recommendations to achieved maximum sustainable benefits and environmental safety

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5.6. Conclusion

The co-application of poultry-litter biochar and Azolla as green manure significantly increased CH_4 and decreased N_2O emissions during early rice growth stages but had no significant impact during later stages. Overall, the co-application of biochar and Azolla significantly decreased seasonal N_2O emissions but did not significantly influence seasonal CH_4 emissions throughout the whole rice growth period. Subsequently, biochar and Azolla co-application significantly increased rice grain yield, and the soil organic C, total N, pH, and EC values. In the presence of Azolla, amendment with biochar significantly decreased both grain yield equivalent CH_4 and N_2O emissions. Although the co-application of biochar and Azolla did not influence the global warming potential, it significantly increased soil C sequestration and decreased net GHG balance. Consequently, the co-application of biochar and Azolla in conjunction with chemical fertilizers during the rice booting stages showed promising potential in increasing grain yield while reducing non-CO_2 GHG emissions. However, it should be noted that our results are based on a pot experiment, spanning a single rice crop season. Long-term field studies should be carried out in the future to confirm our results in field conditions.

CHAPTER VI

6. Poultry-litter Biochar Application in Combination with Chemical Fertilizer and Azolla Green Manure Improves Rice Grain Yield and Nitrogen Use Efficiency in Paddy Soil

6.1. Abstract

Poultry-litter biochar is expected to improve crop productivity. However, its beneficial interaction with chemical fertilizer and/or organic manure on rice grain yield and nitrogen (N) use efficiency is not well-studied. The objective of this study was to determine the effect of poultry-litter biochar (hereinafter biochar) application and its co-treatment with chemical fertilizer and/or Azolla green manure on rice grain yield, nitrogen (N) uptake, and N use efficiency. A pot experiment was conducted with eight treatments with four replications; no amendment (control), chemical fertilizer (NPK), Azolla green manure (Azolla), and NPK + Azolla; without and with biochar amendment. Biochar was the main factor and NPK and Azolla (herein fertilizer N sources) were the sub-factors. The results showed biochar amendment significantly increased grain yield (32.4%), grain N uptake (23.9%), apparent N recovery efficiency (28.1%), agronomic N efficiency (50.0%), internal N utilization efficiency (35.9%), and partial factor productivity of applied fertilizer N (31.3%), and decreased the soil N dependence rate by -15.2% compared with the treatments without biochar amendment. No significant synergistic interactions between poultry-litter biochar and fertilizer N sources were observed on all determined parameters in the present study. Our results suggest that the application of poultry-litter biochar and its co-treatment with chemical fertilizers and/or Azolla green manure is a feasible fertilizer management practice to increase rice grain yield and improve N use efficiency. In addition, co-treatment of poultry-litter biochar and Azolla green manure (Azolla + biochar), has the potential to reduce and/or even replace the basal chemical fertilizer application, increase rice grain yield and N uptake, as well as improve soil fertility, thus reducing related agricultural pollution and production costs.

6.2. Introduction

Nitrogen (N) is the primary nutrient required for crop production. Hence, increased chemical N fertilizer use in rice (*Oryza sativa* L.), coupled with the development of high yielding varieties and improved water management practices, has been key in the increase of rice production to meet the demand from a growing population (Mueller et al., 2012). However, the excessive and indiscriminate use of chemical N fertilizers to meet both the rice cultivar needs as well as maximize yields is the main cause of nutrient imbalances in soils leading to losses in soil fertility due to organic matter depletion (Fageria and Baligar, 2005; Tomar et al., 2020). According to Dawson et al. (2008), N use efficiency (NUE; the ratio of economic yield output to fertilizers input) is dependent on the N uptake by crops, and the soil and fertilizer N supply, as well as the losses of N from soil-plant systems. Notably, the crop N requirement is the primary factor influencing NUE in cereal production (Ladha et al., 2005). Conversely, the NUE for cereal production globally is approximately 30-50% with a costly 67% unaccounted for (Raun and Johnson, 1999). As a result, there is a growing need to adopt alternative sustainable agronomic and soil management practices that not only improve rice crop productivity but also enhance NUE and decrease the associated negative environmental impacts (Mueller et al., 2012; Ding et al., 2018).

Azolla a small aquatic fern commonly seen in lowland rice paddies has traditionally been utilized as green manure for rice due to its N-fixing agronomic significance in relationship with the cyanobacterium *Anabaena azollae* (Liu and Zheng, 1992; Wagner, 1997). Furthermore, in recent years, the agronomic potential of Azolla utilization in lowland rice fields has increased beyond its viability as an alternative nutrient source. For examples, Azolla has been reported to minimize greenhouse gases from continuously flooded rice paddies relative to inorganic fertilizers (Xu et al., 2017; Kimani et al., 2018), successfully reduce water losses through evapotranspiration from flooded surfaces compared to open water surfaces (Kimani et al., 2020b), as well as improve soil fertility through increased soil organic matter base, support nutrient cycling through increased microbial communities and activities, and increase the nutrient retention from the applied inorganic N fertilizers (Yadav et al., 2014; Kollah et al., 2016).

Biochar has gathered research interest as a result of its long-term soil carbon (C) sequestration potential, improvement of agricultural soil, as well as its feasibility as a climate change mitigation option. The viability of biochar as an effective soil amendment is related to but not limited to its contribution to the soil cation exchange capacity, soil organic matter, liming effect of highly acidic soils, improved microbial biomass activities, as well as its overall high recalcitrance to microbial degradation which ensures its long-term soil fertility benefits (Lehmann et al., 2006; Sohi et al., 2010; Ennis et al., 2012). Although the application of biochar in agricultural soils has been reported to contribute positively in terms of increased crop yields and improved agricultural soil quality, these responses have been variable (Jeffery et al., 2011; Biederman and Harpole, 2013). Furthermore, the application of biochar as a sole nutrient source in agricultural soils may be limiting as a result of the biochar's low nutrient composition and low biodegradability nature (Partey et al., 2014). Thus, it is important to explore and evaluate the interactive effects of biochar and other available nutrient sources in agricultural ecosystems.

Recently, studies have confirmed the synergistic effects of biochar and inorganic fertilizers on crop yields, improved soil quality, and NUE (Sohi et al., 2010; Jeffery et al., 2011). However, the interactive effects of biochar derived particularly from animal sources, and organic sources of plant nutrients remain minimally explored. Poultry-litter (a combination of poultry droppings and bedding material), contains higher amounts of plant essential macro and micronutrients and is thus a valuable source of organic fertilizer (Dikinya and Mufwanzala, 2010). However, utilization of poultry-litter biochar in agricultural ecosystems is considered a more effective management practice due to the potential risks of nutrient leaching, N mineralization, excessive phosphorus contamination to surface water, and possible pathogen contamination among other environmental concerns following the application of poultry-litter manure (Chan et al., 2007; Reddy et al., 2008). Nevertheless, the agronomic values as soil amendments of biochar derived from poultry-litter in combination with inorganic and/or organic sources of nutrients have been limitedly reported (Chan et al., 2008; Hass et al., 2012). Therefore, as highlighted above, integrated management approaches of biochar and inorganic and/or organic fertilizers may offer higher agronomic sustainability and impact than the individual use of either biochar, inorganic, or organic fertilizer sources.

The objective of this study was to determine the effects of the application of poultry-litter biochar and its co-treatment with chemical fertilizer (NPK) and/or organic manure (Azolla green manure) on rice grain yield, N uptake, and grain N use efficiency under continuously flooded soil conditions in a single rice cropping season.

6.3. Material and Methods

6.3.1. Characteristics of the Experimental Site

This research was conducted in the main rice cropping season of 2017 at the Experimental Farm of Yamagata University in Tsuruoka, Yamagata Prefecture, located in northeastern Japan (38º44’N, 139º50’E, 16 m a.s.l.). The single rice growth season was from 7th June to 20th September 2017. The daily average air temperature was 22.7 ºC and 6.4 h sunshine time during the rice growth period (Kimani et al., 2020a).

6.3.2. Experiment Design for Poultry-litter Biochar and Fertilizer (Chemical and/or Manure) Treatments

We set-up an in-situ pot experiment with 8 treatments and 4 replications. The 8 treatments, without and with poultry-litter biochar (hereafter biochar) amendment were (1) no amendment (control, soil only), (2) chemical fertilizer (NPK), (3) *Azolla filiculoides* Lam. green manure (hereafter Azolla), and (4) NPK + Azolla. The biochar amendment and/or fertilizer (NPK and/or Azolla) applications for the different treatments are as detailed in **Table 6.1**.

The poultry-litter biochar (a composition of poultry droppings and bedding material) pyrolyzed at 450 ºC -500 ºC under limited-oxygen conditions, was produced and provided by Meiwa Co., Ltd., Kanazawa, Ishikawa, Japan. The properties of the biochar were, 284.5 g kg^{-1} organic C, 26.70 g kg^{-1} total N (TN), 10.0 pH value (H_2O), 2.79 dS m^{-1} electrical conductivity (EC), 2470.0 mg P_2O_5 kg^{-1} available phosphorus (P), and 18.4 and 550.8 mg N kg^{-1} dry weight NH_4^+-N and NO_3^--N, respectively. The Azolla used in this experiment was an introduced species IRRI code FI 1001 (Cheng et al., 2015a, b). It contained 339.9 g kg^{-1} organic C and 33.8 g kg^{-1} TN (Kimani et al., 2020a).

Table 6.1. Summary if the experimental treatments with poultry-litter biochar, chemical fertilizers, Azolla application at the experiment carried out in Tsuruoka, Japan.

Treatment code	Amendments				Total N application
	Biochar incorporation	Basal fertilizer application	Azolla application	Additional fertilizer application	(g pot^{-1})
Control (soil only)	-	-	-	-	-
NPK (chemical fertilizer)	-	0.40 g N, 0.20 g P, and 0.25 g K per pot were applied by KH_2PO_4 and $CO(NH_2)_2$ as basal fertilizer before	-	Top dressing was applied at 49 DAT by KH_2PO_4 and $CO(NH_2)_2$ at 0.20 g N, 0.10 g P, and 0.13 g K per pot.	0.60
Azolla (green manure)	-	-	243 g fresh Azolla (95% water content, 12.2 g dry weight) incorporated as green manure at transplanting to provide 0.40 g N per pot eqv. [Azolla cover grew	Top dressing fertilizer applied as above.	0.60
NPK + Azolla	-	Basal chemical fertilizer applied as above.	Fresh Azolla applied as above.	-	0.80
Control + biochar	66 g per pot dry wt. biochar (20 tons per ha eqv.) mixed with soil at before transplanting.	-	-	-	-
NPK+biochar	Biochar applied as above.	Basal chemical fertilizer applied as NPK treatment shown above.	-	Top dressing fertilizer applied as NPK treatment shown above.	0.60
Azolla + biochar	Biochar applied as above.	-	Fresh Azolla applied as Azolla treatment shown above.	Top dressing fertilizer applied as Azolla treatment shown above.	0.60
NPK + Azolla + biochar	Biochar applied as above.	Basal chemical fertilizer applied as NPK + Azolla treatment shown above.	Fresh Azolla applied as NPK + Azolla shown above.	-	0.80

6.3.3. Soil and Pot Experimental Preparation

The soil used in this experiment was collected from the plough layer (0-15 cm) of a conventional rice field at the University Farm in Tsuruoka, Yamagata. It was classified as an Inceptisols according to the United States Department of Agriculture (USDA) soil taxonomy. It contained 14.5 g kg^{-1} organic C, 1.40 g kg^{-1} TN, 5.24 pH (H_2O, 1:5 w/w), 0.17 dS m^{-1} electrical conductivity (EC), 70.0 mg P_2O_5 kg^{-1} available phosphorus (P), and 24.8 and 101.6 mg N kg^{-1} dry weight NH_4^+-N and NO_3^--N, respectively.

One day before rice transplanting, 7.0 kg soil (4.9 kg dry soil equivalent) was mixed with 66 g pot^{-1} biochar (20 t ha^{-1} equivalent), and 243 g fresh Azolla at 0.40 g N pot^{-1} equivalent (12.2 g Azolla dry weight pot^{-1}), for the biochar and/or Azolla amended treatments, respectively, and 0.87 g KH_2PO_4 and 0.87 g $CO(NH_2)_2$ for the NPK and NPK + Azolla without and with biochar treatments and placed in each experimental pot (19.5-cm diameter, 27.0-cm height, and 0.20-cm thickness). Three rice seedlings (5-week old) per pot were transplanted into the 32 experimental pots. At 49 days after transplanting (DAT), NPK and Azolla only treatments with and without biochar amendment were top-dressed with 0.43 g KH_2PO_4 and 0.43 g $CO(NH_2)_2$ (**Table 6.1**). The Azolla grew and covered on the surface of the Azolla treatments with and without biochar amendment pots was maintained through the experimental period. The flooding water depth in the pots was maintained at 5 cm above the soil surface by continuously adding tap water for the whole rice growth period.

6.3.4. Sample Analyses and Calculation of N use Efficiency

Rice was harvested at maturity (113 DAT) and rice ears and straw were carefully separately and air-dried for one month in a glass-house. After this, the number of panicles were counted and the grains were carefully threshed to measure the grain yield. Grain yields were adjusted and reported on a basis of 14% moisture content (Fageria, 2009). Later, grains were soaked in tap-water and the number of floating and sunken grains counted to determine yield components, including filled spikelet rate (Cheng et al., 2009; 2015a, b). Part of the grain and straw was oven-dried at 70 ºC in a forced air oven for 3 days and ground for measuring C and N contents using Sumigraph NC 220F Analyzer (Sumika Chemical Analysis Service, Ltd., Osaka, Japan) (Nguyen-Sy et al., 2018). Accordingly, grain N uptake (GNU) and straw N uptake as g N pot^{-1} were calculated by grain N and straw N concentration, respectively. The total N uptake (TNU) is the sum of GNU and straw N uptake.

The nitrogen harvest index (NHI) as a percentage was defined as the N uptake in grain (GNU) as a proportion of TNU. Apparent recovery efficiency (ARE_N) was the percentage of N applied recovered in aboveground biomass). Soil N dependent rate (SNDR; the ratio of total N uptake without fertilization to total N uptake with fertilization), agronomic nitrogen efficiency (AE_N, an expression of unit weight increase in grain yield per N applied), physiological N efficiency (PE_N, the unit weight increase in grain yield per unit weight increase in N uptake from N-fertilizer), internal utilization efficiency (IUE_N, the amount of produced grain yield by unit weight plant nutrient accumulation in the total biomass), and the partial factor productivity (PFP_N, unit of grain yield per N applied), were calculated as follows (Fageria and Baligar, 2005; Ye et al., 2007):

$$NHI\ (\%) = \frac{GNU}{TNU} \times 100 \qquad \textit{(1)}$$

$$ARE_N\ (\%) = \frac{N_f - N_u}{N_a} \times 100 \qquad (2)$$

$$SNDR\ (\%) = \frac{N_u}{N_f} \times 100 \qquad (3)$$

$$AE_N\ (g\ g^{-1}) = \frac{G_f - G_u}{N_a} \qquad (4)$$

$$PE_N\ (g\ g^{-1}) = \frac{Y_f - Y_u}{N_f - N_u} \qquad (5)$$

$$IUE_N\ (g\ g^{-1}) = \frac{Y_f - Y_u}{N_a} \qquad (6)$$

$$PFP_N\ (g\ g^{-1}) = \frac{G_f}{N_a} \qquad (7)$$

where GNU and TNU are grain and straw N uptake as previously described above, N_f is the N accumulation by total biomass (grain plus straw) in the fertilized pots (g), N_u is the N accumulation by total biomass (grain plus straw) in the unfertilized pots (g), and N_a is the quantity of N applied (g) for each treatment as shown in **Table 6.1.** G_f and G_u represent the grain yield (g) of the fertilized pots and unfertilized pots, respectively, for each replicate. Y_f and Y_u represent the total biomass (g) of fertilized pots and total biomass of unfertilized pots (g), respectively, for each treatment.

6.3.5. Statistical Analysis

All data were subjected to a two-way analysis of variance (ANOVA) using SPSS 20 software (SPSS Inc., Chicago, IL, USA) to examine the direct and interaction effects of poultry-litter biochar amendment and fertilizer (chemical and/or manure) on rice yield, yield components, and the parameters of N use efficiency. Significant differences among means for the different treatments were compared using Tukey's HSD test at $P < 0.05$ (unless specified otherwise).

6.4. Results

6.4.1. Grain Yield, N Uptake and N Harvest Index Response to Poultry-litter Biochar Amendment

Overall, the average grain yield per pot for the without biochar amended treatments was 14.6 g to 43.7 g, and 21.3 g to 56.7 g for biochar amended treatments, respectively (**Table 6.2**). Compared with the without biochar amended treatments, amendment with biochar significantly increased grain yield by 32.4%. The average total biomass (straw plus grain) for the without biochar was 40.1 g to 105.1 g, and 58.1 g to 130.6 g for with biochar amended treatments, respectively (**Table 6.2**). Compared with the without biochar amended treatments, amendment with biochar significantly increased total biomass by 24.4%. The average percent harvest index values were between 33.8% and 46.6% among all treatments, without significant differences (P=0.064, data not shown).

The N uptake of grain for the without biochar amended treatments was 0.19 g N pot^{-1} to 0.65 g N pot^{-1}, and 0.25 g N pot^{-1} to 0.76 g N pot^{-1} for biochar amended treatments, respectively (**Table 6.2**). Compared with the without biochar amended treatments, amendment with biochar significantly increased rice grain N uptake by 23.9%. Additionally, compared with the without biochar amended treatments, biochar amendment significantly increased total biomass N uptake by 17.3%, and N harvest index by 5.8% (**Table 6.2**).

Table 6.2. Effects of poultry-litter biochar amendment and fertilizer (chemical and/or manure) co-treatment on grain yield and total biomass, and nitrogen (N) uptake and N harvest index.

Treatments		Rice yield		N uptake		N harvest index
		Grain	Total biomass	Grain	Total biomass	
Biochar	Fertilizer	(g pot^{-1})		(g N pot^{-1})		(%)
Without biochar	Control (soil only)	14.6 ± 2.8d	40.1 ± 4.2e	0.19 ± 0.03d	0.32 ± 0.03d	59.6 ± 6.7b
	NPK (chemical fertilizer)	32.1 ± 3.3c	94.9 ± 10.1c	0.44 ± 0.05c	0.76 ± 0.06c	58.7 ± 2.8b
	Azolla (green manure)	42.6 ± 3.7b	99.1 ± 9.1c	0.60 ± 0.06b	0.85 ± 0.09bc	70.5 ± 2.4a
	NPK + Azolla	43.7 ± 2.6b	105.1 ± 2.9bc	0.65 ± 0.02ab	0.96 ± 0.03ab	67.8 ± 1.0ab
With biochar	Control	21.3 ± 5.0d	58.1 ± 5.5d	0.25 ± 0.06d	0.39 ± 0.06d	63.1 ± 6.7ab
	NPK	43.3 ± 3.7b	111.5 ± 8.3bc	0.58 ± 0.07b	0.89 ± 0.07bc	65.6 ± 3.5ab
	Azolla	56.7 ± 4.6a	121.7 ± 8.0ab	0.74 ± 0.05a	1.03 ± 0.05a	72.2 ± 1.6a
	NPK + Azolla	54.8 ± 2.7a	130.6 ± 7.8a	0.76 ± 0.06a	1.08 ± 0.04a	70.5 ± 2.6a
% change by biochar addition		32.4	24.4	23.9	17.3	5.8
ANOVA results						
Biochar (Bio)		**	**	**	**	*
Fertilizer (Fert)		**	**	**	**	**
Bio x Fert		ns	ns	ns	ns	ns

Values are means ± standard deviation (n=4). Different letters following values within the same column indicate significant differences among treatments (Tukey's HSD test: $P < 0.05$). §Total biomass (grain plus straw).

6.4.2. Rice Yield Components Response to Poultry-litter Biochar Amendment

The average number of maximum tiller per pot were 22.8 to 46.0 for the without biochar amended treatments and 25.8 to 47.3 for with biochar amended treatments, respectively (**Table 6.3**). Compared with the no biochar amended treatments, amendment with biochar significantly increased the maximum tiller number on average by 11.1%. The average numbers of productive tiller and panicle per pot at harvest were similar at 14.0 to 32.3 for the without biochar amended treatments, and 19.0 to 33.0 for the biochar amended treatments, respectively (**Table 6.3**). Compared with the without biochar amended treatments, amendment with biochar significantly increased the productive tiller number and panicle per pot on average by 9.7%. The average number of spikelets per panicle was 41.6 to 68.2 for the without biochar amended treatments and 46.0 to 75.8 for with biochar amended treatments, respectively, while the average percentage of filled spikelets were between 62.4% and 74.1% for the without biochar amended treatments and 68.3% to 80.9% for with biochar amended treatments (**Table 6.3**). Compared with the without biochar amended treatments, amendment with biochar significantly increased the number of spikelets per panicle and the percentage of filled spikelets by 18.2% and 5.6%, respectively. The average individual grain weights were between 24.8 mg and 26.3 mg among all treatments, without significant differences (**Table 6.3**).

Table 6.3. Effects of poultry-litter biochar amendment and fertilizer (chemical and/or manure) co-treatment on maximum and productive tiller number, and rice grain yield components.

Treatments		Maximum tiller	Productive tiller	Panicle No.	Spikelet No.	Filled Spikelet	Individual grain
Biochar	Fertilizer	No. per pot	No. per pot	per pot	per panicle	(%)	weight (mg)
Without biochar	Control (soil only)	22.8 ± 2.2e	14.0 ± 0.8b	14.0 ± 0.8c	43.8 ± 5.0cd	72.1 ± 6.8ab	25.6 ± 0.7
	NPK (chemical fertilizer)	46.0 ± 2.7a	32.3 ± 2.9a	32.3 ± 2.9a	41.6 ± 5.0d	68.5 ± 2.8b	24.8 ± 1.7
	Azolla (green manure)	30.3 ± 1.3d	28.3 ± 2.9a	28.3 ± 2.9a	68.2 ± 4.7ab	62.4 ± 6.6b	26.3 ± 1.8
	NPK + Azolla	38.8 ± 4.2bc	32.3 ± 2.2a	32.3 ± 2.2a	58.7 ± 6.5bc	74.1 ± 5.3ab	24.9 ± 0.3
With biochar	Control	25.8 ± 1.0de	19.0 ± 1.8b	19.0 ± 1.8b	46.0 ± 7.9cd	80.9 ± 4.8a	24.9 ± 0.2
	NPK	47.3 ± 2.6a	32.5 ± 1.3a	32.5 ± 1.3a	56.8 ± 6.3bcd	71.5 ± 3.9ab	25.0 ± 0.4
	Azolla	36.3 ± 1.3c	32.8 ± 2.1a	32.8 ± 2.1a	75.8 ± 5.8a	68.3 ± 3.6b	24.8 ± 0.7
	NPK + Azolla	43.8 ± 1.3ab	33.0 ± 1.8a	33.0 ± 1.8a	72.3 ± 11.0ab	71.8 ± 5.6ab	25.4 ± 0.3
% change by biochar addition		11.1	9.7	9.7	18.2	5.6	-
ANOVA results							
Biochar (Bio)		**	**	**	**	*	ns
Fertilizer (Fert)		**	**	**	**	**	ns
Bio x Fert		ns	ns	ns	ns	ns	ns

Values are means ± standard deviation (n=4). Different letters following values within the same column indicate significant differences among treatments (Tukey's HSD test: $P < 0.05$).

6.4.3. Rice Nitrogen (N) Use Efficiency Response to Poultry-litter Biochar Amendment

The percentage of apparent N recovery efficiency (ARE_N) for without biochar amended treatments was 73.4% (NPK), 88.3% (Azolla), and 79.9% (NPK + Azolla), while for with biochar was 95.7% (NPK + biochar), 118.7% (Azolla + biochar), and 95.1% (NPK + Azolla + biochar), respectively (**Table 6.4**). Compared with the without biochar amendment, biochar amendment significantly increased ARE_N by 28.1%. The soil N dependency rate (SNDR) for without biochar treatments was 42.1% (NPK), 37.7% (Azolla), and 33.2% (NPK + Azolla), while for with biochar was 35.7% (NPK + biochar), 30.8% (Azolla + biochar), and 29.3% (NPK + Azolla + biochar), respectively (**Table 6.4**). Compared with the without biochar amendment, biochar amendment significantly decreased SNDR by 15.2%.

The average agronomic N efficiency (AE_N) for without biochar amended treatments was 29.1 g g^{-1} (NPK), 46.6 g g^{-1} (Azolla), and 36.4 g g^{-1} (NPK + Azolla), while for with biochar was 47.8 g g^{-1} (NPK + biochar), 70.2 g g^{-1} (Azolla + biochar), and 50.2 g g^{-1} (NPK + Azolla + biochar), respectively (**Table 6.4**). Compared with the without biochar amended, biochar amended significantly increased AE_N by 50.0%. The average physiological N efficiency (PE_N) was between 102.1 g g^{-1} and 124.7 g g^{-1} among all treatments, without significant differences (**Table 6.4**).

Table 6.4. Effects of poultry-litter biochar amendment and fertilizer (chemical and/or manure) co-treatment on rice N use efficiency components.

Treatments		ARE_N	SNDR	AE_N	PE_N	IUE_N	PFP_N
Biochar	Fertilizer	(%)		(g g^{-1})			
Without biochar	NPK	73.4 ± 13.0b	42.1 ± 6.1a	29.1 ± 8.4c	124.7 ± 25.3	91.2 ± 23.6bc	53.4 ± 5.5c
	Azolla	88.3 ± 14.8b	37.7 ± 5ab	46.6 ± 8.8b	112.3 ± 11.3	98.3 ± 12.4bc	71.0 ± 6.1b
	NPK + Azolla	79.9 ± 6.9b	33.2 ± 3.9ab	36.4 ± 2.6bc	102.1 ± 8.8	81.2 ± 3.7c	54.6 ± 3.3c
With biochar	NPK	95.7 ± 13.2ab	35.7 ± 4.4ab	47.8 ± 8.7b	124.5 ± 3.6	118.9 ± 14.1ab	72.2 ± 6.2b
	Azolla	118.7 ± 10.1a	30.8 ± 3.5b	70.2 ± 8.5a	114.6 ± 5.9	135.9 ± 12.7a	94.5 ± 7.7a
	NPK + Azolla	95.1 ± 4.5ab	29.3 ± 2.2b	50.2 ± 4.1b	119.1 ± 5.7	113.1 ± 4.6ab	68.4 ± 3.3b
% change by biochar addition		28.1	-15.2	50.0	-	35.9	31.3
ANOVA results							
Biochar (Bio)		**	**	**	ns	**	**
Fertilizer (Fert)		**	**	**	ns	*	**
Bio x Fert		ns	ns	ns	ns	ns	ns

Values are means ± standard deviation (n=4). Different letters following values within the same column indicate significant differences among treatments (Tukey's HSD test; ns; not significant, *; $P < 0.05$; **; $P < 0.01$). ARE_N: apparent N recovery efficiency; SNDR: soil N dependent rate; AE_N: agronomic efficiency; PE_N: physiological efficiency; IUE_N: internal utilization efficiency; PFP_N: partial factor productivity.

The average internal N utilization efficiency (IUE_N) for without biochar amended treatments was 91.2 g g^{-1} (NPK), 98.3 g g^{-1} (Azolla), and 81.2 g g^{-1} (NPK + Azolla), while for with biochar was 118.9 g g^{-1} (NPK + biochar), 135.9 g g^{-1} (Azolla + biochar), and 113.1 g g^{-1} (NPK + Azolla + biochar), respectively (**Table 6.4**). Compared with the without biochar amendment, biochar amendment significantly increased IUE_N by 35.9%. In addition, the average partial N factor productivity (PFP_N) for without biochar amended treatments was 53.4 g g^{-1} (NPK), 71.0 g g^{-1} (Azolla), and 54.6 g g^{-1} (NPK + Azolla), while for with biochar was 72.2 g g^{-1} (NPK + biochar), 94.5 g g^{-1} (Azolla + biochar), and 68.4 g g^{-1} (NPK + Azolla + biochar), respectively (**Table 6.4**). Compared with the without biochar amendment, biochar amendment significantly increased PFP_N by 31.3%.

6.5. Discussion

6.5.1. Effect of Poultry-litter Biochar Amendment on Grain Yield and N Uptake of Rice

According to Cheng et al. (2009, 2015a, b), panicle number per area and spikelet number per panicle determined during the rice vegetative period, and the percentage of filled spikelet and individual grain weight determined during the rice reproductive growth period, are the four important yield components that largely determine rice grain yield. In our study, co-treatment of biochar with chemical (NPK) and/or organic (Azolla green manure) fertilizers had no significant effect on the individual grain weight, but on average significantly increased the panicle number per pot (9.7%), spikelet number per panicle (18.2%) and the filled spikelet percentage (5.6%) (**Table 6.3**), and subsequently significantly increased rice grain yield by 32.4% on average compared to the similar treatments without biochar amendment (**Table 6.2**). The higher grain yield might be attributed to the positive effects on grain and total N uptake, 23.9% and 17.3%, respectively, (**Table 6.2**) as a result of improved fertilizer use efficiency in addition to the short-term nutrient's availability within the poultry-litter biochar amendments (Lehmann et al., 2003; Chan et al., 2008). Similarly, Maru et al. (2015) observed significant rice panicle number differences and subsequent grain yield and total grain and dry matter yield attributed to the influences of chicken-litter biochar on nutrient availability and improved nutrient use efficiency.

Furthermore, sole application of biochar or its concurrent amendment with fertilizer (chemical and/or organic), has been reported to positively and significantly impact plant growth through the direct or indirect provision of the necessary nutrients and the significant effects on the physical and chemical properties of soil (Sohi et al., 2010; Oladele et al., 2019; MacCarthy et al., 2020). Thus, other possible reasons for the overall increase in rice grain yield and N uptake in biochar amended treatments may have included: (1). the addition of essential soil micro and macro-nutrients (Dikinya and Mufwanzala, 2010; Hass et al., 2012; Biederman and Harpole, 2013); (2). high availability of water-soluble nutrients as a result of notably high initial EC value of 2.79 dS m^{-1} (Dong et al., 2015); and, (3). the slow-or controlled-release of different forms of N from the chemical and/or organic manure fertilizers applied at basal and/or top-dressing as a result of the biochar's adsorption and desorption capability of applied fertilizers (Hagemann et al., 2017; Shi et al., 2020).

6.5.2. Effect of Poultry-litter Biochar Amendment on N Use Efficiency (NUE) of Rice

According to Baligar et al. (2001) and Fageria (2009), the primary components of nutrient (in this case N) use efficiency (NUE) are the agronomic N efficiency (AE_N), apparent N recovery efficiency (ARE_N), and physiological N efficiency (PE_N), with ARE_N a key index in evaluation of the efficiency of fertilizer management practices. The PFP_N index is important to understand both the long-term productivity trends as well as optimize N fertilizer use efficiency, that is, the total economic outputs relative to the use of all N fertilizer sources including the soil N and applied fertilizer (Olk et al., 1999). In the present study, on average co-treatment of biochar with chemical fertilizer (NPK) and/or organic manure (Azolla green manure) did not significantly influence PE_N, but significantly increased ARE_N by 28.1%, and AE_N by 50.0% compared to the without biochar amended treatments (**Table 6.4**). The reason may be from the greater biochar sorption capacity, which improved N uptake by crop biomass (Huang et al., 2018; Maru et al., 2015; Omara et al., 2020; Steiner et al., 2008). In this study, biochar application significantly and positively influenced the internal nitrogen utilization efficiency (IUE_N; 35.9%) and the partial factor productivity of applied fertilizer (PFP_N; 31.3%), with a significant decrease in the soil N dependency rate (SNDR; 15.2%) compared with without biochar amended treatments (**Table 6.4**). Huang et al. (2018) reported significantly higher IUE of 7% - 10% following continued application of biochar to rice compared to control with a subsequent significant increase in grain yield of 6% and unchanged total N uptake.

In our study, the significant decrease of SNDR (a reflection of the contribution of soil N to plant nutrition) following biochar amendment, reflected the possibility of the increased bioavailability of N for plant use from the presently applied sources of N fertilizers and strengthened reliance of rice growth on these nutrients, and the weakened dependency of rice growth on soil N. Application of chemical and/or organic fertilizer sources in combination with biochar has been reported to largely improve N fertilizer availability and its use efficiency (Jeffery et al., 2011; Maru et al., 2015).

According to Xu et al. (2012), N uptake and PE_N are key parameters in realizing maximum potential grain yield. Sinebo et al. (2004) reported significantly stronger relationships between grain yield and ARE_N as compared to PE_N. In this study, the significant and positive correlation between grain yield and ARE_N at $P < 0.05$ (r = 0.68; with biochar, and r = 0.61; without biochar), as compared to the significant and negative correlation between grain yield and PE_N at $P < 0.05$ (r = -

0.71; for with biochar only, with no significant correlation for the without biochar) (**Table 6.5**), indicated the greater importance of ARE_N to maximizing rice grain yield in our study.

Table 6.5. Correlation coefficients for grain yield, total biomass, N uptake, and N use efficiency of rice grown with biochar amendment and fertilizer (chemical and/or manure) co-treatment (lower left), and without biochar amendment and fertilizer co-treatment (upper right), respectively.

	Grain yield	GNU	Total biomass	TNU	$\mathrm{ARE_N}$	SNDR	$\mathrm{AE_N}$	$\mathrm{PE_N}$	$\mathrm{IUE_N}$	$\mathrm{PFP_N}$
Grain yield		0.93**	0.60*	0.83**	0.61*	-0.68*	0.75**	-0.51	0.10	0.59*
GNU	0.96**		0.49	0.93**	0.64*	-0.80**	0.66*	-0.69*	-0.05	0.42
Total biomass	0.79**	0.89**		0.61*	0.36	-0.54	0.41	0.17	0.53	0.20
TNU	0.92**	0.97**	0.93**		0.61*	-0.85**	0.52	-0.63*	-0.01	0.21
$\mathrm{ARE_N}$	0.68*	0.56	0.31	0.45		-0.76**	0.89**	-0.39	0.52	0.71**
SNDR	-0.77**	-0.79**	-0.65*	-0.76**	-0.54		-0.63*	0.50	-0.22	-0.23
$\mathrm{AE_N}$	0.72**	0.56	0.27	0.45	0.97**	-0.58*		-0.25	0.56	0.88**
$\mathrm{PE_N}$	-0.71*	-0.62*	-0.25	-0.54	-0.61*	0.59*	-0.65*		0.58*	-0.14
$\mathrm{IUE_N}$	0.51	0.41	0.28	0.32	0.93**	-0.40	0.87**	-0.29		0.50
$\mathrm{PFP_N}$	0.60*	0.43	0.16	0.30	0.94**	-0.29	0.93**	-0.58*	0.87**	

Significant at *$P < 0.05$, **$P < 0.01$. GNU: grain nitrogen uptake; TNU: total nitrogen uptake; $\mathrm{ARE_N}$: apparent N recovery efficiency; SNDR: soil N dependent rate; $\mathrm{AE_N}$: agronomic efficiency; $\mathrm{PE_N}$: physiological efficiency; $\mathrm{IUE_N}$: internal utilization efficiency; $\mathrm{PFP_N}$: partial factor productivity.

6.6. Conclusion

Overall, our study results show that amendment with poultry-litter biochar and its co-treatment with chemical and/or manure fertilizer improves the rice N use efficiency and ensures increased grain yield and possible sustainability in the long-term. Notably, the co-treatment of poultry-litter biochar and Azolla green manure and its subsequent growth as a dual crop (Azolla cover) plus chemical fertilizer (NPK) application at the rice booting stage (Azolla + biochar) significantly increased ARE_N, AE_N, and IUE_N efficiencies of N, a probable reflection of the positive effects of poultry-litter biochar and Azolla green manure combination on rice grain yield improvement, enhancement of grain N uptake as well as the N harvest index. Thus, when used in combination with poultry-litter biochar, Azolla application at basal plus top-dressing can reduce and save on chemical fertilizer use to achieve significant yield compared with the conventional chemical fertilizer management practice. In addition, the subsequent significant decrease in SNDR, and the positive influence on soil chemical properties following the co-treatment of Azolla and biochar, emphasizes the need and importance to evaluate nutrient supply and demand.

However, long-term field experiments are necessary to ascertain our findings. Future research studies should seek to propose fertilizer management practices that help in optimizing both the biological and economic efficiency of applied fertilizer sources while at the same time enhancing the long-term soil health of production systems and concurrently mitigating environmental pollution.

CHAPTER VII

7. General Discussion and Conclusions

Rice (*Oryza sativa* L.) is an important source of food for over half of the world's population. With a predicted population growth to nearly 9 billion over the next 20 to 30 years, demand for rice production will have to increase by 25% by 2050 to meet the anticipated increase in population. However, rice agriculture is threatened by the changing global climate as a result of; (1) increasing global temperature, (2) rising sea levels and, and (3) the changes in rainfall patterns and distribution in different regions globally. Additionally, the rising sea levels and temperatures are expected to exacerbate the threat of water scarcity on rice agriculture by increasing the rates of evaporation (E) and evapotranspiration (ET) from open water sources and vegetation, respectively. Conversely, rice agriculture is not just a victim of climate change, but also a contributor. Accordingly, the projected increase in rice production is expected to become less climate-friendly.

First, continuously flooded rice paddies have been recognized as the main sources of the two major potent non-carbon dioxide greenhouse gases (non-CO_2 GHGs)- i.e., methane and nitrous oxide- contributing to climate change and global warming. Methane (CH_4) is the second most important anthropogenic greenhouse gas (GHG) accounting for about 15-20% of the atmospheric radiative forcing. CH_4 is produced through anaerobic degradation of organic matter by archaea bacteria and oxidized by methanotrophic bacteria. It has a global warming potential that is 34 times that of carbon dioxide (CO_2) in 100 years. Nitrous oxide (N_2O) naturally originates in the soil through microbial processes of nitrification and denitrification. N_2O is 298 times more potent in heat-trapping than CO_2 and accounts for about 6.8% of the radiative forcing added to the atmosphere (IPCC, 2013). With the suggested increase in rice production, a subsequent increase in CH_4 emission accompanying this rise in rice production as well as a proportionate increase in N_2O emission following increased nitrogen (N) fertilizer use is undeniably likely. Therefore, research on immediate, feasible, and long-term approaches to mitigate continued emissions of these two non-CO_2 GHGs from conventionally flooded rice ecosystems are necessary.

Second, lowland rice fields are among the major consumers of the non-renewable global freshwater with a subsequently higher consumptive water (herein ET) per unit area higher compared to maize and wheat -two other most important world cereal crops. While crop ET may represent only a small portion of irrigation inflow requirements compared to the higher quantities of water required for land preparation as well as maintaining the flooding water level in the paddy fields, typical ET rates of lowland rice fields range between; (1) 4-5 mm/day in the wet season, (2) 6-7 mm/day in the dry seasons, and (3) about 10-11 mm/day in subtropical regions. Thus, approaches allowing for water, particularly ET loss management are necessary.

Azolla is a floating aquatic fern dominant in lowland paddies whose biological characteristics contribute mainly to N fertilization. Thus, Azolla has for centuries been used as green manure for lowland rice agriculture in China and Vietnam. Recently, with a consistent increase in cover crop adoption in rice agriculture and the understanding of climate change, research on the interaction between cover crops and climate change has gained momentum. Research on the ability of cover crops to; (1) improve soil quality, (2) fix atmospheric N, (3) minimize erosion and N leaching, has been widely documented, the effects of cover crops, in particular, Azolla on ET and the simultaneous CH_4 and N_2O emissions from continuously flooded lowland ecosystems remain scarce and/or contradictory. Agricultural GHG fluxes are complex and heterogeneous and the trade-off relationship between CH_4 and N_2O emissions from paddy fields requires management strategies to mitigate against these emissions simultaneous. This, however, is a daunting task that cannot be achieved by any single management practice but requires different approaches that can be widely implemented both locally by individuals and through large programmes to produce effects on a global scale.

7.1. Azolla as a Water Saver

In Chapter II, we found that Azolla as a cover on the water surface has the potential to reduce ET regardless of the flooding water depth. This was attributed to, but not limited to, its anatomy, horizontal placement of its leaves, and smaller leaf area, which possibly restricted simultaneous ET losses by shielding much of the water surface. Plant physiological traits have been previously reported as key components influencing E, transpiration, and interception losses (Hussey and Odum 1992; Moore and Owens 2012). Additionally, the success of *Azolla* under low flooding water depth in the presence of P to maintain sufficient N accumulation is a conviction that shallow flooding is not only a useful technical improvement over high flooding water depth cultivation but also a new and useful development in growing *Azolla* for agricultural use, especially in adverse water conditions and under the newly proposed water conservation techniques in rice production (Tuong et al 2005; Sujono et al 2011; Darzi-Naftchali and Ritzema 2018).

7.2. Influence of Azolla Cover on CH_4 and N_2O Emissions

In Chapter III, we found that Azolla as a dual crop with rice significantly reduced CH_4 emissions by 34.7% form continuously flooded rice soil planted with rice compared with the rice only treatment. This result was consistent with previous recent studies (Bharati *et al.* 2000; Ma *et al.* 2012; Ali *et al.* 2015; Liu *et al.* 2017). In our study, the moderating effect of floating plants on CH_4 emission from dual Azolla-rice soil ecosystem could be reconsidered due to two main reasons. Firstly, photosynthetically released oxygen by the floating plants into the flooding water, could directly stimulate CH_4 oxidation at the soil-water interface and rhizosphere of the surface layer, indirectly leading to a decrease in CH_4 emission from plant-mediated transport (through the aerenchyma tissues) (Xu et al. 2017). Secondly, the moderating effect on CH_4 emission from dual

Azolla–rice soil could be attributed to the large masses of floating plants covering the flooding water surface of rice soil which could serve as a physical barrier obstructing the diffusion of CH_4 from anaerobic soil to the atmosphere, which provides the other pathways for CH_4 emitting to the atmosphere as ebullition and diffusion (van der Steen et al. 2003). The Azolla cover in this experiment did not affect N_2O emissions from both treatments implying that the Azolla cover did not bring extra N_2O flux from dual Azolla and rice cropping ecosystems. This result was contrary to the results of Chen *et al.* (1997) and Ma *et al.* (2012) who reported N_2O emission from rice paddy affected by Azolla cover. Their results showed that Azolla cover increased N_2O emission from rice paddies due to N-fixation by Azolla providing a source for N_2O production through nitrification and denitrification, especially when the Azolla died.

7.3. Influence of Azolla Incorporation and Dual Cropping on CH_4 and N_2O Emissions

In Chapter IV, we found that incorporation of Azolla as green manure plus its subsequent growth as a cover crop in conjunction with chemical fertilizers (NPK) either at basal or top-dressing, significantly stimulated the total cumulative CH_4 emissions throughout the rice growth period by 31.5% and 43.5% compared with the conventional fertilizer management practice (i.e., NPK treatment). These stimulating effects were consistent with previous findings that Azolla, either incorporated or as a dual crop with rice, increased CH_4 emissions from rice paddy soil pathways (Chen et al. 1997; Adhya et al. 2000; Ying et al. 2000), which was most likely due to the decomposition of the organic amendments by incorporated Azolla. Azolla as green manure significantly increased the cumulative CH_4 emissions during the early rice growth stages (before the ripening stage) compared with the NPK treatment. This was likely attributed to readily available carbon substrates following the incorporation of Azolla. High cumulative CH4 emissions during the late rice growth stages (after-ripening) were ascribed to rice photosynthesis (Aulakh et al. 2001b; Sass and Cicerone 2002).

Incorporation of Azolla as green manure plus its subsequent growth as a cover crop significantly inhibited N_2O emission from flooded paddy soil compared with NPK treatment. Cumulatively high N_2O emissions were recorded during the early rice growth stages in all treatments and on average, at the early rice growth stages, NPK treatment emitted 99.5% N_2O of all growth stages compared to the Azolla amended treatments (97.0% AGM and 96.0% NPK+AGM). There were no significant N_2O emission differences during the late rice growth stages among the three treatments. During the entire rice growth period, AGM and NPK+AGM treatments decreased seasonal N_2O emissions by 3.4 (70.9%), and 4.6 (78.2%) fold relative to NPK treatment, respectively. This reduction in N_2O emissions following Azolla incorporation was likely due to a more effective reduction of N_2O to N_2 during denitrification as a result of the availability of readily decomposable organic matter, in this case, the incorporated Azolla. Availability of organic C has previously been reported to be a critical factor for denitrification (Aulakh et al. 1991b; Baruah and Baruah 2015).

7.4. Influence of Azolla and Biochar Co-treatment on CH_4 and N_2O Emissions

In Chapter V, the co-treatment of poultry-litter biochar and Azolla as green manure significantly increased CH_4 and decreased N_2O emissions during early rice growth stages but had no significant impact during later stages. The significant increase in cumulative CH_4 emissions during early rice growth stages following the addition of biochar is consistent with Knoblauch et al. (2011) and Zhang et al. (2012) who revealed a significant CH_4 emission increase in paddy soils after biochar applications. The CH_4 emission increase was mostly attributed to the increased availability of carbon substrates following the application of Azolla as green manure and/or biochar and their co-application (Jeffery et al., 2016). Unlike in the early growth stages of rice, the application of biochar did not significantly influence cumulative CH_4 emissions during the later stages. This was primarily attributed to the highly stable nature of biochar which causes no significant changes in C availability (Jones et al., 2011). Moreover, the positive priming of soil organic matter and other organic matter inputs by biochar has been observed to persist for the short-term, due to the relatively small amounts of an easily-mineralizable fraction of biochar (Zimmerman et al., 2011). According to Partey et al. (2014) and Saarnio (2015), the labile C pools resulting from root exudates and root litters are thought to be significantly more compared to organic matter and/or biochar labile fractions. Considering this, our observations could partly be ascribed to low soil C availability and supply after 63 DAT following the application of biochar and Azolla.

In our study, the application of Azolla as green manure and/or biochar, and their co-application, did not result in additional N_2O production. Nitrification and denitrification have been identified as the predominant pathways for N_2O production (Charles et al., 2017). According to Miller et al. (2008) availability of easily decomposable organic C and/or NO_3^- stimulates microbial metabolic activity, leading to increased oxygen consumption in the soil, and hence favoring denitrification. The significantly higher effects of Azolla and/or biochar, and their co-application, on N_2O emissions during the early rice growth stages would be explained by the effective reduction of N_2O to N_2 during denitrification due to increased availability of easily decomposable carbon from both Azolla and/or biochar (Cayuela et al., 2014; Miller et al., 2008).

7.5. Influence of Biochar, Azolla, and Chemical Fertilizer and their Co-treatment on Rice Yield and N Use Efficiency

In Chapter VI, we observed significantly higher rice grain yield following biochar amendment relative to the absence of biochar application. This was attributed to the positive effects on grain and total nitrogen (N) uptake as a result of improved fertilizer use efficiency in addition to the short-term nutrient availability within the poultry-litter biochar amendments (Lehmann et al., 2003; Chan et al., 2008). Additionally, compared with no biochar amended treatments, application of biochar significantly increased the apparent N recovery efficiency (ARE_N), decreased the soil N dependency rate (SNDR), and significantly and positively influenced the internal nitrogen utilization efficiency (IUE_N) and the partial factor productivity of the applied fertilizer (PFP_N). The reasons may be from the greater biochar sorption capacity, which improved N uptake by crop biomass (Huang et al., 2018; Maru et al., 2015; Omara et al., 2020; Steiner et al., 2008).

7.6. Influence of Azolla Dual Cropping and/or Incorporation on CH_4 and N_2O Emissions: 1st Year Field Experiment (Tentative Results)

To validate the pot findings in Chapter III and Chapter IV above, we established a field study in a newly constructed rice paddy in Yamagata University Experimental farm, Tsuruoka. The objective of the three-year study (2019-2021) is to verify the potential of Azolla as a cover and both as a cover plus incorporation as green manure on simultaneous CH_4 and N_2O emissions, as well as its influence on rice grain yield. Three treatments were established; NPK (as control), NPK plus Azolla cover only (NPK + Cover), Azolla cover plus incorporation as green manure without chemical fertilizer application (AGM + Cover). Briefly, Gas fluxes were acquired in triplicate for each plot. The gas flux in the fields was measured by a closed-chamber method. Rectangular acrylic chambers (0.45-1.0 m high, 0.16-0.36 m^3 volume) were covered during gas sampling, and the chamber height was adjusted to accommodate the increasing rice height. Eight rice hills were included within the chamber area. The bases of the chambers were inserted to a depth of approximately 2 cm in the soils and were left throughout the cultivation season. Samples for CH_4 and N_2O analysis were collected three times within 30 minutes (0, 15, 30 min) from each chamber. Each chamber was equipped with a rubber septum to allow samples to be taken using a syringe. Each sample taken into the syringe was immediately transferred to a 19-ml evacuated injection vial with a butyl rubber stopper. Flux measurements were made from a metal-walk, which reduced disturbance and prevented the ebullition of gases from the soil surface during the measurements. CH_4 and N_2O concentrations were determined using gas chromatographs equipped with flame ionization detectors (FIDs) and electron capture detectors (ECDs), respectively. These analyses were conducted at the Institute of Agro-Environmental Sciences, NARO.

Our results showed that AGM + Cover significantly decreased grain yield by 37.5% and 38.0% compared with NPK and NPK + Cover, respectively. Cumulatively, AGM + Cover significantly increased total CH_4 emissions by 41.0% (NPK + Cover), with no significant emission differences compared with NPK. AGM + Cover treatment significantly increased the CH_4 grain yield emission equivalent by 96.2% (NPK), and 127.7% (NPK + Cover), and the N_2O grain yield emission equivalent by 579.0% and 169.4%. No significant differences were observed between the

NPK and NPK + Cover treatments (Data not shown). The influence of organic sources on CH_4 and N_2O effluxes in rice paddies has been extensively reported (Kollah et al., 2016). Due to sufficient rainfall during the rice-growing period in the local area (Tsuruoka), irrigation water management practices and measurements of irrigation water use could not be employed or determined, respectively. Adjustments and improvements of notable shortcomings from the first year of study are currently under consideration in the consecutive study years (2020-2021).

7.7. Conclusions

From the sequence of the research studies, the influence of Azolla (*A. filiculoides* Lam.) following its application either; (a) solely as a dual (cover crop) individually or in combination with rice, or (b) as a dual plus incorporation as green manure, and/or (c) as a dual plus incorporation and its co-treatment with poultry-litter biochar on the consumptive water use (herein evapotranspiration, ET), simultaneous methane (CH_4) and nitrous oxide (N_2O) and the rice grain yield and nitrogen use efficiency in constantly flooded rice paddy ecosystems can be summarized as follows:

(1) The application of Azolla as a cover on the flooded water surfaces, regardless of the flooding water depth, has a predicted relative ET reduction efficiency as a result of its physiological characteristics.

(2) Application of Azolla as a dual crop and/or as a dual plus its incorporation as green manure in combination with rice in constantly flooded rice ecosystem can; (i) significantly decrease CH_4 emissions, with no significant effects on the N_2O emissions, likely due (a) to an increase in dissolved oxygen concentration and redox potential at the soil-water interface; enhancing methane oxidation, and (b) no addition N_2O fluxes from the floating Azolla cover, respectively. And (ii) significantly decrease N_2O emissions through the rice growth while contributing significantly to the early rice growth stages CH4 emissions but not during the late rice growth stages.

(3) Co-application of Azolla as green manure and poultry-litter biochar offers a novel approach to increase rice yield while reducing the emissions of CH_4 and N_2O gases at different rice growth stages.

(4) Co-application of Azolla as green manure and its subsequent growth as a dual crop has the potential to reduce and/or even replace the basal chemical fertilizer application, increase rice grain yield and N uptake, as well as improve soil fertility, thus reducing related agricultural pollution and production costs.

Overall, long-term field experiments are necessary to ascertain our findings.

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