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

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Abstract. Sumoharjo, Ma’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 biofilter and perhaps that biomass produced in aquaculture system can be potential for alternative feed. This experiment such a first step of that vision and 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 flow, 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 result 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 amonia biofiltration efficiency which can remove ammonia up to 32.2±3.0% of the total NH\(_3\)-N and NH\(_4^+\)-N (TAN).

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

INTRODUCTION

The main problem in intensification of aquaculture system is water quality decreasing rapidly because high density of fish was reared with high feed input in a less water exchange. Hence, accumulation of fish metabolites, especially amonia, tends to occur in waterbody and build-up to toxic level and effecting fish performance. Wastewater is accumulated while feed is continuously added in a fish culture system (Rafee 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 NH\(_3\)-N and NH\(_4^+\)-N (TAN) increases with the pH of the water because TAN enters the organism as NH\(_3\) and the proportion of NH\(_3\) 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 of ammonia increase glycemia, lipoxxygenase and unsaturated of Erythrocyte Fatty Acids (Liu and Sun Pan 2008). Chronic exposure to high TAN concentrations tends to damage the fish gills, which can contribute to the decreased growth due to the effect on gas exchange efficiency (Handy and Poxton 1993; Nerici et al. 2012).

In Recirculating Aquaculture Systems (RAS), biofilter is a main component and known as low-cost water treatment to keep water quality suitable for fish growth and welfare. Biofilter technology was studied intensively, however, most of them are struggling on bacterial-based biofilters, such as nitrification by nitrifiers and nutrient assimilation by heterotrophics. Smith (2003) categorized biofilter in four main types, i.e. activated sludge, aquatic plant filters, fluidized bed filters, and fix film. *Azolla microphylla* is an aquatic fern. Many reports on *Azolla* had been published, but almost of them were related to its function as natural feed resouces. *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 alga *Anabaena azollae* that fixes nitrogen directly from the atmosfer (Van Howe 1989). So, *Azolla* is probablyable to grow well in a relative low nutrient. 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 in 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 system. The experiment were divided into two part; first was to analyse 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 tank with 1800 liter effective volume were used as experimental group. Particular design of each tank is on the bottom, whereas, geometrically different, i.e. prism, pyramid, and rectangular. Then, on the top of every tank was put three similar trench as biofilter bed with 2 meter in length. At every trench will be got three different water flow rates, i.e., 3 lpm, 5 lpm and 7 lpm. 32 watt of submersible pump was used to supply water from fish tank to the three trench connected in parallel with ¾ inch PVC pipe. Every water flow rate as the treatment was adjusted by up-and-down the outflow head. And synchronization was carried out daily.

Fish species cultivated were Tilapia (*Oreochromis niloticus*), sized 8.3±1.2 g and reared 100 fishes per tank. In every trench were put 50 g *Azolla microphylla*. 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 fish tank passed over the biofilter bed (the trench) once every 2 hours, then the water mixed continuously in the fish tank, so that there were no differences between in and outflow. Samples were taken in fish tank only. Total Amonia Nitrogen (TAN) as nutrient input and removal rates were calculated through mass balance. To estimate TAN input per day in the fish tanks can be calculated based upon the feeding rate (Timmons et al. 2002) as follows:

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

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 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 (NH\(_3\)-N). Lutron portable DO meter model 5510 was used to measure DO. The consentration 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:

\[ DGR \times 100 = \frac{\ln(W_t) - \ln(W_0)}{t} \]

\[ DT = \frac{\ln(2)}{DGR} \]

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 Amonia Nitrogen measured at the final day of the experiment will represent the nutrient output. Thus, in case of this simple RAS, whereas all water are 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) = \((C_i - C_e) \times Q\)

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

Where:
- \(C_i\) : Total Ammonia Nitrogen measured in the fish tank (mg/L x Water Volume x 1000 = g)
- \(C_e\) : Production rate of total ammonia nitrogen (g)
- \(Q\) : Water flow (liter per minutes, lpm)

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

\[
\text{Retained Nitrogen (RN, gram)} = (\text{TKN}_t \times W_t) - (\text{TKN}_o \times W_o)
\]

\[
\text{Retained Nitrogen Efficiency (RNE, %)} = \frac{\text{RN}}{\text{TKN}_t} \times 100
\]

Where:
- \(\text{TKN}_t\) : Total Kjedahl Nitrogen at the end of experiment (g)
- \(\text{TKN}_o\) : Initial Total Kjedahl Nitrogen (g)
- \(W_t\) : Final biomass of Azolla (g)
- \(W_o\) : Initial biomass of Azolla (g)

Data analysis
The means on the Azolla growth rate, doubling time, and nitrogen retension parameters were analyzed using two way analysis of variance (ANOVA, \(\alpha = 0.1\)). 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 the tanks I, II and III were 1519 g, 1504 g, and 1313 g, respectively. Therefore, TAN production of every tank listed in Table 1.

Based on the calculation, TAN production of all the fish tank 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 fish tank.

The TAN production was similar with 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 CO₂excreted via gill by ion exchange along with 500 g fecal solid and 5.5 g PO₄-P. Then, Schneider at 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 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).

<table>
<thead>
<tr>
<th>Tank</th>
<th>Feed consumed (g)</th>
<th>(P_{\text{TAN}}) total (g)</th>
<th>(P_{\text{TAN}}) (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1519</td>
<td>44.7</td>
<td>1.49</td>
</tr>
<tr>
<td>II</td>
<td>1504</td>
<td>44.3</td>
<td>1.48</td>
</tr>
<tr>
<td>III</td>
<td>1313</td>
<td>38.6</td>
<td>1.29</td>
</tr>
</tbody>
</table>

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

Figure 2. Doubling time of Azolla affected by water flow

Figure 3. Doubling time of Azolla biomass affected by grouped-tank

Statistically, the significant difference of Azolla’s biomass production were occured on experimental group-tank factors.Differences in the growth rate of Azolla biomass in each experimental group related to the baseboard designs that allowed better nutrient supply in Tank I and Tank II compared to Tank III. In Tank I and II, the average of 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 is 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 in order 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.
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 (*Lemna minor*) in the same experimental design which reached 25.7% (Sumoharjo 2015). Therefore, it could be as an alternative feed for herbivorous such as Tilapia. The high levels of this protein content are influenced by *Azolla*'s ability to convert nutrients from 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 $P_{\text{TAN}}$ of every tank as experimental group (Figure 4). The results of this experiment showed that the efficiency of the TAN removal by *Azolla* is still lower than the treatment using *Lemna minor* which reached 48%, but still 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 was ranged between 27.3 to 30.7°C, while pH and TAN were tend to decreased during experiment (Figure 5). The proportion of NH$_3$ increases with higher pH. It could be because TAN enters the organism as NH$_3$ (Randall and Tsui 2002). Therefore, the toxicity of TAN (the total NH$_2$-N and NH$_4$-N) increases in line with the increase of pH of the water. Fortunately, pH during experiment tends 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 amonia (NH$_3$) were low and in a tolerable concentration for Tilapia. The toxic level of NH$_3$ 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-ionised ammonia nitrogen over 0.068 mg NH$_3$ was significantly reduced. The specific growth rate and the increasing of the unionised ammonia concentration increased the feed conversion ratio (El-Syafai 2004).

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

<table>
<thead>
<tr>
<th>Source</th>
<th>Degr. of</th>
<th>RN</th>
<th>RN</th>
<th>RN</th>
<th>RN</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td>2</td>
<td>16.4019</td>
<td>8.2009</td>
<td>8.7203</td>
<td>0.034805*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Groups</td>
<td>2</td>
<td>276.2516</td>
<td>138.1258</td>
<td>146.8737</td>
<td>0.000180*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>4</td>
<td>3.7618</td>
<td>0.9404</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>296.4152</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 NH$_3$**
Carbon dioxide (CO₂) is another factor that may affect feed behaviour (Trand-Duy et al. 2008). In an intensive culture systems, CO₂ may not have an adverse effect on fish unless its concentration reaches 100 mg L⁻¹ (Balarin 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 at the first day then decrease to 15.2±1.1 at the 30th day of the experiment (Table 3). CO₂ tended to decrease during the experiment because the turbulences occured in inflow and outflow of the Azolla’s reactor may strip CO₂ to atmosfer. Moreover, algae and Azolla thrived in the reactors play a roles 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 occured in the last day of the experiment. Accumulation of sludge in the Azolla reactor plays 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 coldwater 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 reared 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 achieved). Therefore, harvesting must be done at 15 to 18 days after cultivation. As a biofilter it provides a mini-ecosystem that serves as nutrient controller for the best aquaculture practices. The lower water flow rates 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 potential as feed source for herbivorous fishes.

Table 3. Means and standard deviations of water quality characteristics

<table>
<thead>
<tr>
<th>Water quality parameters</th>
<th>Unit</th>
<th>Tank I</th>
<th>Tank II</th>
<th>Tank III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>°C</td>
<td>29.2±1.2</td>
<td>29.0±2.9</td>
<td>28.8±1.2</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>7.4±0.4</td>
<td>7.3±0.4</td>
<td>7.3±0.3</td>
</tr>
<tr>
<td>DO</td>
<td>mg L⁻¹</td>
<td>4.1±1.4</td>
<td>3.6±1.2</td>
<td>4.0±1.7</td>
</tr>
<tr>
<td>CO₂</td>
<td>mg L⁻¹</td>
<td>21.0±11.4</td>
<td>23.2±13.3</td>
<td>22.0±11.7</td>
</tr>
<tr>
<td>TAN</td>
<td>mg L⁻¹</td>
<td>0.20±0.1</td>
<td>0.21±0.1</td>
<td>0.20±0.1</td>
</tr>
<tr>
<td>NH₃</td>
<td>mg L⁻¹</td>
<td>0.007±0.013</td>
<td>0.004±0.005</td>
<td>0.005±0.007</td>
</tr>
</tbody>
</table>

ACKNOWLEDGEMENTS

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

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