REUSE OF TREATED WASTEWATER FOR RICE CULTIVATION THROUGH CONTINUOUS SUB-IRRIGATION: ASSESSMENT ON GREENHOUSE GAS EMISSIONS AND HEAVY METAL CONTAMINATION IN THE PLANT-SOIL SYSTEM

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SUMMARY

Rice paddy fields are among the most important anthropogenic sources of CH_4 and N_2O, two of the most potent greenhouse gasses (GHGs) in the atmosphere, due to the traditional flooding irrigation and the increasing use of mineral fertilizers in paddy soils. In the shade of water shortage, the reuse of treated wastewater (TWW) for irrigation in rice paddy fields has become a reliable practice that could free up large amounts of fresh water currently used for agricultural irrigation, making this resource available for other purposes. However, it was argued that irrigation with wastewater would increase GHG emissions from rice paddy fields. In addition, irrigation with wastewater has raised concerns about heavy metal contamination in rice plant-soil systems, which could potentially lead to risks to human health. It is therefore necessary to develop a sustainable wastewater irrigation practice that can reduce GHG emissions from rice paddy fields and reduce the use of mineral fertilizers while improving rice productivity without any potential risks to human health.

An innovative rice cultivation system, namely continuous sub-irrigation with TWW (hereinafter, referred to as CSI), has recently been developed to promote the recycling of resources from municipal wastewater treatment plants (WWTPs) and the cost-effective production of forage rice in Japan. This continuous sub-irrigation system has been shown to have attractive advantages in terms of rice productivity and the nutritional quality of rice grains. However, in order to ensure the sustainable adoption of the novel cultivation system, its environmental footprint needs to be thoroughly investigated. This dissertation

therefore acts as a follow-up study based on the body of recent findings, to provide further insight into the practice of CSI with respect to the performance of rice plants, GHG emissions from paddy fields, and heavy metal contamination in the plant-soil system.

A microcosm experiment was conducted in 2018 to investigate emissions of CH_4 and N_2O and yield capacity of a local forage rice, Bekoaoba, between a conventional cultivation fertilized with high doses of mineral fertilizers (Control) and three CSI systems using different water regimes with zero fertilizer use. The examined water regimes included a constant supply rate of 25 L m^{-2} day^{-1} throughout the crop season (R1), a supply rate of either 25 (R2) or 36 L m^{-2} day^{-1} (R3) from 31 to 114 days after transplantation (DAT) combined with a lower rate of 8.3 L m^{-2} day^{-1} for the other growing periods. The results showed that the CSI systems produced higher yields (10.4 – 11.0 t ha^{-1}) with higher rice protein contents (11.3 – 12.8%) than Control (8.6 t ha^{-1} and 9.2%, respectively). All CSI systems markedly reduced CH_4 emissions but the higher supply rates in R1 and R3 significantly increased N_2O emissions compared with Control. The regime R2, which used the appropriate supply rates at the suitable timeframes to meet the N demand of rice plants, was identified as the optimal regime to effectively reduce both CH_4 and N_2O emissions by 84% and 28%, respectively. Furthermore, no adverse effects of TWW irrigation on the accumulation of possible toxic heavy metals, including As, Cr, Cu, Cd, Pb and Zn, was detected in rice grains.

The next phase of this study was to investigate changes in the community structure of CH_4- and N_2O-related soil microorganisms affected by CSI and to link these changes to the gas emissions in order to understand the underlying mechanisms for the GHG mitigation identified in the first experiment. A further experiment was conducted in 2019 to examine two treatments: the CSI system using the optimized water regime R2 and the Control system. The results showed that CSI reduced CH_4 and N_2O emissions by 80% and 66%, respectively, compared with Control. The microbial compositions of methanogenic archaea, methanotrophic, nitrifying and denitrifying bacteria were not significantly affected by the treatments. However, during the reproductive stage, CSI not only markedly inhibited the growth of methanogens in the lower soil layer, but also vastly reduced the abundance of methanotrophs in the upper soil layer, which corresponded significantly to the effective mitigation of CH_4. On the other hand, compared with Control, CSI stimulated a higher abundance of nitrifying and denitrifying bacteria, but this difference did not lead to a marked variation in N_2O emissions between the two treatments, suggesting that the N_2O emission gap between CSI and Control was probably not due to the changes in nitrifying and denitrifying communities, but more likely due to the availability of N in soils and N uptake of rice plants. In addition, soil analysis results showed that CSI significantly increased soil pH, SOM, and SOC ($p < 0.05$) while maintaining soil EC, CEC, N, K, and other macro- and micronutrients at comparable levels ($p > 0.05$) relative to Control. This indicated that CSI was able to effectively fertilize paddy soils despite the elimination of mineral fertilizers. However, a slight decrease in P content in paddy soils under CSI suggested, if necessary, a regular supplementation of P fertilizers. Importantly, the contents of the heavy metals examined in paddy soils were below the maximum permissible levels in agricultural soils recommended by WHO, demonstrating that there was no concern for heavy metal build-up in paddy soils under CSI.

Afterwards, this study expanded its framework to include potential contamination of CuO nanoparticles (NPs) in TWW that could harm rice plants and paddy soils, and subsequently pose risks to human health through the food chain. A follow-up experiment was conducted in 2020 to evaluate the effects of CuO NPs contained in TWW on the rice plant-soil system under CSI. Four hypothetical scenarios of CuO NP contamination in TWW, including 0, 0.02, 0.2, and 2 mg Cu L^{-1} (T1-Control, T2, T3, and T4, respectively) were examined. Another CSI system using TWW contaminated with a bulk source of Cu ($CuSO_4$) at 0.2 mg Cu L^{-1} (T5) was also tested for comparison with T3. The results showed that the contamination of Cu in TWW did not adversely affect rice growth and yield capacity, probably due to the low levels tested. However, a significant accumulation of Cu in paddy soils, roots, and rice grains under T4 compared with the rest of the treatments indicated the concern that Cu could be build up in the plant-soil system under higher levels of CuO NP contamination. Health risk assessment using a Hazard Quotient (HQ) revealed that adults who consume the rice grains harvested in all treatments have a negligible risk of non-cancer health problems caused by Cu.

Overall, this study focused on a number of key scientific issues, such as the efficient reuse of wastewater for irrigation, the reduced use of mineral fertilizers, the mitigation of GHG emissions, and the contamination of heavy metals in rice and paddy soils. The application of CSI in rice paddy fields has been shown to be an innovative solution for the cost-effective recovery of valuable resources from effluents, i.e. plant nutrients and irrigation water, which certainly promotes the transition of WWTPs from pure sewage treatment facilities to an important part of a circular economic model focusing on sustainable agricultural production and consumption, resilient management of water and sanitation, and climate change adaptation.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BAF	Bioaccumulation factor
BW	Body weight
CRD	Completely randomized design
CSI	Continuous sub-irrigation with treated wastewater
DAT	Day(s) after transplantation
DLS	Dynamic light scattering
DO	Dissolved oxygen
EC	Electrical conductivity
EDI	Estimated daily intake
EF	Enrichment factor
FAO	Food and Agriculture Organization
GHG	Greenhouse gas
GWP	Global warming potential
HI	Health index
HQ	Hazard quotient
IR	Ingestion rate
MSD	Mid-season drainage
NP	Nanoparticle
RfD	Oral reference dose
SDG	Sustainable development goal
SOM	Soil organic matter

SPAD	Soil plant analysis development
TC	Total carbon
TF	Translocation factor
TN	Total nitrogen
TOC	Total organic carbon
TWW	Treated wastewater
WHO	World Health Organization
WWTP	Wastewater treatment plant

CHAPTER 1. GENERAL INTRODUCTION

This chapter provides an overview of this research project entitled "Reuse of treated wastewater for rice cultivation through continuous sub-irrigation: Assessment on greenhouse gas emissions and heavy metal contamination in the plant-soil system". It includes the research context and rationales for the study as well as the objectives and scope of the research, followed by a brief description of the potential contribution of the study. In addition, the structure of this dissertation is also outlined here.

1.1 Research Context and Rationales

Resources are becoming increasingly scarce, while population and economic growth have led to a higher demand for resources, putting more stresses on the supply of resource and the environment (Kennedy et al., 2007). Resource stocks are shrinking and resource extraction is causing negative impacts on the environment (Kennedy et al., 2007; Alfonso Pina and Pardo Martinez, 2014); therefore, reuse and recycling of resources has attracted increasing attention around the world. Today, among the emerging problems with the storage of recourses, water scarcity is one of the most critical issues facing us and has become a powerful driver for the expansion of wastewater reuse, particularly in regions with water deficiency and under increasing pressures to utilize all accessible water sources, including municipal wastewater, to replace limited fresh water.

As agriculture is by far the largest user of fresh water, accounting for 70% of global freshwater withdrawals and up to 90% in some developing countries (UNESDOC, 2014), a number of major municipal wastewater reuse schemes for agricultural irrigation have been developed in recent decades. More and more nations have seen irrigation with recycled water/wastewater as an incentive to secure and enhance agricultural production. Municipal wastewater, which is generally constant throughout the year, can provide a stable supply of water, while the availability of fresh water is usually characterized by high seasonal fluctuations or extreme events. As these changes are increasingly possible due to climate change, there has been an interest increasing in the potential for water reuse, while in many countries, not just in arid areas, wastewater has rapidly become the primary low-cost and reliable alternative to conventional water. In the agriculture sector, rice paddy fields are the main users of water and have received extensive irrigation with wastewater throughout the growing season due to their traditional method of flooding irrigation.

In addition to water scarcity, as mentioned above, climate change, which is mainly caused by rising emissions of man-made greenhouse gasses (GHGs), including CH_4 and N_2O, is another major environmental issue facing the planet. Since climate change has a dramatic impact agricultural productivity, including rice production, efforts have been made to reduce the GHG emissions from human activities in general as well as agricultural production, thereby alleviating the adverse effects of climate changes on all aspects of life. In fact, rice paddy fields are among the most important anthropogenic sources of CH_4 and N_2O, and a number of farming practices have been widely studied to reduce GHG emissions from rice paddy fields (Sun et al., 2018; Zhang et al., 2010). However, the increasing production and application of mineral fertilizers in paddy soils has been seen as a key driver of the overall increase in carbon footprint in rice cultivation (Xu et al., 2013; Hasler et al., 2017). Thus, there is a critical need for sustainable rice cultivation systems that can effectively mitigate CH_4 and N_2O emissions and reduce the use of mineral fertilizers while maintaining or improving rice productivity.

In an effort to launch an effective model to promote the circulation of resources from municipal wastewater plants (WWTPs) with a focus on the reuse of treated wastewater (TWW) for agricultural irrigation, innovative rice cultivation systems, namely continuous irrigation with TWW, have been developed for the cultivation of forage rice (*Oryza sativa* L.) in Japan (Pham et al., 2017; Pham et al., 2019; Tran et al., 2019). In such paddy farming systems, TWW is continuously supplied to rice paddy fields during the crop season either through an underground drain pipe (sub-irrigation) or on the soil surface (surface irrigation) and becomes the sole source of irrigation and fertilization of rice plants without the use of mineral fertilizers (Tran et al., 2019). Compared to traditional rice cultivation, these wastewater irrigation practices have been shown to be highly beneficial, since they can reduce the dependence of paddy rice farming on mineral fertilizers, and at the same time reduce the loading of nutrients, particularly N, in WWTP effluents as well as in rice paddy fields into waterways and water-receiving bodies (Pham et al., 2019; Tran et al., 2019; Phung et al., 2020). However, wastewater irrigation in rice paddy fields has previously been claimed to increase the emissions of CH_4 and N_2O from paddy fields due to the high availability of elements in wastewater, in particular organic C and N (Zou et al., 2009). It is therefore essential to investigate the emissions of GHGs from rice paddy fields as affected by the newly-introduced continuous irrigation systems in order to suggest the most appropriate alternative for wastewater irrigation practice to preserve the above-mentioned advantages while avoiding the trade-offs caused by the potential increase in GHG emissions from rice paddy fields. One of the main aims of this research project was therefore the elaboration of CH_4 and N_2O emissions from rice paddy fields under continuous irrigation with TWW.

Since wastewater irrigation is commonly known to increase the metabolic activity of soil microorganisms (Toze, 2006), it has been hypothesized to alter the community structure of soil microorganisms and subsequently to change the emission patterns of CH_4 and N_2O from paddy fields (Zou et al., 2009). In addition, the metabolic activity of soil microorganisms plays an essential role in maintaining soil fertility and soil structure, while eliminating the use of mineral fertilizers under the novel continuous TWW irrigation systems could have a major impact on the availability of plant nutrients and other physicochemical properties of paddy soils. Therefore, the assessment of soil microbial communities relevant to GHG emissions, as well as physicochemical properties of paddy soils influenced by continuous irrigation systems, is of importance in this study.

Moreover, despite the fact that wastewater contains substantial nutrients that are valuable for plant growth and development, it may also contain a wide range of contaminants, including human pathogens, salts, heavy metals, surfactants, pharmaceuticals and a plethora of other potential chemical contaminants (Toze, 2006). Many of the more common environmental risks, such as salinity, nutrient pollution, heavy metal contamination and inorganic and organic contaminants, have been well documented (USEPA, 2004; WHO, 2006), while other emerging contaminants such as pharmaceuticals, cosmeceuticals and metal-based nanoparticles (NPs) have been the focus of research in recent years (Nassiri Koopaei and Abdollahi, 2017; Singh and Kumar, 2020). In fact, the increasing reuse of wastewater for agricultural irrigation highlights the need to draw public attention to adverse effects and evidence-based guidance on the criteria for the reuse of wastewater in order to minimize the potential human and environmental impacts of the above-mentioned reagents, particularly emerging contaminants. Another primary focus of this research project is the potential contamination of heavy metals and metal-based NPs in the rice plant-soil system.

1.2 Aims and Research Scope

The scope of this study has been extended to examine continuous sub-irrigation with TWW (hereinafter, referred to as CSI) in which rice plants are irrigated and simultaneously fertilized with TWW via underground drain pipes installed under the rhizosphere, without taking into account the continous surface irrigation method. There are two overarching aims of this work as follows:

(1) Investigation of CH_4 and N_2O emissions from rice paddy fields under CSI with two specific objectives:

   a. To optimize water regime of CSI for effective mitigation of CH_4 and N_2O emissions from rice paddy fields;
   b. To elaborate the community structure of soil microorganisms related to GHG emissions as affected by CSI.

(2) Evaluation of heavy metal contamination in the plant-soil system and changes in soil physicochemical properties under CSI with the following specific objectives:

   a. To measure the contents of heavy metals in rice grains and paddy soils as influenced by CSI;

b. To assess the effects of CSI on the physicochemical properties of paddy soils;
c. To evaluate the effects of CuO NPs, as potentially emerging contaminants occurring in TWW, on rice growth and their fate in the plant-soil system employing CSI.

1.3 Potential Contribution

This work advances the understanding on effects of the newly-implemented wastewater irrigation practice, CSI, on rice paddy fields with respect to: (1) performance of rice plants, (2) emissions of CH_4 and N_2O from rice paddy fields, (3) changes in the biological and physicochemical properties of paddy soils, and (4) contamination of heavy metals, including emerging contaminants, CuO NPs, in the plant-soil system. The results of this study are expected to contribute to the re-examination of the requirement for wastewater reuse in agricultural irrigation in general as well as CSI for rice paddy farming in particular with regard to potential human and environmental impacts. It also addresses several obstacles in the decision-making process to ensure that the reuse of wastewater is sustainably involved in regional water management.

1.4 The Structure of Dissertation

This dissertation consists of six chapters, including the introduction (Chapter 1), the literature review (Chapter 2), three chapters addressing the above-mentioned objectives (Chapter 3 – 5) and the overall conclusions drawn from the results of this research project (Chapter 6).

Firstly, the introduction overviews the contextual background on the reuse of wastewater as a source of irrigation in rice paddy fields and the specific rationale for this study. The literature review highlights water-related challenges and opportunities for the reuse of wastewater worldwide, while presenting current findings on the CSI system, together with an overview of relevant knowledge and the past work done in conjunction with the objectives of this research project, including GHGs emissions from rice paddy fields irrigated with wastewater, the occurrence of heavy metals in rice plants and paddy soils under wastewater irrigation, and behavior of metal-based NPs in wastewater, plants, and soils. Thereafter, Chapter 3 presents the study "*Emissions of CH_4 and N_2O from rice paddy fields continuously sub-irrigated with treated wastewater*", which included findings on mitigation of the two GHGs from rice paddy fields, yields and nutritional quality of rice grains, as well as the bioaccumulation of potentially toxic heavy metals in rice grains under the influence of CSI system. Subsequently, Chapter 4 puts forward the study "*Linking emissions of CH_4 and N_2O to community structures of relevant soil microorganisms and changes in physicochemical properties of paddy soils under continuous sub-irrigation with treated wastewater*". In this chapter the community structures of soil microorganisms associated with the emissions of CH_4 (methanogenic archaea and methanotrophic bacteria) and N_2O (nitrifying and denitrifying bacteria) have been investigated and linked to the gas fluxes to identify the possible underlying microbial mechanisms for the GHG mitigation figured out in Chapter 3. In addition, this chapter also presents the contents of possibly toxic heavy metals and other physicochemical properties of paddy soils as influenced by CSI. After that, Chapter 5 extends the framework of this dissertation with the study "*Effects of CuO nanoparticles on rice growth and their fate in the plant-soil system under* *continuous sub-irrigation with treated wastewater*", which examined several hypothetical scenarios of the occurrence of CuO NPs as emerging contaminants in TWW and elaborated their effects on the performance of rice plants along with the accumulation of Cu in plants (grains, shoots, and roots) and paddy soils continuously sub-irrigated with CuO NPs-contaminated TWW. Finally, the last chapter summarizes the primary findings and limitations of the present study and outlines recommendations for future work. Subsequently, these main chapters are accompanied by an appendix, which provides supplementary data for this research project, followed by references and additional information about the publications and presentations completed during my Ph.D. course.

CHAPTER 2. LITERATURE REVIEW

This chapter presents perspectives on the reuse of wastewater, highlighting water-related challenges and opportunities for agricultural irrigation with wastewater, followed by an overview of wastewater reuse in paddy rice farming, as well as current findings on the novel continuous sub-irrigation system. In addition, this chapter addresses issues related to GHG emissions from rice fields and the related soil microbial activities in paddy soils. It also outlines the possibility of heavy metal contamination associated with wastewater irrigation with a brief introduction of metal-based NPs as potential emerging contaminants in the rice plant-soil system.

2.1 Perspectives on the Reuse of Wastewater for Irrigation

2.1.1 Water-related challenges and opportunities for wastewater irrigation

Water supplies have been putted under increasing pressure and challenges as a result of the continuous population and economic growth worldwide (WWAP, 2015). Approximately 36% of the global population is agued to be living in water-scare regions, while more than half of it will face water stress conditions by 2050 (HLPW, 2018). Governments around the world have therefore launched a number of water policy options for sustainable water resource management to promote long-term water security and resilience to climatic and non-climatic uncertainties. Recently, the Sustainable Development Goals (SDGs) have added a new dimension to the challenges facing the water supply and sanitation sector (Goal 6), by focusing on sustainability (UNSD, 2020). In fact, as one of the most important factors involved in all aspects of life, including poverty alleviation and social equity, public health and macro-economic performance, etc., water plays a key role, either directly or indirectly, in the achievement of other SDGs (UNSD, 2020). However, there are a number of challenges confronting sustainable water management today, including degraded water quality, inadequate sanitation infrastructure, and unavailability or unequal distribution of water resources, particularly in settlements of low-income people. In addition, these problems present another critical confrontation in order to secure other resources such as food and energy for the continuously increasing population, and to protect both public health and the environment. In this context, wastewater has become a valuable alternative resource, from which water, energy, and plant nutrients can be extracted to help meet the population demands for water, energy, and food production (WWAP 2017).

The above challenges, on the other hand, offer the opportunity to expand and improve sanitary services around the world, in particular wastewater treatment, following a new approach aimed at minimizing the use of resource and promoting the recovery/recycling of resources from WWTPs. In order to integrate sanitation and wastewater treatment services closely with water supply management, it's necessary to change water management paradigms so that wastewater becomes a valuable resource for multiple purposes rather than a burden on society. In fact, wastewater reuse is not a recent innovation and has been part of water management, which has evolved over centuries in the history of mankind (Angelakis, 2015). The reuse of such alternative water resource may well contribute to the conservation of fresh water of high quality and reduce both the environmental pollution and the total cost of water supply. As a result, the reuse of wastewater has been practiced in different sectors, typically in industry and agriculture. For example, wastewater has been reused to maintain the environmental flow and recharge of groundwater, to supply it for urban use and agricultural irrigation, or even as drinking water. Among all sectors of the economy, agriculture is highly water-dependent and increasingly vulnerable to water risks. Agricultural irrigation consumes over 70% of freshwater withdrawals worldwide, accounting for the highest overall reuse of wastewater compared to other sectors (Asano, 1998). Since 1990s, interest in the reuse of wastewater for agricultural irrigation has steadily increased in many parts of the world due to the high water requirements for agricultural production and increasing competition with other water users (Asano and Levine, 1996; Jiménez and Asano, 2008). In addition, wastewater is well known as a valuable source of plant nutrients, which are undoubtedly capable of maintaining soil fertility, bringing enormous benefits to agricultural production (Singh et al., 2012; Carlos et al., 2017).

2.1.2 Continuous sub-irrigation with treated wastewater as an effective wastewater reuse practice for paddy rice cultivation

Rice (*Oryza sativa* L.) is one of the most important crops and has been produced globally over a total cultivated area of more than 167 million hectares with an annual output of 782 million tons (FAOSTAT, 2018). As a semi-aquatic plant, rice cultivation is generally characterized by flood irrigation and typically requires vast amounts of water during its entire crop season, accounting for 50% of the global irrigation water in agriculture sector and more than 70% of the total irrigation water in Asia (Tuong and Bouman, 2003). Due to the high demand for water consumption, irrigation with wastewater is an attractive alternative for paddy rice cultivation, which has been widely practiced in many countries over a long period of time and intensively investigated for its pros and cons (Yoon et al., 2001; Wang et al., 2006; Kang et al., 2007; Papadopoulos et al., 2009; Chung et al., 2011; Son et al.,2013; Jung et al., 2014; Nyomora, 2015; Xu et al., 2017).

There are a number of undeniable advantages with regard to the reuse of wastewater or TWW for rice cultivation. A number of studies have shown that rice yield from TWW-irrigated paddy fields could be 35 – 55% higher than that of the groundwater-irrigated fields (Toze, 2006), while the reuse of wastewater could reduce the total cost of rice production by 8.8 – 11.9% (Papadopoulos et al., 2009) and reduce the use of chemical N fertilizers by approximately 45% without yield loss (Xu et al., 2017). The increased grain yield and reduced usage of N fertilizers were likely attributed to significant supply of plant nutrients derived from the irrigation wastewater (Kang et al., 2007; Jung et al., 2014; Xu et al., 2017), which could simultaneously improve soil fertility and increase the metabolic activity of soil microorganisms (Toze, 2006).

In Japan, rice is the staple food, so rice production has played a key role in the Japanese society, as in other rice exporters in Asia, such as China, Thailand, and Viet Nam. Rice paddy fields in Japan are often referred to as the country's cultural and environmental indicators, while water for paddy irrigation has conventionally been drawn from streams, natural lakes, or man-made reservoirs. Nevertheless, the rice production in Japan has been in an urgent need to reduce costs by improving the efficiency and market competitiveness, while facing a number of challenges (Nanseki et al., 2016), for instance, the decline in total rice farming production over the last 3 decades. In particular, the total area of rice harvested in 2018 (147×10^4 ha) was continuously reduced by 9, 17, and 29% compared with that in 2010, 2000, and 1990, respectively (FAOSTAT, 2018).

In the shade of water shortage and other emerging environmental problems, water management in rice paddy farming in Japan also requires an integrated transition to consider new ideas for the recognition of water as a circulating resource that fosters the re-allocation and reuse of alternative water sources, such as effluents from WWTPs. Recently, attempts have been made to build a model for the effective circulation of resources from WWTPs (Figure 2.1). In this model, WWTPs are not only facilities dedicated solely to the treatment of wastewater, but also to the recovery of valuable resources such as irrigation water, plant nutrients, and organic fertilizers that can be recycled into agricultural production. This model can contribute to the protection of the environmental by further purifying WWTP effluents through rice paddy fields as natural wetlands and at the same time by reducing the use of mineral fertilizers, thereby reducing the loads of plant nutrients, particularly N from WWTPs and rice paddy fields into waterways and water-receiving bodies (Pham et al., 2019; Tran et al., 2019).

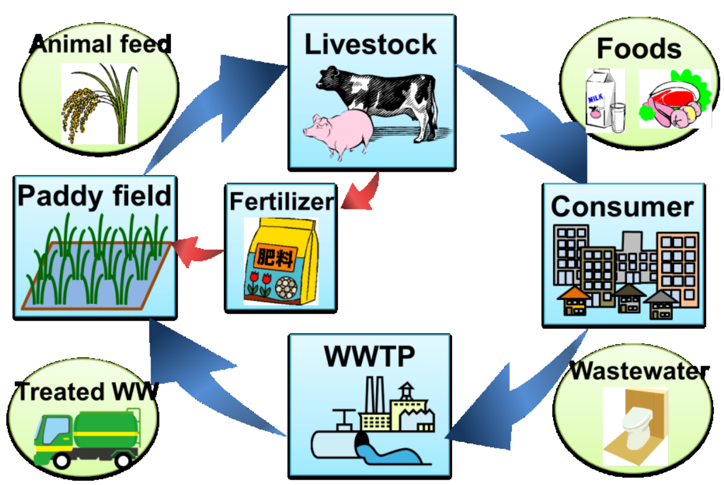

Figure 2.1 Concept of resource circulation involving urban (consumers) and rural areas (rice and livestock famers) with a focus on cultivation of forage rice through irrigation with TWW (Source: Pham and Watanabe, 2017).

In order to initiate this model and to promote cost-effective production of forage rice in Japan, an innovative rice cultivation practice using CSI has been newly developed and demonstrated with a number of attractive results (Pham et al., 2017; Pham et al., 2019; Tran et al., 2019). The CSI system was originally examined in a bench-scale experiment in Tsuruoka City, Japan in 2015 (Pham et al., 2017), in which a number of irrigation practices, including a continuous surface irrigation (Figure 2.2) and a continuous sub-irrigation system (CSI, Figure 2.3) were examined. It was firstly shown that, compared with other examined farming systems, including the continuous surface irrigation system and a conventional rice cultivation fertilized with high doses of mineral fertilizers, rice paddy fields using CSI at a flowrate of 25 L m^{-2} day^{-1} resulted in the highest rice yield (9 t ha^{-1}), shoot dry weight (12.4 t ha^{-1}), and superior rice protein content (13.1%) (Pham et al., 2017). Interestingly, such the high yield of protein-rich rice was achieved under TWW irrigation only with a supplementation of a P fertilizer while eliminating the application of all other mineral fertilizers. Furthermore, the CSI system could substantially purify effluents from WWTPs by removing 80 – 90% of N contained in the irrigation TWW compared with the continuous surface irrigation system (58%) at the same flowrate (Pham et al., 2017).

Those were the first promising results that allowed further studies to focus on optimizing continuous irrigation with TWW for cultivation of forage rice in Japan. Particularly in a follow-up experiment, Pham et al. (2019) completely eliminated the use of mineral fertilizers, including the P fertilizer, and found that the highest rice yield (14.1 t ha^{-1}), shoot dry weight (12.9 t ha^{-1}), and rice protein content (14.6%) were also achieved from the paddy fields employing the CSI system. In line with the previous work, the continuous sub-irrigation system was expected to contribute to environmental protection by removing 85

– 90% of N from TWW, more effectively than the continuous surface irrigation system, which showed a removal efficiency of approximately 63% (Pham et al., 2019).

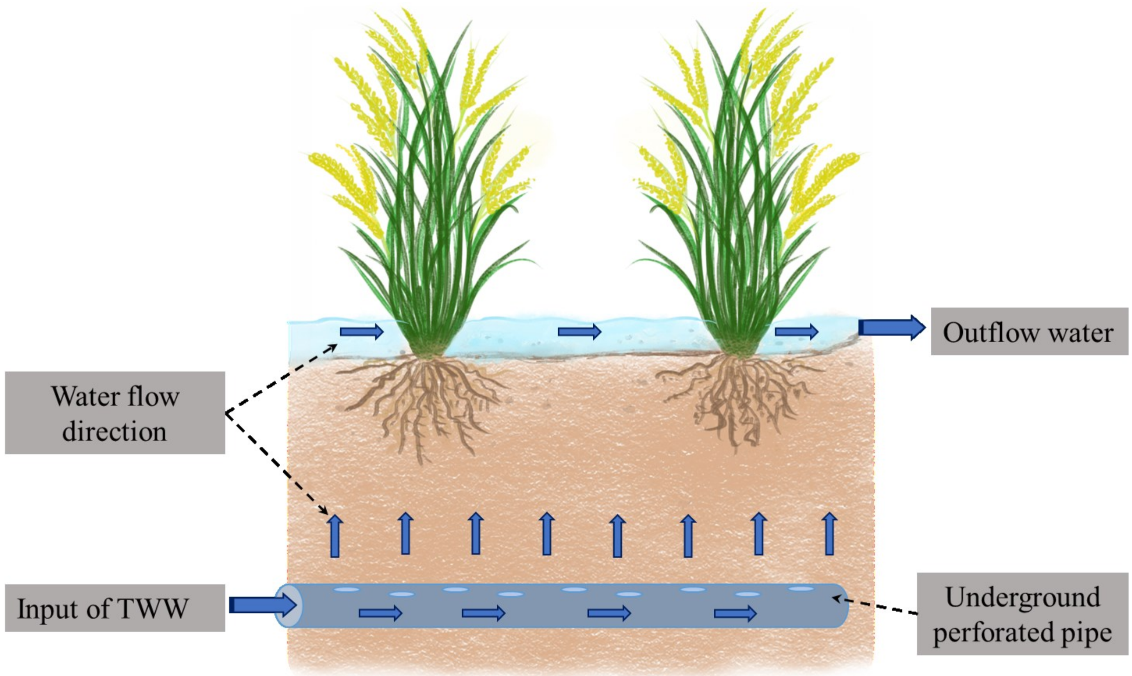

Figure 2.2 Schematic illustration of continuous sub-irrigation with TWW (CSI) in rice paddy fields in which TWW is continuously supplied via underground drain pipes permanently installed below the rhizosphere. The TWW infiltrates the soil layers upwards to the soil surface and finally flows out of the paddy fields through the outlets set at 5 cm above the soil surface to maintain the water standing level.

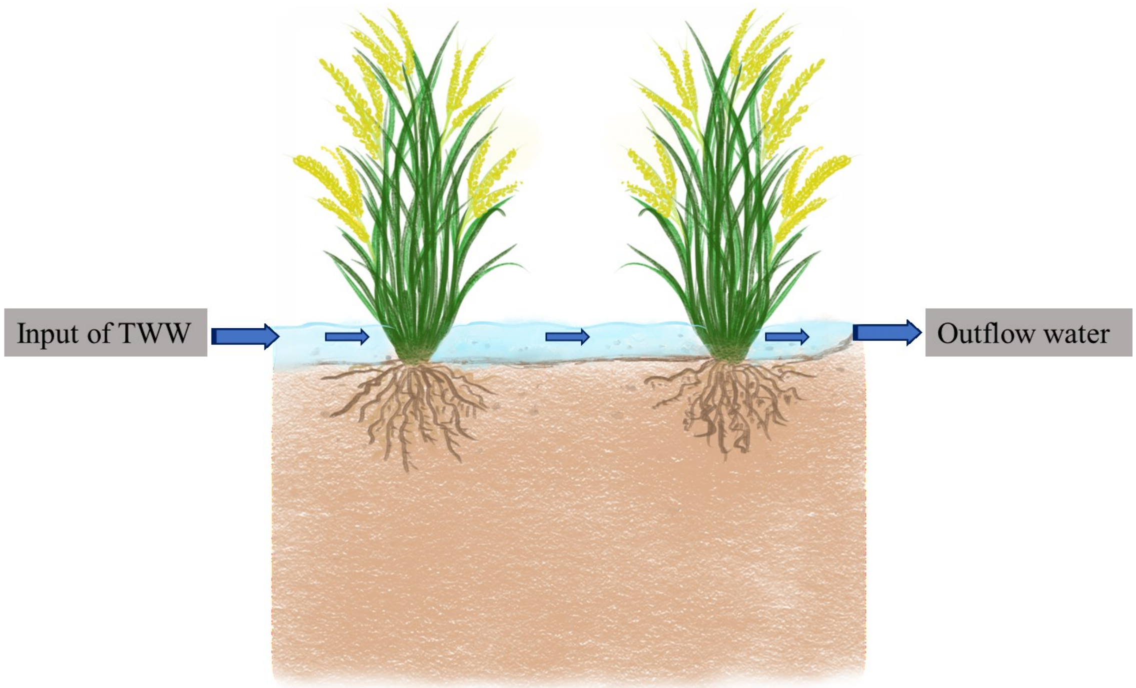

Figure 2.3 Schematic illustration of continuous surface irrigation with TWW in rice paddy fields, in which TWW is continuously supplied into the rice paddy fields via inlets set on the soil surface, spreads over the paddy fields, and finally flows out of the fields through outlets set at 5 cm above the soil surface at the opposite side of the inlets.

Subsequently, based on the above-mentioned results from the bench-scale experiments, Tran et al. (2019) conducted two consecutive pilot-scale experiments to verify the feasible adoption of both continuous irrigation systems in a constructed paddy fields within the local WWTP. The test field consisted of 2 plots, each with a surface area of 75 m^2. Although only P-fertilizer was used in 2016 and no mineral fertilizer was supplemented in the 2017 season, the yields obtained were comparable to those recorded in normal paddy fields (7.3 t ha^{-1}). In particular, the rice yields from the plots using CSI systems (9.1 and 7.5 t ha^{-1}) were significantly higher than those from the plots under continuous surface irrigation system (7.5 and 7.1 t ha^{-1}) in 2016 and 2017, respectively. Interestingly, there was no significant difference between the two fields in rice protein content, and these contents (11.6 – 13%) were significantly higher than those (8.8%) observed in normal paddy fields. This indicated that rice grains under these TWW irrigation practices was more favorable to animal husbandry since it could reduce the use of costly protein feeds such as imported soymeal. These studies have shown the high feasibility of adopting CSI to achieve high production and good nutritional quality of forage rice, which could promote the effective reuse of TWW in rice cultivation and enhance the self-sufficient feed ratio for sustainable dairy farming in Japan (Tran et al., 2019). Furthermore, these alternative farming systems are expected to improve farmland efficiency, which inevitably ensures domestic livestock production and stops the increase in abandoned farmland that has been observed in Japan since the 1970s (Cheng et al., 2017).

2.2 Greenhouse Gas Emissions from Rice Paddy Fields

2.2.1 Emissions of CH_4 and N_2O from rice paddy fields

One of the major environmental problems facing the world today is climate change, which is primarily caused by increasing emissions of man-made GHGs. CH_4 and N_2O are the two most important GHGs from agriculture, with global warming potentials (GWP) of 28 and 265 CO_2-equivalents (CO_2-eq.), respectively, over a 100-year time horizon (IPCC, 2013). Atmospheric concentrations of CH_4 and N_2O have increased rapidly from the pre-industrial levels of 722 and 270 ppb to the present levels of 1830 and 324 ppb, respectively (Myhre et al., 2013). Reducing GHG emissions to the atmosphere is therefore urgently needed to mitigate the adverse effects of climate change.

Rice paddy fields are significant anthropogenic sources of these two GHGs, accounting for approximately 19% of the global CH_4 emissions and 11% of the total agricultural emissions of N_2O (IPCC, 2007). The increasing production of rice to meet the growing demand of the global population is likely to lead to increased emissions of CH_4 and likely requires higher inputs of mineral fertilizers, especially N fertilizers, which inevitably intensifies emissions of N_2O to the atmosphere as well (Gagnon et al., 2011). The emission of these GHGs from paddy fields depends mainly on a number of microbial-mediated processes in soils, i.e. production and oxidation of CH_4, nitrification, and denitrification, and on numerous gas transport pathways, i.e. plant-mediated transport, molecular diffusion, and ebullition. The following sections will discuss in details the mechanisms of CH_4 and N_2O emissions from paddy fields associated with their respective microbial communities in paddy soils.

2.2.2 Emission mechanisms of CH_4 and relevant soil microbial communities

Methanogenic archaea (methanogens) are the main vectors behind CH_4 emissions from rice paddy fields. These microorganisms thrive well under anaerobic conditions and are responsible for the harvesting and transformation of organic carbons into CH_4, the process called methanogenesis (Le Mer and Roger, 2001; Conrad, 2007; Bloom, 2012). Approximately 70 – 80% of the atmospheric CH_4 is derived from biological origin. In rice paddy soils, straws from rice plants are generally the main inputs of organic materials for methanogenesis, especially during the early weeks of the crop season. These materials normally build up and decompose during the rainy season or inundation periods, and become the main source of methanogenic substrates that are readily transformed into CH_4 by methanogens and released into the atmosphere (Naser et al., 2007). Other sources of organic carbon, such as newly-produced plant residues or root exudates, will become more important during the later periods of the crop season. Once CH_4 is produced in anaerobic soil layers, it is then transported vertically to the atmosphere through three main pathways (Wassmann et al., 1993), including: (1) the diffusion of dissolved CH_4 into flooded waters, (2) the formation of bubbles triggered by soil fauna and crop management procedures, and (3) the plant transport via aerenchyma systems (Figure 2.4).

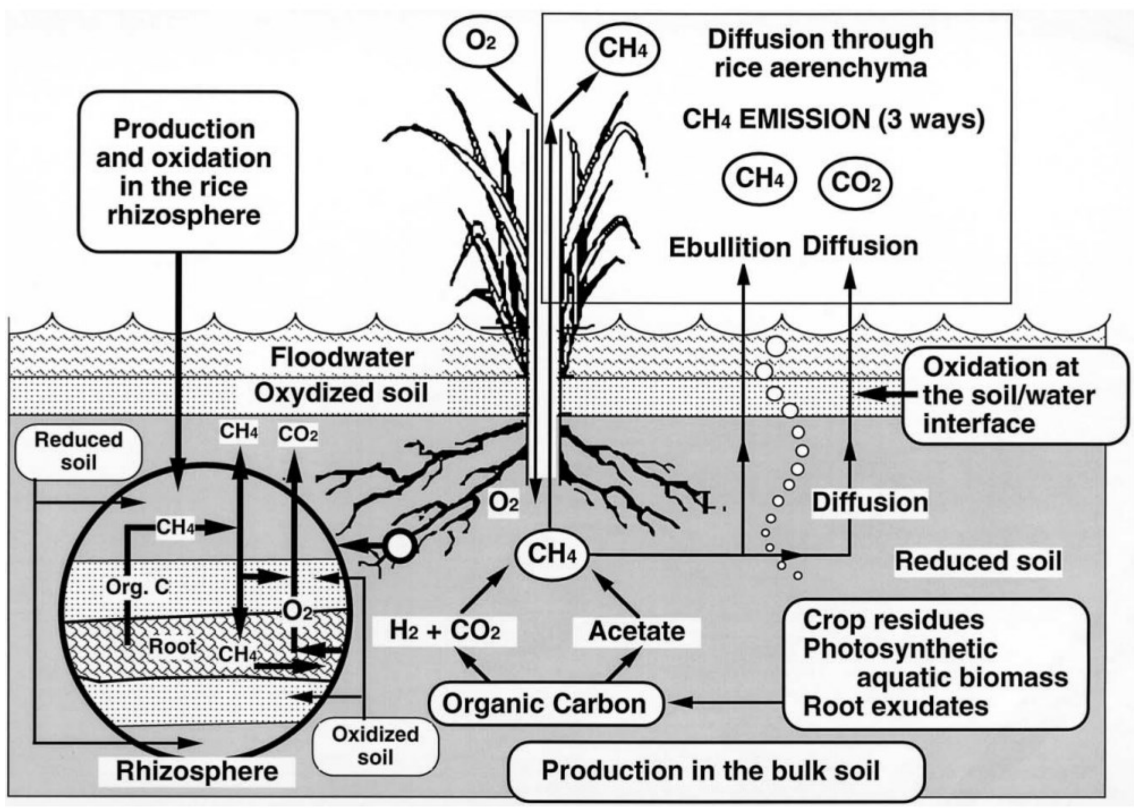

Figure 2.4 Schematic illustration of the production, consumption and transfer of CH_4 to the atmosphere from rice paddy fields (Source: Le Mer and Roger, 2001).

However, not all the CH_4 produced in the rhizosphere is released into the atmosphere. Particularly, under aerobic conditions in a thin oxidized soil layer on the soil-water interface (Figure 2.4), a portion of CH_4 is consumed by methanotrophic bacteria (methanotrophs) prior to the release to the atmosphere (Conrad, 2007). The emission of CH_4 is therefore strongly dependent on the balance between the production (methanogenesis) and the consumption (methanotrophy) of CH_4 in paddy soils. Accordingly, a CH_4 source is generally an environment in which the balance is positive, leading to the emission of CH_4. Since rice paddy soils are characterized by their submerged state, where anaerobic condition predominates, the balance between the production and consumption of CH_4 is usually positive, making rice paddy soils a significant source of this potent GHG (Le Mer and Roger, 2001).

All the methanogenic archaea that have been identified so far belong to the phylum *Euryarchaeota* within the domain Archaea (Conrad and Claus, 2005; Whitman et al., 2006; Lyu and Liu, 2018), and have been included in many orders and families (Figure 2.5). These archaea gain their energy by producing CH_4 from simple substrates such as H_2, CO, formate, and some alcohols like isopropanol or ethanol, which are oxidized and then CO_2 is reduced to CH_4. In addition, CH_4 can also be formed by reducing the methyl groups in acetate, methanol, trimethylamine, and dimethylsulfide, part of which is oxidized to CO_2 to generate the electrons required to reduce the methyl group to CH_4. These reactions are thermodynamically exergonic under normal condition and can therefore operate in nature once the concentration of the substrates is sufficiently large.

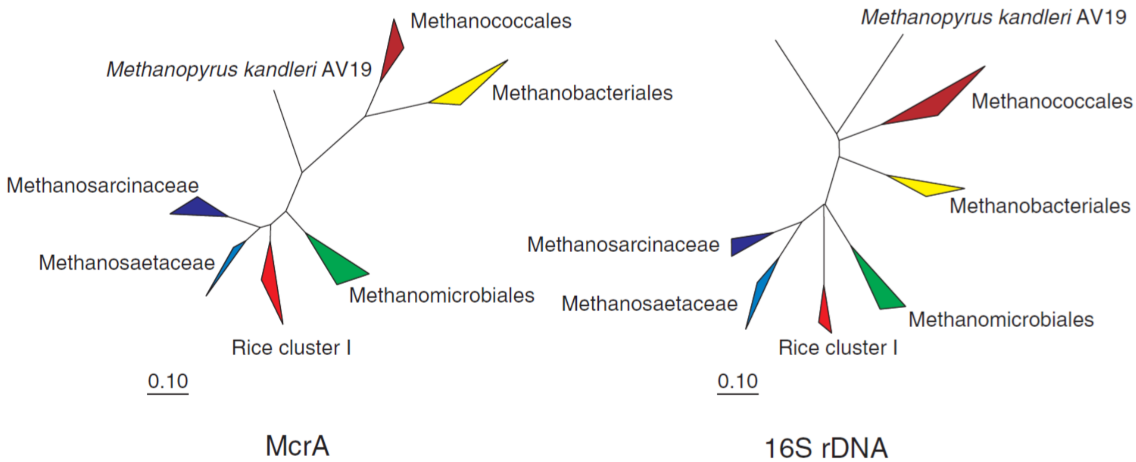

Figure 2.5 Phylogeny of methanogenic archaea classified based on either the methyl-coenzyme M reductase (*mcr*A) or the 16S rRNA gene (16S rDNA) (Adapted from Conrad et al., 2006).

Methanotrophic bacteria, on the other hand, belong to *Proteobacteria* and are conventionally divided into two groups: Type I and Type II (Bowman, 2006). Type I methanotrophs belong to the class Gammaproteobacteria, family *Methylococcaceae* and comprise the following genera: *Methylococcus*, *Methylocaldum*, *Methylomicrobium*, *Methylosphaera*, *Methylomonas*, *Methylobacter*, *Methylosarcina*, *Methylothermus*, and *Methylohalobius*. Type II methanotrophs belong to the class Alphaproteobacteria, family *Methylocystaceae* with 4 genera: *Methylocystis*, *Methylosinus*, *Methylocella*, and *Methylocapsa*. The distinction between these two methanotrophic groups has been well documented so far. For example, Type I and Type II methanotrophs differ not only in phylogenetic affiliation but also in a number of biochemical characteristics, such as the C assimilation pathway (Type I with ribulose monophosphate pathway and Type II with serine pathway) or the dominant phospholipid fatty acids (unsaturated PLFAs in Type I with 16 and 14 C atoms and that in Type II with 18 C atoms). However, commonly in both methanotrophic groups, both of them activate CH_4 with a methane monooxygenase (MMO), which is an enzyme capable of oxidizing the C-H bond and results in the formation of methanol: $CH_4 + O_2 + 2\ [H] \rightarrow CH_3OH + H_2O$ (Dalton, 2005; Murrell et al., 2000). Within this dissertation, CH_4 emissions from rice paddy fields using CSI systems will be revealed in Chapter 3, while their linkage to the community structure of the methanogenic archaea and methanotrophic bacteria in paddy soils under the influence of CSI will be further discussed in Chapter 4.

2.2.3 Emission mechanisms of N_2O and related soil microbial communities

Emissions of N_2O are generally dependent on and involved in the cycling and transforming of N in soils, including nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA), anaerobic NH_4 oxidation (Anammox), etc. (Figure 2.6). Typically, in an oxidized soil layer, NH_4^+ released from fertilizers and/or mineralized from organic matter is oxidized to NO_2^- by primary nitrifiers (usually *Nitrosomonas sp.*), then NO_2^- is oxidized to NO_3^- by secondary nitrifiers (*Nitrobacter sp.*) (Butterbach-Bahl et al., 2013). Subsequently, NO_3^- and NO_2^- can be diffused to a reduced soil layer where these compounds are reduced stepwise to gaseous end products (NO, N_2O, and N_2) by denitrification that is mediated by anaerobic bacteria in an oxygen-limiting environment (Butterbach-Bahl et al., 2013). In addition to the interface between the oxidized and reduced soil layers, the nitrification-denitrification process can also occur in the rice rhizosphere. DNRA may not occur significantly in rice paddy soils, but Anammox may potentially occur if specific conditions permit (Ishii et al., 2011). Furthermore, N_2O produced by nitrification and incomplete denitrification may be reduced to N_2 by N_2O-reducing microorganisms (Ishii et al., 2011). While the importance of nitrification and denitrification in rice paddy soils has been well studied, the microbes responsible for these processes have not been well understood until recently. In addition, the contributions of the newly-discovered processes, such as archaeal ammonia oxidation, fungal denitrification, anaerobic methane oxidation coupled with denitrification, and Anammox (Nakaya et al., 2009), to the cycling of N in paddy soils have not been well studied. As briefly mentioned above, there are a variety of biotic and abiotic processes that produce N_2O, however it is widely considered that microbial nitrification and denitrification are the key drivers of N_2O emissions, since these microbial metabolic pathways have a wealth of processes that produce or consume N_2O.

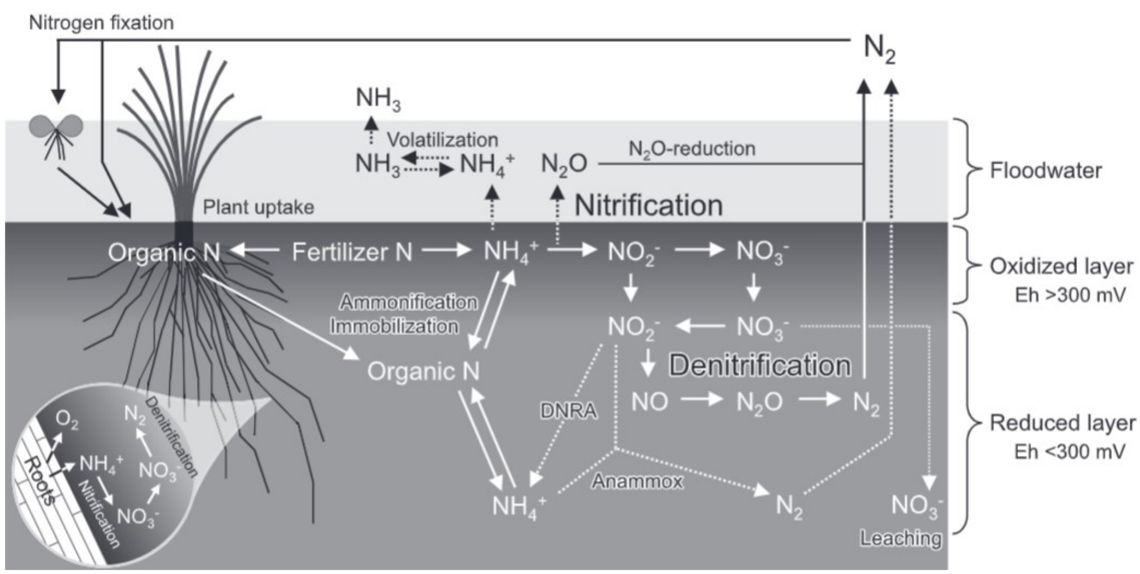

Figure 2.6 Schematic illustration of N-cycling processes in rice paddy soils (Adopted from Ishii et al., 2011).

In general, denitrification is widely considered a key process contributing to N_2O emission (Knowles, 1982). Denitrification is highly dependent on the presence of available C compounds, because organic C is used as an energy supply for heterotrophic denitrifiers

(Stevens et al., 1998; Morley and Baggs, 2010). The availability of C and N will usually increase the rate of denitrification; however, higher denitrification rates do not always result in higher N_2O emissions from soils as denitrifying bacteria primarily produce two gases, N_2O and N_2. It is important to consider that not only the abundance of C and N, but also the ratio between the available C and NO_3, i.e. the ratio between electron donors and receivers, is a key indicator that regulates the release of N_2O in soils via denitrification (Richardson et al., 2009). In this dissertation, given the existing gaps in the literature, N_2O emissions from rice paddy fields under the effects of CSI will be elaborated in Chapter 3, while their relationship with the community structure of the nitrifying and denitrifying bacteria in paddy soil will be further discussed in Chapter 4.

2.3 Risks of Heavy Metal Contamination under Wastewater Irrigation

Wastewater reuse for crop irrigation has certain advantages as mentioned in the previous sections, such as supplying essential nutrients and organic matter, saving water and nutrients, and reducing water pollution, etc. However, irrigation with wastewater might cause a number of environmental and human health risks, which are attributed to undesirable pathogens and chemical pollutants contained in wastewater. Of all the contaminants, heavy metals are toxic pollutants that strongly limit the beneficial use of wastewater (Petrus and Warchol, 2005; Chen et al., 2013; Chen et al., 2015; Albalawned et al., 2016). The build-up of toxic elements in soils and crops by irrigation with wastewater results in soil pollution, and in turn, affects food safety and human health (Khan et al., 2008; Singh et al., 2010; Khalid et al., 2017). Under the framework of this study, this section will concentrate on the risks of contamination with heavy metals and metal-based NPs in plants and soils.

2.3.1 Heavy metal contamination in rice plants and paddy soils

Contamination of agricultural soils and crops by heavy metals have been considered one of serious environmental problems due to their non-biodegradable nature and long biological life, as well as their possible impacts on human health (Radwan and Salama, 2006; Khan et al., 2010). Depending on the source of origin, wastewater can contain various heavy metals in different concentrations. In fact, it has been documented that even after passing through WWTPs, TWW may still contain significant amounts of heavy metals, and such elements can easily be absorbed in all forms of life through the food chain (Niehoff et al. 2002; Muchuweti et al. 2006). For example, Chen et al. (2009) analyzed the total contents of heavy metals in wastewater-irrigated soils in the Eastern suburb of Beijing, China and found remarkable high total concentrations of Cd, Cr, Ni, Zn and Cu (0.81, 78.3, 31.8, 71.5 and 25.5 mg kg^{-1}, respectively) in the upper layers of the soils relative to a reference soil (0.36, 53.8, 18.7, 51.9 and 16.0 mg kg^{-1}, respectively). Another research performed in a wastewater-irrigated field in the suburb of Shenyang City, China, also reported that total concentrations of Cd, Ni, Cu and Pb (2.66, 241.59, 69.24 and 88.05 mg kg^{-1}, respectively) were substantially higher in top soils irrigated with wastewater than those in a reference soil (Zhang et al., 2008). Similarly, an investigation undertaken in Hanoi, Viet Nam, showed that a paddy soil irrigated with wastewater, which was polluted with wastes from various industrial plants, had average total concentrations of Cu, Zn, Pb, and Cd (202, 192, 159, and 4 mg kg^{-1}, respectively) higher than the permissible levels of the Vietnamese standards (Nguyen et al., 2008).

It is well known that soils irrigated with wastewater accumulate heavy metals such as Cd, Zn, Cr, Ni, Pb, and Mn in surface soil layers, and, as a result of repeated use of wastewater, the capacity of soils to retain heavy metals is reduced and the soils can release heavy metals into ground water or soil solution, from which the metals are available for plant uptake (Sharmaet al., 2007). Accordingly, one possible pathway of dietary uptake of the trace metals could be through consumption of crops or crop products that were irrigated with contaminated wastewater. In general, heavy metal contamination of foodstuffs is the first indicator of food safety and quality (Radwan and Salama, 2006; Khan et al., 2010), because heavy metal-contaminated foods have toxic effects on human health and may seriously deplete some vital nutrients in the body. This may further responsible for decreasing immunological defenses, intrauterine growth retardation, impaired psychosocial faculties, malnutrition-related disabilities, and high prevalence of upper gastrointestinal cancer rates (Arora et al., 2008). For example, Cd and Pb have been found to have carcinogenic effects (Jaishankar et al., 2014), while other elements, such as Cu and Zn, are important nutrients for human health, but can be toxic at high concentrations (Rahman et al., 2014). Particularly, Zn may decrease immune function and high-density lipoprotein levels (Harmanescu et al., 2011), while Cu excess may cause acute stomach and intestinal aches and liver damage (Rahman et al., 2014). When plants are exposed to excess heavy metals in soils, they exhibit symptoms of phytotoxicity such as inhibition of seed germination, reduced plant height, decreased tillering, stunted root and shoot growth, leaf chlorosis and necrosis, inhibited chlorophyll metabolism, and lower fruit and grain yields. As most of the above-mentioned adverse effects of heavy metals on physiological and agronomical parameters are related to the fundamental photochemical reactions in plants (Rahman et al., 2007), such exposure can even lead to plant death.

Metal contamination in paddy soils and subsequent accumulation in various parts of the rice plant including rice grains have been reported from different countries. Accumulation of heavy metals, such as Cd, Zn, Cu, Pb, Cr, Ni, Fe, Zn, Co, Hg and As, in different parts of rice plants (roots, straw, hull, and grains) was also widely reported at varying degrees. Although rice grains accumulate the least amount of toxic metals compared to other parts like hull, straw, and roots, people who take rice as the staple food for daily energy requirement are potentially exposed to the potentially toxic metals via rice consumption (Solidum et al., 2012). Since rice consumption is a main route of human exposure to heavy metals in rice paddy fields, appropriate preventive and remedial measures should be enforced in the locals with potential risk of metal contamination, especially in wastewater-irrigated areas. Findings on the accumulation of heavy metals in the rice plant-soil systems utilizing CSI will be presented and discussed further in Chapter 3 and Chapter 4.

2.3.2 Metal-based nanoparticles as emerging pollutants

Nanoparticles in general and metal-based NPs in particular have gained increasing attention for their peculiar quantum-size properties and high unique surface areas. Nanotechnology offers great social benefits, with potentially extensive use of NPs in numerous fields of industry, such as semiconductor, pharmacy, etc. Due to their special properties, metal-based NPs are integrated into a wide range of household products (Gao et al.,2012), and the most widely found metal-based NPs in day-to-day consumer goods are Ag, TiO_2, ZnO, CuO, and Au (Woodrow, 2011).

In water environments, a number of modeling studies have predicted that NPs are generally present at very low concentrations from mg L^{-1} to μg L^{-1} (Boxall et al., 2007; Muller and

Nowack, 2008; Gottschalk et al., 2009; Neal et al., 2011), however, there is still a shortage of evidence on realistic concentration ranges of NPs in natural aqueous systems. This is mainly attributed to the complex behaviors of NPs in suspensions. For instance, aggregation and sedimentation of NPs may occur naturally in the presence of suspended or dissolved substances (i.e. nature organic matter) in water, which would favor the removal of NPs from suspensions (Grassa et al., 2002). However, the combined effect of pH, ionic strength, electrolyte species and concentrations, and other water characteristics will either result in NP aggregation by charge neutralization, bridging, electrical double layer compression and other processes (Zhang et al., 2002), or could cause NPs to be more stable in suspensions (Hyung et al., 2006; Keller, 2010). Consequently, it is always possible that uptake of NPs by plants and animals, and subsequently biomagnification of NPs in the food chain readily happen.

Attempts have been made around the world to track the occurrence of NPs in water as well as in wastewater streams, and it has been reported that approximately 10% of NPs pass through WWTPs to enter surface waters (Limbach et al., 2008). In recent years, the presence of NPs in wastewater has been repeatedly documented (Pachapur et al., 2016; Baranidharan and Kumar, 2018). Accordingly, NPs can affect plant and human health during the reuse of wastewater for irrigation purpose (Mara et al., 2007; Qadir et al., 2010; Singh and Kumar, 2014). Therefore, information on the uptake of these NPs in edible plants is of significant importance. Within the scope of this research project, findings on the interaction of a metal-based NPs, CuO NPs, with rice plants and paddy soils employing CSI is presented and discussed in Chapter 5.

CHAPTER 3. EMISSIONS OF CH_4 AND N_2O FROM RICE PADDY FIELDS CONTINUOUSLY SUB-IRRIGATED WITH TREATED WASTEWATER

This chapter presents the findings on emissions of CH_4 and N_2O from forage paddy fields using CSI for both irrigation and fertilization. An optimized water regime under continuous sub-irrigation system is recommended in order to achieve high yields of protein-rich rice with successful mitigation of the two GHGs. The results of this chapter were published in an article by Springer Nature in the journal Scientific Reports, while some of the results were presented in Kofu City, Japan, at the 53rd Annual Conference of the Japan Society of Water Engineering (JSWE) on 7 - 9th March 2018, and in Berlin, Germany, at the 12th IWA International Conference on Water Reclamation and Reuse on 16 - 20th June 2019.

3.1 Introduction

The production of forage rice (*Oryza sativa* L.) has recently been promoted by the Japanese government to reduce the cost of domestic animal husbandry by reducing the use of imported feedstuffs, which may be of unstable supply and highly priced depending on the global markets. Efforts have been made to support the production of forage rice and at the same time reduce the scarcity of fresh water in agriculture through the reuse of TWW (Tran et al., 2017). Most recently, CSI was developed as a novel practice for effective reuse of TWW from local WWTPs for the cultivation of high nutritional quality forage rice (Pham et al., 2017; Pham et al., 2019; Tran et al., 2019), which received a great deal of attention with a number of positive findings as discussed in Chapter 2.

However, forage rice varieties have been reported to emit more CH_4 than common staple rice (Cheng et al., 2017), which was likely due to increased production of biomass when grown under similar growth conditions. As a result, the current promotion of forage rice production in Japan is likely to indicate a possible increase in CH_4 emissions from the country's agricultural sector. In addition, wastewater irrigation was claimed to increase both CH_4 and N_2O emissions from rice paddy fields due to the high availability of elements in irrigation wastewater, such as organic C and N (Zou et al., 2009). In particular, Zou et al. (2009) reported that paddy fields irrigated with sewage water emitted CH_4 and N_2O at 27 – 33% and 68 – 170% respectively, compared with conventional river water-irrigated fields. The above circumstances suggest that irrigation of forage rice fields with TWW could result in a synergistic effect to increase the emissions of GHGs to the atmosphere. However, there were also contrasting findings that TWW irrigation under straw return practices could reduce CH_4 and N_2O emissions from paddy fields by 24.5 – 26.6% and 37 – 39%, respectively, compared to tap water irrigation (Xu et al., 2017). Due to differences in reported GHG emissions from paddy fields under different water and fertilization practices, GHG emissions from forage paddy fields using CSI requires a thorough investigation.

It is well known that GHG emissions from paddy fields are also influenced by agronomic practices, such as water regime, N fertilization, and C input (Zou et al., 2009; Riya et al., 2015a, b; Xu et al., 2017). Given that the continuous sub-irrigation system uses TWW as the sole source for both water and fertilization management (Pham et al., 2019; Tran et al., 2019), such an irrigation regime would likely lead to significant shifts in both water and nutrient inputs, which would subsequently influence the GHG emissions and the performance of rice plants. The objective of this chapter was therefore to estimate emissions of CH_4 and N_2O and to optimize the CSI water regime in order to minimize the GHG emissions from forage rice fields. In addition, the yield of grains, the nutritional quality of rice, and the accumulation of heavy metals in rice grains as affected by CSI have also been elaborated.

3.2 Methodology

3.2.1 Experimental design

A microcosm experiment was conducted in 2018 at Yamagata University, Tsuruoka City, Japan, using four growing containers (36 cm in height, 30 cm in width, 60 cm in length) to simulate paddy fields of 0.18 m^2 as shown in Figure 3.1a. A perforated drainage pipe (21 cm in diameter) was installed at the bottom of each container and covered with a 5 cm layer of gravel. The containers were filled with 30 kg of loamy soil (air dry, 20% moisture) with a pH of 5.5, an EC of 9.3 mS m^{-1}, and a total C, N, P, and K content of 20 g kg^{-1}, 1710, 920 and 1839 mg kg^{-1} respectively. Four hills of the local forage rice, cv. Bekoaoba, were transplanted in each container at a plant density of 15 × 30 cm (Figure 3.1a).

Four treatments were examined, including one under conventional farming practices (Control) and three CSI systems, each with a different water regime (R1, R2, and R3; Figure 3.2). The TWW used for CSI treatments was collected from the local WWTP and monitored weekly for its physicochemical properties using the standard methods described in a previous study (Pham et al., 2017). The basic characteristics of TWW have been shown in Table A.1.

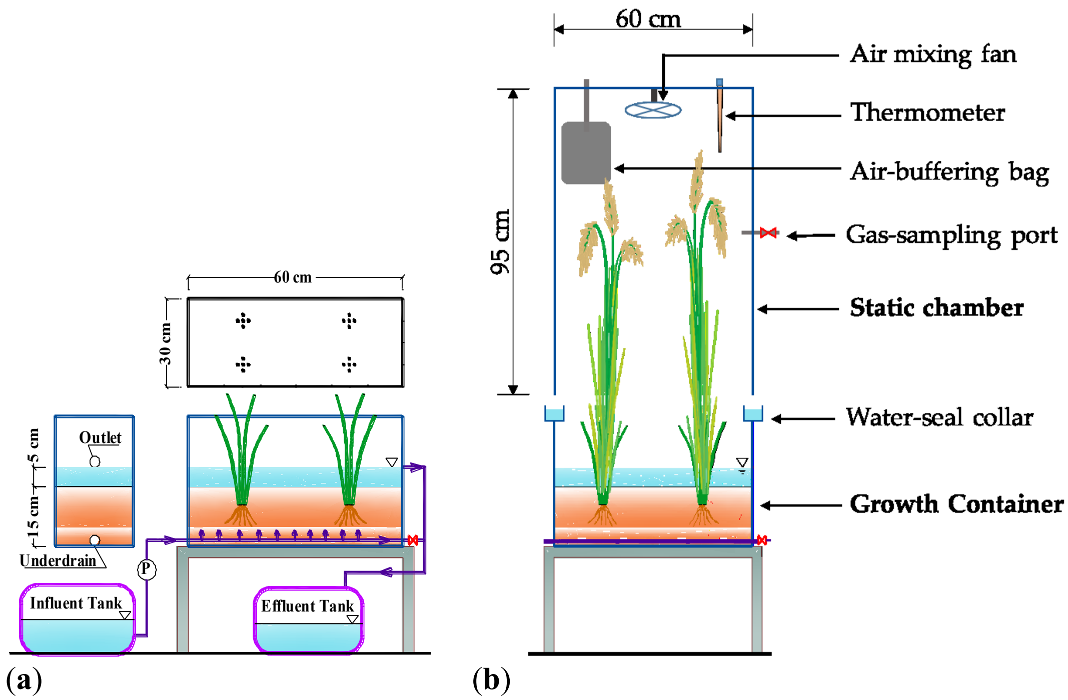

Figure 3.1 Schematic illustration of the experimental growth container equipped with continuous sub-irrigation systems (a) and the top chamber used for gas sampling (b).

Control treatment was fertilized with 160 kg N - P_2O_5 - K_2O ha^{-1} as basal at 1 day before transplantation and 100 kg N - K_2O ha^{-1} at the panicle-initiation stage. The fertilizer doses were adjusted on the basis of the surface area of the containers, while daily watering involved manually adding tap water to maintain 5 cm of standing water above the soil surface. Three treatments using CSI systems (R1, R2, and R3; Figure 3.2) were established by pumping TWW stored in the influent tank into the containers through the perforated pipes from which TWW infiltrated the soil layer and eventually overflowed into the effluent tanks through the outlets installed at 5 cm above the soil surface (Figure 3.1a).

Three CSI systems were controlled by electric pumps (EYELA Cassette Tube Pump SMP-23) with a constant supply rate of 25 L m^{-2} day^{-1} throughout the crop season (R1); a supply rate of either 25 (R2) or 36 L m^{-2} day^{-1} (R3) from 31 to 114 days after transplantation

(DAT) combined with a lower rate of 8.3 L m^{-2} day^{-1} for other growing periods (Figure 3.2). Irrigation with TWW was initiated at 3 DAT and maintained continuously throughout the crop season, with the exception of 1 week of mid-season drainage (MSD) that is conventionally practiced in the local. The CSI systems were not supplemented with exogenous fertilizers; therefore, TWW was the only source of water and fertilization inputs. Total N inputs of R1, R2, and R3 were approximately 811, 575, and 778 kg N ha^{-1}, respectively, which were theoretically estimated based on the supply rates (Figure 3.2) and the N concentrations in TWW during the crop season (Table A.1).

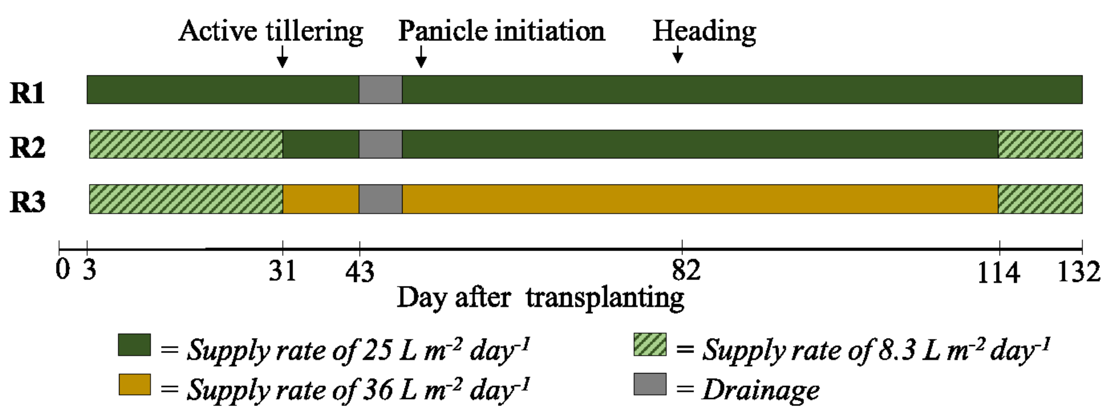

Figure 3.2 Schematic illustration of the examined water regimes (R1, R2, and R3) using the CSI system during the growth period.

3.2.2 Measurement of grain yield and quality of rice

Grain yield was measured at harvest and presented as the weight of brown rice, adjusted to a moisture content of 14%. The nutritional quality of the grains harvested was assessed on the basis of the rice protein content, which was calculated by multiplying the conversion factor of 5.95 by the total N content of brown rice (Jones, 1941). The N content of brown rice was analysed by an automatic high-sensitivity NC analyser (Sumigraph NC-220F, SCAS, Japan). Concentrations of possible toxic heavy metals, including As, Cr, Cu, Cd, Pb, and Zn, in brown rice were determined by the standard wet-digestion method (MOE, 2001) followed by either an atomic absorption spectrometer (AAS Model AA7000 equipped with hydride generator, Shimadzu Corporation, Japan) for As, or an inductively coupled plasma mass spectrometer (ICP-MS) (Elan DRC II, PerkinElmer, Japan) for the other elements.

3.2.3 Gas sampling and analysis

Gas samples were collected during the growing season (from May to September 2018) using the manually-operated closed chamber method (Minamikawa et al., 2015). In this experiment, I used transparent chambers (top chambers) (95 cm in height, 30 cm in width, 60 cm in length) which could be mounted on the growing containers on water-seal collars for every gas-sampling event (Figure 3.1b). Each chamber was equipped with a single battery-driven fan to homogenize the air inside, an air buffer 2-L plastic bag to compensate for pressure changes, a thermometer to monitor temperature changes during the gas sampling period, and a gas sampling port equipped with a 3-way stopcock valve to collect the samples (Figure 3.1b).

Gas samples were collected weekly from 10:00 to 11:00 am. A 50-mL plastic syringe with a 3-way stopcock valve was used to collect gas samples from the top chambers at 0, 15, and 30 minutes after deployment. The collected gas samples were immediately transferred to 10-mL air-evacuated glass vials and transported to the laboratory for the measurement of CH_4 and N_2O concentrations using a gas chromatograph (Shimadzu GC-2014, Kyoto, Japan). Fluxes and seasonal cumulative emissions of CH_4 and N_2O were estimated following standard procedures (Minamikawa et al., 2015). Briefly, CH_4 and N_2O fluxes were calculated by examining linear increases in CH_4 and N_2O concentrations in the top chambers over time. Seasonal cumulative emissions of CH_4 and N_2O from all chambers were calculated directly from the flux. In order to estimate the net GWP of each treatment, seasonal cumulative emissions of CH_4 and N_2O were converted to CO_2-eq. using radiative forcing potential relative to CO_2 over a 100-year time horizon of 28 for CH_4 and 265 for N_2O (IPCC, 2013). Greenhouse gas intensity (GHGI) was calculated by dividing the net GWP by the grain yield (Zhang et al., 2010).

3.2.4 Statistical analysis

Four rice plants transplanted into each of the experimental chambers were treated as four replicates for analysis of the grain yields, the rice protein contents and the concentrations of the heavy metals examined. The data were subjected to analysis of variance (ANOVA), and the means of significant treatment effects were compared using Tukey's honestly significant difference test (HSD) at a 5% probability level using IBM SPSS Statistics 24.0. The differences in CH_4 and N_2O emissions among the four treatments were assessed based on a single data point basis.

3.3 Results

3.3.1 Grain yield and rice quality

Although the treatments using CSI systems (R1, R2, and R3) were not supplemented with mineral fertilizers, their grain yields were not significantly different ($p > 0.05$) from those achieved in Control (Figure 3.3a). The lack of fertilizers in three CSI treatments did not reduce the production of rice grain, but tended to increase the yield compared to the use of fertilizers in the Control treatment. In particular, the TWW-irrigated treatments produced higher yields (10.1 – 11 t ha^{-1}) than the Control (8.6 t ha^{-1}), irrespective of the water regime used. The yields of R1, R2, and R3 averaged 10.5 t ha^{-1}, 22% higher than that of Control (Figure 3.3a).

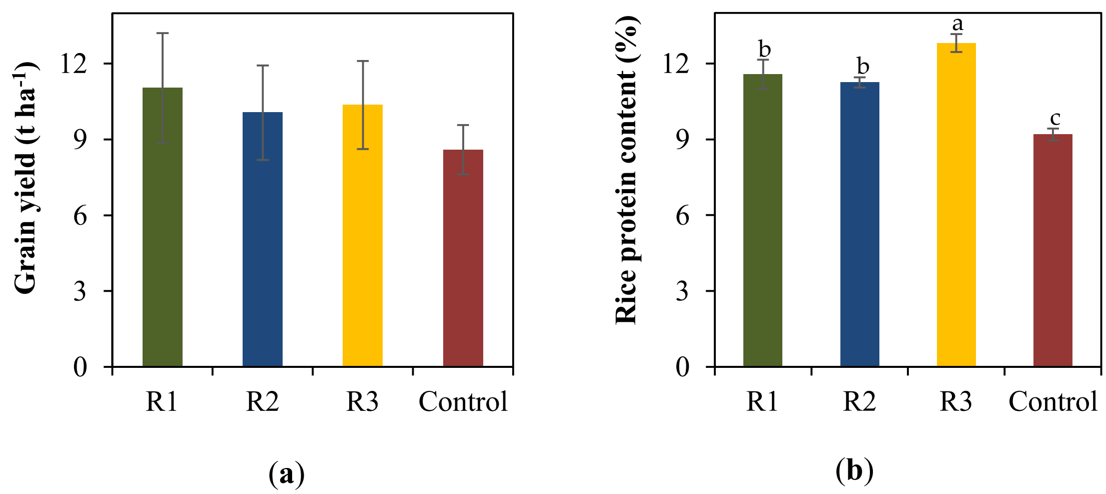

Figure 3.3 Grain yield (a) and rice protein content (b) under different cultivation systems. Error bars present standard deviations (n = 4). Different letters indicate significant difference ($p < 0.05$), whereas similar letters or no letter indicate no significant difference.

Rice quality was generally assessed based on it nutritional quality and the concentrations of heavy metals in brown rice. The nutritional quality of rice is assessed based on its protein content, which is especially important for feedstuffs. The protein content responded differently to the four cultivation treatments (Figure 3.3b). The highest protein content (12.8%) was observed in R3, followed by R1 (11.6%) and R2 (11.3%), while the lowest (9.2%) was observed in Control.

Most of the heavy metal elements examined (with the exception of Cr and Pb) responded differently to the treatments ($p < 0.05$), as indicated by their bioaccumulation in brown rice (Table 3.2). Compared to Control, all three CSI treatments tended to decrease the concentrations of As, Cr, and Cu, but slightly increased the concentrations of Cd, Pb, and Zn.

Table 3.1 Concentration of heavy metals in brown rice.

Element (mg kg^{-1})	Treatment				ML*	SD**
	R1	R2	R3	Control		
As	0.18 ± 0.01 ^{ab}	0.16 ± 0.03 ^{b}	0.18 ± 0.01 ^{b}	0.21 ± 0.01 ^{a}	0.35	2
Cr	0.04 ± 0.01	0.05 ± 0.02	0.05 ± 0.01	0.06 ± 0.02	NA	NA
Cu	5.01 ± 0.52 ^{bc}	4.61 ± 0.41 ^{c}	5.82 ± 0.26 ^{a}	5.51 ± 0.12 ^{ab}	NA	NA
Cd	0.03 ± 0.01 ^{ab}	0.02 ± 0.01 ^{b}	0.04 ± 0.01 ^{a}	0.02 ± 0.01 ^{b}	0.4	1
Pb	0.07 ± 0.05	0.07 ± 0.02	0.06 ± 0.01	0.06 ± 0.01	0.2	3
Zn	21.7 ± 0.65 ^{ab}	21.9 ± 1.86 ^{a}	23.3 ± 1.58 ^{a}	18.6 ± 0.94 ^{b}	NA	NA

*Maximum levels for contaminants and toxin in foods (FAO/WHO, 2017); **Japanese standard for animal feed (FAMIC, 2019); NA: not available; Different letters in a row indicate significant difference ($p < 0.05$), whereas similar letters and no letter indicate no significant difference among treatments.

In particular, the concentration of As decreased by 17% under the CSI treatments. Although the concentration of As did not differ among R1, R2 and R3 ($p > 0.05$), it was noteworthy that the lowest As concentration was observed under R2 (0.16 mg kg^{-1}). Similarly, CSI treatments decreased Cr concentration (0.04 – 0.05 mg kg^{-1}) compared to Control (0.06 mg kg^{-1}), but this difference was not significant ($p > 0.05$). The same trend was observed for Cu, where TWW irrigation (R1 and R2) decreased Cu concentration compared to Control, except for a slight increase with R3. The lowest concentration of Cu (4.61 mg kg^{-1}) was observed in R2 and was 20% lower than that in Control (5.51 mg kg^{-1}, Table 3.1). Concentrations of Cd increased slightly with CSI under the water regimes R1 and R3 (0.03 and 0.04 mg kg^{-1}, respectively), while R2 remained the same as in Control (0.02 mg kg^{-1}). Similarly, the concentration of Pb increased slightly under R1 and R2, but this increase was not significant ($p > 0.05$), whereas R3 resulted in the same level as Control. It is interesting that the three CSI treatments significantly increased the concentration of Zn (21.7 – 23.3 mg kg^{-1}) compared to Control (18.6 mg kg^{-1}) ($p < 0.05$). Overall, all the examined elements were observed at concentrations below the thresholds for contaminants and toxins in foods recommended by FAO/WHO (2017) and the Japanese standard for the concentration of heavy metals in animal feed (Table 3.1). Among three CSI treatments, the water regime R2 likely resulted in the lowest accumulation of the examined heavy metals in rice grains.

3.3.2 Fluxes of CH_4 and N_2O from the paddy soils

Emissions of CH_4 were not affected by the different cultivation methods from the beginning of the crop season to the heading stage, approximately 80 DAT, whereas substantial fluctuations were observed during the flowering and ripening phases (96 DAT onwards, Figure 3.4a). Before the heading stage, there was no significant difference in CH_4 flux among the four treatments (0.05 – 1.76 mg CH_4 m^{-2} h^{-1}). However, the treatments did have a considerable effect as the rice plants began to flower (Figure 3.4a). Typically, the Control exhibited the highest increase until peaking (13.1 mg CH_4 m^{-2} h^{-1}) at ~103 DAT. Subsequently, CH_4 emissions decreased gradually at the end of the ripening stage, although at a higher level than those observed under any CSI treatments. The constant supply rate of 25 L m^{-2} day^{-1} over the entire crop season in R1 (Figure 3.2) emitted remarkably more

CH_4 than the water regimes R2 and R3 under which CH_4 emissions were likely to be at the same levels (Figure 3.4a). The highest seasonal average flux of CH_4 (4.75 mg CH_4 m^{-2} h^{-1}) was observed in Control, followed by R1 (1.4 mg CH_4 m^{-2} h^{-1}) and R3 (0.79 mg CH_4 m^{-2} h^{-1}), while the lowest was observed in R2 (0.76 mg CH_4 m^{-2} h^{-1}).

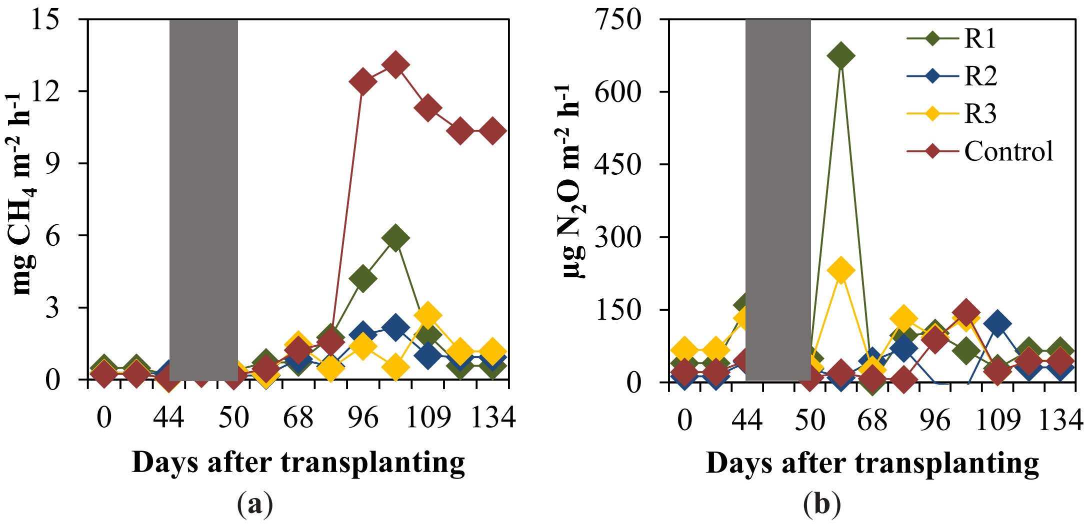

Figure 3.4 Fluxes of CH_4 (a) and N_2O emissions (b) under different cultivation systems. Gray belts indicate the mid-season drainage (MSD).

The water status was generally divided into two conditions throughout the crop season: a non-waterlogged period during the MSD and waterlogging during the growth period (Figure 3.2). N_2O emissions were high during the MSD and for ~3 days thereafter (Figure 3.4b). Drainage resulted in two high-peak fluxes in R1 (628.4 and 674.4 µg N_2O m^{-2} h^{-1}) and R3 (294.8 and 231.7 µg N_2O m^{-2} h^{-1}), while only one peak flux was observed in R2 and Control (112.4 and 293.3 µg N_2O m^{-2} h^{-1}, respectively). As a result, fluctuations in N_2O emissions were observed among the four treatments, regardless of the cultivation practice used. Seasonal N_2O fluxes averaged 155.3 and 101.6 µg N_2O m^{-2} h^{-1} for R1 and

R3, which were 163% and 72% higher than that of Control (59.2 μg N_2O m^{-2} h^{-1}), respectively. The lowest average flux was recorded in R2 (38.8 μg N_2O m^{-2} h^{-1}), which emitted 35% less than in Control.

3.3.3 *Seasonal cumulative emissions and global warming potential*

The seasonal cumulative emissions of CH_4 and N_2O are shown in Table 3.2. CSI systems considerably reduced CH_4 emissions, regardless of the water regime used. Compared to Control (146.89 kg CH_4 ha^{-1}), CSI treatments decreased CH_4 emissions by 70%, 84%, and 83% with R1 (44.77 kg CH_4 ha^{-1}), R2 (24.17 kg CH_4 ha^{-1}), and R3 (25.06 kg CH_4 ha^{-1}), respectively. However, CSI increased N_2O emissions by 170% and 110% for R1 (3.6 kg N_2O ha^{-1}) and R3 (2.81 kg N_2O ha^{-1}), respectively, compared to Control (1.34 kg N_2O ha^{-1}). Conversely, R2 (0.96 kg N_2O ha^{-1}) decreased N_2O emissions by 28% compared with the control. Overall, although all three continuous irrigation regimes could reduce CH_4 emissions, R1 and R3 had a trade-off by increasing N_2O emissions. Only R2 could effectively reduce both CH_4 and N_2O emissions.

Irrespective of water regimes, CSI considerably decreased the net GWP over a 100-year time horizon compared with Control (Table 3.2). The net GWPs of R1, R2, and R3 averaged 1529 ± 643 (kg CO_2-eq ha^{-1}), which was 66% lower than that of the Control (4468 kg CO_2-eq ha^{-1}). This result suggests that CSI would substantially diminish the radiative force of forage rice paddy fields. The modified regimes R2 and R3 reduced the net GWP of R1 by 58% and 35%, respectively. Overall, the lowest net GWP (932 kg CO_2-eq ha^{-1}) among the four examined treatments was observed in R2 (Table 3.2).

Table 3.2 Seasonal cumulative emissions of CH_4 and N_2O, global warming potentials, and greenhouse gas intensities under different cultivation systems.

Treatment	**Cumulative emissions** (kg ha^{-1})		**Net GWP**	**GHGI**
	CH_4	N_2O	(kg CO_2-eq ha^{-1})	(kg CO_2-eq t^{-1})
R1	44.77	3.60	2209	206 ± 41 ^{b}
R2	24.17	0.96	932	95 ± 16 ^{c}
R3	25.06	2.81	1446	142 ± 23 ^{bc}
Control	146.89	1.34	4468	525 ± 65 ^{a}

*Different letters in the column indicate significant difference (p<0.05), whereas similar letters indicate no significant difference among treatments.

GHGI is an index commonly used to represent the efficiency of rice production systems by linking grain yield with the corresponding net GWPs, for which the effects of producing a certain grain yield on the climate during cultivation is estimated. Since no significant difference was observed among grain yields (Figure 3.3a), the GHGIs of the four treatments followed the same trend observed in the net GWP, in which the highest (525 ± 65 kg CO_2-eq t^{-1}) was recorded in the control and the lowest (95 ± 16 kg CO_2-eq t^{-1}) in R2.

3.4 Discussion

3.4.1 High yield of protein-rich rice without the use of mineral fertilizers

The higher grain yields achieved in the CSI systems were probably attributed to the continuous supply of plant nutrients, particularly N, contained in the TWW at high concentrations during the growing season (Table A.1). As the most yield-limiting nutrient in rice production, N is generally applied through high doses of mineral fertilizers to ensure high levels of rice production, especially for high-yielding cultivars such as the forage rice Bekoaoba used in this study (Riya et al., 2015a, b). In this experiment, the continuous sub-irrigation systems supplied large amounts of N into R1, R2, and R3 (approximately 811, 575, and 778 kg N ha^{-1}, respectively), which were 3.1, 2.2, and 3.0-fold higher than that supplemented by the fertilizers in Control (260 kg N ha^{-1}). As a result, the rice plants under CSI maintained considerably higher leaf greenness, which was measured using a chlorophyll meter and expressed as SPAD values (Pham et al., 2019), compared with the rice plants under the control during the flowering and grain filling stages (around 80 DAT onwards, Figure 3.5). Since the SPAD value is strongly associated with N status in leaves (Ata-Ul-Karim et al., 2016), it is one of the best indicators of photosynthetic activities in rice plants. Generally, starch and sugar accumulate at high levels in rice culms and leaf sheaths before flowering, and the accumulated carbohydrates are translocated into rice grains during the grain filling stage, which causes the rice culms and leaves to senesce and turn yellowish (Yoshida, 1981). By delaying the senescence and maintaining effective photosynthesis, more carbohydrates could be produced and be available for grain filling, subsequently resulting in higher grain yields under the CSI treatments. These results suggest that the N derived from TWW could sufficiently substitute for the N supplemented by mineral fertilizers, which is consistent with those of a previous study (Sun et al., 2013), which demonstrated that N-rich wastewater was as effective as commercial N fertilizer at achieving optimum rice yields. This was further supported by previous studies, which reported that CSI removed 85 – 90% of the N available in TWW (Pham et al., 2019), and the utilisation of the newly-absorbed N until the late growth period is critical for producing the high yields of high-yielding rice varieties (Ida et al., 2009).

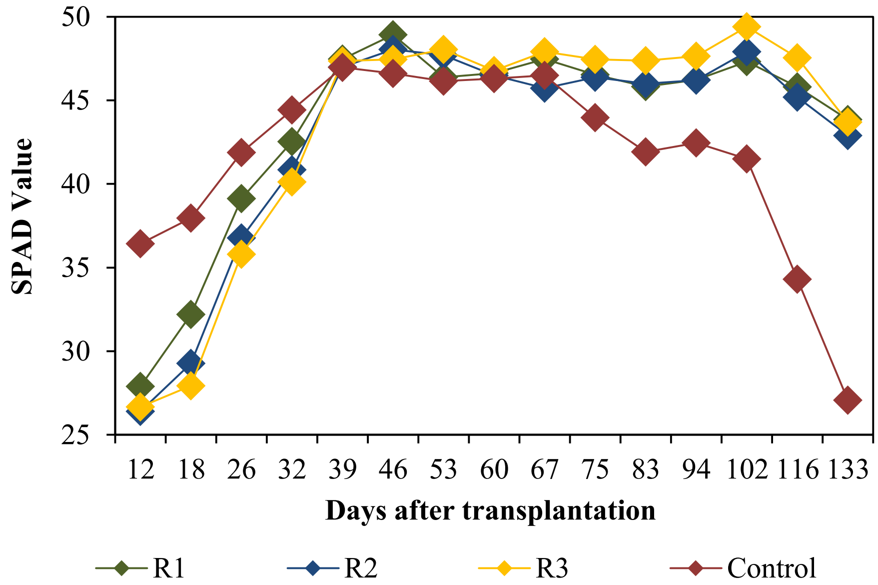

Figure 3.5 Leaf greenness (SPAD value) of the rice plants under the examined treatments during the growing period.

Water regime R1 was the preferred water scheme demonstrated in the previous study (Pham et al., 2019); however, continuous irrigation at a constant rate of 25 L m^{-2} day^{-1} throughout the growing season probably reduced the efficient use of N due to the variable N demand of rice plants in different growth stages (Yoshida, 1981). In the present study, this water regime has been modified into R2 and R3 to test for an optimal regime. The lower supply rate of 8.3 L m^{-2} day^{-1} was used during the early growth stage and near maturity when the rice plants generally had a low N-absorbing capacity (Yoshida, 1981). A higher supply rate of either 25 L m^{-2} day^{-1} or 36 L m^{-2} day^{-1} was used in R2 or R3, respectively, from 31 to 114 DAT (Figure 3.2), which was a period of high N demand, when active tillering, panicle initiation, heading, and grain filling occurred consecutively with a high N-use efficiency to maximize tiller number, increase panicle size, improve filled-grain percentage, and enhance grain weight (Yoshida, 1981). Compared with the original water regime R1, R2 and R3 did not cause significant yield loss ($p > 0.05$, Figure 3.3a), suggesting an opportunity to apply suitable supply rates at appropriate timings to meet the N demands of the rice plants. The better N assimilation during the late growth period also explained the increased rice protein content observed in R1, R2, and R3 (Figure 3.3b). The highest protein content obtained in R3 ($p < 0.05$) can be attributed to the higher supply rate of 36 L m^{-2} day^{-1} maintaining the most efficient photosynthesis in the rice plants when the maturity approached (Figure 3.5).

Overall, this experiment has provided solid evidences to suggest that CSI is an effective mean for the reuse of WWTP effluents to produce high yields of protein-rich rice without using exogenous mineral fertilizers. This result agreed with those of the previous studies mentioned in Chapter 2 (Jung et al., 2014; Tran et al., 2019; Pham et al., 2019), suggesting a cost-effective strategy for recycling water and plant nutrients that reduces both the demand for mineral fertilizers and the amount of nutrients discharged into surface water bodies. Furthermore, eliminating the use of mineral fertilizers will not only decrease the adverse effects of the environment but will also increase the profits for farmers (Papadopoulos et al., 2009; Jung et al., 2014). Relative to the constant supply rate in R1, combining more suitable supply rates with relevant timings in R2 and R3 could maintain the high yielding capacity and high rice protein content (Figure 3.3).

3.4.2 No risk of heavy metal contamination in rice grains

The accumulation of heavy metals by crops irrigated with wastewater has generally been considered an environmental problem as discussed in Chapter 2. However, in this experiment, there was no notable adverse effect of TWW irrigation on the accumulation of the possible toxic elements, including As, Cr, Cu, Cd, and Pb, in rice grains. This result is in accordance with those of other studies (Kang et al., 2007; Jung et al., 2014; Pham et al., 2017), which reported that the chemical compositions of rice irrigated with TWW were within the common range observed in conventional paddy fields. The slight increase in Zn content in the rice grains under CSI treatments compared with that under Control (Table 3.1) was probably due to the higher concentration of Zn in TWW relative to other elements (Table A.1). Interestingly, Zn is one of the most essential micronutrients for humans, and thus, attempts have been made to improve Zn content in rice (Swamy et al., 2016). The lower concentrations of As, Cr and Cu observed under CSI treatments relative to Control were probably attributed to the continuous overflow of water that might carry these elements out of the paddy soils. Eventually, although minor variation in the concentrations of the examined elements was observed among three CSI treatments, and between those and Control, all the concentrations were below the maximum limits recommended by FAO/WHO (2017) and the Japanese standard (FAMIC, 2019) (Table 3.1), suggesting that the rice grains harvested from the paddy fields using CSI are safe to use as feedstuffs.

3.4.3 Optimized waster regime for effective mitigation of CH_4 and N_2O emissions

The production of CH_4 in rice fields generally results from the anaerobic decomposition of organic matter in rhizosphere soil. In contrast with a previous study (Zou et al., 2009), which reported an increase in CH_4 emissions from paddy fields irrigated with wastewater, the CSI systems in this experiment considerably reduced the seasonal emissions of CH_4 by 70 – 84% compared with the conventional cultivation (Table 3.2). This decrease was probably due to the substantial amounts of dissolved oxygen (DO) maintained in the TWW

(Table A.1) being continuously supplied into the rhizosphere as TWW was pumped into the deep soil layers, which might subsequently inhibit methanogen communities and their activities regarding CH_4 production. The high peaks of CH_4 fluxes recorded in all treatments during the grain filling stage were mainly attributed to the higher availability of C substances in the paddy soils as a result of enhanced root exudation during the flowering time. The highest exudation rates were observed during the grain filling stage compared to the other growth stages (Aulakh et al., 2001). Since root exudates provide C substrates for methanogenesis in soils, the higher root exudation during the flowering time could greatly stimulate CH_4 emissions in the following stages (Aulakh et al., 2001). Among three CSI treatments tested in the present study, R2 was the most effective in terms of CH_4 mitigation (Figure 3.4, Table 3.2), probably owing to the lowest input amount of available C accompanied by the lowest irrigation rates (Figure 3.2).

The emissions of N_2O in Control, which contributed 8% to the net GWP, were substantially low compared with the CH_4 emissions. This result agrees with those of other studies that reported negligible N_2O emissions in flooded paddy fields under conventional cultivation (Denmead et al., 1979; Smith et al., 1982). The high peaks of N_2O fluxes observed in all treatments during the MSD were consistent with the common phenomenon in paddy fields under the field drainage that promotes N_2O emissions due to enhanced nitrification-denitrification processes under favourable conditions (Zou et al., 2009; Riya et al., 2015a). The additional peaks recorded in R1 and R3 within 3 days afterwards were likely due to the high N concentration in the soils and surface water when TWW was re-supplied into the experimental containers. The N_2O emissions from R1, R2, and R3 contributed to 43%, 27%, and 51% of the net GWP, respectively, probably due to the high N concentration in the TWW continuously supplied (Table A.1). The lowest emission of N_2O recorded in R2 relative to the other CSI treatments (Table 3.2) was mainly attributed to the lowest N input accompanied by the lower irrigation rates. It is likely that the lower fertilization in R2 ensured the efficient use of nutrients by rice plants, leaving very little residual N for nitrification and denitrification, thereby reducing the N_2O emissions. The higher N_2O emissions from R1 and R3 were essentially due to the enhanced nitrification/denitrification processes induced by the considerable N contained in TWW and the higher supply rates (Figure 3.2). Furthermore, the rich sources of organic matter supplied by TWW could also benefit N-cycling bacterial communities (Zou et al., 2009), subsequently increasing N_2O emissions. Overall, these results indicate that R2 is the most effective mitigation water regime under CSI that can overcome the trade-off between N_2O and CH_4 emissions compared to R1 and R3.

Efficient cultivation practices must involve producing the optimum rice yield along with low environmental footprints. Prior studies have reported many potential practices to increase rice yield and simultaneously mitigate GHG emissions from paddy fields (Sun et al., 2018; Zhang et al., 2010). In the present study, CSI considerably decreased the net GWPs primarily owing to the considerable decrease in the CH_4 emissions (Table 3.2). The combination of two supply rates in R2 and R3 (Figure 3.2) tended to decrease the seasonal CH_4 and N_2O emissions (Figure 3.4), and subsequently reduce the net GWPs compared to the constant supply rate in R1. The lowest net GWP and GHGI attained in R2 is attributed to its most effective minimization of both CH_4 and N_2O emissions. The results have shown that appropriately matching the lower (8.3 L m^{-2} day^{-1}) and higher (25 L m^{-2} day^{-1}) supply rates with the periods of low and high N demand of rice plants (Figure 3.3), respectively, leads to R2 being the optimized irrigation regime for CSI to reduce the GHG budget of rice paddy fields without significant yield loss or protein reduction.

3.5 Summary

CSI could produce high yields of protein-rich rice through the effective reuse of TWW as the sole source for both irrigation and fertilisation. Importantly, by employing the optimal water regime (R2), CSI can effectively mitigate CH_4 and N_2O emissions from rice paddies. The practice of recycling valuable plant nutrients contained in TWW to meet the high nutrient demand of forage rice production instead of applying high doses of mineral fertilizers demonstrates the potential to minimize the dependence of rice cultivation on fertilizers, which would simultaneously mitigate GHG emissions and promote sustainable rice paddy farming. Importantly, the results in this study definitely motivate the local farmers to adopt the continuous sub-irrigation systems for effective reuse of TWW in their paddy rice farming.

CHAPTER 4. LINKING EMISSIONS OF CH_4 AND N_2O TO COMMUNITY STRUCTURES OF RELEVANT SOIL MICROORGANISMS AND CHANGES IN SOIL PHYSICOCHEMICAL PROPERTIES UNDER CONTINUOUS SUB-IRRIGATION WITH TREATED WASTEAWTER

This chapter presents an investigation on soil microbial communities related to CH_4 and N_2O emissions from paddy fields and physicochemical properties of paddy soils as affected by CSI. By linking the abundance of relevant microbes to GHG emissions, this chapter identifies the potential underlying microbial mechanisms for GHG mitigation induced by CSI as identified in Chapter 3. In addition, potential of heavy metal contamination in paddy soils, soil fertility and other characteristics of paddy soils have been also elaborated herein. Part of this chapter was used for a manuscript submitted to the journal Soil Biology and Biochemistry by Elsevier (under review), while some findings were presented at the Water and Environment Technology Conference held online by the Japan Society of Water Engineering (JSWE) on 7 - 8th November 2020.

4.1 Introduction

A number of studies have been conducted to investigate emissions of CH_4 and N_2O with respect to the structure of the relevant microbial communities in paddy soils as influenced by different chemical and organic fertilization practices (Fan et al., 2016; Yuan et al., 2018; Liu et al., 2019; Kong et al., 2019). However, no report is available on the assessment of such soil microbial communities associated with the emissions of these two GHGs from paddy fields under irrigation with wastewater. On the other hand, CSI systems examined in this research project uses TWW as the sole source for both irrigation and fertilization with zero use of mineral fertilizers, as discussed in Chapters 2 and 3, but no research has been done so far to understand possible changes in the physicochemical properties of paddy soils using the continuous sub-irrigation system. Conventionally, fertilization with mineral fertilizers plays an important role in maintaining soil fertility and crop productivity, therefore the elimination of mineral fertilizer usage seen in CSI systems could have an enormous impact on plant nutrition as well as other characteristics of paddy soils.

Since wastewater irrigation is generally known to increase the metabolic activity of soil microorganisms (Toze, 2006), this wastewater reuse practice may cause changes in the structure of the soil microbial community, and subsequently alter the emission patterns of CH_4 and N_2O from paddy soils (Zou et al., 2009). As discussed in Chapter 2, emissions of CH_4 and N_2O from rice paddy fields are closely linked to the activities of methanogenic, methanotrophic, nitrifying, and denitrifying microbial communities in paddy soils. I hypothesize that CSI has significant effects on the abundance of these microbial communities in paddy soils, which probably results in the reduced CH_4 and N_2O emissions figured out in Chapter 3. In addition, the metabolic activity of soil microorganisms also plays a key role in maintaining the fertility of paddy soils. Changing the metabolic activities of soil microbial communities may also cause changes in soil fertility and other soil properties, indicating the need to elaborate physicochemical properties of paddy soils as affected by CSI.

This chapter therefore presents a follow-up study with specific objectives as follows: (1) to investigate the community structure of the above-mentioned microbes in paddy soils;

(2) to explore the potential underlying microbial mechanism for the CSI-induced GHG mitigation; and (3) and to investigate soil physicochemical properties of paddy soil under CSI versus a conventional cultivation practice.

4.2 Methodology

4.2.1 Experimental design

A microcosm experiment was conducted in 2019 at Yamagata University, Tsuruoka City, Japan, to elaborate the performance of CSI versus a conventional rice cultivation (Control) with respect to the specific objectives set out above. The growing containers described in Chapter 3 were again used to simulate paddy fields of 0.18 m^2. Each treatment consisted of three replicates, so, six containers were used, each filled with 30 kg of loamy soil (air-dried, 20% moisture) and transplanted with four hills of rice seedlings on 27th May 2019.

The soil was newly collected from the experimental paddy field in the university farm, air-dried and sieved through 2 mm before use. Rice seeds of the local forage rice Bekoaoba were sown in a nursery bed tray 35 days before transplantation. The TWW used in this experiment was collected from the local WWTP and monitored weekly for its physicochemical properties. The average basic characteristics of the irrigation TWW during the experimental period were shown in Table A.2.

Consistent with the control described in Chapter 3, the control treatment in this experiment was also applied with mineral fertilizers at 160 kg N - P_2O_5 - K_2O ha^{-1} as basal and 100 kg N - K_2O ha^{-1} as topdressing at 1 day before transplantation and at the panicle initiation stage (52 DAT), respectively. Daily irrigation was carried out by manually adding tap water to maintain 5 cm of standing water above the soil surface. The CSI treatment was not supplemented with exogenous fertilizers and was equipped with the continuous sub-irrigation system using the optimized water regime R2 as identified in Chapter 3.

4.2.2 Gas sampling and analysis

Gas samples were collected from each of the three replicates per treatment once a week over the growing period from 27th May to 1st October 2019, except for the MSD period during which the gas samples were collected once every two days. The sampling protocol and sample analysis were done following the standard methods as thoroughly described in Chapter 3.

4.2.3 Soil sampling

For microbial analysis, the paddy soils from CSI and Control were sampled at six time points, which were representative of the different soil conditions and growth stages of rice plants during the crop season: pre-transplantation, maximum tillering, panicle-initiation, booting, grain filling and ripening phases (0, 43, 52, 64, 107, and 126 DAT, respectively). Samples were collected in each container using a soil core (1.5 cm in diameter × 15 cm in depth) from which the upper and lower layers of paddy soils (0 – 1 and 12 – 15 cm, respectively) were collected separately. The respective soil layers of the three replicates were mixed to form one composite sample of each treatment, placed in a 50 mL commercial centrifuge tube (SuperClear^{TM} Ultra High Performance Centrifuge Tubes, VWR international, USA) and then transferred to the laboratory and stored immediately at -80 °C.

For analysis of soil physicochemical properties, the paddy soils were sampled (0 - 20 cm) on 1st October 2019 after rice harvest. The samples were then oven-dried at 80 ºC for 48 h.

Subsequently, the soil samples were ground by ceramic mortars and passed through either a 250-μm sieve for the analysis of total nitrogen (TN) and total carbon (TC), or a 2-mm sieve for the analysis of other physiochemical properties of paddy soils.

4.2.4 DNA extraction and PCR assays

Soil DNA was extracted from 24 samples (2 treatments × 2 layers × 6 time points) using 0.25 g of the frozen soils as the input for a DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) following the instructions of the manufacturer. Next, DNA concentration was determined using Qubit 4 Fluorimeter (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and total DNA extracts were stored at -80 °C until further analysis.

The 24 total DNA extracts were all used for Illumina MiSeq 16S rRNA gene sequencing. Amplicon library preparation and Illumina MiSeq sequencing were performed by Fasmac Co., Ltd. (Atsugi, Japan). The following universal primers were used: U515F (ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGCCAGCMGCCGCGGTAA) and U806R (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT). They targeted the V4 hypervariable regions of the archaeal and bacterial 16S rRNA genes and were used for the first 16S rRNA gene amplification (Miya et al., 2015). Operational taxonomic units (OTUs) were defined at the sequence similarity level of 97 %, and a representative sequence from each OTU was assigned to a taxonomic identity using the Quantitative Insights into Microbial Ecology (QIIME) software package (Caporaso et al., 2010). Compositions of the archaeal and bacterial communities (at the genus level) in the soil samples were determined by classifying the taxa of each OTU using the Greengenes database at Fasmac Co., Ltd.

In order to evaluate the total loads of archaeal and bacterial communities in the soil samples, two real-time PCR (qPCR) assays targeting the 16S rRNA genes of the archaeal and bacterial DNA were performed, respectively, using a CFX96 Touch Real-Time Detection System (Bio-Rad Laboratories, Inc. Hercules, CA, USA). The primers and probes used for the assays were previously developed by Nadkarni et al. (2002) and Yu et al. (2005). The standards ($10^2 – 10^6$ gene copies) for the qPCR assays for archaea were prepared using the strain NBRC110930 *Haloarchaeobius iranensis*, while those for the qPCR assay of bacteria were prepared using the strain NBRC3301 *Escherichia coli* K12. The reaction conditions for the amplification of archaea were as follows: 95 ℃ for 10 min, followed by 40 cycles at 95 ℃ for 20 s and 60 ℃ for 1 min; in contrast, the conditions for bacterial amplification were as follows: 50 ℃ for 2 min and 95 ℃ for 10 min, followed by 50 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. The abundance of the methanogenic, methanotrophic, nitrifying and denitrifying genera was calculated based on their proportions compared to the quantity of total archaeal and bacterial communities in the soil samples.

4.2.5 Analysis for soil physicochemical properties

Basic properties of paddy soils were analysed following the standard methods. In particular, the analysis of soil texture was carried out by determining the percentage of sand, silt and clay contents in the soil samples using the standard hydrometer method (Bouyoucos, 1962)**.** Soil pH and EC were measured at a soil-to-water volume ratio of 1:5 in milli-Q water using a pH meter (HM-30R, DKK-TOA Corportation) and a pH/Conductivity meter (Horiba pH/Cond meter D-054), respectively (Elfanssi et al., 2018). Soil cation exchange capacity (CEC) of the soil samples were measured by using the ammonium acetate method. The detailed procedure for the ammonium acetate method is described in ASTM D7503 (ASTM 2010).

The loss on ignition (LOI) method was used to estimate SOM using a sample mass of at least 20 g, which was previously dried at 105 ºC about 24 hours and then ignited at 550 ºC for 3 hours (Hoogsteen et al., 2015). The SOM was calculated as follows:

$$\text{SOM (\%)} = \frac{\text{W1 - W2}}{\text{W1}} \times 100$$

where W1 and W2 is the soil weights at 105 and 550 °C, respectively.

Soil organic carbon (SOC) was estimated according to Hoogsteen et al. (2015), using the following equation:

$$\text{SOC (\%)} = \alpha_T \times (\text{LOI}_T - b_T \times C)$$

where α_T is the carbon content of SOM in 550 ºC ($\alpha_T = 0.56$ kg kg^{-1}), LOI_T is the mass loss of SOM, b_T is the clay correction factor for structural water loss ($b_T = 0.09$ kg kg^{-1}), and C is the clay content (%).

Soil TN and TC were analyzed an automatic high-sensitivity Sumigraph NC-220-F Analyzer (Sumika Chemical Analysis Service Ltd., Tokyo, Japan). In order to measure contents of other elements, the soil samples were digested using the standard wet-digestion method (MOE, 2001). The content of P was determined by molybdate blue–ascorbic acid colorimetric method using HACH DR/890 Colorimeter, while total contents of macro and micro nutrients (i.e. K, Mg, Ca, Na, B, Mn, Fe, Ni, Cu, Zn, Mo) and possible toxic heavy metals (i.e. Cr, Cd, Pb, As) was determine by AAS or ICP-MS as descripted in Chapters 3.

4.2.6 Statistical analysis

Differences in the examined data between CSI and Control were evaluated using Student's *t* tests at a significance level of 0.05. The Pearson correlation analysis was carried out between the fluxes of CH_4 and N_2O and the abundance of the relevant microbial communities, using the data derived from the 6 time points across the treatments. All statistical analyses were performed using IBM SPSS Statistics 24.0.

4.3 Results

4.3.1 Fluxes of CH_4 and N_2O from the paddy soils

The patterns in daily fluxes of CH_4 were similar between the two cultivation treatments examined. CH_4 emissions were not notably affected by the treatments during the vegetative phase (0 − 56 DAT), and thereafter, substantial fluctuations were observed during the reproductive stage (64 DAT onwards, Figure 4.1a). In the first growth stage, CH_4 emissions from paddy fields were negligible (< 1.37 mg CH_4 m^{-2} h^{-1}), regardless of the treatments. However, the gas fluxes from both Control and CSI treatments markedly increased from the start of the booting stage (63 DAT) and peaked at 20.5 and 4.3 mg CH_4 m^{-2} h^{-1} during the flowering period (86 and 94 DAT, respectively). Subsequently, the fluxes from Control decreased sharply to their minimum (11.1 mg CH_4 m^{-2} h^{-1}) at the end of the crop season, while those from CSI decreased gradually and remained at considerably lower levels (2.1 – 3.3 mg CH_4 m^{-2} h^{-1}) during the late growing period. Overall, CSI showed a seasonal mean flux of 1.1 ± 1.4 mg CH_4 m^{-2} h^{-1}, which was markedly lower than that of Control (5.5 ± 7.2 mg CH_4 m^{-2} h^{-1}).

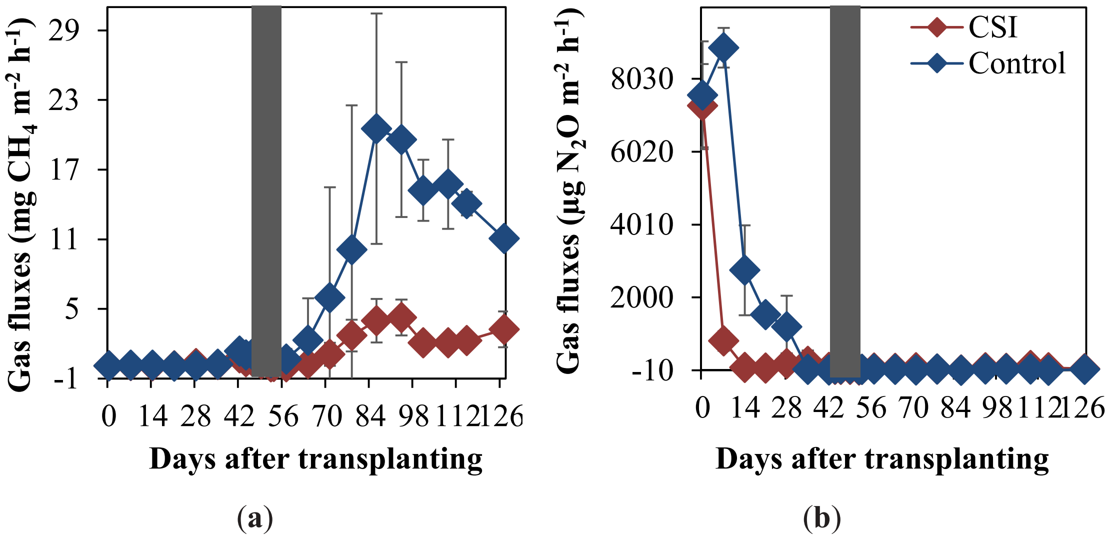

Figure 4.1 Fluxes of CH_4 (a) and N_2O (b) from the paddy fields under the examined treatments during the crop season. Gray belts indicate the mid-season drainage (MSD).

The emissions of N_2O were high and fluctuated dynamically during the first 5 weeks after transplantation, thereafter they relatively approached a plateau until the end of the crop season (Figure 4.1b). The highest flux of N_2O (7290 µg m^{-2} h^{-1}) under CSI was recorded at the beginning the crop season (0 DAT) and the fluxes decreased dramatically until reaching a low point of 52 µg m^{-2} h^{-1} before rising again and reaching a peak of 242.9 µg m^{-2} h^{-1} at 35 DAT. Meanwhile, starting with 7578.6 µg m^{-2} h^{-1}, the N_2O fluxes under Control reached the highest peak of 8887.9 µg m^{-2} h^{-1} at 7 DAT, before being dramatically reduced to 22 and then −2.6 µg m^{-2} h^{-1} at 35 and 42 DAT, respectively. Subsequently, the fluxes remained slightly fluctuating, regardless of the treatments. The seasonal average emission rate of N_2O under Control (1013.3 µg m^{-2} h^{-1}) was approximately 2.4-fold higher than that (423.8 µg m^{-2} h^{-1}) observed under the CSI system.

4.3.2 Seasonal cumulative emissions and global warming potential

The total cumulative GHG emissions during the crop season were shown in Table 4.1. In line with the findings in Chapter 3, CH_4 emissions under Control (212.8 kg ha^{-1}) was tremendously reduced by 80% by CSI (42.3 kg ha^{-1}). At the same time, CSI also significantly reduced the N_2O emissions recorded in the control treatment (32.7 kg ha^{-1}) by 66%. As a result, the GWP of the paddy fields under CSI (4149 kg CO_2-eq ha^{-1}) was effectively attenuated by 72% compared to that (14632 kg CO_2-eq ha^{-1}) of the conventional cultivation.

Table 4.1 Cumulative emissions of CH_4 and N_2O, and their net global warming potentials under the two examined cultivations during the crop season.

Treatment	Cumulative emissions		Global warming potential (kg CO_2-eq ha^{-1})
	CH_4 (kg ha^{-1})	N_2O (kg ha^{-1})	
CSI	42.3 ± 14.3 ^{b}	11.2 ± 2.5 ^{b}	4149 ± 470 ^{b}
Control	212.8 ± 86.9 ^{a}	32.7 ± 4.6 ^{a}	14632 ± 1971 ^{a}

Mean ± Standard deviation of the three replicates (n=3). Different letters in the same column indicate significant difference (p<0.05).

4.3.3 Community structure of methanogens and methanotrophs in the paddy soils

The methanogenic community consisted of 7 archaeal genera in all soil samples, as shown in Figure 4.2, in which the *Methanolinea* was irregularly found only in the lower soil layer. In the upper soil layer with CSI treatment, *Methanocella* and *Methanobacterium* were the predominant genera, accounting for 37% and 28% of the community, respectively, followed by *Methanosarcina* (16%), *Methanosaeta* (10%), and *Ca. Methanoregula* (8%). *Methanospirillum* had the lowest abundance and was occasionally found at negligible loads

(~1%, Figure 4.2a). This trend, with similar relative abundance of the genera, was also observed with Control in the same soil layer, with the following genera (arranged in the order of dominance): *Methanocella* (38%), *Methanobacterium* (27%), *Methanosarcina* (16%), *Methanosaeta* (10%), *Ca. Methanoregula* (7%), and *Methanospirillum* (< 1%). Similarly, the relative abundance of methanogens between CSI and Control treatments in the lower soil layer was consistent (Figure 4.2b). The three most abundant methanogens were *Methanobacterium*, *Ca. Methanoregula*, and *Methanocella* (~21 – 26%), followed by *Methanosaeta* and *Methanosarcina* (14 – 16%), while the rest of the genera that were accounted for had negligible contributions (< 1%).

The methanotrophic community consisted of 6 bacterial genera as presented in Figure 4.3, in which *Ca. Methylomirabilis* was detected only in the lower soil layer. Consistently in both the upper and lower soil layers, the most dominant methanotrophic bacteria were *Methylosinus* and *Crenothrix,* regardless of the treatments. In the upper soil layer, the copy numbers of *Methylosinus* averaged 55% and 53% for CSI and Control treatments, respectively, whereas *Crenothrix* had average copy numbers of 35% and 37% for the two treatments, respectively (Figure 4.3a). In contrast to the upper soil layer, the growth of *Crenothrix* was stimulated in the lower layer, accounting for 54% and 56% of the methanotrophic communities treated with CSI and Control, respectively. The relative abundance of *Methylosinus* treated with CSI (31%) was comparable to that of Control (33%) (Figure 4.3b). Contrastingly, the rest of the methanotrophic genera had uniform contributions at minor proportions (< 10%) to the total methanotrophic bacterial communities, regardless of the cultivation treatments. Thus, although there was no notable distinction between CSI and Control treatments in the microbial composition, there was an apparent variation in the composition of the identified genera between the upper and lower soil layers in both cultivation systems.

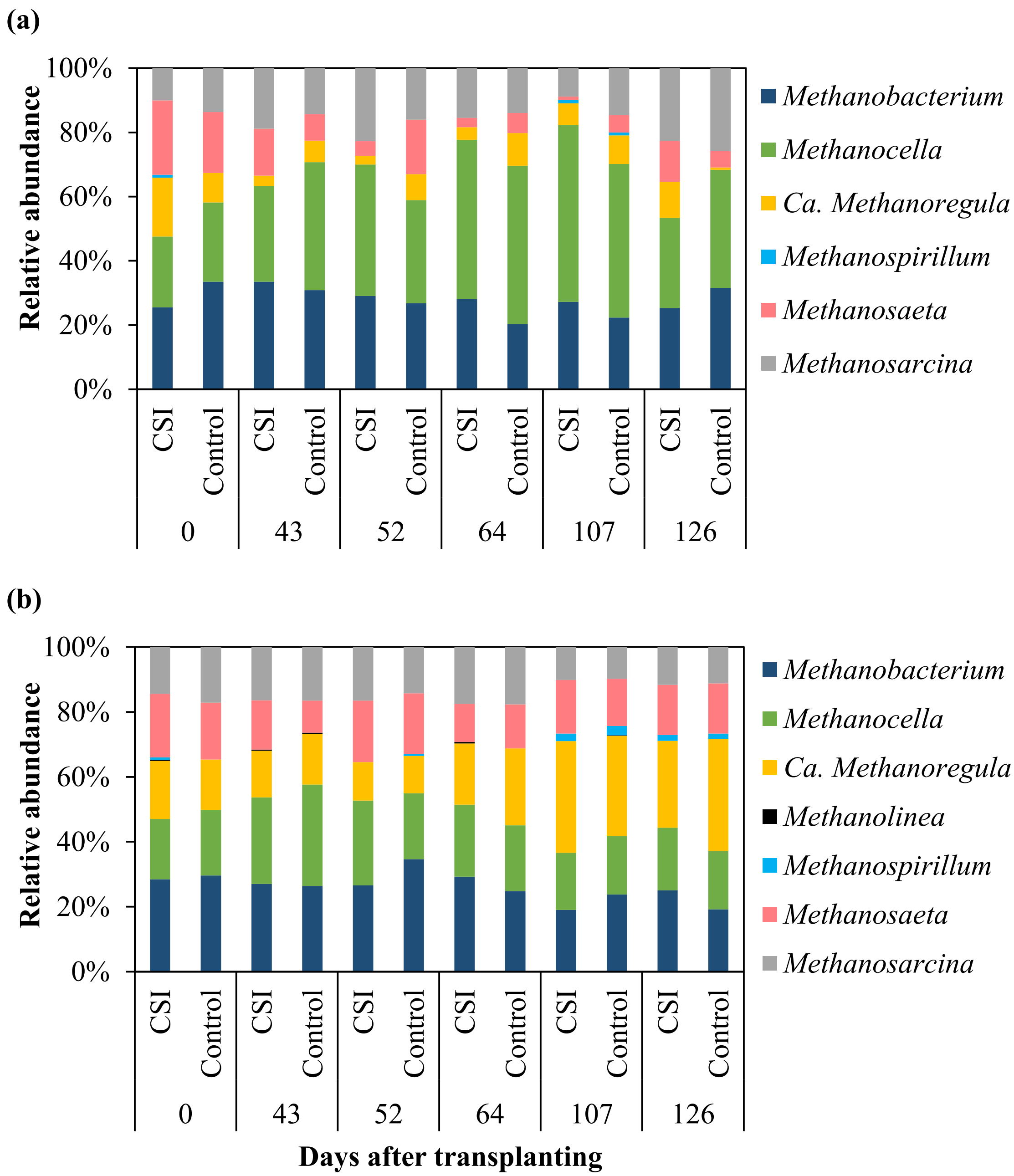

Figure 4.2 Taxonomic composition of methanogenic archaea in the upper (a) and lower layers (b) of the paddy soils under the examined treatment during the crop season.

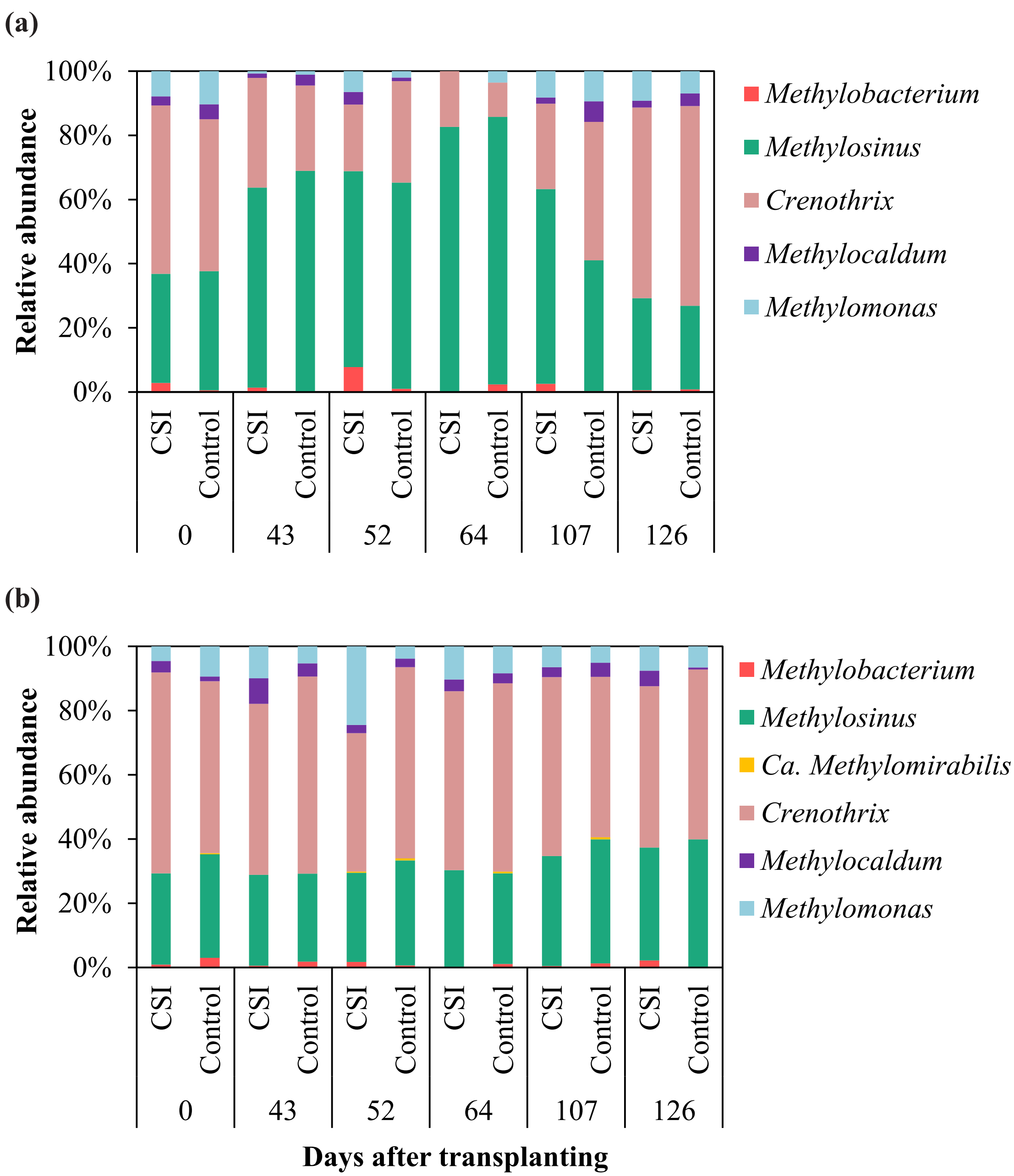

Figure 4.3 Taxonomic composition of methanotrophic bacteria in the upper (a) and lower layers (b) of the paddy soils under the examined treatment during the crop season.

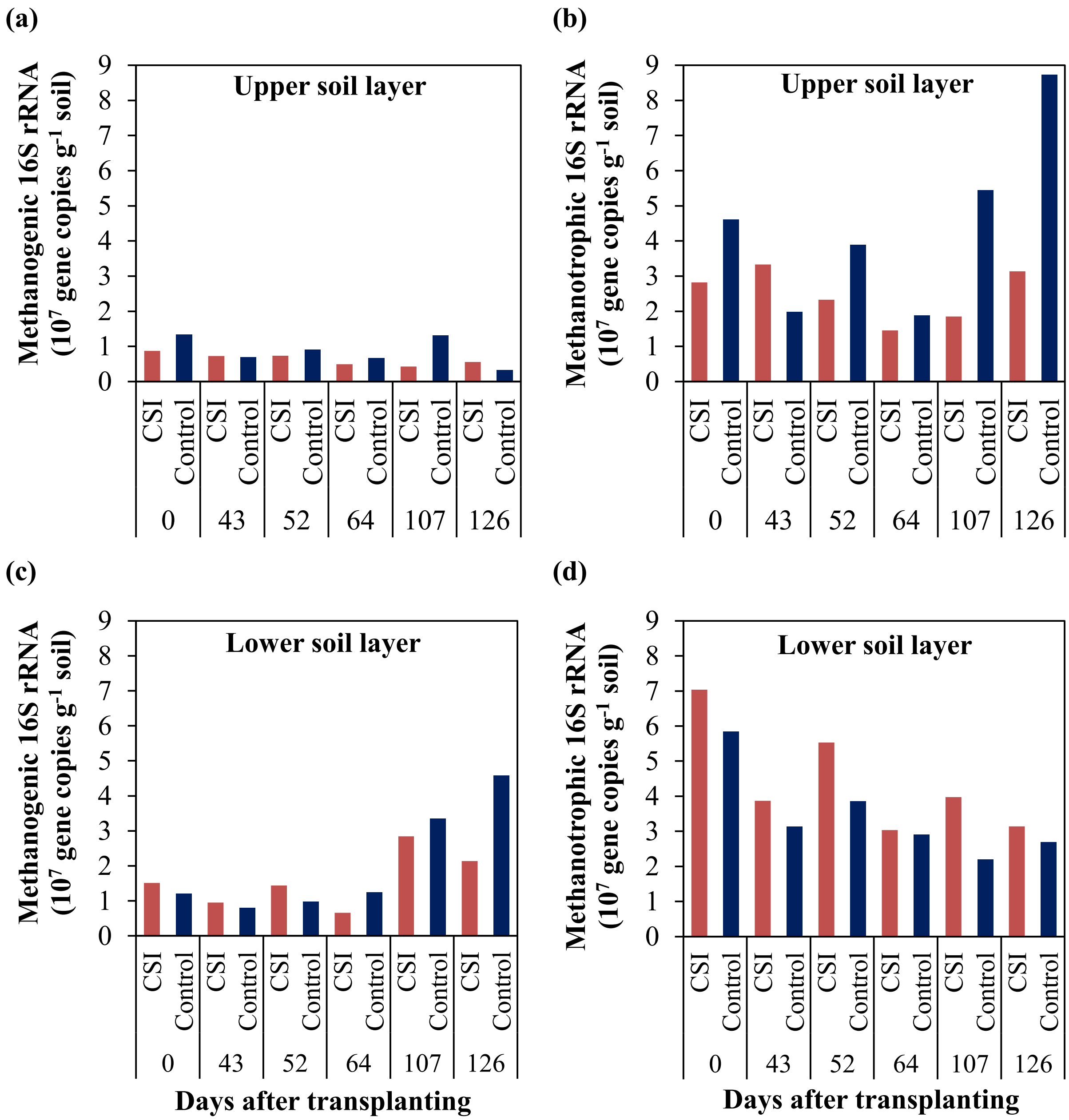

Figure 4.4 Abundance of methanogenic archaea (a and c) and methanotrophic bacteria (b and d) in the upper and lower layers of the paddy soils, respectively, under the examined treatments during the crop season.

The methanogenic and methanotrophic community abundance varied considerably during the entire crop season (Figure 4.4). Consistently in both treatments, the abundance of the methanogenic archaea was markedly lower than that of the methanotrophic bacteria, especially in the upper soil layer, where the methanogen loads (3.3×10^6 – 1.3×10^7 gene copies g^{-1} soil) were vastly smaller than those of the methanotrophs (1.5×10^7 – 8.7×10^7 gene copies g^{-1} soil). In addition, the target microbes generally had higher abundance in the lower soil layer than in the upper layer, regardless of the cultivation systems. Across the six sampling time points, CSI tended to have a lower abundance of methanogens than Control. Particularly, the copy numbers of the methanogenic archaeal 16S rRNA genes recorded in the upper and lower soil layers with CSI treatment, averaged 6.4×10^6 and 1.6×10^7 gene copies g^{-1} soil, respectively, which were 27 and 20% lower than those (8.8×10^6 and 2.0×10^7 gene copies g^{-1} soil) observed with Control (Figure 4.4a, c). It was noteworthy that the gap in the numbers of the methanogenic archaea between CSI and Control treatments were more prominent during the reproductive stage (from 64 DAT onwards, Figure 4.5a, c). For the methanotrophic bacteria, however, CSI treatment immensely reduced the average abundance of the methanotrophs (2.5×10^7 gene copies g^{-1} soil) by more than 43% relative to Control (4.4×10^7 gene copies g^{-1} soil) in the upper soil layer (Figure 4.4b). In contrast, the methanotrophic community in the lower soil layer with CSI treatment (4.4×10^7 gene copies g^{-1} soil) was 18% more abundant than that (3.4×10^7 gene copies g^{-1} soil) with Control (Figure 4.4d). Overall, there were noticeable dynamic changes in the abundance of the soil microbial communities with CSI treatment in contrast to Control throughout the crop season.

4.3.4 Community structure of nitrifiers and denitrifiers in the paddy soils

There were 3 and 24 genera identified as nitrifying and denitrifying bacteria in the soil samples throughout the crop season, as shown in Figures 4.5 and 4.6, respectively. Among the nitrifying bacteria, *Nitrospira* was the most dominant genera accounted for approximately 98% in CSI and 96 – 98.5% in Control, regardless of the soil layers (Figure 4.5). The other genera, *Nitrosovibrio* and *Arthrobacter*, were accounted for negligible proportions and not detected at all the sampling time points throughout the growing period. The denitrifying genera in the upper soil layer were slightly more diversity than that in the lower soil layer (Figure 4.6). In particular, the denitrifying community in the upper soil layer consisted of 24 genera, 4 of which were not found in the lower soil layer, namely *Microvirgula*, *Comamonas*, *Stenotrophomonas*, and *Bacillus*. The lower soil layer, on the other hand, had 22 denitrifying genera, 2 of which were not detected in the upper soil layer, including *Shewanella* and *Paracoccus*.

In the upper soil layer, the most dominant denitrifying genera was *Hyphomicrobium* accounted for 27 and 25% under CSI and Control, respectively, followed by *Streptomyces* (11.7 – 10.8%), *Rhodobacter* (15 – 7.1%), *Burkholderia* (7.8 – 27.8%), *Azospirillum* (11.1 – 6.3%), and *Paenibacillus* (7.3 – 7.4%), whereas other genera were accounted for minor proportions (< 5%, Figure 4.6a). In the lower soil layer, *Hyphomicrobium* was the most dominant denitrifying genera, accounted for 52 and 56% under CSI and Control treatments, respectively, followed by *Streptomyces* (15.4 – 17.3%), *Burkholderia* (10.5 – 9.2%), and *Paenibacillus* (5.8 – 9.1%). Other genera were accounted for negligible proportions (< 3.3%, Figure 4.6b). In both soil layers, there was no notable distinction in the microbial compositions observed between CSI and Control.

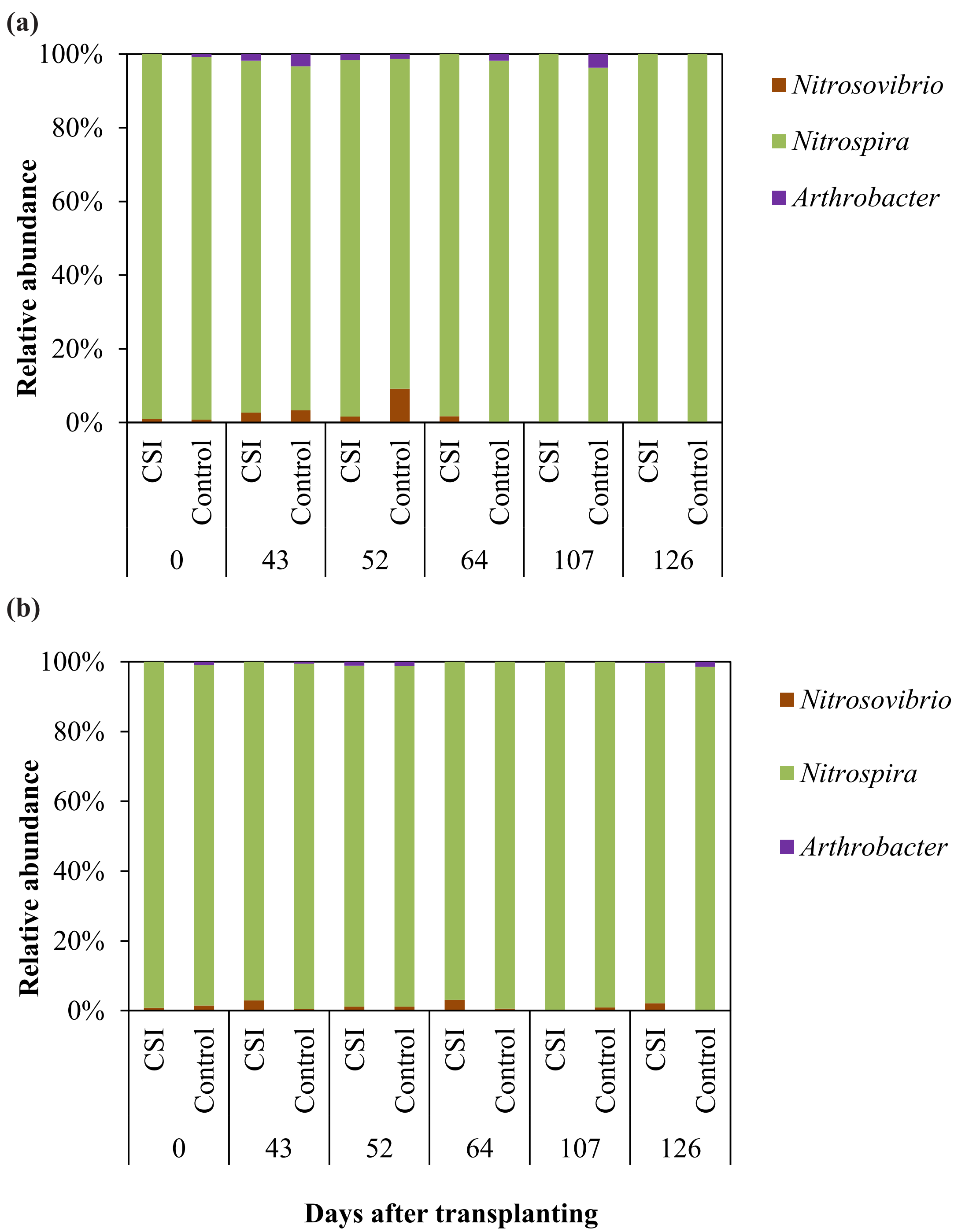

Figure 4.5 Taxonomic composition of nitrifying bacteria in the upper (a) and lower layers (b) of the paddy soils under the examined treatment during the crop season.

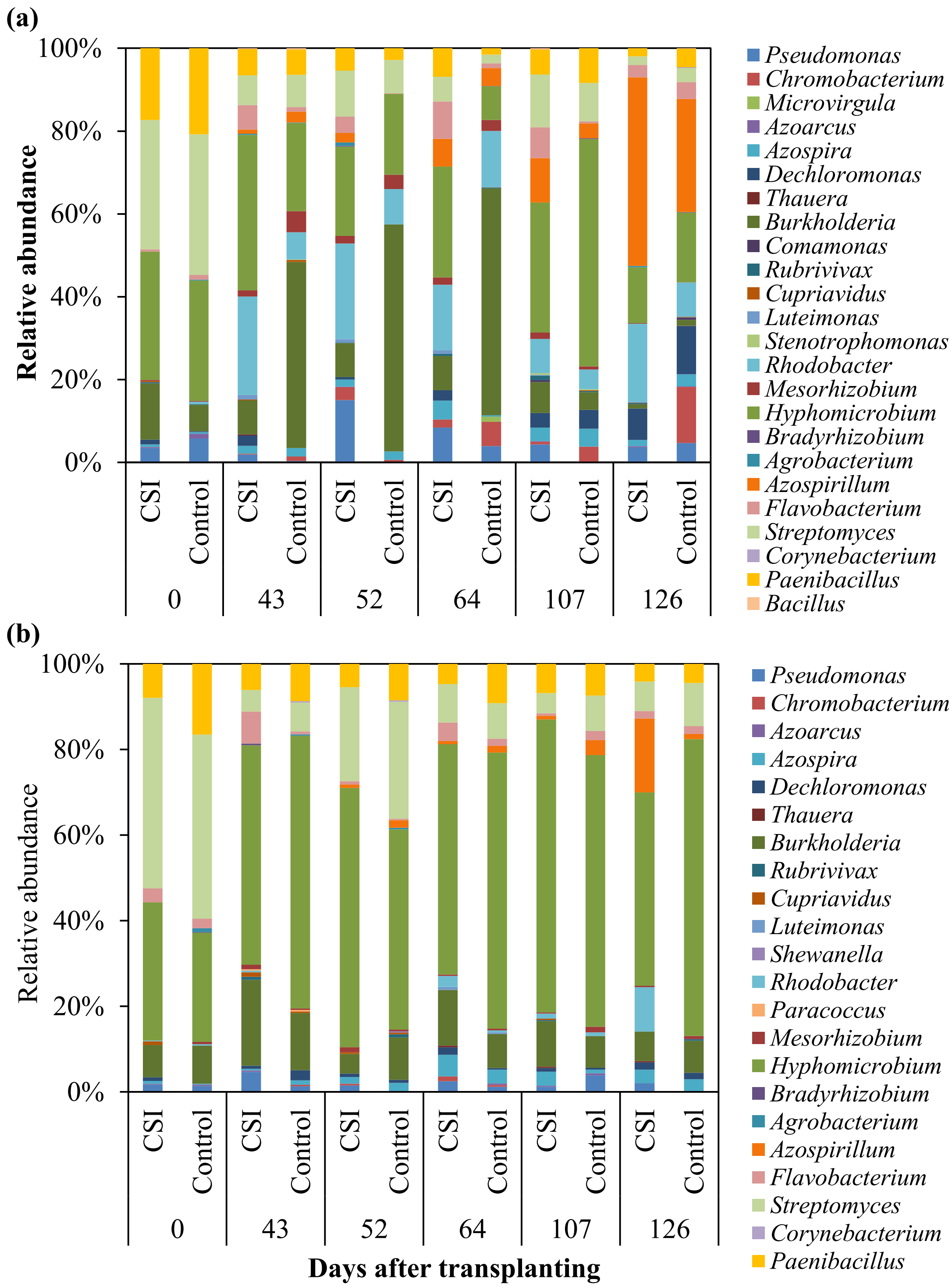

Figure 4.6 Taxonomic composition of nitrifying bacteria in the upper (a) and lower layers (b) of the paddy soils under the examined treatment during the crop season.

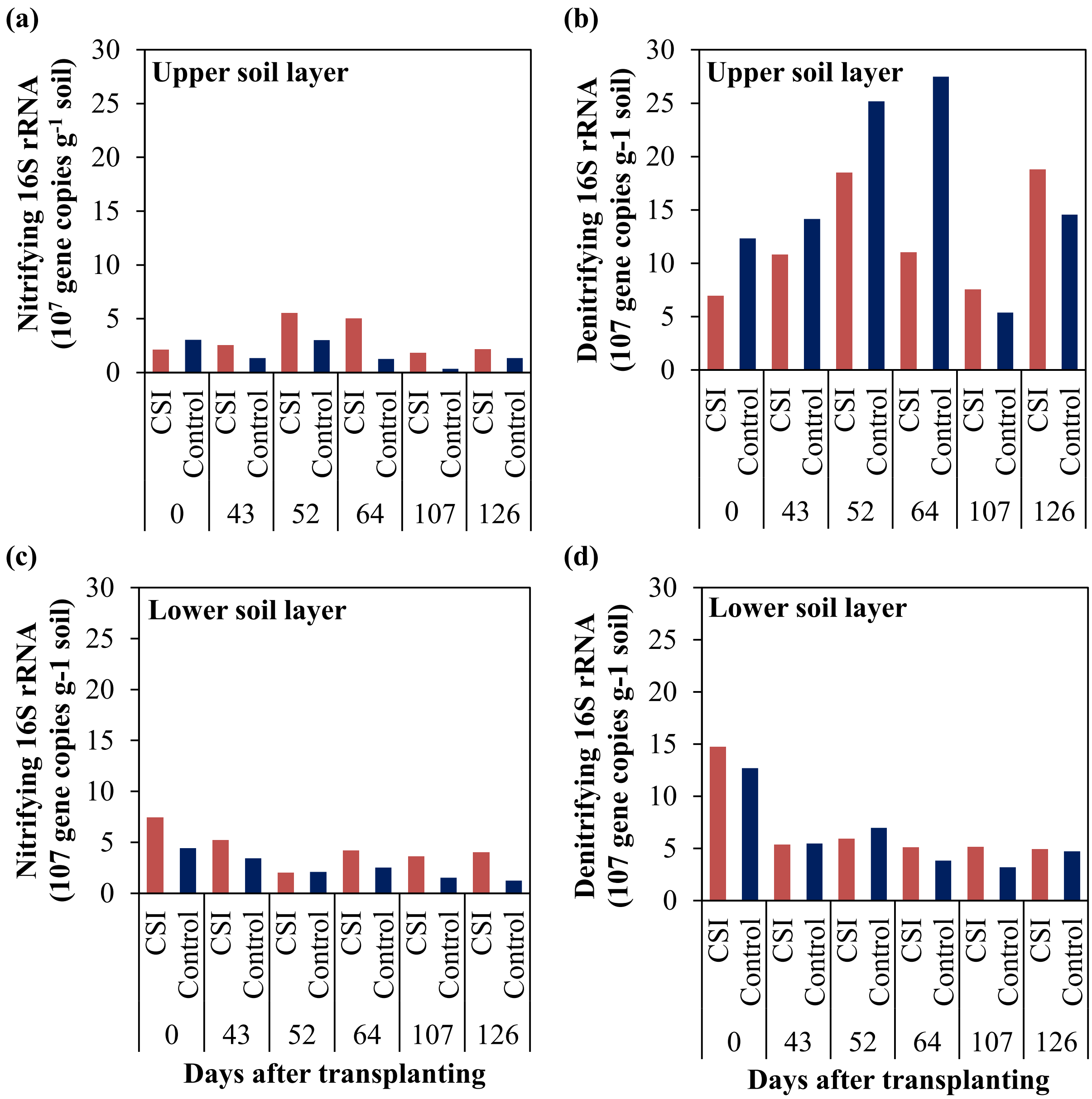

Figure 4.7 Abundance of nitrifying (a and c) and denitrifying bacteria (b and d) in the upper and lower layers of the paddy soils, respectively, under the examined treatments during the crop season.

The abundance of nitrifying and denitrifying communities varied notably during the entire crop season (Figure 4.7). Consistently in both treatments, the abundance of the nitrifying bacteria was remarkably lower than that of the denitrifying community, especially in the upper soil layer where the amounts of nitrifies in CSI and Control (3.2×10^7 and 1.7×10^7 gene copies g^{-1} soil, respectively) were vastly smaller than those of the denitrifies in CSI (1.2×10^8 gene copies g^{-1} soil) and in Control (1.7×10^8 gene copies g^{-1} soil) (Figure 4.7a, b). A similar trend was observed in the lower soil layer, where the abundance of nitrifies under CSI and Control (4.4×10^7 and 2.5×10^7 gene copies g^{-1} soil) were markedly lower than those (6.9×10^7 and 6.1×10^7 gene copies g^{-1} soil) of denitrifies in both treatments, respectively (Figure 4.7c, d). Overall, across the six sampling time points, the abundance of nitrifies under CSI was higher than that under Control in either upper or lower soil layers. In the case of denitrifiers, however, CSI had a lower abundance of the target microbes in the upper soil layer while possessing a higher abundance of microbes in the lower soil layer compared to Control (Figure 4.7b, d).

4.3.5 Physicochemical properties of the paddy soils

The basic characteristics of paddy soils under the two examined treatments were presented in Table 4.2. In respect to the texture of paddy soils at the end of the crop season, there was a slight variation in the soil faction between CSI and Control treatments. In particular, the silt content in paddy soils with CSI (23.7%) was higher than that (22.8%) under Control, while there was a minor reduction in the sand content with CSI when compared to Control. However, these differences were statistically insignificant ($p > 0.05$). There was no difference in the clay content (5.37%) between paddy soils under the two treatments. In terms of the chemical properties, relative to Control, CSI significantly increased soil pH, SOM, and SOM ($p < 0.05$) while maintaining EC and CEC comparable levels ($p > 0.05$). Compared to the initial soil prior to the crop season, soil pH under Control treatment was significantly lower pH ($p < 0.05$), whereas that under CSI was slightly higher ($p > 0.05$).

Both CSI and Control significantly reduced soil EC and CEC of the initial soil. In addition, while CSI increased the contents of SOC and SOM, the Control treatment significantly lowered the initial soil variables.

Table 4.2 Basic properties of the soil before (initial) and after the experiment under the examined cultivation treatments.

Indicator	Unit	Initial soil	CSI	Control
Clay	%	3.67 ± 0.03	5.37 ± 1.36 a	5.37 ± 1.36 a
Silt	%	29.18 ± 0.42	23.71 ± 3.25 a	22.80 ± 0.42 a
Sand	%	67.15 ± 0.44	70.92 ± 2.00 a	71.83 ± 0.95 a
pH	-	5.78 ± 0.13	6.05 ± 0.28 ^{a}	5.39 ± 0.12 ^{b*}
EC	dS m^{-1}	0.09 ± 0.00	0.06 ± 0.01 *	0.07 ± 0.03
CEC	$cmol_c$ kg^{-1}	9.25 ± 0.03	7.98 ± 0.16 *	7.94 ± 0.27 *
SOC	%	2.79 ± 0.01	2.91 ± 0.01 ^{a*}	2.63 ± 0.06 ^{b*}
SOM	%	4.99 ± 0.02	5.24 ± 0.02 ^{a*}	4.75 ± 0.12 ^{b*}

Different letters in a row indicate significant differences ($p < 0.05$) between CSI and Control, whereas the asterisks indicate significant differences ($p < 0.05$) between the initial soil and the soils after the experiment.

Table 4.3 shows the contents of macro and micronutrients in paddy soils as influenced by the two cultivation treatments. There was no significant difference in the contents of N and K ($p > 0.05$) between CSI and Control, while CSI significantly decreased P content ($p < 0.05$) compared to Control. However, it was noteworthy that CSI treatment decreased the contents of P and K by 14.4% and 7.5%, respectively. In the case of Ca and Mg, there was no notable difference between CSI and Control treatments. There was no significant difference in the content of the macronutrients (N, P, K, Ca, Mg) between the paddy soils sampled at the harvest time and the initial soils, regardless of cultivation treatments. With regards to the contents of micronutrients in paddy soils, CSI slightly increased the contents of Fe (15.6 g kg^{-1}) and Zn (57.9 g kg^{-1}) while reducing the content of B (2.0 mg kg-1) in paddy soils compared to Control (15.5 g kg^{-1}, 57.9, and 2.0 mg kg^{-1}, respectively). Both treatments maintained Mn and Mo at exactly the same contents relative to the initial soil while notably increasing Cu content in the initial soil at the same extent. However, all variations in the contents of micronutrients were not significant ($p > 0.05$).

Table 4.3 Contents of plant nutrients in the paddy soils before (initial) and after the experiment under the examined cultivation treatments.

Indicator	Unit	Initial soil	CSI	Control	ML[1]
N	g kg^{-1}	1.46 ± 0.02	1.52 ± 0.07	1.44 ± 0.05	-
P	g kg^{-1}	0.88 ± 0.11	0.77 ± 0.07 ^{b}	0.9 ± 0.04 ^{a}	-
K	g kg^{-1}	3.17 ± 0.49	2.83 ± 0.16	3.06 ± 0.07	-
Ca	g kg^{-1}	2.89 ± 0.31	2.73 ± 0.05	2.82 ± 0.03	-
Mg	g kg^{-1}	5.43 ± 0.32	5.57 ± 0.19	5.54 ± 0.05	-
Fe	g kg^{-1}	14.7 ± 1.2	15.6 ± 0.2	15.5 ± 0.4	-
Mn	g kg^{-1}	0.3 ± 0.02	0.3 ± 0.001	0.3 ± 0.008	-
B	mg kg^{-1}	2.8 ± 1.5	2.0 ± 0.2	2.3 ± 0.2	-
Mo	mg kg^{-1}	0.3 ± 0.02	0.3 ± 0.01	0.3 ± 0.01	-
Cu	mg kg^{-1}	9.0 ± 0.5	9.9 ± 0.2	9.9 ± 0.2	100
Zn	mg kg^{-1}	55.1 ± 4.8	57.9 ± 1.6	57.3 ± 0.6	300

Mean ± Standard deviation of the three replicates (n=3). Different letters in the same row indicate significant difference (p<0.05). ML: Maximum permissible limits in agricultural soil (Kamunda et al., 2016).

The possible toxic heavy metals examined in this study included As, Cd, Cr, and Pb, and their contents in paddy soils were shown in Table 4.4. CSI significantly reduced the content of As in paddy soils compared with Control treatment ($p < 0.05$). On the other hand, it tended to increase the Cr content, although this difference was not significant ($p > 0.05$). Furthermore, there was no significant difference in Cd and Pb between CSI and Control treatments ($p > 0.05$), but both treatments significantly increased the Cd content compared with the initial soil ($p < 0.05$). Despite the variation in the accumulation of the examined elements between CSI, Control and the initial soil, all the elements were recorded at levels much lower than the maximum permissible limits in agricultural soils (Kamunda et al., 2016).

Table 4.4 Contents of the examined heavy metals in the paddy soils before (initial) and after the experiment under the examined cultivation treatments.

Indicator	Unit	Initial soil	CSI	Control	ML[1]
As	mg kg^{-1}	6.4 ± 0.3	5.8 ± 0.3 [b]	6.4 ± 0.1 [a]	20
Cd	mg kg^{-1}	0.11 ± 0.01	0.13 ± 0.00 *	0.13 ± 0.00 *	3
Cr	mg kg^{-1}	23.8 ± 2.2	28.6 ± 2.8	24.9 ± 1.4	100
Pb	mg kg^{-1}	12.0 ± 0.7	12.5 ± 0.3	12.4 ± 0.2	100

Different letters in a row indicate significant differences ($p < 0.05$) between CSI and Control whereas the asterisks indicate significant differences ($p < 0.05$) between the initial soil and the soils after the experiment. ML: Maximum permissible limits in agricultural soil (Kamunda et al., 2016).

4.4 Discussion

4.4.1 Relationship between CH_4 emissions and the methanogenic and methanotrophic communities

It is well established that methanogens are obligate anaerobic microbes that produce CH_4 as an essential component of their energy metabolism, and rice paddy fields are among the most common habitats for their growth and development (Conrad, 2007). Currently, there are 33 archaeal genera representing 13 families within 6 orders that were identified as methanogens (Kim and Whitman, 2014). The methanogenic genera identified in the present study were those commonly found in rice paddy fields, sewage, sludge, soil, and freshwater sediments (Kim and Whitman, 2014). In contrast to methanogens, methanotrophs are generally classified as aerobic bacteria that can utilize CH_4 as their sole energy source (Op den Camp et al., 2009). There are 19 methanotrophic genera that have previously been described (Stein et al., 2012; Op den Camp et al., 2009), and they are frequently found at the oxic/anoxic interfaces of environments such as wetlands, aquatic sediments, and rice paddy fields, where they oxidize 10 – 90% of the CH_4 produced by methanogens in anoxic zones before the gas reaches the atmosphere (Op den Camp et al., 2009; Conrad, 2007). The methanotrophic genera identified in this study along with the above-mentioned methanogenic genera have previously been reviewed in detail (Kim and Whitman, 2014; Stein et al., 2012; Op den Campt et al., 2009; Conrad, 2007). In this study, there was no differential impact on the relative abundance of these microbes when CSI and Control treatments were compared (Figure 4.2 and 4.3). This was probably owing to the waterlogged conditions that were predominant consistently in both treatments throughout the crop season, thereby determined the soil microbial compositions in a similar fashion, despite their difference in the fertilization practices. This was in accordance with Mentzer et al. (2006) who found that flooding had a remarked greater effect than nutrient loading on altering both the composition and the functional components of soil microbial communities. This explanation was further supported by the similarity in the soil Eh and soil temperature between the examined cultivation systems during the crop season (Figure A.1). Being unique ecosystems, rice paddy fields contain various dynamic sub-habitats in which methanogenic and methanotrophic communities are differentially influenced by the soil layer profile (Conrad, 2007). The explicit dissimilarity between the upper and lower soil layers in the community structures of both microbial communities observed in this study (Figure 4.2, 4.3 and 4.4) was further supported by the non-uniform relationship between the CH_4 fluxes and the abundance of methanogenic and methanotrophic communities in the two soil layers, as indicated by the Pearson's correlation analyses shown in Table 4.5.

Table 4.5 Correlation coefficients between CH_4 fluxes and the methanogenic archaea and methanotrophic bacteria in the upper and lower soil layers across the crop season.

Parameter	**CH_4 Fluxes**	**M1**	**M2**	**M3**	**M4**
CH_4 Fluxes	1	0.15	0.70*	0.82**	-0.58
M1	0.15	1	0.13	-0.19	0.29
M2	0.70*	0.13	1	0.75**	-0.21
M3	0.82**	-0.19	0.75**	1	-0.33
M4	-0.58	0.29	-0.21	-0.33	1

M1 and M2, Methanogens and Methanotrophs in the upper soil layer, respectively; M3 and M4, Methanogens and Methanotrophs in the lower soil layer, respectively. ** Correlation is significant at $p < 0.01$; * Correlation is significant at $p < 0.05$.

The possible explanation for this dissimilarity was likely due to the specific characteristics of the different layers in paddy soils (Conrad, 2007). In particular, the gas fluxes across the crop season had a significantly positive relationship with the total abundance of methanotrophic bacteria (r = 0.7, $p < 0.05$) in the upper soil layer while possessing no significant correlation with the methanogenic community in the same soil layer, which was most likely owing to the shallow oxic surface of the flooded soil in the upper layer that is suitable for the growth of aerobic methanotrophs but not for anaerobic methanogens (Conrad, 2007). This also explained the lower abundance of methanogens in the upper soil layer compared with the lower layer, regardless of the treatments (Figure 4.4a, c). In the lower layer of paddy soils, however, the gas fluxes had no significant correlation with the methanotrophic community, while consistently possessing a strongly positive relationship ($p < 0.01$) with the total abundance of methanogenic community (r = 0.8). This phenomenon was primarily attributed to the anoxic and reduced conditions in the deep soil layer, which is suitable for the growth and development of anaerobic methanogens, but not for aerobic methanotrophs (Conrad, 2007). Previously, the significantly positive relationships between CH_4 fluxes and the methanogenic and methanotrophic communities were also reported in paddy fields by Lee et al. (2014), who found that CH_4 emissions were positively and significantly correlated with the transcripts of *mcr*A and *pmo*A genes, which were used as phylogenetic markers and a mean to calculate the abundance of methanogens and methanotrophs, respectively (Seo et al., 2014). However, these results were in contrast with Kong et al. (2019), who reported that CH_4 fluxes were significantly and positively related to *mcr*A gene copy numbers but negatively related to *pmo*A gene copy numbers.

In respect to the methanogens in the lower soil layer, the higher copy number of the methanogenic 16S rRNA genes with CSI treatment during the vegetative growth period was likely attributed to the higher input of TOC in the irrigation TWW (Table A.2), compared to the tap water used in Control. However, this difference did not translate into a notable variation in CH_4 fluxes, which were comparable between CSI (0.09, 0.44, and -0.004 mg CH_4 m^{-2} h^{-1}) and Control treatments (0.09, 1.04, and 0.37 mg CH_4 m^{-2} h^{-1}) across the first three sampling times during the vegetative stage of the rice plants (0, 44, and 52 DAT, respectively). These CH_4 fluxes were tremendously lower, when compared to those measured during the reproductive period (around 64 DAT onwards, Figure 4.1a) regardless of the treatments, which was in line with the lower and higher abundance of the methanogens in the former and later growth periods, respectively (Figure 4.4c). This was possibly because of the lower availability of the C substances in paddy soils in the first growth duration, compared with the following reproductive growth period.

Watanabe et al. (1999) demonstrated that almost 100% of CH_4 produced in the early period of rice growth originated from rice straw and soil organic matter (SOM). In this microcosm experiment, the soil was homogenized and all plant residues and other organic matter were carefully removed before potting, thus leaving the experimental soil with a relatively low content of organic matter, consequently there was less C substrate for the methanogenesis process in the rhizosphere. In addition, the low availability of C substances was also due to the decrease in the release of photosynthetic products and exudates from the rice root systems that were not fully developed from the early growth to the tillering period of the rice plants. Later in the season, however, plant photosynthesis became a more important source for CH_4 production. The high peaks recorded for CH_4 fluxes from both CSI and

Control treatments during the grain filling period (around 86 DAT onwards, Figure 4.1a), were most likely attributed to a high availability of C substances in the paddy soils. These C substances primarily originated from organic root exudates and decaying root debris (Lu et al., 2000), which were at the highest rates during the flowering period (about 80 DAT), when compared to the other growth stages of rice plants (Aulakh et al., 2001). Since the photosynthetic products derived from the root exudates were the main substrates contributing to 65 – 70% of total CH_4 emissions during this period (Watanabe et al., 1999), higher root exudation in the flowering stage could greatly stimulate the fluxes of CH_4 during the following growth period of the rice plants (Aulakh et al., 2001). During this time (from 64 DAT onwards), the lower abundance of the methanogens with CSI, relative to Control, was likely to be a result of the lower amounts of C substances that were available for the microbial metabolism. Consistent with the findings in Chapter 3, CSI could postpone the physiological senescence of rice plants as maturity approached, by maintaining high levels of leaf greenness, which were measured using the Soil Plant Analysis Development (SPAD) chlorophyll meter, compared to the Control (Figure 4.8).

As discussed previously in Chapter 3, by delaying senescence and maintaining effective photosynthesis, the rice plants under CSI are likely to have diminished the rate of root exudation and decay in the rhizosphere, leaving fewer readily available C substrates for methanogenesis. Thus, the CSI-induced mitigation of CH_4 was attributed to its inhibition of the growth of the methanogenic community in the lower soil layer during the reproductive growth period, which was likely the result of limited root exudation and root decay in the rhizosphere.

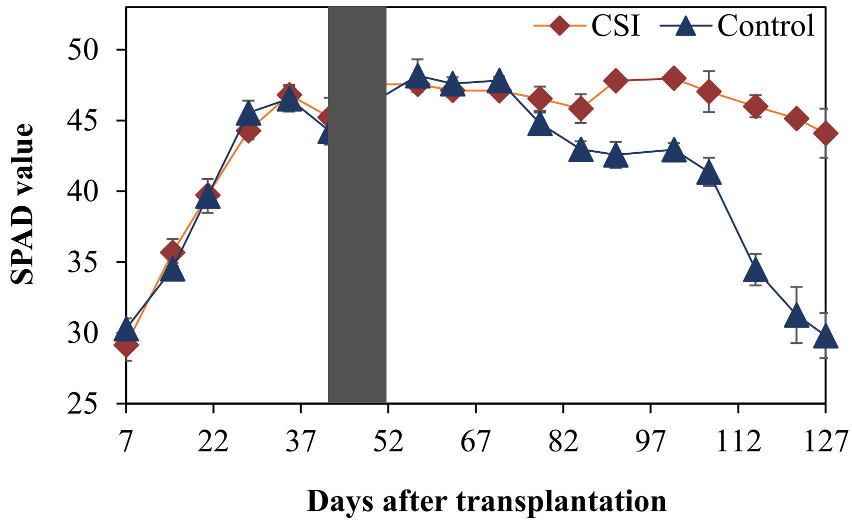

Figure 4.8 Soil plant analysis development (SPAD) value of the rice leaves under the examined treatments during the crop season. The SPAD values are the means within each treatment (n = 3). The grey belt indicates the time of the MSD period, while the error bars denote the standard deviation of the means.

With regards to the methanotrophic bacteria in the upper soil layer, relative to Control, the CSI treatment vastly reduced the abundance of microbial communities across the season, with an exception at 43 DAT. Especially, the difference was extremely prominent in the late growth period (107 – 126 DAT), when the number of methanotrophs in Control was increased by approximately 3-fold compared with CSI (Figure 4.4b). In rice paddy fields, methanotrophs are important CH_4 consumers, oxidizing substantial parts of CH_4 produced in the lower soil layer (Conrad, 2007); thus, the methanotrophy appeared to have positive responses to methanogenesis. Therefore, more CH_4 produced in the lower soil layer and diffused into the upper soil layer could be conducive to a stimulated methanotrophy process, linked with a higher abundance of the methanotrophic community in the upper soil layer.

Overall, the reduction of CH_4 emissions from the paddy fields under CSI was clearly linked with the CSI-induced inhibition of methanogenic community growth in the lower layer of the paddy soils, while the reduced abundance of the methanotrophic community was probably due to lower CH_4 production in the deep soil layer and subsequently lower CH_4 diffusion into the upper layer of the paddy soils.

4.4.2 Relationship between N_2O emissions and the nitrifying and denitrifying communities

The nitrifying microbial community consists of 8 bacterial genera identified as autotrophic nitrifiers and 5 microbial genera classified as heterotrophic nitrifiers (Robertson and Groffman, 2007). On the other hand, there are more than 60 genera of denitrifying microorganisms identified so far (Laurent et al., 2007). In this study, *Nitrosospira* was dominant within the nitrifying bacteria regardless of both treatments and soil layers. This result was a common phenomenon in most soils, especially acid soils (Prosser, 2011; Robertson and Groffman, 2007). Since denitrification is performed by a wide range of soil bacteria, a relatively higher number of denitrifying genera found in this study compared to the nitrifying community was reasonable. Interestingly, the N_2O fluxes had a significantly positive relationship with both the nitrifying and denitrifying bacteria in the lower soil layer while there was no such correlation in the upper soil layer (Table 4.6). In the lower soil layer, the higher abundance of nitrifying and denitrifying bacteria under CSI across the crop season could be attributed to higher C availability due to high TOC and OM in the TWW used for the irrigation in CSI treatment (Table A.2). However, this difference in the microbial communities did not result in a marked difference in the gas emissions between CSI and Control treatments.

Table 4.6 Correlation coefficients between N_2O fluxes and the nitrifying and denitrifying bacteria in the upper and lower soil layers across the crop season.

Parameter	N_2O Fluxes	N1	D1	N2	D2
N_2O Fluxes	1	0.04	-0.32	0.65*	0.95**
N1	0.04	1	0.15	0.12	0.18
D1	-0.32	0.15	1	-0.4	-0.24
N2	0.65*	0.12	-0.4	1	0.7*
D2	0.95**	0.18	-0.24	0.7*	1

N1 and D1, Nitrifiers and Denitrifiers in the upper soil layer, respectively; N2 and D2, Nitrifiers and Denitrifiers in the lower soil layer, respectively. ** Correlation is significant at $p < 0.01$; * Correlation is significant at $p < 0.05$.

Compared with the CH_4 emission, the low N_2O emission might be the result of slowly-processed nitrification in submerged soils, as nitrifying bacteria are obligate aerobes. Denitrification is generally regulated by the availability of O_2, soil C, and NO_3^- (Robertson and Groffman, 2007), but under the waterlogged condition in paddy fields, it was mainly limited by NO_3^- provided by nitrifiers. As a result, once nitrification is limited at the low concentration of O_2, denitrification is accordingly also low, then a low N_2O emission was recorded. The markedly high N_2O fluxes from both CSI and Control treatments during the first 4 weeks after transplantation were highly likely to be attributed to the surplus N supply that exceeded the demand for young rice plants at that time, rather than to other factors, including the abundance of nitrifying and denitrifying bacteria communities in the paddy soils. Where NH_4^+ uptake is high, the nitrification rates will be low (Robertson and Groffman, 2007). In Control, the supply of N in basal usually exceeds the demand for young rice plants during the first growing periods, resulting in much higher fluxes of N_2O

in the first month of the crop season. Although CSI may increase the availability of NH_4^+ in the paddy soil, it is likely to accelerate nitrification; however, NH_4^+ supplies usually did not exceed the plant demand, and nitrifiers are relatively poor competitor of NH_4^+ in the soil solution. The higher abundance of nitrifying and denitrifying bacteria in CSI is likely to result in the higher N_2O fluxes under CSI at the following sampling time points (70.1, 3.0, 44.4, 127.1, 35.5 μg N_2O m^{-2} h^{-1}) compared to Control (-2.64, 20.0, 17.5, 29.3, 20.8 μg N_2O m^{-2} h^{-1}, respectively). However, these emissions were negligible at a later time compared to the first month, and the total emission of N_2O under CSI was still much lower compared to the control treatment. Generally, wetlands like rice paddy fields are well known to have rich organic material sources, thereby having a great potential to function as the sinks of N due to their ability to support denitrification. In many cases, these sink areas retain reactive N produced in source areas. For example, the buffer zones next to streams can be managed to keep NO_3 moving out of crop fields in groundwater (Lowrance et al., 1984). This NO_3 can be stored in plant tissue or in SOM as organic N or can be denitrified to N gas and thereby released to the atmosphere, preferably as N_2, a nonreactive form, rather than N_2O (Lowrance et al., 1984). In this study, with remarkably lower N_2O emissions (Table 4.1) combined with the effective assimilation of N to produce high rice yield of protein-rich rice as identified in Chapter 3, rice paddy fields employing CSI may be considered a sink of N that has a high potential to remove reactive N from the environment, preventing it from moving into adjacent ecosystems. This was supported by a very high efficiency of N removal identified in this experiment (Figure A.2) as well as in the previous studies (Pham et al., 2017; Pham et al., 2019; Tran et al., 2019). Overall, it possible that the differences in N_2O emissions between CSI and Control were not likely attributed to the variations in the nitrifying and denitrifying communities, but were more dependent on the availability of N in soils and N uptake of rice plants over the entire crop season.

4.4.3 Enhanced soil fertility and no risk of heavy metal contamination in paddy soils

In respect to the basic properties of paddy soils, there were clear effects of CSI versus Control on the examined variables. Either CSI or Control treatments did not significantly change the soil texture, which was probably due to the fact that the soil composition of sand, silt and clay was relatively constant and not easily changed over a short period of time. Acidification in the soils under Control treatment due to the use of mineral fertilizers was a common phenomenon not only in rice paddy soils, but also in other soils. Interestingly, CSI increased the soil pH, which was in line with other studies reporting an increase of soil pH under wastewater irrigation due to high organic matters contained (Kunhikrishnan et al., 2017; Jahany and Rezapour, 2020). Organic substances are amphoteric substances that could act either as an acid or as a base. In this condition, the increase in soil pH under CSI may be due to the mineralization of organic substances, thereby releasing OH^- ions through ligand exchange. Other possible reasons may be due to the addition of basic cations (Ca, Mg, and Na) from the irrigation TWW used in the CSI system (Table A.2). Increasing soil pH to near neutral conditions would increase nutrient mineralization and reduce the solubility of heavy metals in soils, leading to an optimum concentration of nutrients and a low concentration of heavy metals in soil solution for plant uptake (Neina, 2019). Thus, while mineral fertilizers used in Control have acidified the soils, CSI may have neutralized soil pH and may provide a better nutrient uptake for rice plants. The lower EC recorded under CSI treatment compared to the initial soil was likely due to the release of salt ions. This could be explained by a higher concentration of Na (2-fold) in the effluent water than that in the influent TWW (Table A.3). Previously, low soil EC values were also reported after the crop season by Chaganti et al. (2020), who found that irrigation can be used to remove salts from the soil profile. However, it has generally been argued that irrigation with wastewater could increase soil salinity compared to fresh or tap water due to the high concentration of salts in wastewater (Muyen et al., 2011; Bedbabis et al., 2014; Elgallal et al., 2016; Jahany and Rezapour, 2020) and possibly worsen soil productivity and reduced crop yields. In this experiment, soil EC in both CSI or Control treatments was below the salinity threshold (4 dS m^{-1}), implying that there was no concern about the increase of soil salinity when CSI was adopted. The decreasing of CEC in both treatments compared with the initial soil was likely due to a reduction in the exchangeable K (Table 4.3) which may be due to a leaching through the effluent water in CSI treatment or to the bonding of clay and mineral particles in Control. The slightly higher CEC under CSI compared with that under Control was consistent with previous studies (Abd-Elwahed, 2019; Jahany and Rezapour, 2020) which reported that irrigation with wastewater could increase soil CEC compared to fresh or tap water. This was probably attributed to an increase in inputs of organic matter from the irrigation TWW. Higher CECs have been well known to potentially improve soil productivity, nutrient retention capacity, and the ability to keep groundwater from cation pollution. As a result, CSI seemed to make paddy soils more favorable compared with Control. This was further supported by the higher SOC and SOM in paddy soils under CSI treatment. As SOC provides energy and substrates for microbial metabolism, higher SOC and SOM can stimulate soil biological diversity and enzyme activities (Sharma and Singh, 2019), thus enhancing soil fertility.

The higher SOM and SOC under CSI were likely due to the high concentration of TOC in TWW (Table A.2) as well as the continuous irrigation practice that supplied and enhanced organic matters in paddy soils during the entire crop season.

With regard to plant nutrients, although most of the variables are comparable between the two treatments, it was noteworthy that CSI tended to increase N in paddy soils compared to Control. This may have been due to a large amount of N in TWW (Table A.2) significantly increasing the content of TN in soils, which was consistent with a number of previous reports (Arienzo et al., 2009; Abd-Elwahed, 2019). In addition, the higher soil N content under CSI was in accordance with the above-mentioned increase in soil pH induced by CSI. In general, N is naturally derived from plant and animal residues in various stages of decay, either inorganic or organic forms. Mineralization of organic matter releases N and hydroxide ions that can increase soil pH as described above. Although the difference in soil N content between CSI and Control was not statistically significant ($p > 0.05$) in this experiment, this still demonstrated that TWW irrigation alone could provide sufficient N for plant growth and development, given the high yield of rice and the higher protein content accumulated in rice grains identified in this current experiment (Table A.4) as well as the prior findings (Chapter 3). However, the zero use of mineral fertilizers in CSI treatment was probably the reagent for the lower contents of P and K in paddy soils under CSI compared with Control. Such lower P and K contents were also found in a previous study (Tran et al., 2019) and may be due to the low concentrations of these elements in the irrigation TWW (Table A.2). The higher level of P in paddy soils under Control compared to either the initial soil or the soil under CSI was the result of the high input of P fertilizers, which could have led to precipitation of P in paddy soils (Palansooriya et al., 2020). On the other hand, comparable levels of micronutrients between CSI and Control treatments, and between them and the initial soil, have shown that neither TWW irrigation nor mineral fertilizer application have had an impact on the micronutrient pool in paddy soils. Micronutrients are as important as macronutrients for rice plants, but they are only required and absorbed in small quantities by plants. Overall, despite zero use of mineral fertilizers, CSI was able to effectively maintain the soil nutrient content while increasing the organic matter content in paddy soils compared to the mineral fertilizer application.

The heavy metals examined in this study are non-essential and generally toxic for plants and animals at high levels (Phung et al., 2020). It was noteworthy that CSI surprisingly reduced As concentration of the initial soil while Control maintained it unchanged after the growing period. This was probably due to the effects of the continuous irrigation practice that could wash As ions out of paddy soils through the outflow of water effluents. In addition, Suriyagoda et al. (2018) and Kumarathilaka et al. (2018) have shown that high organic matter contains several amounts of organic acid which could reduce bioavailability of As by their binding and/or forming an insoluble complex with As in the soil. It was further documented that the content of As increased at a low pH (Abbas et al., 2018) especially under flood conditions (Wan et al., 2019). CSI maintained a higher pH compared to Control as mentioned above, which may limit the availability of As in the paddy soils to some extent. Cr is commonly present in soil in the form of either Cr(IV) or Cr(III). It was well documented that soil pH and SOM were among the most influential factors in the availability of Cr in soil. The increase in SOM can facilitate the reduction of Cr (IV) to Cr (III) which can then be completely precipitated in the soils with a pH > 5.5 (Shahid et al., 2017; Ertani et al. 2017). In view of the changes in soil pH between the two treatments

(Table 4.2), the higher Cr content in CSI treatment was reasonable. In the case of Cd and Pb, the contents of these elements in CSI and Control treatments increased significantly compared to the initial soil. The increase of Cd under Control could be the result of the application of mineral fertilizers containing phosphate (Kurbier et al., 2019; Qin et al., 2020) whereas the occurrence of Cd in the irrigation TWW (Table A.2) possibly caused the accumulation of Cd in the TWW-irrigated soil. It was generally considered that accumulation of heavy metals in the wastewater-irrigated crops was an environmental problem because these possible toxic metals tend to accumulate in the soil and could subsequently become bioavailable for crops (Toze, 2006). Although there were notable variations in the examined elements between the two treatments, the accumulation of these elements in paddy soils under either CSI or Control were clearly below the maximum permissible levels in agricultural soils recommended by WHO (2017) (Table 4.4), indicating that there was no concern about heavy metal build-up in paddy soils using CSI.

4.5 Summary

In this chapter, I examined the emission of CH_4 and N_2O linking with the community structure of methanogens, methanotrophs, nitrifiers, and denitrifiers in paddy soils as influenced by CSI for the first time in the literature. This work has verified the effectiveness in reducing CH_4 and N_2O emissions from rice paddy fields employing CSI as figured out in the prior experiment. By linking GHG fluxes with the abundance of the relevant microbes in paddy soils, the experiment has already highlighted the possible underlined microbial mechanism of GHG mitigation as influenced by CSI.

In addition, CSI has been showed to make a slightly increase in soil N content and a significantly increase in SOC and SOM while maintaining the other macro and micronutrients at comparable levels as Control treatment. The elimination of mineral fertilizers in CSI treatment appeared to be an attractive practice to reduce the production cost of forage rice, was well as to reduce the dependence of rice paddy farming on mineral fertilizers. Importantly, this experiment showed that there was no risk of heavy metal accumulation in paddy soils using CSI. However, continuous monitoring of these potentially toxic heavy metals is needed in a long-term experiment to prevent accidental contamination.

CHAPTER 5. EFFECTS OF CUO NANOPARTICLES ON RICE GROWTH AND THEIR FATE IN THE PLANT-SOIL SYSTEM UNDER CONTINUOUS SUB-IRRIGATION WITH TREATED WASTEWATER

This chapter presents findings on the performance of rice plants and changes in paddy soils subject to the CSI system in which the irrigation TWW contained CuO NPs as emerging pollutants at several hypothetical levels of contamination. By simulating different scenarios for the occurrence of CuO NPs in TWW, this chapter raises concern about the reuse criteria for TWW in agricultural irrigation in general as well as in rice paddy farming through the CSI system in particular under the circumstances of widespread use of NPs in the world.

5.1 Introduction

Increased production and widespread use of nanomaterials in different industries and products has resulted in inevitable release of metal-based NPs into the environment (Peralta-Videa et al., 2011), and therefore their effects on organisms and the environment have been of increasing concern worldwide (Tamez et al., 2019). As mentioned in Chapter 2, metal-based NPs can enter the soil through atmospheric deposition, sewage irrigation, soil amendment with sewage sludge, use of fertilizers and pesticides, etc., which is likely to make the soil, particularly farmland, a sink of NPs (Peng et al., 2020). Repeated reports of the occurrence of metal-based NPs in the water environment, especially effluents from WWTPs (Pachapur et al., 2016; Baranidharan and Kumar, 2018), suggest a critical need to revisit the wastewater reuse criteria for these emerging contaminants in order to identify and overcome emerging challenges in the decision-making process of regional and global water management.

In the previous chapters, efforts have been made to understand the advantages and possible disadvantages that CSI could cause to the environment in terms of GHG emissions and the accumulation of toxic heavy metals in rice plants and paddy soils. Adopting such irrigation practice provides an opportunity for intensive reuse of TWW in rice paddy fields, which could potentially expose rice fields to metal-based NPs and could subsequently harm plant and human health. Therefore, information on the interaction of metal-based NPs with rice plants and paddy soils using CSI is of considerable importance in order to ensure the sustainability of the CSI system.

CuO NPs have been widely used among different types of metal-based NPs due to their application in daily necessities and therefore likely to occur in sewage systems (Singh and Kumar, 2020). To date, there is a remarkable knowledge gap with respect to the occurrence of CuO NPs in rice paddy fields (Tamez et al., 2019). Most of the current studies have investigated interactions between CuO NPs and rice plants during short-term seed germination or seedling growth, in hydroponic solutions, or under very high NP exposure levels that are unlikely occur under real circumstances (Shaw and Hossain, 2013; Peng et al., 2020; Yang et al., 2020). More work is therefore needed in longer timeframes, under natural soil conditions and under a more realistic reflection of possible environmental exposures of CuO NPs to rice plants.

In this chapter, a microcosm experiment was conducted to provide insights into the performance of rice plants and the possible build-up of Cu in rice plant-soil systems under the exposure of CuO NPs contained in TWW at a number of hypothetical concentrations that are more likely to occur under realistic conditions. The specific objectives are: (1) to understand the effects of CuO NPs on plant growth, grain yield, and rice nutritional quality; (2) to measure the accumulation of Cu in rice plants and paddy soils using the CSI system; and (3) to estimate possible risks to human health through rice consumption.

5.2 Methodology

5.2.1 Experimental materials

CuO NPs in a powder form with a particle size < 50 nm (CAS No. 1317-38-0) and bulk Cu ($CuSO_4$, CAS No. 7758-98-7) were purchased from Sigma Aldrich, Ltd., Japan. The TWW used for irrigation was collected from the local WWTP indicated in the previous chapters. Rice seedlings (30-day old) of Bekoaoba and paddy soils used in this experiment were prepared at Yamagata University Farm as described in Chapter 3.

5.2.2 Experimental design and plant growth condition

A microcosm experiment was carried out at Yamagata University, Tsuruoka City, Japan, using Wagner pots (Φ252 x 300 mm) equipped with a CSI system. In this experiment, CSI systems were modified to suit the experimental pots and effectively simulate the conditions of rice paddy fields in which TWW stored in an influent tank was continuously fed into the bottom of the pots, filtered through the soil layers, and overflowed out of the pots through the outlets set at 5 cm above the soil surface (Figure 5.1).

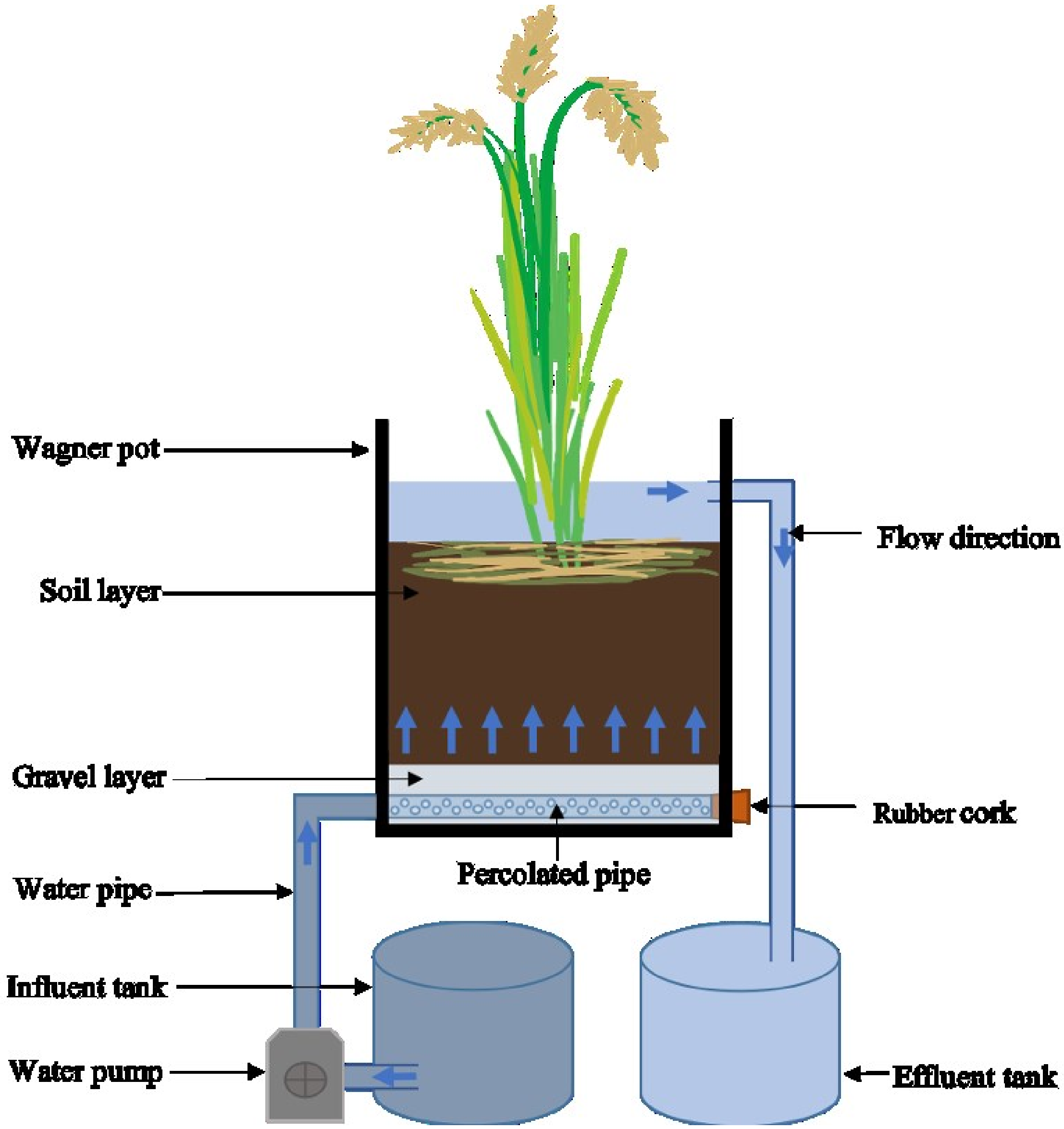

Figure 5.1 Schematic illustration of an experimental pot equipped with a continuous sub-irrigation with TWW (CSI) system.

Each pot was filled with 7.5 kg of the prepared soil and watered with TWW for stabilization for 3 days before transplantation. Five uniform seedlings were transplanted into one pot as a single rice hill on May 20th, 2020. Three DAT, TWW at different levels of Cu contamination, either as CuO NPs or as $CuSO_4$, were continuously supplied into the respective pots at a constant flow rate of 1.25 L day^{-1} (equivalent to 25 mm day^{-1}) until harvesting (approximately 133 DAT), except for a MSD at the end of the vegetative stage. All the pots were not supplemented with mineral fertilizers, and TWW served as a single source of moisture and nutrients for rice plants. The pots were placed in an open environment on a bench with a transparent roof to prevent unwanted rainfall.

Table 5.1 The examined treatment carried out in the present experiment.

Treatment	Irrigation water	Irrigation system	Contaminant source	Added levels (mg L^{-1})	Added Cu concentration (mg L^{-1})
T1	TWW	CSI	None	-	-
T2	TWW	CSI	CuO NPs	0.025	0.02
T3	TWW	CSI	CuO NPs	0.25	0.2
T4	TWW	CSI	CuO NPs	2.5	2
T5	TWW	CSI	$CuSO_4$	0.5	0.2

The CuO NP powder was directly poured into TWW at 4 concentrations, including 0 (T1-Control), 0.025 (T2), 0.25 (T3), and 2.5 mg CuO NPs L^{-1} (T4), corresponding to 0, 0.02, 0.2, and 2 mg Cu L^{-1} added to TWW, respectively. These concentrations were selected with a consideration for the Cu limit (3.0 mg L^{-1}) established for the effluent standards (FAO, 2014). In order to achieve well mixed dispersion and minimize aggregation and agglomeration, the NP solutions were shaken and ultrasonic (100W, 30 KHz) for 45 minutes prior to irrigation. In addition, the $CuSO_4$ powder was added to TWW in order to prepare a solution of 0.2 mg Cu L^{-1} (T5) which was used to compare with the CuO NP suspension at the same contamination level (T3). Overall, 5 treatments were examined in this experiment as shown in Table 5.1. The treatments were laid out in a completely randominzed design (CRD) with 3 replicates.

5.2.3 Characterization of CuO NPs and $CuSO_4$ in treated wastewater

The hydrodynamic size and the stability of the CuO NPs in the TWW used in T2, T3, and T4 were characterized by the Zetasizer Nano ZS (Malvern Panalytical Ltd.). The hydrodynamic diameter values of the NPs in the irrigation TWW were found to range from 369.8 ± 51.81 to 796.3 ± 117.3 d.nm, while the zeta potential of the NPs in TWW was fairly consistent at -17.9 – -18.0 mV (Table 5.2).

Table 5.2 Characterization of CuO NPs in the TWW used in the examined treatments

Treatment	**CuO NPs (*mg/L*)**	**Cu (*mg/L*)**	**Zeta potential (*mV*)**	**Z-average (*d.nm*)**
T1	-	-	-	-
T2	0.025	0.02	-17.9 ± 0.96	410.3 ± 104.6
T3	0.25	0.2	-20.1 ± 2.47	369.8 ± 51.81
T4	2.5	2	-18.0 ± 0.1	796.3 ± 117.3
T5	-	-	-	-

In order to investigate the roles of the dissolved Cu ions in causing effects on rice plant-soil systems, the contents of total dissolved Cu ion in the suspensions of CuO NPs and the $CuSO_4$ solution were determined. First, after preparation of the irrigation TWW containing either CuO NPs or $CuSO_4$ as mentioned above, 1 L of the prepared TWW was sampled and stored separately in 10 bottles of 100-mL. These 100 ml samples were centrifuged at 11500 rpm for 30 minutes after ultrasonic vibration dispersion. Then the supernatants were filtered through 20-nm glass filters. Subsequently, the total content of Cu in the filtrates was analyzed by a portable colorimeter (HACH DR/890 Portable Colorimeter, HACH, USA) following the instructions of the manufacture. The concentrations of Cu ions released from the added CuO NPs or $CuSO_4$ were estimated by subtracting the total Cu concentrations in the prepared TWW by the concentration of Cu in the original TWW. The Cu released from the added sources were shown in Figure 5.2.

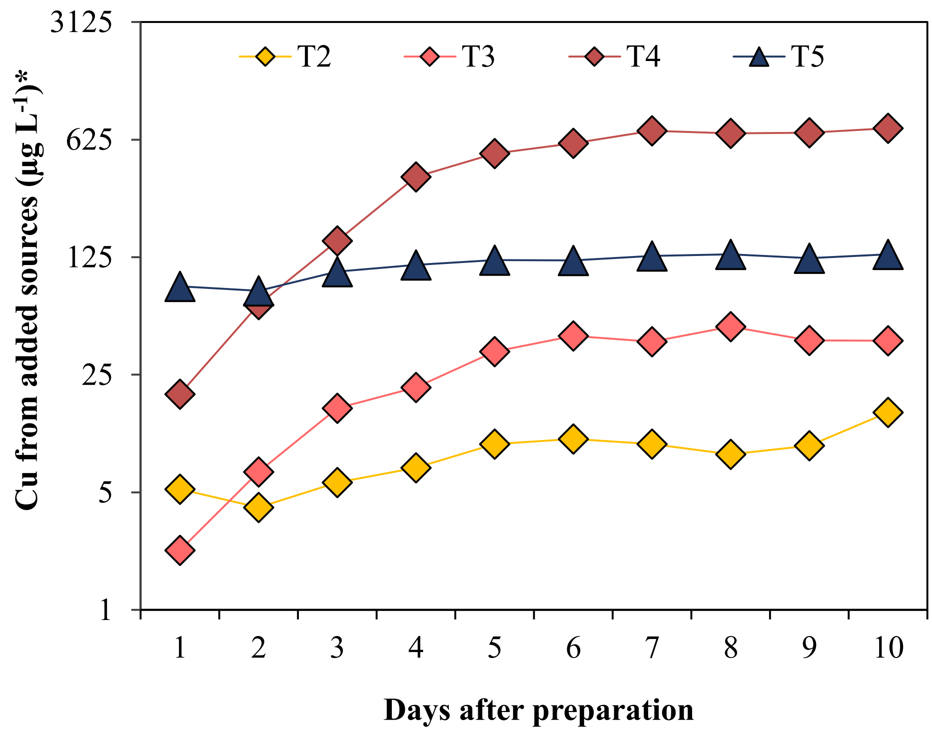

Figure 5.2 Concentrations of Cu ions released from the added CuO NPs or $CuSO_4$ in the irrigation TWW during 10 days after addition.

5.2.4 Monitoring of plant growth, grain yield, and rice protein content

Rice growth parameters, including plant height, leaf greenness (SPAD value) and tiller number, were monitored weekly while shoot dry weight, root dry weight, grain yield, yield components and rice protein content were measured at harvest time in accordance with the standard protocols (Yoshida et al., 1976). Harvested grains were air-dried at room temperature for 10 days prior to de-hulling. Grain yields and rice protein content were assessed as described in Chapter 3.

5.2.5 Measurement of Cu accumulation in rice plants and paddy soils

Brown rice and dry shoot samples were ground with the help of a lab miller, while dry root samples were cut into small pieces (< 1 cm). Samples of paddy soils were also collected from each pots after harvesting, then oven-dried at 105 °C for 48 h, ground manually using ceramic mortars and passed through a 2-mm sieve before further analysis. All the prepared samples were then digested using the same methods and protocols as described in Chapters 3 and 4.

5.2.6 Calculation of translocation, enrichment, and bioaccumulation factor

Translocation factor (TF) or mobilization ratio (Barman et al., 2000; Gupta et al., 2008) of Cu was calculated to determine the relative translocation of Cu from paddy soils to roots, from roots to shoots, and from shoots to grains using the following equation:

$$\mathrm{TF} = \frac{\text{Cgrain or shoot or root}}{\text{Cshoot or root or soil, respectively}}$$

where $C_{\text{grain or shoot or root or soil}}$ represents the concentrations of Cu in brown rice, shoots, roots, and paddy soils, respectively.

Bioaccumulation factor (BAF), the ratio of the concentration of Cu in grains to that in the corresponding paddy soils, was calculated for each treatment to quantify the bioaccumulation effect of rice plants on the uptake of Cu from paddy soils (Liu et al., 2005). The BAF was calculated as follows:

$$\mathrm{BAF} = \frac{\mathrm{Cgrain}}{\mathrm{Csoil}}$$

Enrichment factor (EF) was calculated to derive the degree of accumulation of Cu in contaminated paddy soils (T2, T3, T4, and T5) from uncontaminated soil (T1) as follows (Kisku et al., 2000):

$$\mathrm{EF} = \frac{\mathrm{Csoil\ (T2, T2, T4, or\ T5)}}{\mathrm{Csoil\ (T1)}}$$

5.2.7 Assessment of possible human health risk

Although the rice variety used in this study is intended as a feed rather than a staple food for human consumption, estimating the potential health risk due to ingestion of the harvested rice was expected to provide a meaningful reference for the future adoption of the CSI system in the cultivation of staple rice. Potential health risks associated with long-term consumption of contaminated rice was estimated using the United States Environmental Protection Agency (US EPA) Hazard Quotient (HQ) as follows:

$$\mathrm{HQ} = \frac{\mathrm{EDI}}{\mathrm{RfD}}$$

where EDI represents estimated daily intake of Cu through rice consumption, and RfD is the oral reference dose of Cu. When the HQ is valued at < 1, there is no obvious risk. Conversely, when measured at ≥ 1, the exposed population will experience health risks (Zheng et al., 2007).

The EDI was calculated using the following equation (Llobet et al., 2003):

$$\mathrm{EDI} = \frac{\mathrm{Cgrain} \times \mathrm{IR}}{\mathrm{BW}}$$

where IR is ingestion rate of rice in Japan at the average value of 308 g person^{-1} day^{-1} and BW is the individual body weight of a Japanese adult at 60 kg (MHLW, 2019).

In the absence of an oral RfD for Cu, the US EPA converted the 1.3 mg Cu L^{-1} EPA action level for drinking water to an RfD of 0.04 mg Cu kg^{-1} day^{-1}, assuming 2 L day^{-1} of water consumption and a 70 kg body weight (US EPA, 2017). In this study, the RfD of Cu was 0.044 mg kg^{-1} day^{-1}, considering the water consumption of 2 L day^{-1} and the body weight of 60 kg for Japanese adults.

5.2.8 Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) and the means of significant treatment effects were compared using Tukey's honestly significant difference test at the 5% probability level. All statistical analyzes were performed using IBM SPSS Statistics 24.0.

5.3 Results

5.3.1 Plant growth, grain yield, and rice protein content

In the examined treatments, the addition of Cu to TWW, either in the form of CuO NPs or $CuSO_4$, tended to increase number of tillers, number of panicles and shoot and root weight, while the addition of CuO NPs (T2, T3, and T4) was likely to reduce plant height compared to Control (T1) and T5 (Table 5.3). In particular, tiller number in Control was approximately 7.5% lower than the average tiller number observed in T2, T3 and T4, while only 3.3% lower than that in T5. The same trend was observed in panicle number, in which T4 remarkably increased panicle number under Control by 14%. In terms of root weight, T2, T3, and T4 increased root weight of Control by 6.3 – 42%, while T5 had a root weight of 24% higher than that in Control. It was also noteworthy that CuO NPs tended to reduce plant height compared to T1 and T5. However, all these differences were statistically insignificant ($p > 0.05$).

Table 5.3 Growth indicators of the rice plants as affected by the examined treatments.

Treatment	**Tiller number (*tillers/hill*)**	**Plant height (*cm*)**	**Panicle number (*panicle/hill*)**	**SPAD at harvest**	**Shoot weight (*g/hill*)**	**Root weight (*g/hill*)**
T1	48.7 ± 2.1	97.5 ± 2.1	35.7 ± 5.5	37.1 ± 2.1	62.6 ± 2.4	12.6 ± 2.8
T2	52.7 ± 6.7	95.8 ± 2.0	38.0 ± 2.0	36.1 ± 3.4	62.5 ± 1.9	17.9 ± 2.9
T3	49.0 ± 1.0	96.6 ± 2.6	38.7 ± 1.5	36.7 ± 1.6	64.1 ± 3.3	13.4 ± 4.1
T4	55.3 ± 0.6	94.1 ± 3.9	40.7 ± 1.5	37.2 ± 2.1	62.7 ± 1.2	15.1 ± 2.5
T5	50.3 ± 2.1	97.5 ± 2.8	37.0 ± 1.7	35.1 ± 2.6	63.5 ± 4.9	15.6 ± 3.5

The yield of brown rice and rice protein content was shown in Figure 5.3. In the same manner to the growth parameters, there was no statistically significant difference in rice yield and rice protein content among the treatments. However, it was noted that the addition of Cu in the irrigation TWW tended to increase rice yield compared to Control, regardless of the sources of Cu. In particular, T2, T3, T4 and T5 increased the rice yield of Control (74 g $hill^{-1}$) by 13.5, 8, 12.2, and 9.5%, respectively. In the case of rice protein content, the addition of Cu to the irrigation water either by CuO NPs (T2, T3, T4) or $CuSO_4$ (T5) tended to slightly reduce the protein contents of brown rice recorded in Control.

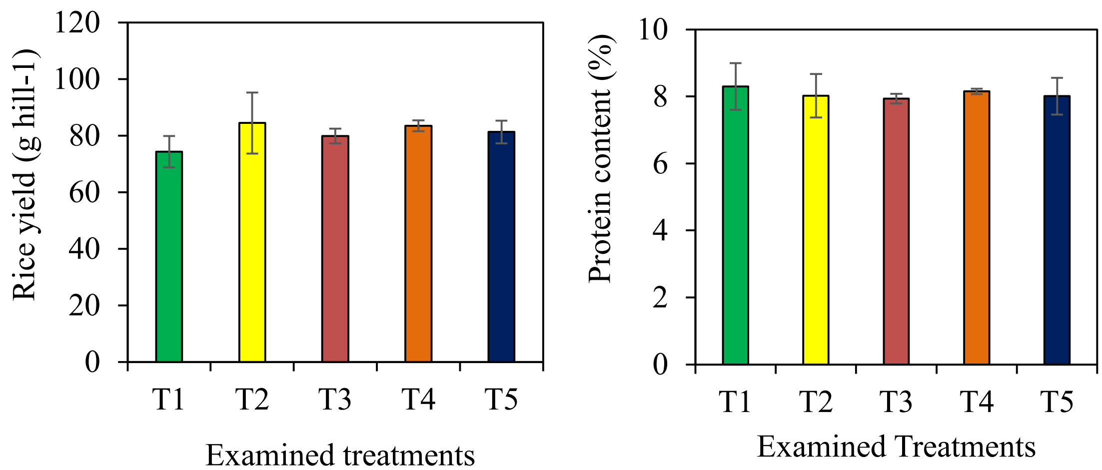

Figure 5.3 Rice yield (a) and rice protein content (b) as affected by the examined treatments.

Overall, despite notable variations in the parameters examined, the Cu contamination in the irrigation TWW either by CuO NPs or $CuSO_4$ did not cause significant effects on rice growth, yield capacity and the nutritional quality of grains.

5.3.2 Bioaccumulation of Cu in the rice plants and the paddy soils

Statistically significant differences in the content of Cu in brown rice, shoots, roots and paddy soils were observed among the treatments ($p < 0.05$), while such significant differences were not observed in the Cu contents of rice shoots ($p > 0.05$) as shown in Table 5.4. The results generally show that Cu accumulated more in roots than in shoots and grains. In both rice grains and roots, the highest content of Cu was recorded in T4, which received the highest quantity of CuO NPs added to the irrigation TWW. Among three treatments supplemented with CuO NPs, the higher the addition of CuO NPs, the higher Cu contents in rice grains and roots were observed. Interestingly, at the same concentration of Cu added (0.2 mg L^{-1}), there was no significant difference between T5 and T3 in all parts of rice plants as well as in paddy soils ($p > 0.05$). Despite the relatively low accumulation of Cu in roots, T1 and T2 had remarkably higher levels of Cu in shoots compared to the other treatments, although this difference was not significant ($p > 0.05$). The addition of Cu by either CuO NPs or $CuSO_4$ significantly increases the content of Cu accumulated in paddy soils compared to Control, especially at the highest CuO NP contamination in T4 ($p < 0.05$).

Table 5.4 Total content of Cu in the rice grains, shoots, roots, and paddy soils at the end of the crop season.

Treatment	**Cu contents in different samples** (mg kg^{-1})			
	Grain	Shoot	Root	Soil
T1	5.9 ± 0.7^{ab}	110.7 ± 67.3	34.3 ± 4.0^{b}	16.5 ± 0.6^{b}
T2	4.6 ± 0.9^{b}	92.9 ± 58.8	49.6 ± 8.6^{b}	17.3 ± 0.9^{ab}
T3	5.1 ± 0.8^{b}	24.2 ± 10.0	155.5 ± 76.9^{b}	18.2 ± 0.9^{ab}
T4	7.5 ± 0.8^{a}	18.2 ± 3.9	504.8 ± 212.5^{a}	19.5 ± 1.0^{a}
T5	4.9 ± 0.6^{b}	39.9 ± 7.5	303.9 ± 43.5^{ab}	17.9 ± 1.0^{ab}

TF is one of the main components of human exposure to potential toxic heavy metals through the food chain. TF of Cu from soils to roots ($TF_{soil-root}$) was the highest in T4 followed by T5, and T3 and T2, while $TF_{soil-root}$ recorded in Control was the lowest. Interestingly, T5 had a $TF_{soil-root}$ of 16.95, almost twice that of T3, which had the same Cu contamination level. In contrast, TF from roots to shoots ($TF_{root-shoot}$) was highest in Control, followed by T2, while the rest of the treatments, T3, T4, and T5, had relatively low $TF_{root\text{-}shoot}$ values. In the case of TF from shoots to grains ($TF_{shoot\text{-}grain}$), the highest value was recorded in T4, followed by T3 and T5, while the lower ends were observed in T2 and T1. All of the above-mentioned differences were statistically significant ($p < 0.05$, Table 5.5).

Table 5.5 Translocation factors of Cu from soils to roots, roots to shoots, and shoots to grains as affected by the examined treatments.

Treatment	**Translocation factor**			**Enrichment factor***
	Soils to roots	Roots to shoots	Shoots to grains	
T1	2.08 ± 0.32^{c}	3.39 ± 2.30^{a}	0.07 ± 0.04^{c}	1.0 ± 0.04^{b}
T2	2.89 ± 0.64^{bc}	1.96 ± 1.23^{ab}	0.06 ± 0.03^{c}	1.05 ± 0.06^{ab}
T3	8.45 ± 3.75^{bc}	0.17 ± 0.74^{b}	0.23 ± 0.09^{b}	1.10 ± 0.06^{ab}
T4	25.91 ± 11.14^{a}	0.04 ± 0.02^{b}	0.42 ± 0.06^{a}	1.18 ± 0.06^{a}
T5	16.95 ± 1.64^{ab}	0.14 ± 0.05^{b}	0.13 ± 0.03^{bc}	1.08 ± 0.06^{ab}

EFs of paddy soils under T2, T3, T4, and T5 were estimated using T1 as the reference soil for a non-contaminated soil. In this study, the maximum EF (1.18) was recorded in T4, followed by T3, T5, and T2 (1.1, 1.08, 1.05, respectively). Since EF values for all the contaminated soils were higher than 1 (Table 5.5), indicating the enrichment of Cu in paddy soils due to the contamination of Cu in the irrigation water.

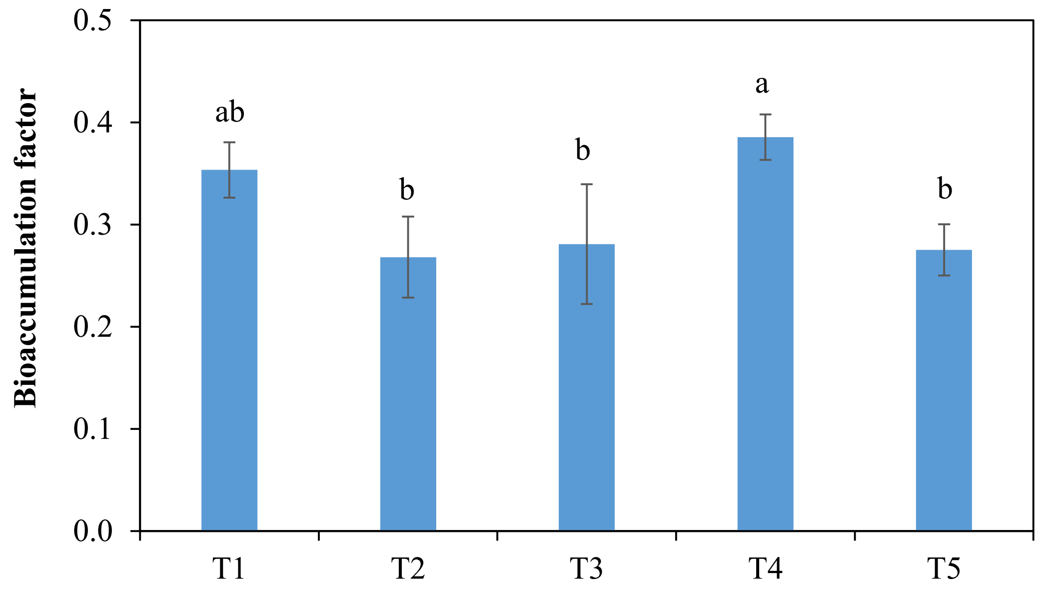

Figure 5.4 Bioaccumulation factor of Cu across the examined treatments. Different letters indicate significant ($p < 0.05$, n = 3).

BAF of Cu transferred from paddy soils to rice grains was shown in Figure 5.4. There was a significant variation in BAFs among the treatments with the highest value was accounted for T4, while T2, T3, and T5 had lower BAF values than T1 ($p < 0.05$).

5.3.3 Estimation of dietary intake and exposure risk

The highest EDI of Cu was recorded under T4 (0.039), comparable with that observed under Control (0.03) ($p > 0.05$) and significantly higher ($p < 0.05$) than EDI values under T2, T3, and T5 (0.024 – 0.026). As a result, the risk estimate (i.e. HQ value) for adults was found to be higher under T4 (0.88), followed by T1 (0.68), while other treatments, T2, T3, and T5, had HQ values at a relatively lower range of 0.54 – 0.59 (Table 5.6).

Table 5.6 Estimation of daily intake and risk (in term of hazard quotient) for the case scenario of inadvertent ingestion of contaminated rice grains.

Treatment	EDI ($mg\ kg^{-1}\ day^{-1}$)	RfD ($mg\ kg^{-1}\ day^{-1}$)	HQ
T1	0.030 ± 0.003^{ab}	0.044	0.68 ± 0.07^{ab}
T2	0.024 ± 0.005^{b}	0.044	0.54 ± 0.11^{b}
T3	0.026 ± 0.004^{b}	0.044	0.59 ± 0.10^{b}
T4	0.039 ± 0.004^{a}	0.044	0.88 ± 0.09^{a}
T5	0.025 ± 0.003^{b}	0.044	0.57 ± 0.07^{b}

5.4 Discussion

5.4.1 Effects of CuO NPs on rice performance and the paddy soils

Cu is an essential micronutrient for plant growth and plays an important role in many physiological processes, such as photosynthesis, mitochondrial respiration, carbohydrate distribution, N reduction and fixation, protein metabolism, gene regulation, signal transduction, etc. (Hopkins, 1999; Hänsch and Mendel, 2009). However, it is also well known that Cu is toxic to plants at higher levels. In this study, the effects of CuO NPs on rice plants were evaluated in comparison with the effects of a bulk Cu source, $CuSO_4$. Previously, a number of studies have been conducted to investigate phytotoxicity of CuO NPs on rice plants. For example, Yang et al. (2015) reported that CuO NPs inhibited elongation of rice roots at 2000 mg L^{-1}. In a follow-up study, Yang et al. (2020) demonstrated that under 7 days of exposure to CuO NPs at 62.5, 125, and 250 mg L^{-1}, the growth rate of rice seedlings was significantly reduced under hydroponic conditions. In addition, Costa and Sharma (2016) also reported that biomass of rice plants grown in 1000 mg L^{-1} CuO NPs suspension decreased by 31% on fresh weight and by 14% on a dry weight basis. In contrast to those findings, the present study showed comparable parameters of rice growth and yield capacity under contamination of either CuO NPs or $CuSO_4$ versus Control. Since phytotoxicity of CuO NPs in rice plants was dose-dependent (Yang et al., 2020), combined, the concentrations of CuO NPs tested in this study were much lower compared to previous studies (Yang et al., 2015; Yang et al., 2020) and were likely under the resistance of rice plants, these Cu contamination levels were not sufficient to result in clear phytotoxicity of CuO NPs. This was further supported by a slight increase in most of the plant growth parameters, including grain yield, tiller number, panicle number, shoot weight of rice plants irrigated with the Cu-contaminated TWW. This could possibly be attributed to the fact that Cu ions released from CuO NPs and $CuSO_4$ (Figure 6.2) could have been used by rice plants as micronutrients for normal growth and development (Hänsch and Mendel, 2009). Low concentrations of input Cu source could have served as a source of nutrient to rice plants rather than toxins. Interestingly, the exposure of the bulk Cu (T5) at the same concentration (0.2 mg Cu L^{-1}) did not result in any difference compared to the exposure of CuO NPs (T3) to rice plants. The slight decrease in rice protein content induced by Cu addition was probably due to the fact that the excess Cu content may have led to an ectopic binding of proteins, thereby disrupting the protein structures (Yang et al., 2006). However as mentioned above, this decrease was statistically insignificant. Overall, no visible toxic symptom was observed in rice plants under the exposures to either CuO NPs or $CuSO_4$, but the addition of Cu appeared to increase the growth and yield capacity of rice plants.

On the other hand, the difference in the internalized accumulation of Cu in the different parts of rice plants indicated a significant variation in the uptake and translocation of Cu as affected by the treatments. In particular, the content of Cu was also found to be higher in roots than in shoots and grains of most of the treatments except T1 and T2, which is corroborated by findings from previous studies (Jarvis et al., 1976; Leita et al., 1993). Yang et al. (2002) also reported that the accumulation of Cu was higher in roots, while a small fraction (10%) of the absorbed Cu was translocated to stems. The high concentrations of Cu in roots were reasonable, since the vast majority of Cu ions are bound by scavenging proteins such as metallothioneins immediately after absorption to prevent Cu from accumulating in a toxic form. This may also occur due to large amount of mucilage secreted by root tips and root hairs that contribute to the adsorption of NPs. However, part of the imported Cu may bypass this system and is captured by small binding proteins, so-called copper chaperones (O'Halloran and Culotta, 2000; Huffman and O'Halloran, 2001), which spare Cu from the detoxification systems and guide it to the target sites in the cells (Hänsch and Mendel, 2009). The higher accumulation of Cu in roots under T2, T3, T4, and T5 compared to Control was also consistent with previous studies (Costa and Sharma, 2016; Lidon and Henriques, 1998), most likely due to higher availability of Cu in soils through irrigation with the contaminated TWW. Across the systems under the treatments with CuO NPs, the higher the input of CuO NPs, the higher the accumulation of Cu in rice roots. The higher content of Cu in roots under T5 compared to T3 was probably due to the higher release of Cu ions from $CuSO_4$ compared to that derived from CuO NPs as shown in Figure 5.2. The majority of Cu accumulated in roots indicated that only a small portion of Cu was transported upwards, except in the cases of T1 and T2 which had a higher content of Cu in shoots compared to those in roots. The high content of Cu in T1 and T2 shoots was probably due to the fact that the concentration of Cu in the TWW used in these two treatments was much lower than in the rest treatments, thus increasing the absorption and transport of Cu upwards as an essential micronutrient rather than a toxicant as mentioned above. In rice grains, Cu concentrations ranged from 4.6 and 7.5 $\mu g\ g^{-1}$, which did not exceed the maximum permissible limit of 10 $\mu g\ g^{-1}$ (Hang et al., 2009), indicating that there was no serious accumulation of Cu in rice grains, although the highest Cu contamination (2 mg L^{-1}) increased the accumulation of the element in rice grains at the highest level of 7.5 $\mu g\ g^{-1}$.

The food chain (soil-plant-human) is mainly known as one of the major pathways for human exposure to soil contaminants, and soil-to-plant transfer is one key process of human exposure to toxic heavy metals (Zhuang et al., 2009). The higher the TF values, the greater the mobility/availability of metals (Khan et al., 2008; Dean, 2007). In plants, the metal translocation process is a crucial factor in determining the distribution of metals in different plant tissues (Xiong, 1998). The significant differences in TFs among the treatments examined herein suggested significant effects of CuO NPs and $CuSO_4$ added to TWW. Particularly, the highest $TF_{soil\text{-}root}$ in T4 was attributed to the highest input of CuO NPs in the irrigation water. Interestingly, $TF_{soil\text{-}root}$ in T5 was 2-fold higher than that in T3 despite the same Cu input. This was most likely due to the higher dissolution of $CuSO_4$ compared to CuO NPs as shown in Figure 5.2. $TF_{soil\text{-}root}$ in T1 and T2 was relatively low due to the low concentration of Cu in the input water. However, as soon as Cu entered the plant system, rice plants in T1 and T2 had a higher $TF_{root\text{-}shoot}$ compared to the rest treatments, probably due to the lower Cu concentration in the TWW used in these two treatments, in which rice plants in T1 and T2 could have absorbed and transported Cu upwards as an essential micronutrient and not as a toxicant. In the case of $TF_{shoot\text{-}grains}$, the higher input of CuO NPs led to a higher translocation of Cu to grains. The comparison between the effects of $CuSO_4$ (T5) and CuO NPs (T3) showed that CuO NPs had a higher translocation capability into rice grains compared to the bulk Cu. This may suggest the existence of CuO NPs themselves in rice plants, especially in the grains.

In paddy soils, the impacts of Cu addition on the accumulation of Cu in paddy soils were evident. Importantly, the slightly higher content of Cu in T3 soils compared to that in T5 soils indicated a higher retention of Cu in the NP form relative to the bulk Cu. However, the content of Cu in paddy soils recorded under the current examined treatments was within the globally range of normal soils (13 – 24 mg kg^{-1}) and was below the mean content of Cu in paddy soils (20.7 mg kg^{-1}) (Satpathy et al., 2014). EF values for all the examined treatments were greater than 1, indicating higher availability and distribution of Cu in contaminated soils, which could subsequently increase the accumulation of Cu in the plants grown in those soils (Gupta et al., 2008). The trend in BAFs was in the ranking order of T4 > T1 > T3 > T5 > T2, and all were lower than 1, indicating that rice plants absorbed Cu but did not intensively accumulate the metal in rice grains.

5.4.2 Possible impacts of CuO NPs on human health via rice consumption

Consumption of rice has been identified as one of the major pathways of human exposure to toxic heavy metals accumulated in rice grains (Satpathy et al., 2014). This study presents only HQs of Cu and all HQs under the examined treatments were found to be smaller than 1, indicating no concern for non-cancer-based health effects in adults under the consumption of rice grains. As a result, the health risk of Cu exposure through rice consumption was generally assumed to be safe. However, local residents may be at risk from a combination of several toxic heavy metals at the same time (Liu et al., 2011). This requires further investigation on HQs of other elements in rice grains under the same treatments.

5.4.3 Revisit the criteria for wastewater reuse in agricultural irrigation

Four scenarios of Cu contamination in TWW were simulated, taking into account the FAO effluent standard, i.e. all the simulated contaminations of Cu were below the effluent limit for Cu (3 mg L^{-1}). Since no adverse effect of the added Cu on the performance of rice plants has been observed and the risk to human health associated with the consumption of rice grains is likely to be negligible, it is possible to reuse TWW which meet the FAO effluent standard for agricultural irrigation, in particular for paddy rice irrigation via the CSI system.

5.5 Summary

This study was carried out using a pot experiment to determine the effects of CuO NPs contained in TWW on rice plants under the CSI system compared to the effects of a bulk Cu source, $CuSO_4$. TWW contaminated with either CuO NPs or $CuSO_4$ did not have significant effects on rice growth, yield capacity and nutritional quality of rice, likely due to low levels of contamination. In addition, there was no concern about the risks to human health associated with consumption of the rice grown under the CSI system. Overall, this study has shown that it is safe to use TWW, which meet the FAO effluent standard, for irrigation in rice paddy fields using the novel continuous sub-irrigation system.

CHAPTER 6. CONCLUSIONS

This chapter sets out the overall conclusions drawn from the findings of the previous chapters. It also discusses the limitations of the experiments carried out in this dissertation and makes recommendations for future studies.

6.1 Primary Findings

Findings from the consecutive experiments conducted herein have addressed the objectives of this Ph.D. study and provided insights into the performance of CSI in rice paddy fields on a number of important issues, including yield capacity and nutritional quality of rice grains, the efficient use of wastewater for irrigation, the use of mineral fertilizers, GHG mitigation, and the contamination of heavy metals as well as CuO NPs in rice plants and paddy soils. The main conclusions of the study are as follows:

1. CSI produced high yield of rice and a superior protein content in rice grains without the need for mineral fertilizers. This implied a promising practice for the sustainable reuse of WWTP effluents for low-cost production of protein-rich rice, which could minimize the dependence of rice production on mineral fertilizers and simultaneously purify WWTP effluents through effective assimilation of plant nutrients contained in TWW, particularly N, during the entire crop season.
2. CSI effectively reduced the emissions of CH_4 and N_2O from rice paddy fields, thus reducing the environmental footprint of paddy rice cultivation. An optimized water regime using CSI has been identified with the highest effectiveness in GHG

mitigation without compromising crop productivity and the nutritional quality of rice grains.

3. CSI significantly affected the community structure of methanogens, methanotrophs, nitrifiers, and denitrifiers in paddy soils, and these changes appeared to be closely correlated with CH_4 and N_2O emission patterns from paddy fields. The potential underlying mechanism for CSI-induced GHG mitigation was also proposed, in which the reduction of CH_4 emissions was attributed to the inhibition effect of CSI on the abundance of methanogenic and methanotropic communities in paddy soils, while the reduced emission of N_2O was most likely due to the effective plant uptake of N, leaving less N for nitrification/denitrification.
4. Despite zero use of mineral fertilizers, CSI could increase soil fertility by increasing soil pH, SOM, SOC and N content in paddy soils while maintaining unchanged other soil physicochemical properties, such as soil EC, CEC and other macro and micronutrients. However, a slight decrease in P content in paddy soils under CSI suggested regular soil analysis and supplementation of exogenous P fertilizers when needed. There was no risk of accumulation of the possible toxic heavy metals, such as As, Cd, Cu, Cr, Pb and Zn, in rice grains and paddy soils under CSI, indicating the safe use of TWW for paddy rice irrigation by the continuous sub-irrigation system.
5. There was no adverse effect of CuO NPs and $CuSO_4$, which were included in TWW used in the continuous sub-irrigation system, on rice growth and yield capacity, and there was no concern for non-cancer-based health effects on adults under rice consumption. This indicated that the reuse of effluents that met the FAO effluent

standard to irrigate rice paddy field was relatively safe for plants, soils, and human. However, a significant increase in the accumulation of Cu in rice grains, roots and paddy soils contaminated with high levels of CuO NPs may raise concerns about Cu contamination in the plant-soil system and subsequently, may have an impact human health through the food chain under long-term TWW irrigation, which is always potentially exposed to CuO NPs as well as other metal-based NPs in the sewage system.

6.2 Limitations of This Study and Future Work

There have been some limitations encountered in this study. First, CSI systems have been examined in microcosm scales rather than in real field conditions, which may limit the adoption of CSI in the real world. Follow-up experiments should therefore be carried out in field scales to verify the advantages of CSI as identified in the microcosm experiments discussed in this dissertation, thus motivating local farmers to adopt CSI in their rice farming systems.

Secondly, the abundance and composition of soil microbial communities examined in this study was estimated using one soil composite for each treatment rather than with multiple microbial assays. Combined, patterns of GHG emissions from rice paddy fields are strongly associated with changes in other soil physicochemical properties throughout the crop season, including soil pH, DO, porosity, N status, and SOM (Wang et al., 2019; Fan et al., 2015; Seo et al., 2014). In view of these limitations, follow-up research should be extended to include gas emissions linked to dynamic changes in microbial communities, as well as variations in soil physicochemical properties in real-scale paddy fields, throughout the crop season.

Third, the effects of CuO NPs on rice plant-soil systems have been evaluated in a single crop season, which may not reflect the actual circumstances of the potential contamination of CuO NPs as well as other metal-based NPs in sewage systems and, subsequently, their fate in paddy fields once CSI is employed over a long period of time. Therefore, the long-term effects of metal-based NPs on rice plants and paddy soils under CSI should be investigated in order to avoid the build-up of heavy metals in the rice paddy ecosystem.

Forth, the health risk assessment conducted in this study focused solely on the contamination of Cu in rice grains without taking into account other possible toxic heavy metals such as As, Cd, Cr, Pb and Zn, thus possibly underestimating human health risks associated with the rice consumption. Therefore, the future study should also estimate the HQs of not only Cu but also the heavy metals mentioned above in order to assess the hazard index (HI), which is a tool for long-term risk assessment of residues of toxin in food commodities, thereby ensuring the safety of CSI in rice paddy cultivation.

APPENDIX

Table A. 1 Basic properties of the TWW used for irrigation during the 2018 crop season.

Parameters	Unit	May	Jun	Jul	Aug	Sep	Mean
pH		7.5	7.2	7.5	7.4	7.0	7.3
EC	mS m^{-1}	70	63	68	61	54	63
DO	mg L^{-1}	4.7	4.3	3.2	3.4	4.6	4.0
TOC	mg L^{-1}	6.2	5.8	6.0	5.5	5.4	5.8
TN	mg L^{-1}	37	33	27	22	21	28
TP	mg L^{-1}	0.7	0.5	0.4	0.6	0.9	0.6
K	mg L^{-1}	9.2	9.5	11.0	9.5	9.2	9.7
As	µg L^{-1}	0.2	0.4	0.2	0.4	0.5	0.3
Cr	µg L^{-1}	1.0	0.6	0.9	0.5	0.6	0.7
Cu	µg L^{-1}	9.4	8.7	8.5	7.9	8.1	8.5
Cd	µg L^{-1}	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Pb	µg L^{-1}	5.4	4.2	2.9	1.3	0.6	2.9
Zn	µg L^{-1}	42	49	56	50	30	45

Table A. 2 Basic properties of the TWW used for irrigation during the 2019 crop season.

Properties	**Unit**	**May**	**Jun**	**Jul**	**Aug**	**Sept**	**Mean**	**RL**[1]
pH		7.2	7.1	6.5	7.3	6.5	6.9	6.5 – 8.4
EC	dS m^{-1}	0.7	0.6	0.6	0.6	0.6	0.6	<0.7
DO	mg L^{-1}	1.4	1.5	2.5	2.2	2.2	1.9	NA
ORP	mV	310.0	273.0	269.5	262.0	269.0	276.7	NA
TN	mg L^{-1}	31.5	28.2	26.0	27.1	28.5	28.3	60
TOC	mg L^{-1}	6.9	4.3	6.1	6.1	5.8	5.8	NA
P	mg L^{-1}	0.2	0.1	0.1	0.3	0.3	0.2	15
K	mg L^{-1}	17.1	17.1	18.2	18.6	18.5	17.9	30
Mg	mg L^{-1}	1.8	1.7	1.9	2.4	2.0	2.0	NA
Ca	mg L^{-1}	20.1	21.4	23.3	27.7	26.2	23.7	NA
Na	mg L^{-1}	66.0	61.1	65.0	56.6	60.9	61.9	<69
B	µg L^{-1}	77.5	78.8	66.1	67.7	62.2	70.5	<700
Mn	µg L^{-1}	30.6	50.7	46.1	50.0	50.4	45.6	200
Fe	µg L^{-1}	88.9	83.9	72.6	115.9	97.6	91.7	5000
Cu	µg L^{-1}	29.1	21.1	24.9	25.5	18.2	23.7	200
Zn	µg L^{-1}	70.0	54.9	57.4	54.7	49.1	57.2	2000
Mo*	µg L^{-1}	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7	10
Cr	µg L^{-1}	0.9	0.6	0.8	5.2	1.3	1.8	100
Cd*	µg L^{-1}	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	10
Pb	µg L^{-1}	0.7	0.9	0.7	0.9	0.9	0.8	5000
As	µg L^{-1}	0.1	0.2	0.5	0.3	0.4	0.3	100

Table A. 3 Basic properties of the effluent water under CSI during the 2019 crop season.

Properties	Unit	June	July	August	September	Mean
pH		6.7	7.5	8.1	8.1	7.6
EC	dS m^{-1}	1.21	0.46	0.58	0.75	0.75
DO	mg L^{-1}	5.0	4.7	4.2	4.4	4.6
ORP	mV	289.5	242.9	278.2	237.3	262.0
TN	mg L^{-1}	8.0	1.8	1.2	0.9	3.0
TOC	mg L^{-1}	29.1	6.3	9.5	10.7	13.9
P	mg L^{-1}	1.0	1.6	0.0	0.0	0.7
K^*	mg L^{-1}	10.1	1.2	< 0.3	< 0.3	5.7
Mg	mg L^{-1}	7.9	3.1	3.6	3.3	4.5
Ca	mg L^{-1}	50.8	19.7	31.2	28.6	32.6
Na	mg L^{-1}	123.5	58.1	129.9	112.3	105.9
B	µg L^{-1}	47.9	45.7	50.5	48.2	48.1
Mn	µg L^{-1}	558.5	17.5	2.6	3.3	145.5
Fe	µg L^{-1}	134.5	138.5	268.4	434.9	244.1
Cu	µg L^{-1}	18.7	7.3	7.7	8.1	10.5
Zn	µg L^{-1}	66.6	35.5	59.1	41.2	50.6
Mo^*	µg L^{-1}	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7
Cr	µg L^{-1}	1.3	1.0	0.9	1.2	1.1
Cd^*	µg L^{-1}	0.5	< 0.1	< 0.1	< 0.1	0.5
Pb	µg L^{-1}	1.2	0.8	1.1	0.6	0.9
As	µg L^{-1}	0	0	0	0.0	0.0

Table A. 4 Yield, yield components, and rice protein content as affected by the examined treatments in the 2019 crop season.

Treatment	Panicles number m^{-2}	Filled grains panilce^{-1}	1000-grain weight (g)	Yield (t ha^{-1})	Protein (%)
CSI	551.3 ± 23.6 ^{a}	62.3 ± 2.3 ^{a}	35.2 ± 0.4	10.7 ± 0.7	11.1 ± 0.01 ^{a}
Control	683.8 ± 30.9 ^{b}	54.8 ± 1.6 ^{b}	34.5 ± 0.1	11.4 ± 0.2	9.3 ± 0.43 ^{b}

Different letters in the same column indicate significant difference ($p < 0.05$), while no letter indicate non-significant difference ($p > 0.05$).

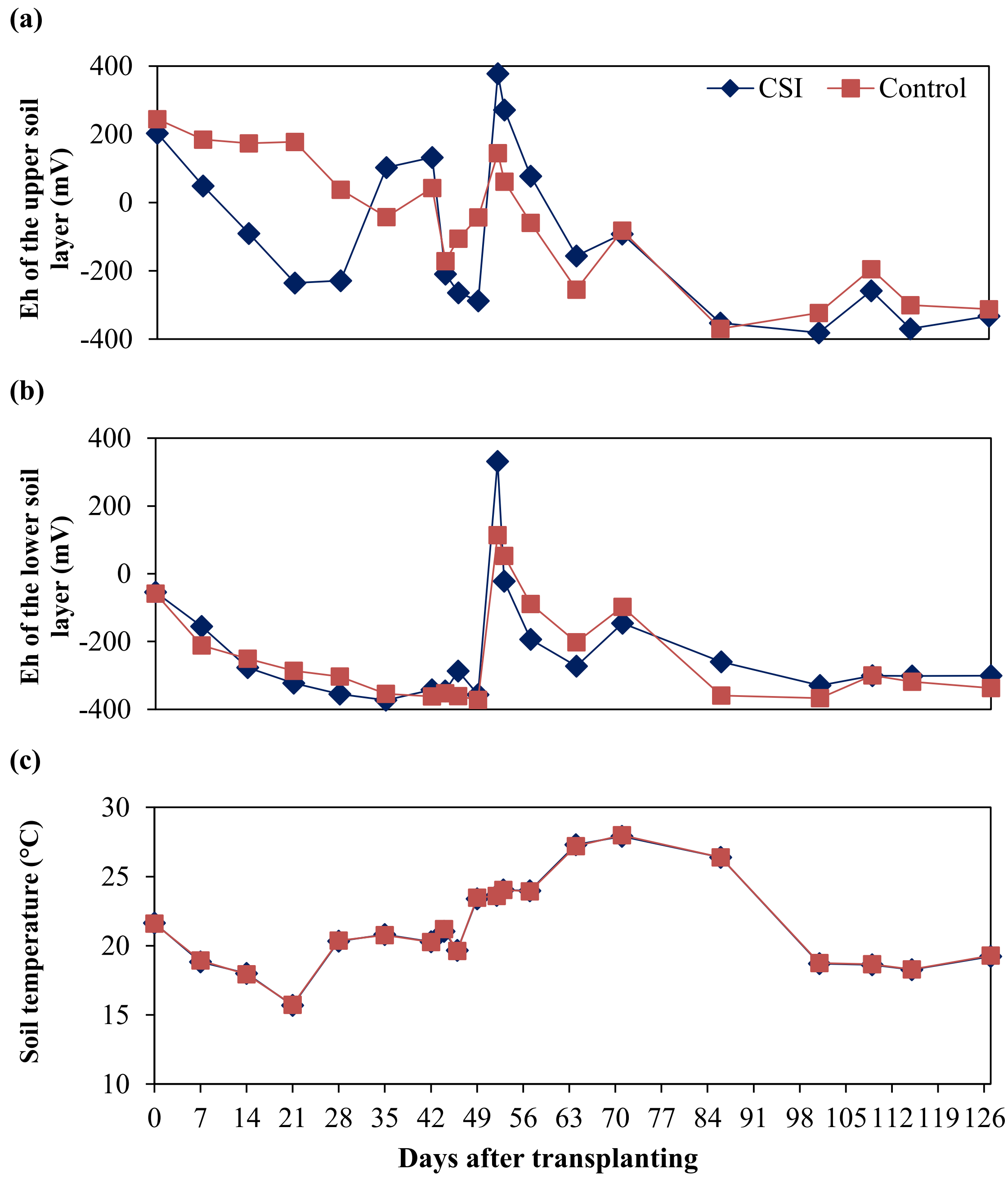

Figure A. 1 Soil Eh in the upper (a) and lower layers (b) and soil temperature (c) in the paddy fields under the two examined cultivation practices during the crop season.

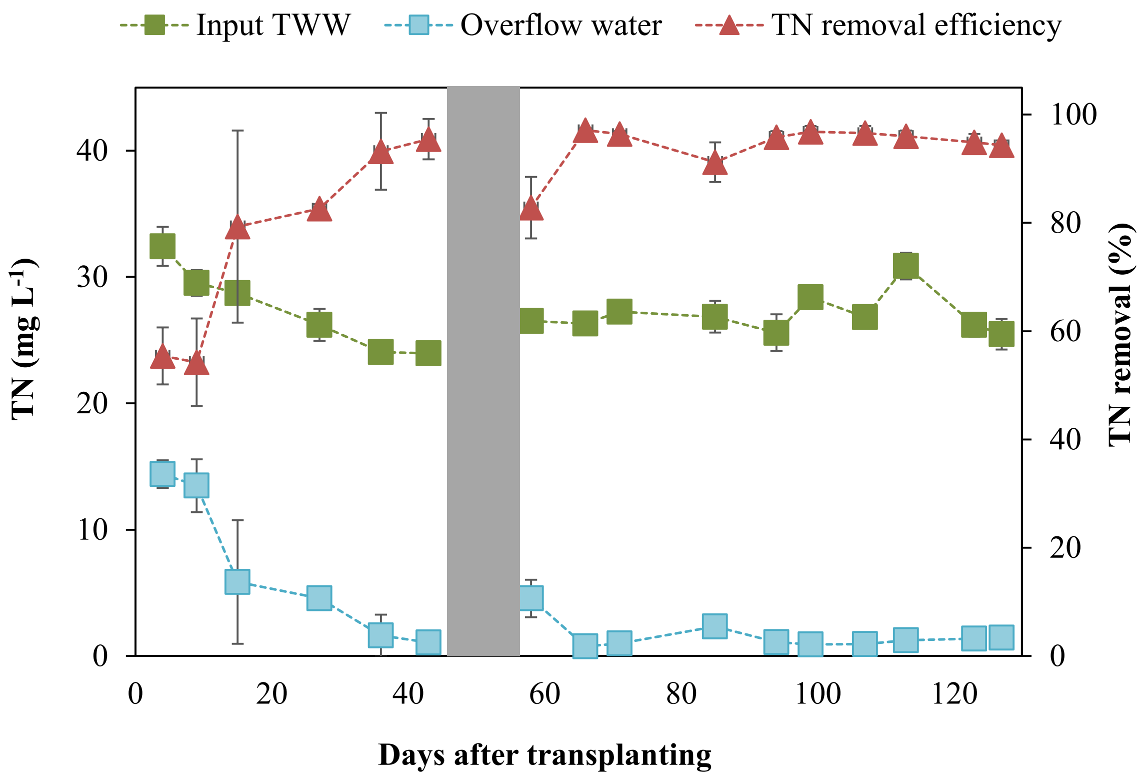

Figure A. 2 Concentration of total N (mg L^{-1}) in the irrigation TWW and effluent water from CSI treatment, and N removal efficiency (%) of the CSI system during the crop season. The grey belt indicates the MSD period.

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ACHIEVEMENTS

Journal Papers

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Awards

1. First Price Award in the Young Water Reuse Professionals Development and Integration Workshop, at the 12th IWA International Conference on Water Reclamation and Reuse, Berlin Germany, 16 – 20th June 2019. Awarded presentation: "*Development of a natural treatment coupled with UV disinfection for cost-effective reuse*".

International Conferences

1. ○Luc Duc Phung, Dung Viet Pham, Shuhei Masuda, Fumiaki Takakai, Masateru Nishiyama, Toru Watanabe. Reduction of greenhouse gas emissions from paddy fields in response to continuous irrigation with treated municipal wastewater. The 12th IWA International Conference on Water Reclamation and Reuse, Berlin, Germany, 16-20th June 2019.
2. Hiroyuki Arichi, ○ Toru Watanabe, Luc Duc Phung, Masateru Nishiyama, Hiroyuki Kato, Dung Viet Pham. High yield and nutritional quality of forage rice (Oryza sativa) achieved by continuous irrigation of treated municipal wastewater without synthetic fertilizers in pilot- and real-scale experiments. The 12th IWA International Conference on Water Reclamation and Reuse, Berlin, Germany, 16-20th June 2019.
3. ○Luc Duc Phung, Dung Viet Pham, Lanh Danh Tran, Toru Watanabe. Cultivation of Forage Rice Using Treated Municipal Wastewater. The 1st International Symposium on Recent Trend/Technology of Food Security and Management in Asia, Tsuruoka, Japan, 15th March 2019.

4. ○Luc Duc Phung, Dung Viet Pham, Toru Watanabe. Reduction of Greenhouse Gas Emissions from Paddy Fields in Response to Continuous Irrigation with Treated Wastewater. Symposium on Management of Land and Water Resources: Studies in Europe and Asia, Tsuruoka, Japan, 21st November 2018.
5. ○Lanh Danh Tran, Dong Duy Pham, Luc Duc Phung, Dung Viet Pham, Masateru Nishiyama, Toru Watanabe. High yield and nutritional quality of rice (Oryza sativa) for animal feed achieved by continuous irrigation of treated municipal wastewater. PAWEES & INWEPF International Conference 2018, Nara, Japan, 20-22nd November 2018.
6. Dong Pham Duy, Dung Pham Viet, ○Luc Duc Phung, Toru Watanabe. High yield and nutritional quality of a forage rice (Oryza sativa) achieved by continuous irrigation of treated municipal wastewater without supplementation of synthetic fertilizers. International Symposium on C and N Dynamics by Land Use and Management Changes in East and Southeast Asian Countries, Tsuruoka, Japan, 10-12th September 2018.
7. ○Toru Watanabe, Luc Phung Duc, Lanh Tran Danh, Megumi Ichikawa, Masateru Nishiyama, Dong Pham Duy, Dung Pham Viet. Cultivation of protein-rich forage rice with continuous irrigation of treated municipal wastewater. International Symposium on C and N Dynamics by Land Use and Management Changes in East and Southeast Asian Countries, Tsuruoka, Japan, 10-12th September 2018.
8. ○Lanh Tran Danh, Luc Phung Duc, Dung Pham Viet, Dong Pham Duy, Masateru Nishiyama, Toru Watanabe. A pilot-scale experiment of treated municipal wastewater irrigation to achieve high yield and nutritional quality of forage rice.

International Symposium on C and N Dynamics by Land Use and Management Changes in East and Southeast Asian Countries, Tsuruoka, Japan, 10-12th September 2018.

Domestic Conferences

1. 〇Luc Duc Phung, Shuhei Masuda, Dung Viet Pham, Fumiaki Takakai, Toru Watanabe, More protein-rich rice with less greenhouse gas emissions under continuous sub-irrigation with treated wastewater, The Water and Environment Technology Conference (WET2020-online), 7-8th November 2020.
2. 〇Luc Duc Phung, Dung Viet Pham, Ayumi Ito, Toru Watanabe, Replacing synthetic fertilizers in rice cultivation with composted sewage sludge and liquid fertilizers extracted from surplus activated sludge，第 54 回日本水環境学会年会，令和２年３月１６～１８日，盛岡市
3. 〇渡部徹，Phung Duc Luc，宮澤優彰，増田周平，高階史章，Pham Viet Dung，下水処理水を連続灌漑する水田からの温室効果ガスの放出，第 54 回日本水環境学会年会，令和２年３月１６～１８日，盛岡市
4. 〇竹田壮太，増田周平，大野剛，高階史章，岡野邦宏，宮田直幸，Phung Luc，Pham Dung，渡部徹，酒造好適米栽培における下水処理水の最適利用方法の開発に向けた実験的検討，第 54 回日本水環境学会年会，令和２年３月１６～１８日，盛岡市
5. 〇Luc Duc Phung, Dung Viet Pham, Masateru Nishiyama, Toru Watanabe，Continuous irrigation with treated municipal wastewater effectively reduces

greenhouse gas emissions from paddy fields，第 53 回日本水環境学会年会，平成３１年３月７～９日，山梨県甲府市

6. ○Lanh Danh Tran，Luc Duc Phung，Dong Duy Pham，Atsushi Sasaki，Dung Viet Pham，Masateru Nishiyama，Toru Watanabe，Pilot-scale experiment using treated municipal wastewater irrigation to achieve high yield and nutritional quality of feed rice，第 53 回日本水環境学会年会，平成３１年３月７～９日，山梨県甲府市

7. ○渡部徹，Pham Viet Dung，Phung Duc Luc，Mila Siti Fatimah，Muhammad Agung Basyar，伊藤歩，汚泥濃縮液と汚泥コンポストを併用した高タンパク飼料用米の栽培，第 57 回下水道研究発表会，令和２年８月１８～２０日，大阪市

8. ○北林怜，増田周平，高階史章，岡野邦宏，大野剛，Phung Duc Luc，Pham Viet Dung，渡部徹，酒米栽培における下水処理水の投入負荷量が生育特性および環境負荷に及ぼす影響，令和元年度土木学会東北支部技術研究発表会，令和２年３月７日，秋田市

9. ○渡部徹，Phung Duc Luc，宮澤優彰，西山正晃，増田周平，高階史章，Pham Viet Dung，下水処理水灌漑で飼料用米を栽培する水田からの温室効果ガス放出と関連微生物群の解析，令和元年度土木学会東北支部技術研究発表会，令和２年３月７日，秋田市

10. ○竹田壮太，増田周平，児玉雅，岡野邦宏，Phung Duc Luc，Pham Viet Dung，渡部徹，下水処理水の投入負荷量が酒造好適米の生育特性および

品質におよぼす影響，第５６回下水道研究発表会，令和元年８月６日，横浜市

11. 〇市川恵，Phung Duc Luc，Tran Danh Lanh，Pham Viet Dung，渡部徹，高タンパク飼料用米栽培における下水汚泥コンポストの有用性，平成 30 年度土木学会東北支部技術研究発表会，平成３１年３月２日，仙台市

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