Biomass production of *Azolla microphylla* as biofilter in a recirculating aquaculture system

SUMOHARJO1,*, MOHAMMAD MA’RUF2,**, IRWAN BUDIARTO3,***

1Laboratory of Aquaculture System and Technology, Faculty of Fisheries and Marine Science, Universitas Mulawarman. Jl. Gunung Tabur, Kampus Gunung Kelua, Samarinda 75116, East Kalimantan, Indonesia. Tel./fax: +62-541-748648, *email: sumoharjo@gmail.com
2Laboratory of Aquaculture Environment, Faculty of Fisheries and Marine Science, Universitas Mulawarman. **email: maruff68@gmail.com
3Aquaculture Department, Faculty of Fisheries and Marine Science, Universitas Mulawarman. ***email: budianto.irwan12@gmail.com

Manuscript received: 1 April 2018. Revision accepted: 20 May 2018.

Abstract. Sumoharjo, M. ’Ruf M. Budiarto I. 2018. Biomass production of *Azolla microphylla* as biofilter in a recirculating aquaculture system. *Asian J Agric* 2: 14-19. This study utilized macrophyte (*Azolla microphylla* Kaulf.) as a biofilter and investigated that biomass produced in an aquaculture system could potentially be an alternative feed. This experiment was aimed to determine the *Azolla* microphylla growth rate and its efficiency in removing ammonia from a simple recirculating aquaculture system. The experimental units were set up in three different water flows, i.e., 3 lpm, 5 lpm, and 7 lpm onto the three different geometrically baseboard of Tilapia (*Oreochromis niloticus*) growing tanks (prism, rectangular and limas). The results showed that water flow did not give significant effect (P < 0.10) on the growth rate of *Azolla*. The lower water flow (3 lpm) resulted in the highest ammonia biofiltration efficiency, which can remove ammonia up to 32.2±3.0% of the total NH3-N and NH4+-N (TAN).

Keywords: *Azolla microphylla*, ammonia, biofiltration, recirculating, water flow

**INTRODUCTION**

The main problem in the intensification of aquaculture systems is water quality decreasing rapidly because of a high density of fish being reared with a high feed input in less water exchange. Hence, accumulation of fish metabolites, especially ammonia, tends to occur in waterbody and build-up to toxic level and affecting fish performance. Wastewater is accumulated, while feed is continuously added in a fish culture system (Rafiee and Saad 2005).

In an intensive land-based fish farming system, the toxicity of excreted nitrogenous compounds is often a limiting factor (Bradfield 1985; Brune et al. 2003, Nerici et al. 2012). The toxicity of the total NH3-N and NH4+-N (TAN) increases with the pH of the water because TAN enters the organism as NH3 and the proportion of NH3 increases with higher pH (Randall and Tsui 2002; Nerici et al. 2012). When environmental TAN level increases, the excretion of ammonium by aquatic animals decrease and the ammonium levels in the blood and tissues rise (Nerici et al. 2012). Long-term exposure to ammonia increases glycemia, lipoygenase and unsaturated Erythrocyte Fatty Acids (Liu and Sun Pan 2008). Chronic exposure to high TAN concentrations tends to damage the fish gills, which can contribute to decreased growth because of gas exchange efficiency (Handy and Paxton 1993; Nerici et al. 2012).

In Recirculating Aquaculture Systems (RAS), biofilter is the main component and known as low-cost water treatment to keep water quality suitable for fish growth and welfare. Biofilter technology was studied intensely, however, most of them are struggling on bacterial-based biofilters, such as nitrification by nitrifiers and nutrient assimilation by heterotrophs. Smith (2003) categorized biofilters into four main types, i.e., activated sludge, aquatic plant filters, fluidized bed filters, and fixed film.

*Azolla microphylla* is an aquatic fern. Many reports on *Azolla* have been published, but almost all of them are related to its function as a natural feed resource. *Azolla* was recommended by FAO (2009) as feed-in small-scale aquaculture and had been used as a main component in food for tilapia (Fiogbé et al. 2014). According to Lumpkin and Placknett (1980) and Van Hove (1989), *Azolla* under good conditions presents high productivity and protein content (generally 20-30%, on a dw basis).

Growing *Azolla* seems easy (Datta 2011) because of its endosymbiotic blue algae, *Anaabaena azollae*, that fixes nitrogen directly from the atmosphere (Van Howe 1989). So, *Azolla* is probably able to grow well in a relatively low nutrient environment. However, reports on *Azolla* as biofilter to remove nitrogen from the fish culture water are rare. Even though, as a macrophyte, *Azolla* should be served as a phototrophic converter at the trophic level. So, it has great potential as a biofilter for maintaining water quality in RAS as well as providing an alternative feed for growing-fishes.

This study focused on utilizing *Azolla* as biofilter in RAS. The experimental units were designed in integration of fish tank and *Azolla* growing bed to meet a series of recirculating systems. The experiment was divided into two parts; first was to analyze the effect of different water flow on *Azolla* growth rates, and the second one was to determine the optimum biomass of *Azolla* for converting nitrogen from fish waste.
MATERIALS AND METHODS

Experimental unit configuration

The experimental units were a pilot scale. Three types of tanks, with 1800-liter effective volume, were used as an experimental group. The design of the bottom of each tank varied geometrically, i.e., prism, pyramid, and rectangular. On the top of every tank were three similar trenches that had a 2-meter long biofilter bed. Every trench had three different water flow rates, i.e., 3 lpm, 5 lpm and 7 lpm. A 32 watt submersible pump was used to supply water from each fish tank to the three trenches, connected parallelly with ¾ inch PVC pipe. The water flow rate, as the treatment was adjusted, was done by the outflow head. Synchronization was carried out daily.

The fish species cultivated were tilapia (Oreochromis niloticus), sized 8.3±1.2 g and had 100 fishes per tank. In every trench, 50 g Azolla microphylla was added. The fish were fed ad satiation with floating pellets (CP. Prima 781-3, 31-33% raw protein).

Nutrient budgeting

In the recirculating system, the complete water from the fish tank passed over the biofilter bed (the trench) once every 2 hours, then the water was mixed continuously in the fish tank, so that there were no differences between in and outflow. Samples were taken in fish tank only. Total Ammonia Nitrogen (TAN) as nutrient input and removal rates were calculated through mass balance. To estimate TAN input per day from within the fish tanks the following can be calculated based upon the feeding rate (Timmons et al. 2002):

\[ P_{TAN} = F \times PC \times 0.092 \]

Where:
- \( P_{TAN} \): Production rate of total ammonia nitrogen, (kg/day)
- \( F \): Feed rate (kg/day)
- \( PC \): Protein concentration in feed (decimal value)

The constant in the ammonia generation equation assumes that protein is 16% nitrogen, 80% nitrogen is assimilated by the organism, 80% assimilated nitrogen is excreted, and 90% of nitrogen is excreted as TAN+10% as urea. In addition, the nitrogen in feces is not removed from the system but collected in the filter bed until the end of the experiment.

Water quality

Water quality parameters were monitored every three days such as temperature, pH, dissolved oxygen (DO), Total Ammonia Nitrogen (TAN), and Un-ionized Ammonia Nitrogen (NH3-N). Lutron portable DO meter model 5510 was used to measure DO. The concentration of TAN was determined using TAONSUN spectrophotometer (Suzhou Taonsun Scientific Instruments, China).

Biomass calculation

Biomass growth of Azolla cultivated in biofilter units (gutter/trench) is expressed as doubling time (day\(^{-1}\)) which is calculated according to daily growth rate (DGR, %/g/day) (Zonnenveld et al., 1991) as follows:

\[
 DGR \ (\%/g/day) = \frac{\ln(W_t) - \ln(W_0)}{t} \times 100 \\
 DT \ (day^{-1}) = \frac{\ln 2}{DGR} 
\]

Where:
- DGR: daily growth rate (%/g/day)
- \( \ln \): logarithmic natural
- \( W_t \): final biomass of Azolla (g)
- \( W_0 \): initial biomass of Azolla (g)
- DT: Doubling Time

Total Ammonia Nitrogen measured on the final day of the experiment will represent the nutrient output. Thus, in case of this simple RAS, whereas all the water is recirculated and there is no discharge, nutrient removal rate can be calculated with mass balance equation (Al Hafedh et al., 2003) as follows:

\[
 \text{Waste Loading Rate (g/m}^3\text{ per day)} = C_i \times Q 
\]
Waste Removal Rate (g/m³ per day) = (Ci – Ce) × Q

Removal efficiency (E) = \frac{\text{waste removal rate}}{\text{waste loading rate}} × 100

Where:
Ci : Total Ammonia Nitrogen measured in the fish tank (mg/L x Water Volume x1000 = g)
Ce : P_{TAN} (Production rate of total ammonia nitrogen (g)
Q : Water flow (liter per minute, lpm)

Retained nitrogen of Azolla is expressed as gram and can be calculated by using the following formulae:

Retained Nitrogen (RN, gram)= (TKNₓ W₁) - (TKNₓ W₀)

Retained Nitrogen Efficiency (RNE, %) = \frac{RN}{P_{TAN}}

Where:
TKN : Total Kjedahl Nitrogen at the end of experiment (g)
TKN₀ : Initial Total Kjedahl Nitrogen (g)
W₁ : Final biomass of Azolla (g)
W₀ : Initial biomass of Azolla (g)

Data analysis

The means on the Azolla growth rate, doubling time, and nitrogen retention parameters were analyzed using two-way analysis of variance (ANOVA, α = 0.1). The analysis was done using STATISTICA 8.0.

RESULTS AND DISCUSSION

Nutrient input and biomass production of Azolla

Total feed consumed by the fish in tanks I, II, and III were 1519 g, 1504 g, and 1313 g, respectively. The TAN production of every tank is listed in Table 1.

Based on the calculations, TAN production of all the fish tanks were evenly 3% of the total feed input. For example, tank I released 1.49 g TAN per day in 1800 liter of water. It means that 0.83 mg.L⁻¹ of TAN was added and diluted in the water of the fish tank.

The TAN production was similar to the assumption of Colt (1991) that waste output of fish consuming 1000 g feed and 250 g O₂ are 30 g of TAN and 340 g CO₂; excreted via gill by ion-exchange along with 500 g fecal solid and 5.5 g PO₄-P. Then, Schneider et al. (2005) stated that the Fish-Biomass-Converter retains 20-50% feed N and 15-65% feed P. This means that 50-80% feed N and 35-85% feed P are discharged as waste.

Fish waste that was released in the water column was then recirculated and served as nutrient input for Azolla. The treatment with water flows showed no significant difference (P < 0.1) among 3 lpm, 5 lpm, and 7 lpm on doubling time of Azolla's biomass (Figure 2).

Statistically, the significant difference in Azolla's biomass production occurred on experimental group-tank factors. Differences in the growth rate of the Azolla biomass in each experimental group related to the baseboard designs. This allowed better nutrient supply in Tank I and Tank II compared to Tank III. In Tanks I and II, the average biomass growth was doubled from the initial population that occurred every 10.44 and 10.88 days while in Tank III was every 20.13 days (Figure 3).

The result is inversely proportional to the TAN reduction pattern, which means that during the 1st day until the 20th day there was active ammonia assimilation by Azolla, and at the peak multiplication of the biomass the assimilation rate of N decreases, resulting in the TAN concentration in the water to rise again.

Azolla has been known to have the ability to fix nitrogen from the air, so that it can survive and keep growing under low nutrient conditions in the water. However, from the results of this study, there is a correlation between minimal TAN concentration and the rate of assimilation of N by Azolla. The TAN concentration in water should remain at a value of > 0.1 mg.L⁻¹ to maintain the rate of assimilation of N. If the TAN concentration <0.1 mg.L⁻¹ the assimilation of N tends to be slower or even stopped so that in this phase will result in cessation or decrease in growth rates of Azolla.

### Table 1. Feed consumption and TAN Production (P\text{\textsubscript{TAN}}) in 30 days

| Tank | Feed consumed (g) | P\text{\textsubscript{TAN}} total (g) | P\text{\textsubscript{TAN}} (g/day) |
|------|------------------|-----------------------------------|-------------------------------|
| I    | 1519             | 44.7                              | 1.49                          |
| II   | 1504             | 44.3                              | 1.48                          |
| III  | 1313             | 38.6                              | 1.29                          |

### Figure 2. Doubling time of Azolla affected by water flow

### Figure 3. Doubling time of Azolla biomass affected by grouped-tank
Based on the results of *Azolla* Total Kjeldahl Nitrogen (TKN) analysis on the 30th day showed that the average *Azolla* protein content in each treatment was different but not significant (P> 0.10). The level of protein content present in *Azolla* in this study was relatively good (28.8 %) compared to the results of the tests with the duckweed (*Lemma minor*) in the same experimental design which reached 25.7% (Sumoharjo 2015). Therefore, it could be an alternative feed for herbivorous fish such as tilapia. The high levels of this protein content are influenced by *Azolla*‘s ability to convert nutrients from the water into *Azolla* biomass. The nitrogen retention by *Azolla* showed considerable value in each trial and showed significant differences between treatments as well as groups (Table 2).

The highest nitrogen retention was achieved by the 3 lpm treatment of 11.27 ± 6.95 gN, followed by the 5 lpm treatment of 9.23 ± 7.28 gN, and the lowest was the 7 lpm treatment which only retained N of 7.97 ± 6.22 g.

**TAN removal efficiency**

The TAN conversion rates were determined as overall retained nitrogen of *Azolla* from P<sub>TAN</sub> of every tank as part of the experimental group (Figure 4). The results of this experiment showed that the efficiency of the TAN removal by *Azolla* was still lower than the treatment using *Lemma minor* which reached 48% but was much higher than *Spyrogyra* sp which retained 2.91% N of TAN produced by tilapia (Sumoharjo 2015). Determining how much TAN removal will greatly determine the potential level of the use of a phototrophic organism as a biofilter for the use of water quality management in RAS.

**Water quality and nitrogen dynamics**

Water quality characteristics, such temperature ranged between 27.3 to 30.7°C, while pH and TAN tended to decrease during experiment (Figure 5). The proportion of NH₃ increased with higher pH. It could be because TAN enters the organism as NH₃ (Randall and Tsui 2002). Therefore, the toxicity of TAN (the total NH₃-N and NH₄-N) increased in line with the increase of pH of the water. Fortunately, pH during the experiment tended to decline from 7.9 ± 0.2 at the beginning to 6.6 ± 0.2 at the end of experiment. So that the proportion of un-ionized ammonia (NH₃) was low and in a tolerable concentration for Tilapia. The toxic level of NH₃ for short-term exposure usually are reported in between 0.6 to 2 mg.L⁻¹, while the maximum tolerable concentration is to be 0.1 mg.L⁻¹ (Pillay 1992). Moreover, the specific growth rate (SGR) of tilapia exposed to un-ionized ammonia nitrogen over 0.068 mg NH₃ was significantly reduced. The specific growth rate and the increase of the unionized ammonia concentration increased the feed conversion ratio (El-Syafai 2004).

**Table 2. Variance analysis of retained nitrogen by *Azolla***

| Source      | Degr. of | RN | MS  | F       | p       |
|-------------|----------|----|-----|---------|---------|
| Treatments  | 2        | 16.4019 | 8.2009 | 8.7203 | 0.034805* |
| Groups      | 2        | 276.2516 | 138.1258 | 146.8737 | 0.000180* |
| Error       | 4        | 3.7618   | 0.9404  |         |         |
| Total       | 8        | 296.4152 |         |         |         |

Note: *: Significant difference on 90% of reliability

**Figure 4. TAN Removal efficiency**

**Figure 5. Water quality characteristics: A. Temperature, B. pH, C. TAN, D. The proportion of NH₃**
Carbon dioxide (CO₂) is another factor that may affect feed behavior (Trand-Duy et al. 2008). In an intensive culture system, CO₂ may not have an adverse effect on fish unless its concentration reaches 100 mg.L⁻¹ (Balair and Heller 1982). Nile tilapia can tolerate CO₂ concentration above 20 mg.L⁻¹ (Wedemeyer 1996). In this study, CO₂ was 30.2±3 mg.L⁻¹ on the first day then decrease to 15.2±1.1 on the 30th day of the experiment (Table 3). CO₂ tended to decrease during the experiment because the turbulences that occurred in inflow and outflow of the Azolla reactor may strip CO₂ to atmosphere. Moreover, algae and Azolla thrived in the reactors play a role in removing CO₂ out of the system.

Concentration of dissolved oxygen (DO) during the experiment ranged between 2.2 to 6.2 mg.L⁻¹ (Table 3). The lowest DO concentration occurred on the last day of the experiment. Accumulation of sludge in the Azolla reactor played a role in decreasing DO gradually. This may happen because there is no sludge disposal from the system. DO should be maintained above 3.0 ppm and 5.0 ppm for warm and cold-water fish, respectively (Buttner et al. 1993). However, most species of fish are distressed when DO falls to 2-4 mg.L⁻¹ (Floyd 2003).

The lower extreme value of DO (less than 0.8 mg.L⁻¹) was obtained from an experiment in which there were no significant differences between the yields of Nile tilapia raised in ponds with two aeration regimes (Teichert-Coddington and Green 1993). Thus, practical threshold of DO for Nile tilapia was not higher than 10% of saturation (0.8 mg.L⁻¹ at 26°C) (Trand-Duy et al. 2008).

In conclusion, Azolla microphylla can be grown well in RAS. The assimilation rate of TAN by Azolla decreased after its peak biomass production (when doubling time was achieved). Therefore, harvesting must be done 15 to 18 days after cultivation. As a biofilter, it provides a mini-ecosystem that serves as nutrient controller for aquaculture practices. The lower water flow rate the higher nitrogen retention, although, there was no significant effect of water flow rates on the Azolla growth response. It has enough protein content, hence has potential as feed source for herbivorous fishes.

### Table 3. Means and standard deviations of water quality characteristics

| Water quality parameters | Unit | Tank I | Tank II | Tank III |
|--------------------------|------|--------|---------|----------|
| Temperature              | °C   | 29.2±1.2 | 29.0±2.0 | 28.8±1.2 |
| pH                       |      | 7.4±0.4 | 7.3±0.4 | 7.3±0.3  |
| DO                       | mg.L⁻¹ | 4.1±1.4 | 3.6±1.2 | 4.0±1.7  |
| CO₂                      | mg.L⁻¹ | 21.0±11.4 | 23.2±13.3 | 22.0±11.7 |
| TAN                      | mg.L⁻¹ | 0.20±0.1 | 0.21±0.1 | 0.20±0.1 |
| NH₃                      | mg.L⁻¹ | 0.007±0.013 | 0.004±0.005 | 0.005±0.007 |

**ACKNOWLEDGEMENTS**

The authors would like to thank the Faculty of Fisheries and Marine Science, Mulawarman University, Samarinda, Indonesia for the financial support through operational funding for research and development, Mulawarman University, the year 2017.

**REFERENCES**

Al Hafedh YS, Alam A, Alam MA. 2003. Performance of plastic biofilter media with different configurations in a water recirculation system for the culture of Nile tilapia (Oreochromis niloticus). J Aquacult Eng 29: 139-154.

Balair JD, Haller RD. 1982. The intensive culture of tilapia in tanks, raceways and cages. In: Muir JR, Roberts RJ. (eds.), Recent Advances in Aquaculture. Westview Press Inc., Colorado.

Bradfield AE. 1983. Laboratory studies of energy budgets. In: Tyler P, Calow P (eds.), Fish Energetics: New Perspectives. John Hopkins University Press, Baltimore.

Brunske DE, Schwartz G, Eversole AG, Collier JA, Schwieder TE. 2003. Intensification of pond aquaculture and high rate photosynthetic systems. Aquacult Eng 26: 65-86.

Bultner JK, Soderberg RW, Terlizzi DE. 1993. An Introduction to Water Chemistry in Freshwater Aquaculture. Massachusetts: NRC Fact Sheet No. 170.

Côt J.1991. Aquaculture production system. J Anim Sci 69: 4183-4192.

Datta SN. 2002. Culture of Azolla and its efficacy in diet of Laevo rohita. Aquaculture 232: 117-127.

FAO (Food and Agriculture Organization of the United Nations). 2009. Use of algae and aquatic macrophytes as feed in small-scale aquaculture: A Review. FAO-Fisheries and Aquaculture Technical Paper, 531. Food and Agriculture Organization of the United Nations. Rome, Italy.

Frogé BDJC, Miché C, Van Howe. 2014. Use of a natural aquatic fern, Azolla microphylla, as a main component in food for the omnivorous - phyltoplanktonophagous tilapia, Oreochromis niloticus L. J Appl Ichthyol 20: 517-520.

Floyd RF. 2003. Oxygen for fish production. FACT Sheet FA-27. Extention-Institute of Food and Agricultural Science. University of Florida, Gainesville, FL.

Handy BD, and Poston MG. 1993. Nitrogen pollution in matriculture: toxicity and excretion of nitrogenous compounds by marine fish. Rev Fish Biol Fish 3 (3): 205-241.

Liu TL, Sun PB. 2008. Effect Ammonium on Blood Characteristic and Lypoxygenase Activities in Cultured Tilapia. Department of Food Science. National Taiwan Ocean University, Taiwan.

Lumpkin TA, Plucknett DL. 1980. Azolla: Botany, physiology and use as a green manure. Econ Bot 34: 111-134.

Neric C, Silva A, Merino G. 2012. Effect of two temperatures on ammonia excretion rates of Seriola dumerilii (Palm fish) juveniles under rearing conditions. Aquacult Eng 46: 47-52.

Pillay TVR. 1992. Aquaculture and the Environment. 1st ed. Cambridge University Press, Cambridge.

Rafe G, Saad CR. 2005. Nutrient cycle and sludge production during different stages of red tilapia (Oreochromis sp.) growth in a recirculating aquaculture system. Aquaculture 244: 109-118.

Randall D, Tsui T. 2002. Ammonia toxicity in fish. Mar Poll Bull 45: 17-23.

Schneider O, Sereti V, Eding EH, Verrhet JAJ. 2005. Analysis of nutrient flows in integrated intensive aquaculture systems. J Aquacult Eng 32: 379-401.

Smith M. 2003. Biological Filters for Aquaculture. L.S. Enterprises, Fort Fort Myers, FL, USA.

Sumoharjo. 2015. Performance of duckweed (Lemma minor) as biofilter in recirculating aquaculture system. J Trop Fish Sci 21: 86-94.
Teichert-Coddington D, Green BW. 1993. Tilapia yield improvement through maintenance of minimal oxygen concentrations in experimental grow-out ponds in Honduras. Aquaculture 118: 63-71.
Timmons MB, Ebeling JM, Wheaton FW, Summerfelt ST, Vinci BJ. 2002. Recirculating Aquaculture Systems, 2nd ed. Cayuga Aqua Ventures, New York.
Tran-Duy A, Schrama JW, Van Dam AA, Vereth JAJ. 2008. Effects of oxygen concentration and body weight on maximum feed intake, growth and hematological parameters of Nile tilapia, Oreochromis niloticus. Aquaculture 275: 152-162.
Van Hove C. 1989. Azolla and its Multipurpose Uses with Emphasis on Africa. FAO, Rome.
Wedemeyer GA. 1996. Physiology of fish in intensive culture. Chapman and Hall, New York.
Zonnenveld A, Huisman EA, Boon JH. 1991. The Principles of Aquaculture. PT. Gramedia Pustaka Utama, Jakarta. [Indonesian]