A training report of

JICA's KCCP for the Project for Development of the Duckweed Holobiont Research Values towards Thailand BCG Economy (Be-HoBiD)

on

Wastewater treatment using duckweed holobiont

May 29 – September 7, 2023

Submitted by

Ms. Karnjana Ruenpham

Research Assistant, Faculty of Science, Kaseartsart University

Introduction

Duckweeds are small, free-floating aquatic plants widely distributed in freshwater ecosystems, belonging to the *Lemnaceae* family and consist of five genera: *Spirodela, Landoltia, Lemna, Wolffia,* and *Wolffiella*. “Duckweeds” is the collective term used to refer to all members of this family. These duckweeds are likely to serve as the primary producers in freshwater ecosystems. They could be used for culinary purposes, animal feed, functional food, biofuel production, and wastewater treatments.

According to the morphology of duckweeds, which have leaves (usually called fronds) and elongated fine roots that provide a habitat for many other microorganisms, the assemblage of a duckweed host and many other microbes is called holobiont. This duckweed holobiont plays a crucial role in duckweed growth and wastewater treatment, in which bacteria associated with duckweed can improve its performance and production. Therefore, the purposes of this course are:

- To learn a series of basic techniques and knowledge necessary for studying duckweed culture.
- To learn a series of basic techniques and knowledge necessary for studying wastewater treatment using duckweed holobiont.
- To learn a series of basic techniques and knowledge necessary for studying the isolation of benefit bacteria for wastewater purification and enhancement of duckweed holobiont-based wastewater treatment.

The course duration started from May 29 to September 7, 2023, at the Interdisciplinary Center for River Basin Environment, University of Yamanashi.

1. Duckweed culture (May 29 – June 14, 2023)

Due to their fast-growing ability, sterile duckweed culture (including *Spirodela polyrhiza*, *Lemna minor*, and *Wolffia globosa*) should be subcultured periodically within a growth chamber with regulated temperature, humidity, and a light-dark cycle, as shown in Figure 1. The used media include Hoagland’s medium and a 1/10 strength solution of Hutner’s medium (1/10 Hutner’s medium). Duckweed culture grown in 1/10 Hutner’s medium exhibits greater growth due to its richer nutrient content compared to Hoagland’s medium. Therefore, the choice of media depends on the purpose of the study. 1/10 Hutner’s medium is suited for maintaining a healthy duckweed culture, while Hoagland’s medium is suited for investigating the benefit bacteria associated with duckweed species.

Figure 1. Duckweed culture in a growth chamber with regulated temperature, humidity, and a light-dark cycle.

2. Wastewater treatment using duckweed holobiont (June 15 – 29, 2023)

To demonstrate wastewater treatment using the duckweed holobiont, *Spirodela polyrhiza* is transferred to the beakers containing wastewater. These treatments are cultured in a growth chamber for five days. Each day, the growth of *S. polyrhiza* is monitored and the water quality is assessed by collecting samples for later analysis of Total Organic Carbon (TOC) and ammonium-nitrogen content.

As the wastewater is nutrient-rich, *S. polyrhiza* from both beakers can grow rapidly within five days. The water surface on day 5 (Figure 2B) has more duckweed canopy than at the beginning of the experiment (Figure 2A). When observed from a side view, as shown in Figure 2C, *S. polyrhiza* forms more than two or three layers. These observations suggest that *S. polyrhiza* can thrive and grow quickly within wastewater. However, the concentration of wastewater is the limiting factor, and further studies are needed.

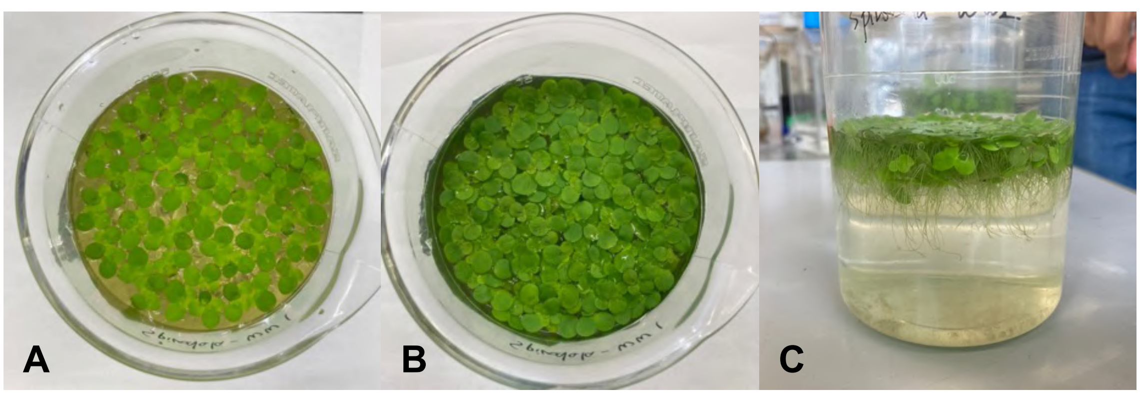

Figure 2. Wastewater treatment using *Spirodela polyrhiza* holobiont on (A) day 0 and (B and C) day 5.

To investigate the wastewater treatment ability of duckweed holobiont, the water quality is assessed each day for Total Organic Carbon (TOC) and ammonium-nitrogen content. TOC refers to the organic carbon dissolved in water, serving as an energy source for bacteria. This bacterial consumption can lead to a reduction in dissolved oxygen in the water body. Similarly, a high nitrogen concentration can lead to eutrophication, which also reduces dissolved oxygen levels. Ammonium-nitrogen or ionized ammonia is used to indicate nitrogen contamination in wastewater.

Figure 3 demonstrates the results of water quality indicators that the TOC and ammonium content decrease each day, suggesting that duckweed holobiont can be effectively used for wastewater treatment.

However, this investigation only supports the wastewater treatment ability of duckweed holobiont. The specific bacteria strains and the wastewater purification mechanism are still unknown, and further studies of duckweed-holobiont-based wastewater treatment are needed.

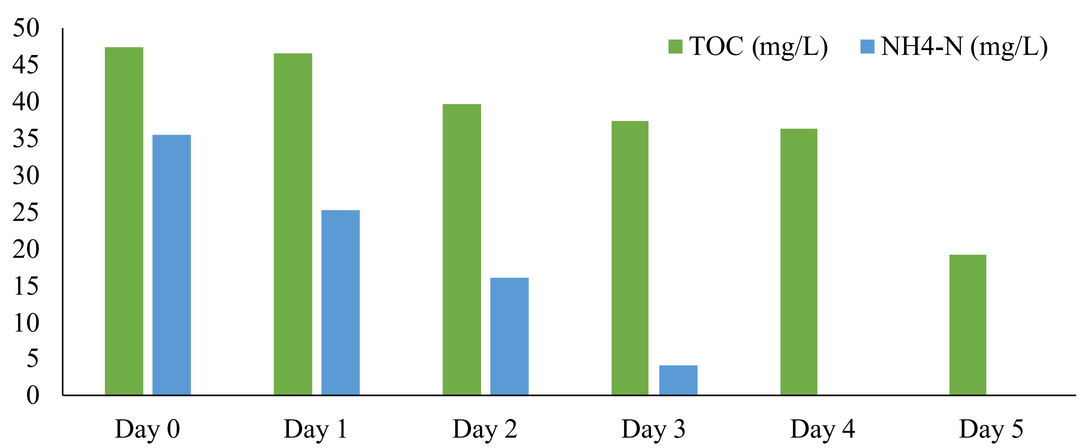

Figure 3. Results from water quality assessments of wastewater treatment using duckweed holobiont.

3. Enhancement of duckweed holobiont-based wastewater treatment (July 30 – September 7, 2023)

To enhance duckweed holobiont-based wastewater treatment, associated bacteria are isolated from duckweed co-culture with environmental water. Sterile *S. polyrhiza*, *L. minor*, and *W. globosa* are transferred into flasks, each containing filtered environmental water from two sampling sites. All flasks are cultured in a growth chamber for ten days.

Figure 4 demonstrates that the duckweed canopy on day 10 is denser than on day 0, with *S. polyrhiza* (Figure 4A), *L. minor* (Figure 4B), and *W. globosa* (Figure 4C) fully floating on the water surface. The presence of these duckweed species in low-nutrient environmental water suggests that environmental bacterial communities might play essential roles in duckweed growth. Among these bacteria associated with duckweed species, there might be benefit bacteria that promote wastewater purification.

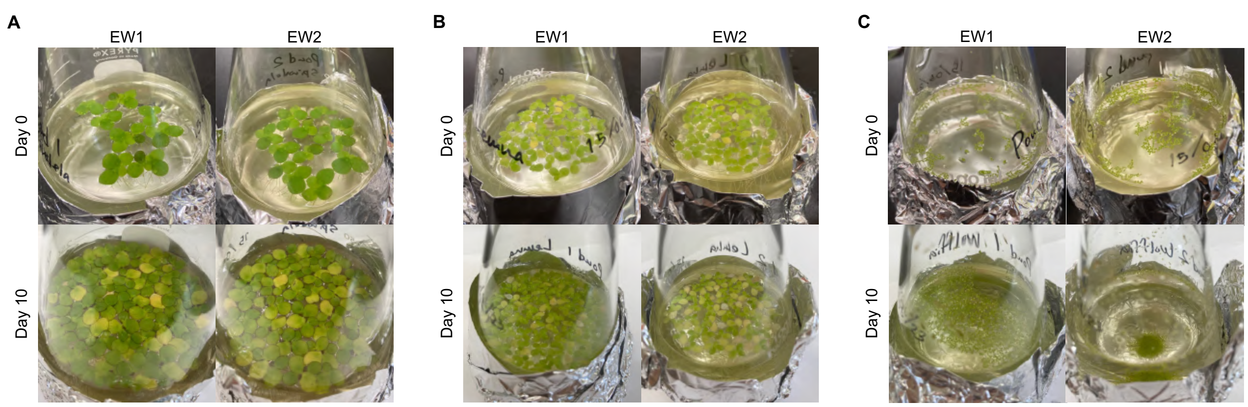

Figure 4. Co-culture of duckweed species with environmental bacterial communities. (A) *Spirodela polyrhiza,* (B) *Lemna minor*, and (C) *Wolffia globosa* are co-cultured with bacterial communities collected from two different sites: environmental water 1 (EW1) and environmental water 2 (EW2).

After ten days of co-culture, the bacteria associated with duckweed species are isolated through serial dilution and spread plating on R2A agar plates. The bacterial plates are incubated for two weeks, allowing fast-growing and slow-growing bacteria to develop and capture the entire bacterial population. Figure 5 shows the bacterial colonies grown on R2A plates, with higher concentrations exhibiting more bacterial colonies.

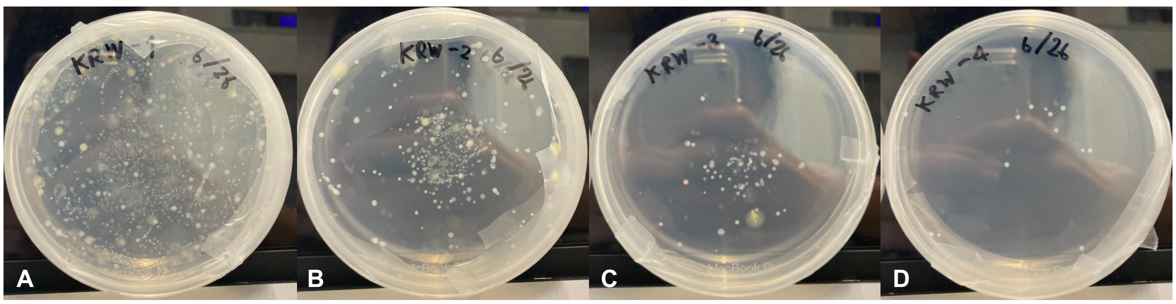

Figure 5. Bacterial colonies isolated from duckweed species growing on R2A plates from serial dilutions at (A) 10^{-1}, (B) 10^{-2}, (C) 10^{-3}, and (D) 10^{-4} concentrations.

After colonies have grown, the single colonies from each plate are picked and streaked onto new R2A plates. Subsequently, each plate is re-streaked to obtain a single strain culture, as shown in Figure 6.

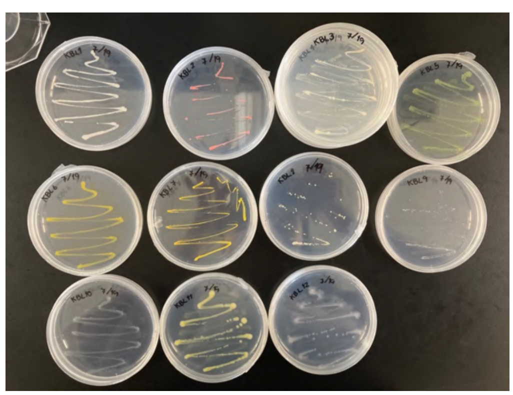

Figure 6. Single bacterial strains isolated from duckweed growing on R2A plates.

The single bacterial strains isolated from duckweed species are stored in 15% glycerol for long-term storage and further studies. Fresh bacterial cultures are prepared for DNA extraction (Figure 7A) and 16S amplification using PCR (Figure 7B) to identify the bacterial strains. Due to the training duration, sequencing of the 16S PCR product is necessary, and the identification of bacteria is part of future studies. Once the identification is completed, the next step is to confirm the capabilities of the benefit bacteria, such as promoting duckweed growth and improving wastewater purification.

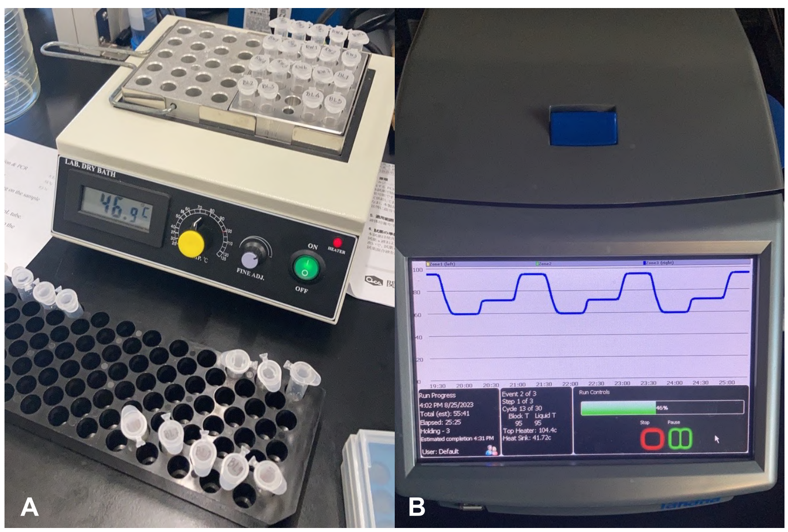

Figure 7. Identification of bacteria associated with duckweed using (A) DNA extraction and (B) 16S amplification.

However, the experiment involving the duckweed co-culture with a confirmed Plant Growth-Promoting Bacteria (PGPB) is a part of this training. The sterile *S. polyrhiza* cultured in Hoagland's media is inoculated with PGPB for 6, 12, and 24 hours. Once the inoculation is completed, transfer *S. polyrhiza* into freshly prepared Hoagland's media and culture them in a growth chamber for two weeks.

The results show that the duckweed inoculated with PGPB (Figures 8B – 8D) is well-grown compared to the duckweed cultured in Hoagland's media (Figure 8A). Duckweed inoculated with PGPB for 24 hours shows a greater number of fronds, with more inoculation time resulting in a higher number of fronds. These observations suggest that PGPB can promote duckweed growth in a low-nutrient environment.

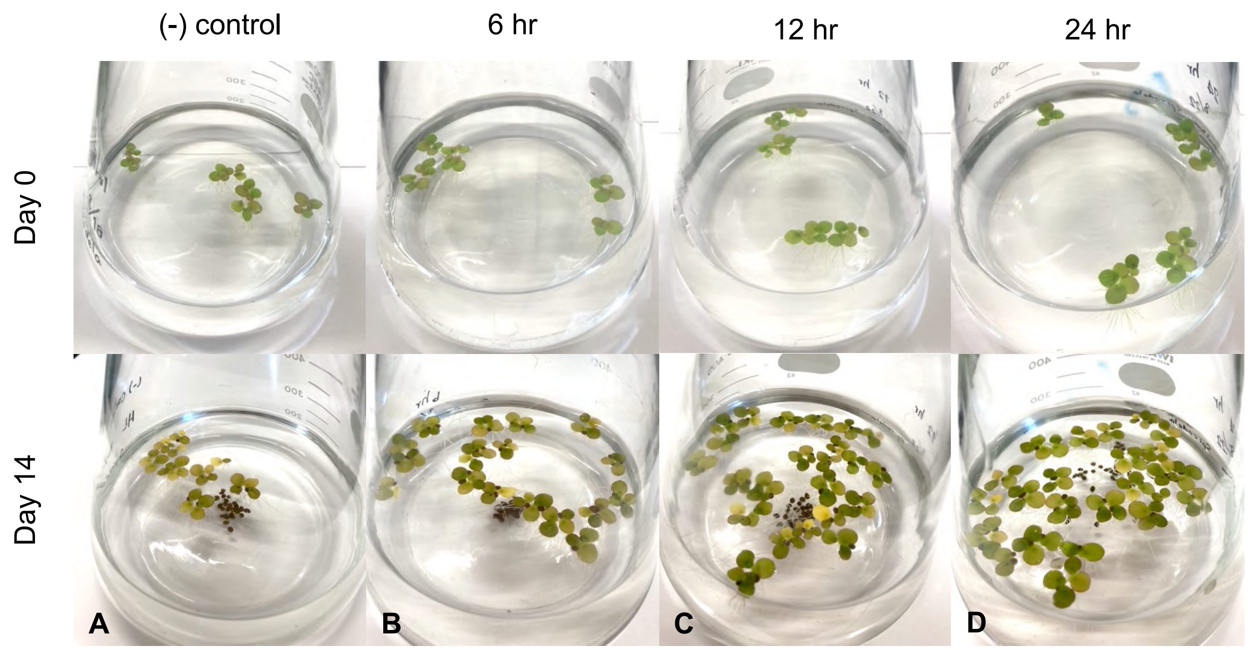

Figure 8. Duckweed co-culture with plant growth-promoting bacteria.

Moreover, dark green and sunken structures are observed in every culture of day 14 (Figure 8). These structures are called turions, which are the starch-rich dormant fronds. Duckweed species form turions to withstand environmental stress, such as a low-nutrient environment and the winter. When the environmental conditions are suitable for survival, these turions germinate and float to the water surface again.

Acknowledgment

I would like to express my deepest gratitude to the Science and Technology Research Partnership for Sustainable Development (SATREPS) and the Japan International Cooperation Agency (JICA) for giving me the opportunity to gain precious experience in research and allowing me to continue my Ph.D. studies in wastewater treatment using duckweed-holobiont. Furthermore, this training would not have been possible without Prof. Tadashi Toyama, Prof. Kazuhiro Mori, and lab members, who provided me with guidance and assistance. These valuable experiences will support both my future studies and duckweed-holobiont research in Thailand.

Besides gaining laboratory experience, I was deeply impressed by Japanese culture, society, and architecture. During my stay in Japan, I had the opportunity to visit many remarkable places in both Kofu and Tokyo. Exploring these locations left an impression on me and will remain in my memory.