Dan A. 2026. Chapter 2: Optimization of Substrate-Plant Combinations in Horizontal Subsurface Flow Constructed Wetlands and Their Microbial Responses: Enhancement of Livestock Wastewater Treatment and Greenhouse Gas Emission Reduction. In Thi Thuong Nguyen and Rongxuan Wang (eds.), *Perspectives on Wastewater Treatment Technology for Sustainable Pig Farming: Comparative Studies on Problem Structures in China, Japan, and Vietnam*. Osaka: Asia-Japan Research Institute, Ritsumeikan University. ISBN 978-4-910550-16-9

Chapter 2
Optimization of Substrate-Plant Combinations in Horizontal Subsurface Flow Constructed Wetlands and Their Microbial Responses: Enhancement of Livestock Wastewater Treatment and Greenhouse Gas Emission Reduction

Dan A

Abstract: In this study, six different substrate-plant combinations of horizontal subsurface flow constructed wetlands (CWs) were implemented using two functional substrates (ceramsite and blast furnace slag) and three plant conditions (*Canna indica*, *Typha orientalis*, and unplanted). The primary aim was to evaluate these CWs for their ability to purify livestock wastewater and to measure their greenhouse gas emission fluxes. Subsequently, through 16S rRNA gene sequencing and molecular biological analysis, the mechanisms influencing livestock wastewater purification and greenhouse gas emission reduction in these CW combinations were elucidated. The results showed that the removal efficiencies of chemical oxygen demand (COD), total nitrogen (TN), ammonia nitrogen (NH_4-N), and nitrate nitrogen (NO_3-N) in livestock wastewater by the CWs ranged from 33–44%, 70–87%, 82–98%, and 25%–80%, respectively. The emission fluxes of Carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) varied between 2.05–88.8 mg/m^2/h, 0.12–7.55 μg/m^2/h, and 2.19–8.89 mg/m^2/h, respectively. Although the unplanted CWs produced lower greenhouse gas emission,

the planted CWs demonstrated superior wastewater purification performance. Notably, the CWs featuring ceramsite and *Canna indica* (CS+CI-CWs) exhibited the best overall performance in both wastewater treatment and greenhouse gas reduction. This was partly due to the higher abundance of aerobic and anaerobic bacteria in the CS+CI-CWs, which facilitated simultaneous nitrification and denitrification processes, thereby providing favorable conditions for the complete conversion of nitrogen to N_2. Furthermore, the increased abundance of nosZ gene in the CS+CI-CWs promoted N_2 production, effectively reducing N_2O emission flux and thus lowering the global warming potential.

Keywords: *horizontal subsurface flow constructed wetlands, substrate-plant combinations, livestock wastewater purification, greenhouse gas reduction, microbial response*

1. Introduction

CWs remove pollutants from water primarily through plant uptake, substrate adsorption, and microbial transformation (de Oliveira Souza et al. 2023). Due to their stable treatment performance, low cost, and low energy consumption, they have been widely used in recent years for treating various types of wastewaters, including livestock wastewater (Hu et al. 2020; Parde et al. 2021). However, CWs involve biotransformation processes of carbon and nitrogen, which inevitably release greenhouse gases such as CO_2, CH_4, and N_2O, thus having a negative environmental impact (Cocozza et al. 2023). Particularly, livestock wastewater, characterized by high organic loads and high nutrient content, is more prone to greenhouse gas production, exacerbating global warming (Hu et al. 2020). Therefore, it is necessary to understand how substrate-plant combinations in CWs can be

optimized to balance the purification efficiency of livestock wastewater with the control of greenhouse gas emissions.

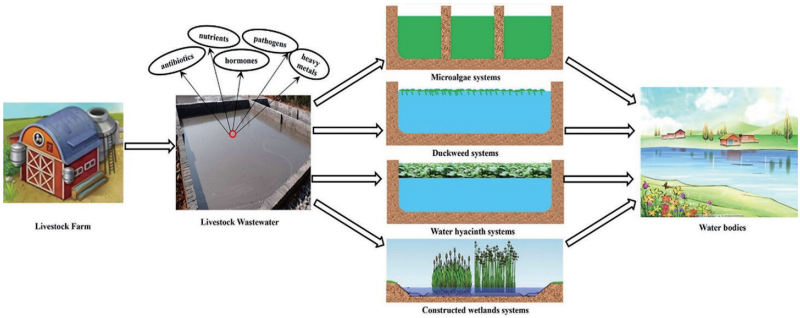


Figure 1. Sustainable livestock wastewater treatment via phytoremediation: Current status and future perspectives. Source: (Hu et al. 2020)

The presence of plants in CWs not only helps remove pollutants from water but also plays a crucial role in regulating the production, consumption, and transmission of greenhouse gases (Sun et al. 2013). Research on the impact of plants on greenhouse gas emissions shows significant differences, sometimes even yielding contradictory conclusions (Gu et al. 2015; Xu et al. 2023). Furthermore, different types of plants vary in their effects on greenhouse gas emissions from CWs (Maltais-Landry et al. 2009). The substrate is also a core component of CWs and plays an indispensable role in the wastewater purification process (Mlih et al. 2020). The type of substrate and the order in which it is filled can affect the pH level of the wetland system, plant growth, and the structure of the microbial community (Ding et al. 2018), thereby indirectly influencing the emission of greenhouse gases. There is significant interaction among these factors, and the combined effects of these interactions determine the purification function and

greenhouse gas emission capabilities of the CWs (Chen et al. 2020). Hence, it is necessary to reveal how the substrate-plant combinations in CWs affect the treatment of livestock wastewater and the flux of greenhouse gas emissions.

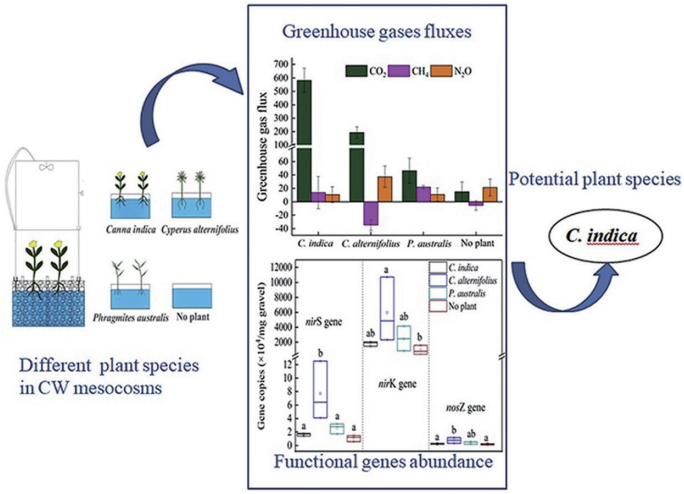


Figure 2. Greenhouse gas emissions and wastewater treatment performance by three plant species in subsurface flow CW mesocosms. Source: (Chen et al. 2020)

The aims of this study are to (a) compare the pollutant removal efficiencies and greenhouse gas emission fluxes in different CWs, and to select substrate-plant combinations that excel in both water purification and greenhouse gas reduction; (b) combine 16S rRNA gene sequencing with molecular biology analysis to explain how these combinations of CWs affect the purification of livestock wastewater and the reduction of greenhouse gas emissions.

2. Materials and Methods

The synthetic livestock wastewater used in this experiment was artificially prepared, and the formulation of its components is as follows. The prepared synthetic wastewater was stored in a storage tank and was fed into the CWs in batches with a hydraulic retention time of 24 hours. The average concentrations of COD, TN, NH_4-N, and NO_3-N in the synthetic wastewater are 348±40.6 mg/L, 25.5±4.36 mg/L, 19.7±3.72 mg/L, and 11.5±2.50 mg/L, respectively. These values are close to the known compositions of real livestock wastewater (Knight et al. 2000), indicating that the synthetic wastewater effectively simulates the real wastewater.

The CW was set up on January 22, 2022, within the Zhongkai University of Agriculture and Engineering in Guangzhou, China. The wetland units utilized polyethylene tanks; common substrates such as sand, pebbles, and gravel were used as the base, while novel substrates such as blast furnace slag (BFS) and ceramsite (CS) were used as functional substrates. Common wetland plants *Canna indica* and *Typha orientalis* were selected as test plants. Based on the different functional substrates and plant species, six CW treatments were set up (Figure 3): CS-CW and BFS-CW with ceramsite and blast furnace slag as functional substrates, respectively. CS+CI-CW and BFS+CI-CW were planted with *Canna indica*, CS+TO-CW and BFS+TO-CW were planted with *Typha orientalis*, and CS+UP-CW and BFS+UP-CW had no plants. The planting density was 12 plants per unit. Each treatment had two replicates.

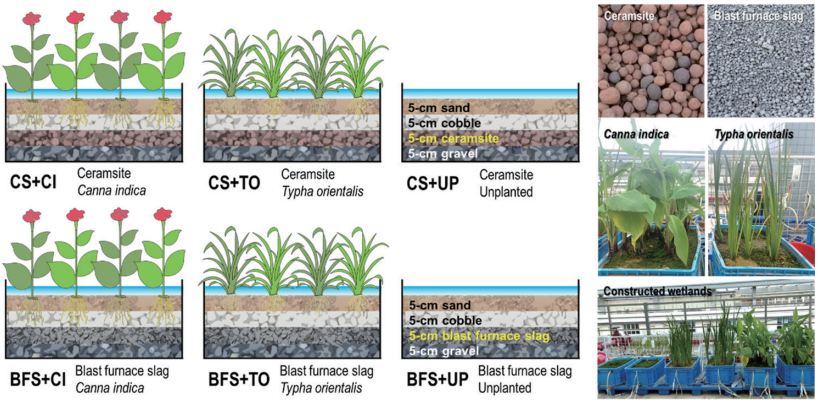


Figure 3. Schematic diagram and pictures of the horizontal subsurface flow CWs. Source: Author

Water samples were collected from April 1 to June 22, 2022, totalling 20 sampling events. The physicochemical parameters measured in the water samples were water temperature and pH. The pollutants measured were concentrations of COD, TN, NH_4-N, and NO_3-N. Greenhouse gas samples were collected from February 28 to June 24, 2022, totalling 15 sampling events. The gases measured were CH_4, CO_2, and N_2O concentrations, which were then converted to corresponding gas emission fluxes. After the collection of gas and water samples, microbial samples were collected on June 28, 2022, for 16S rRNA sequencing. The microbial samples were taken from the biofilm of the substrate near the rhizosphere of the planted systems and from the corresponding locations of the substrate in the unplanted systems.

3. Results and Discussion

(1) Purification of Livestock Wastewater

Figure 4 shows the removal efficiencies of water quality parameters in different CWs: COD, TN, NH_4-N, and NO_3-N. The average removal efficiencies for COD, TN, NH_4-N, and NO_3-N are 38±5%, 80±7%, 92±6%, and 62±20%, respectively. All CWs demonstrate high nitrogen and low carbon removal efficiencies for livestock wastewater. Among these, the type of substrate does not significantly affect the pollutant removal performance of the CWs. However, planted systems, particularly those with *Canna indica*, significantly enhance the removal efficiencies of TN and NO_3-N.

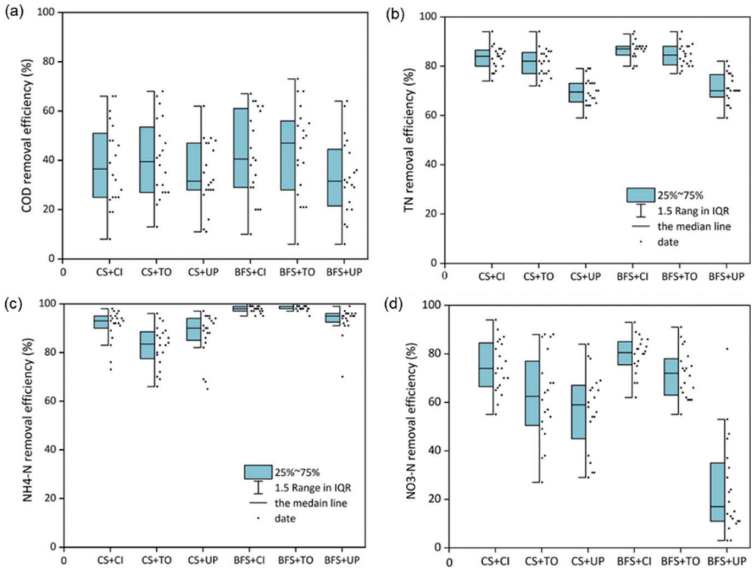


Figure 4. Removal efficiencies of water quality parameters in different CWs: (a) COD, (b) TN, (c) NH_4-N, and (d) NO_3-N. Source: Author

By comparing the correlation between the top 10 most abundant bacterial phyla and environmental factors, it was found that the relative abundance of *Bacteroidota* is significantly negatively correlated with

the removal efficiency of NH_4-N ($p < 0.05$), while *Chloroflexi* and *Planctomycetota* are significantly negatively correlated with the removal efficiency of TN ($p < 0.05$). Generally, these bacteria are mainly associated with the decomposition of organic matter and are not greatly related to nitrogen transformation processes. However, the high carbon and nitrogen pollution load in livestock wastewater, along with the low carbon and high nitrogen removal effectiveness in CWs, leads to an extremely high C/N ratio (30–60) in the wetlands. Although a high C/N ratio is favorable for denitrification reactions, it may lead to a drop in O_2 levels, thereby affecting the nitrification process and other oxygen-dependent biochemical reactions.

By comparing the correlation between the top 10 most abundant bacterial genera and environmental factors, we found that the relative abundances of *Ralstonia* and *Sediminibacterium* are significantly negatively correlated with the removal of TN ($p < 0.05$), while the relative abundance of *Sphingomonas* is significantly negatively correlated with the removals of COD and TN ($p < 0.05$), as well as the emission fluxes of CO_2 and N_2O ($p < 0.05$). These bacteria have the highest relative abundances in unplanted systems. On one hand, these bacteria may compete with nitrogen-transforming microbes for ecological niches and O_2 consumption, thus impacting the removal effectiveness of COD and TN. This phenomenon is particularly pronounced in unplanted systems lacking plant roots and root oxygenation. On the other hand, the decreased removal performance of organic matter and nitrogen in unplanted systems leads to reduced production of CO_2 and N_2O during decomposition, hence the removal efficiencies of COD and TN show a significant positive correlation with the emission fluxes of CO_2 and N_2O ($p < 0.05$).

(2) Reduction of Greenhouse Gas Emission

Figure 5 shows the emission fluxes of greenhouse gases: CO_2, CH_4, and N_2O. The average emission fluxes for CO_2, CH_4, and N_2O are 46.3±37.1 mg/m^2/h, 2.99±2.76 μg/m^2/h, and 5.52±2.84 mg/m^2/h, respectively. All CWs demonstrate high CO_2 and N_2O emission and low CH_4 emission. The type of substrate does not significantly affect the greenhouse gas emission flux in CWs, but the global warming potential (GWP) of CS-CWs is less than that of BFS-CWs. The presence of plants increases the greenhouse gas emission flux and enhances the GWP. The greenhouse gas emission flux and GWP of CI-CWs are lower than those of TO-CWs.

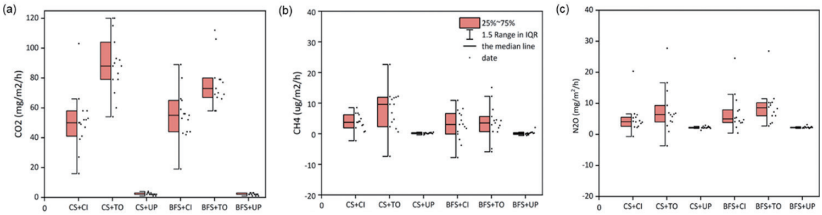


Figure 5. Emission fluxes of greenhouse gases in different CWs: (a) CO_2, (b) CH_4, and (c) N_2O. Source: Author

The pmoA and mcrA genes encode enzymes that respectively participate in the conversion of CH_4 to methanol and the reduction of compounds to produce CH_4. Both the abundance of the pmoA gene and the pmoA/mcrA ratio are higher in CS-CWs than in BFS-CWs; within CS-CWs, the unplanted systems show significantly higher pmoA gene abundance and pmoA/mcrA ratios compared to planted systems; correspondingly, these CWs with higher pmoA gene abundance and pmoA/mcrA ratios also have lower CH_4 emission fluxes. Methanotrophs prefer neutral to slightly acidic environments, and the typically higher

alkalinity of blast furnace slag is not conducive to the growth and activity of these bacteria, resulting in a reduced abundance of the pmoA gene.

The nosZ gene encodes a nitrous oxide reductase that participates in the production of N_2, while the nirS and nirK genes encode nitrite reductases involved in the production of N_2O. The abundance of the nosZ gene and the nosZ/(nirS+nirK) ratio are higher in CS-CWs than in BFS-CWs; these ratios and abundances are higher in unplanted systems than in planted systems; correspondingly, these CWs with higher nosZ gene abundance and nosZ/(nirS+nirK) ratios also have lower N_2O emission fluxes. Blast furnace slag is alkaline and contains heavy metals that hinder the growth of microbes like denitrifying bacteria. In contrast, ceramsite balances pH well and doesn't contain heavy metals, making it better for microbial growth. This is why the nosZ gene is more abundant in CS-CWs. Also, plants can increase oxygen in the rhizosphere, possibly limiting anaerobic denitrifying microbes. In unplanted systems, the lower oxygen conditions can better support these microbes, enhancing nosZ gene activity and reducing N_2O emissions.

4. Conclusion

(a) In summary, the bacterial community structure in CWs is influenced by the combination of different substrates and plants. The presence of ceramsite and plants promotes bacterial enrichment, while the diversity of rhizosphere bacteria is primarily affected by the species of plant. Unplanted systems enrich more specific bacteria, which may inhibit the removal of organic matter and nitrogen by competing for limited ecological niches and dissolved oxygen, thereby directly reducing the emissions of CO_2 and N_2O. Among these, CS+CI-CWs exhibit the best performance in terms of diversity and abundance of

bacterial communities, providing favorable conditions for the effective biodegradation of pollutants in livestock wastewater.

(b) Systems with ceramsite and those without plants have higher nosZ gene abundance and nosZ/(nirS+nirK) ratios, which help reduce N_2O emissions. Although unplanted systems have lower greenhouse gas emissions, their effectiveness in purifying livestock wastewater is also lower. Overall, CS+CI-CWs perform the best in both livestock wastewater treatment and greenhouse gas reduction. This is partly due to the higher abundance of aerobic and anaerobic bacteria in CS+CI-CWs, which facilitates the simultaneous processes of nitrification and denitrification, providing favorable conditions for the complete transformation of nitrogen to N_2. Moreover, the higher nosZ gene abundance in CS+CI-CWs promotes the production of N_2, effectively reducing N_2O emission flux and thereby lowering the GWP.

References

Chen, X., H. Zhu, B. Yan, B. Shutes, D. Xing, G. Banuelos, R. Cheng and X. Wang. 2020. Greenhouse Gas Emissions and Wastewater Treatment Performance by Three Plant Species in Subsurface Flow Constructed Wetland Mesocosms. *Chemosphere*, 239, 124795.

Cocozza, C., C. Di Iaconi, S. Murgolo, A. Traversa, F. De Mastro, M. De Sanctis, V. G. Altieri, C. Cacace, G. Brunetti and G. Mascolo. 2023. Use of Constructed Wetlands to Prevent Overloading of Wastewater Treatment Plants. *Chemosphere*, 311(2), 137126.

de Oliveira Souza, A., J. K. Brasil Bernardelli, L. de Carvalho Silva, F. Perretto, A. Nagalli, V. Mymrin, F. Hermes Passig and K. Q. de Carvalho. 2023. Plasterboard Sheet Applied in Vertical-Flow Constructed Wetlands: Nutrients Removal, Microbial Diversity, and Mechanisms. *Journal of Water Process Engineering*, 53, 103811.

Ding, X., Y. Xue, Y. Zhao, W. Xiao, Y. Liu and J. Liu. 2018. Effects of Different Covering Systems and Carbon Nitrogen Ratios on Nitrogen Removal in Surface Flow Constructed Wetlands. *Journal of Cleaner Production*, 172, 541–551.

Gu, X., K. Chen and C. Fan. 2015. Preliminary Evidence of Effects of *Phragmites australis* Growth on N_2O Emissions by Laboratory Microcosms. *Ecological Engineering*, 83, 33–38.

Hu, H., X. Li, S. Wu and C. Yang. 2020. Sustainable Livestock Wastewater Treatment Via Phytoremediation: Current Status and Future Perspectives. *Bioresource Technology*, 315, 123809.

Knight, R. L., V. W. E. Payne Jr., R. E. Borer, R. A. Clarke Jr. and J. H. Pries. 2000. Constructed Wetlands for Livestock Wastewater Management. *Ecological Engineering*, 15(1-2), 41–55.

Maltais-Landry, G., R. Maranger, J. Brisson and F. Chazarenc. 2009. Greenhouse Gas Production and Efficiency of Planted and Artificially Aerated Constructed Wetlands. *Environmental Pollution*, 157(3), 748–754.

Mlih, R., F. Bydalek, E. Klumpp, N. Yaghi, R. Bol and J. Wenk. 2020. Light-Expanded Clay Aggregate (LECA) as a Substrate in Constructed Wetlands: A Review. *Ecological Engineering*, 148, 105783.

Parde, D., A. Patwa, A. Shukla, R. Vijay, D. J. Killedar and R. Kumar. 2021. A Review of Constructed Wetland on Type, Treatment and Technology of Wastewater. *Environmental Technology & Innovation*, 21, 101261.

Sun, H., C. Zhang, C. Song, S. X. Chang, B. Gu, Z. Chen, C. Peng, J. Chang and Y. Ge. 2013. The Effects of Plant Diversity on Nitrous Oxide Emissions in Hydroponic Microcosms. *Atmospheric Environment*, 77, 544–547.

Xu, P., M. Jiang, I. Khan, M. Shaaban, J. Zhao, T. Yang and R. Hu. 2023. The Effect of Upland Crop Planting on Field N_2O Emission from Rice-Growing Seasons: A Case Study Comparing Rice-Wheat and Rice-Rapeseed Rotations. *Agriculture, Ecosystems & Environment*, 347, 108365.

2. Dr. Dan A



Chapter 2. Optimization of Substrate-Plant Combinations in Horizontal Subsurface Flow Constructed Wetlands and Their Microbial Responses: Enhancement of Livestock Wastewater Treatment and Greenhouse Gas Emission Reduction

Dr. Dan A is an Associate Professor at Zhongkai University of Agriculture and Engineering, China, with a Ph.D. in Environmental Engineering from the University of Osaka, Japan. Her research focuses on the rhizosphere micro-interface processes of emerging pollutants and their molecular response mechanisms. She also explores ecological remediation technologies for agricultural non-point source pollution, including livestock wastewater. Dr. Dan has successfully led 9 national, provincial, and municipal projects and has published 36 research papers in these areas.