International Journal of Agricultural Technology 2021 Vol. 17(1):325-336
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Appropriate stocking density for growth of watermeal (*Wolffia arrhiza*) and its efficiency of total ammonia nitrogen removal

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Sirirustananun, N. and Jongput, B. (2021). Appropriate stocking density for growth of watermeal (*Wolffia arrhiza*) and its efficiency of total ammonia nitrogen removal. International Journal of Agricultural Technology 17(1):325-336.

Abstract Watermeal was cultivated at six different stocking densities as 0 (control), 0.5, 1.0, 1.5, 2.0 and 2.5 g fresh weight (fw)•l^{-1} for two weeks. Results indicated that all watermeal treatments showed optimal growth during the first week. Watermeal cultivated at 2.5 g fw•l^{-1} revealed the highest growth performance but specific growth rate (SGR) was not significantly differed when compared to the other stocking densities. During the second week, all treatments were recorded as no growth with negative averaged fw gain and SGR. The cultivation of watermeal at 1.5 g fw•l^{-1} showed significantly higher of total ammonia nitrogen (TAN) removal rate (6.34±0.24 %•day^{-1}•g^{-1}) than watermeal was cultivated at 2.5 g fw•l^{-1} and the control treatment. The starting stocking density of watermeal at 1.5 g fw•l^{-1} was determined the most suitable for growth and water quality treatment.

Keywords: Khai-nam, Watermeal, Starting stocking density, Ammonia absorption

Introduction

Watermeal is classified in the genus *Wolffia* that includes the smallest flowering plants in the world. Species of *Wolffia* have no roots and no true leaves, they are free-floating with connected plants such as *Spirodela* and *Lemna* species. Their leaf-like body is called a thallus. In abundant water, roundish thalli can grow up to 1.3 mm in diameter. *Wolffia* are found in warm regions and subtropical global zones with 11 species identified worldwide (Ivan and Katya, 2013; Pandey and Verma, 2018). *Wolffia* spp. as aquatic plants are commonly found in Thailand, Laos, Myanmar and other Asian nations, *Wolffia globosa* (L.) and *Wolffia arrhiza* (L.) are two species that are discovered in Thailand (Chareontesprasit and Jiwyam, 2001; Ruekaewma *et al*., 2015; Appenroth *et al*., 2018). *W. arrhiza* is the smallest vascular plant or

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tracheophyte that reproduces by shedding unicellular haploid spores, a process known as gemmation. The abnormal leaf-like body is called a frond. During unfavorable environmental conditions of autumn and winter, fronds become motionless and sink to the bottom of the water as a turion. In a eutrophic lake, *Wolffia* changes its way of feeding from photoautotrophic to heterotrophic which will turn out its own energy by chemical change or absorb it from the atmosphere within the style of dissolved carbon. *Wolffia* can be considered as the most evolutionarily advanced species of Lemnaceae (Czerpak *et al*., 2004; Czerpak and Piotrowska, 2005; Khvatkov *et al*., 2015; Pandey and Verma, 2018).

W. arrhiza has long been used as a vegetable in Myanma, Laos and Thailand, especially in the northeast and north of Thailand, where it is known as Khai-nam according to the oval shape as eggs of water. Other Thai names are Pum, Kai-pum and Kai-nhae. Khai-nam is traditionally used in local menus. *Wolffia* spp. provide high nutrition, particularly protein, animal feed and human consumption (Bhanthumnavin and McGarry, 1971; Suppadit *et al*., 2008; Ruekaewma *et al*., 2015; Appenroth *et al*., 2018). Nutritionally, *Wolffia* spp. consist of protein at 20-30%, starch 10-20%, fat 1-5% and fiber 25%. Essential amino acid content is closed to the requirements of preschool-aged children according to the standards of the World Health Organization (Appenroth *et al*., 2018). Apart from human food, *Wolffia* are added as a protein substitute for soybean meal in formulated diets for animal food or fish feed to reduce costs (Ariyaratne, 2010; Chantiratikul *et al*., 2010; Pradhan *et al*., 2019). Moreover, it has been interested in bioenergy or biorefining and bioactivities (Tipnee *et al*., 2017; Heenatigala *et al*., 2018). Other benefits of watermeal include water treatment and nutrients removal due to fast reproduction rate, and ability to absorb large amounts of nitrogen and phosphorus. It can also fix nitrogen from the atmosphere and grow in water with restricted nitrogen content (Fujita *et al*., 1999; Suppadit *et al*., 2008; Baidya and Patel, 2018).

Wolffia spp. cultivation in Thailand had recored as a manure, BG-11 media and N-P-K fertilization (Sricharoen *et al*., 2001; Sricharoen *et al*., 2002; Rowchai and Somboon, 2007). Researchers examined the development of suitable watermeal culture systems for mass production of hygienic human food and application as hydroponic fertilizer which showed the highest fresh weight, dry weight and chlorophyll content (Ruekeawma *et al*., 2015; Damna *et al*., 2017).

This experiment aimed to determine the appropriate stocking mass of *W. arrhiza* cultivation and its efficiency of TAN removals which are useful information for the development of watermeal cultivation and biological water treatment.

Materials and methods

Experimental site and watermeal collection

The experiment was conducted at the Aquaculture Unit of the Agricultural and Industrial Technology Faculty, Phetchabun Rajabhat University, Thailand. Watermeal was collected from natural water in Tabo district, Amphoe Mueang, Phetchabun, Thailand during October 2019. Nutritional values are shown in Table 1. After gathering, the watermeal was soaked in cleaned water. Before experiment, the watermeal was acclimated in water that had total ammonia nitrogen (TAN) content ranged from 0.75-1.0 mg•l^{-1} for two days.

Table 1. Nutritional values of watermeal used in the initial experiment

Analyzed item	Mean ±SD
Crude protein (%)	16.56±0.35
Crude fat (%)	2.12±0.16
Crude fiber (%)	11.49±0.12
Ash (%)	18.34±4.61
Calcium (%)	1.30±0.20
Phosphorus (%)	0.25±0.005
Gross energy (kcal/kg)	3,591.23±47.72

Experimental operation

Experiment was performed in Completely Randomized Design which consisted of six treatments in triplicate. Treatments were 0 (control treatment), 0.5, 1.0, 1.5, 2.0 and 2.5 g fw•l^{-1}. Culturing for two weeks, watermeal was fed at each treatment density in 2 l clear containers which contained water with total ammonia nitrogen (TAN) between 0.75-1.0 mg•l^{-1}. Ammonium chloride (NH_4Cl) was used as the nutrient source for watermeal growth. The biomass was weighed and recorded on the first and seventh days in each week, while densities were adjusted to the basal stocking densities after weighing according to the method of Pereira *et al*. (2006). At three and four day intervals in each week, light density, water temperature, dissolved oxygen (DO), pH and TAN of water samples were determined. TAN in the water was analyzed using the indophenol blue-hypochlorite method (American Public Health Association, 1998). The specific growth rate (SGR) of watermeal was estimated by the formula:

$$SGR\ (\%\bullet day^{-1}) = 100[\ln(fw)-\ln(iw)]/t$$

Where fw is final wet weight and iw is initial wet weight of watermeal during the experiment and t is the experimental period (days) (Macchiavello and Bulboa, 2014). TAN removed from water was calculated as the difference between the initial and final concentrations in water on the third day of the first and second weeks. TAN removal rates were calculated according to the equation:

TAN removal rates ($\%\bullet day^{-1}\bullet g^{-1}$) = $100[(iTAN-fTAN)/(t\times iw\times iTAN)]$

Where iTAN and fTAN are initial TAN and final TAN in water during the experimental period, t is the experimental period (days) and iw is the initial wet weight of watermeal.

Statistical analysis

Three replications of each treatment were evaluated for means and standard deviations. Differences among treatments were tested using one-way ANOVA and Duncan's multiple range test at 95% confidence interval. All data were processed with IBM SPSS statistics version 21.

Results

Watermeal growth

The difference of starting stocking densities was significantly affected on growth of watermeal. After two weeks of cultivation, results indicated that the average fw gain and SGR of watermeal in all treatments were highest in the first week and decreased in the second week (Figures 1 and 2). In the first week, watermeal was cultivated at 2.5 g $fw\bullet l^{-1}$, the highest stocking density level showed 3.24±0.32 g and 6.51±0.51 $\%\bullet day^{-1}$ of average fw gain and SGR, respectively which were higher than other treatments (Table 2 and 3). However, SGR showed not significantly different ($p>0.05$) when compared with 0.5, 1.0, 1.5 and 2.0 g $fw\bullet l^{-1}$ (Table 3). In the second week, results indicated that watermeal cultivated at all treatments showed no growth and recorded negative average fw gain and SGR (Figure 1 and 2, Table 2 and 3).

At the end of the experiment, the effect of different stocking densities on growth of watermeal showed significant differences between treatments in average fw gain ($p<0.05$). Average fw gain of watermeal cultivated at 2.5 g $fw\bullet l^{-1}$ (2.79±0.35 g) was significantly higher than the other treatments but SGR (2.88±0.25 $\%\bullet day^{-1}$) showed no significant difference ($p>0.05$) when compared with 0.5, 1.0, 1.5 and 2.0 g $fw\bullet l^{-1}$ (Tables 2 and 3).

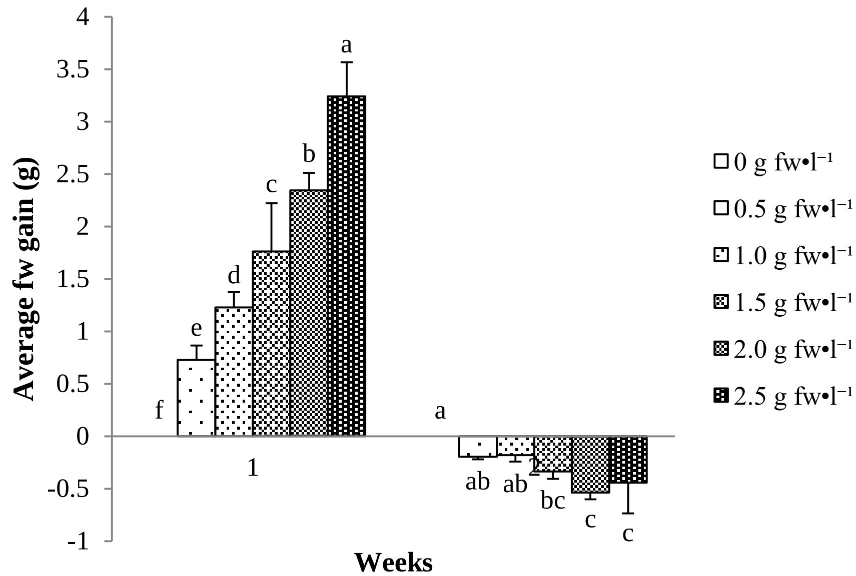

Figure 1. Average fw gain (g) of watermeal cultivated at 0, 0.5, 1.0, 1.5, 2.0 and 2.5 g fw•l^{-1}

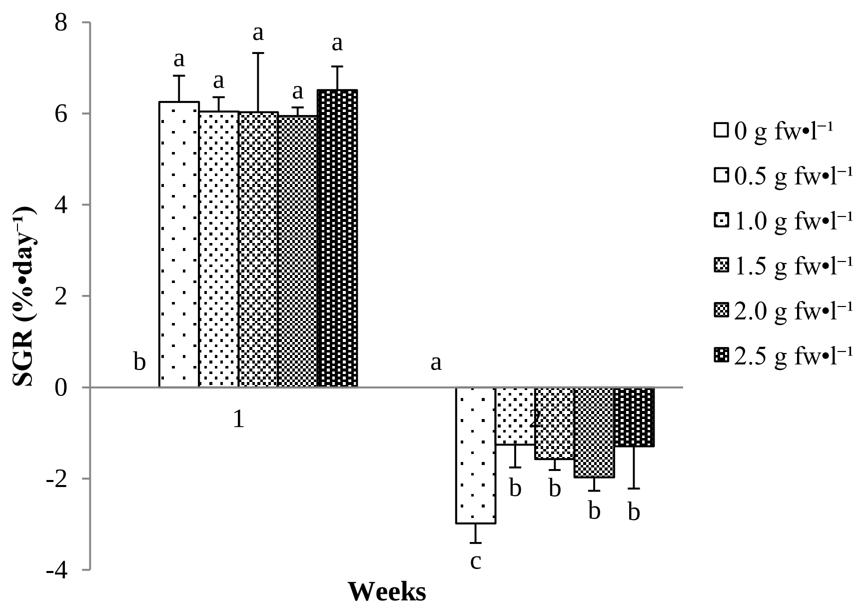

Figure 2. SGR (%•day^{-1}) of watermeal cultivated at 0, 0.5, 1.0, 1.5, 2.0 and 2.5 g fw•l^{-1}

Table 2. Average fw gain (g) of watermeal was grown at 0, 0.5, 1.0, 1.5, 2.0 and 2.5 g fw•l^{-1}

Stocking density level (g fw•l^{-1})	Week		End of experiment
	First	Second	
0	0^{f}	0^{a}	0^{e}
0.5	0.72 ± 0.13^{e}	-0.19 ± 0.02^{ab}	0.53 ± 0.11^{d}
1.0	1.22 ± 0.14^{d}	-0.18 ± 0.06^{ab}	1.04 ± 0.18^{c}
1.5	1.76 ± 0.46^{c}	-0.33 ± 0.07^{bc}	1.42 ± 0.53^{bc}
2.0	2.34 ± 0.16^{b}	-0.53 ± 0.06^{c}	1.80 ± 0.11^{b}
2.5	3.24 ± 0.32^{a}	-0.44 ± 0.29^{c}	2.79 ± 0.35^{a}

Difference of superscripts in a same column means significant difference between treatments ($p<0.05$). fw = fresh weight.

Table 3. SGR (%•day^{-1}) of watermeal was grown at 0, 0.5, 1.0, 1.5, 2.0 and 2.5 g fw•l^{-1}

Stocking density level (g fw•l^{-1})	Week		End of experiment
	First	Second	
0	0^{b}	0^{a}	0^{b}
0.5	6.25 ± 0.57^{a}	-2.98 ± 0.42^{c}	2.40 ± 0.28^{a}
1.0	6.04 ± 0.31^{a}	-1.25 ± 0.50^{b}	2.64 ± 0.26^{a}
1.5	6.02 ± 1.29^{a}	-1.57 ± 0.24^{b}	2.51 ± 0.80^{a}
2.0	5.94 ± 0.19^{a}	-1.97 ± 0.29^{b}	2.39 ± 0.07^{a}
2.5	6.51 ± 0.51^{a}	-1.29 ± 0.92^{b}	2.88 ± 0.25^{a}

Difference of superscripts in a same column means significant difference between treatments ($p<0.05$). SGR = specific growth rate, fw = fresh weight.

TAN removal

Findings indicated that the difference of stocking densities on total ammonia nitrogen (TAN) removal showed significant differences ($p<0.05$) between treatments. The efficiency of TAN removals in all treatments were the highest in the first week (within seven days) and decreased in the second week. In the first week, decrease in TAN and TAN removal per day of watermeal cultivated at 2.5 g fw•l^{-1} were 1.56±0.02 mg•l^{-1} and 0.52±0.008 mg•l^{-1}•day^{-1}, respectively. There were significantly higher than the other treatments ($p<0.05$). However, TAN removal rate of watermeal cultivated at 1.5 g fw•l^{-1} (6.34±0.24%•day^{-1}•g^{-1}) was significantly higher than watermeal cultivated at 2.5 g fw•l^{-1} ($p<0.05$) but showed no significant differences ($p>0.05$) when compared with 0.5, 1.0 and 2.0 g fw•l^{-1} (Table 4). In the second week, the efficiency of TAN removal decreased about 39.33±6.41 to 65.04±4.41% of the first week (Table 5).

Table 4. Efficiency of TAN removal by watermeal was grown at different stocking densities in the first week of the experiment

Parameter	Stocking density levels (g fw•l^{-1})					
	0	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**
TAN removal (mg•l^{-1})	0.08±0.005^{f}	0.39±0.04^{e}	0.68±0.05^{d}	1.04±0.005^{c}	1.35±0.04^{b}	1.56±0.02^{a}
TAN removal per day (mg•l^{-1}•day^{-1})	0.02±0.001^{f}	0.13±0.01^{e}	0.22±0.01^{d}	0.34±0.001^{c}	0.45±0.01^{b}	0.52±0.008^{a}
TAN removal rate (%•day^{-1}•g^{-1})	CC	6.23±0.05^{a}	6.15±0.04^{a}	6.34±0.24^{a}	6.17±0.08^{a}	5.61±0.04^{b}

Difference of superscripts in a same row means significant difference between treatments ($p<0.05$), CC = could not be calculated due to no data (no watermeal), Fw = fresh weight, TAN = total ammonia nitrogen.

Table 5. Efficiency of TAN removal by watermeal was grown at different stocking densities in the second week of the experiment

Parameter	Stocking density levels (g fw•l^{-1})					
	0	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**
TAN removal (mg•l^{-1})	0.13±0.04^{d}	0.19±0.01^{d}	0.34±0.02^{c}	0.38±0.02^{bc}	0.44±0.05^{b}	0.68±0.06^{a}
TAN removal per day (mg•l^{-1}•day^{-1})	0.04±0.01^{d}	0.06±0.06^{d}	0.11±0.09^{c}	0.12±0.09^{bc}	0.14±0.01^{b}	0.22±0.02^{a}
TAN removal rate (%•day^{-1} •g^{-1})	CC	5.23±0.50^{a}	4.64±0.21^{ab}	3.51±0.13^{c}	2.99±0.42^{c}	3.68±0.48^{bc}
Removable efficiency decreasing (%)	CC	39.33± 6.41^{a}	45.57±2.08^{ab}	59.99± 3.09^{c}	65.04± 4.41^{c}	52.70±6.59^{bc}

Difference of superscripts in a same row means significant difference between treatments ($p<0.05$), CC = could not be calculated due to no data (no watermeal), Fw = fresh weight, TAN = total ammonia nitrogen.

During the experiment, water temperature, pH, DO, light density and TAN were 28.25±0.07 to 31.85±2.19 ℃, 7.50±0.24 to 7.79±0.007, 5.70±0.14 to 6.00±0.00 mg•l^{-1}, 4,560±463.86 to 9,795±265.76 lux and 0.47±0.38 to 1.34±0.02 mg•l^{-1}, respectively.

Discussion

Watermeal was used in the experiment contained a low level of protein due to natural water has low level of ammonia nitrogen that affects the accretion of crude protein in plants according to the knowledges of Food and Agriculture Organization of the United Nations (1997) and Hasan and Chakarbarti (2009) presented crude protein content of duckweed depending on the ammonia nitrogen in water.

The highest stocking density at 2.5 g fw•l^{-1} gave the highest average fw gain and SGR, possibly because of primary duckweed reproduction via asexual vegetative budding where each frond can produce a new plant (Koschnick *et al*., 2014), thus many population of duckweed can produce many new plants. For fast harvesting, watermeal cultivation at a high stocking density is recommended (Kongban, 2014).

However, SGR of all treatments revealed no significant difference because starting stocking densities did not affect the early stage of watermeal growth but the balance between water volume and water surface area highly affects on growth performance (Food and Agriculture Organization of the United Nations, 1997). Stocking density or degree of crowding will be impact growth when watermeal has grown. The degree of crowding is largely a function of duckweed growth along with the availability of nutrients, temperature and light. Duckweed growth increased steadily until plants completely covered the water surface and then remained constant. Exponential growth of duckweed occurred until the plants become overcrowded or utilized all the available nutrients. High density of plants intercepts light transmission and inhibits growth. Optimal density is a cover that is complete but still provides enough space to accommodate rapid growth of the colony (Hasan and Chakarbarti, 2009). The recommended starting stocking density in this study was 1.5 g fw•l^{-1} in accordance with various suggested as 0.39 g•l^{-1} (Ruekaewma *et al*., 2015), 0.59 g•l^{-1}(Damna *et al*., 2017), 0.63-0.84 g•l^{-1}(Kongban, 2014) and 12 g•l^{-1} (Suppadit *et al*., 2008) that are between 0.39 to 12 g•l^{-1}.

After 7 days or 1 week, watermeal growth turned negative; plants started to die but still absorbed TAN for maintenance, thus TAN removal showed in Table 5. The control treatment (no watermeal) showed TAN removal due to nitrogen has been denitrified and released into the atmosphere as nitrous oxide (N_2O) or denitrogen gas (N_2) (Fe and He, 2015).

Our study noticed that at high stocking density showed high ability of TAN removal as reported by Suppadit *et al*. (2008) found that TAN removal by watermeal increased as stocking density level increased. They showed that when different starting biomasses of watermeal and treatment time were applied, concentration of TAN in the water decreased as biomass and treatment time increased. The highest TAN removal per day by watermeal in this trial ranged between 0.13 to 0.52 mg•l^{-1}•day^{-1}, conforming to results presented by Fu and He (2015) for total nitrogen (TN) absorption of five aquatic plants; *Lythrum salicaria*, *Alisma orientale*, *Acorus calamus*, *Monochoria korsakowii* and *Sagittaria sagittifolia* at around 0.20 to 0.45 mg•l^{-1}•day^{-1}. Results were also agreeable with Su *et al.* (2019) for the presence of single plants such as *Colocasia tonoimo*, *Dysophylla sampsonii*, *Eichhornia crassipes*, *Eleocharis plantagineiformis*, *Hydrocotyle vulgaris*, *Ipomoea aquatica*, *Rotala indica*, *Salvinia natans* and *Typha orientalis* where NH_4^+-N concentration decreased at 0.14 to 0.18 mg•l^{-1}•day^{-1}. Nevertheless, our results conflicted with Suppadit *et al.* (2008) who studied effluent treatment from shrimp farms using watermeal (*W. arrhiza*). Their result showed that the highest TAN removal by watermeal was 0.071 mg•l^{-1}•day^{-1} at 12 g•l^{-1} stocking density during 30 days of treatment time. This low TAN removal rate was possibly the result of a long treatment time with no harvesting. During the experimental period, water temperature, pH and light intensity still remained optimum for duckweed growth that should be between 17.5 to 30 ℃ water temperature (Hasan and Chakarbarti, 2009), 6.5 to 7.5 pH (Food and Agriculture Organization of the United Nations, 1997; Hasan and Chakarbarti, 2009), and 5,000 to 10,000 lux light intensity (Rowchai and Somboon, 2007).

In conclusion, starting stocking densities showed no significant differences in SGR. Growth of watermeal was highest in the first week and then declined in the second week. Starting biomass had no effect on growth but efficiency of TAN removal by watermeal cultivated at 1.5 g fw•l^{-1} was significantly higher than at 2.5 g fw•l^{-1}. Thus, stocking density at 1.5 g fw•l^{-1} is suitable for growth and water quality treatment. In addition, harvesting of biomass should be conducted every seven days to maintain optimal growth rate and TAN absorption.

Acknowledgements

The author would like to offer particular thanks to Institute of Research and Development and Faculty of Agricultural and Industrial Technology of Phetchabun Rajabhat University for permitting the experimental operations.

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(Received: 9 May 2020, accepted: 30 December 2020)