



RESEARCH ARTICLE

REVISED Effect of different biofloc starters on ammonia, nitrate, and nitrite concentrations in the cultured tilapia *Oreochromis niloticus* system [version 3; peer review: 2 approved]

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Abstract

Background: High stocking density and intensive feeding in aquaculture systems lead to the accumulation of organic waste, which results in an increase in ammonia, nitrite, and nitrite concentrations in culture media. Biofloc is a potential technology to overcome this problem. The starter is a crucial carbon source for bacteria in the formation of biofloc. The objective of the present study aimed to explore the best starter of biofloc in a red tilapia *Oreochromis niloticus* culture system.
Methods: A completely randomized design with four levels of treatment was used in this study. The tested starter was (A) control treatment, biofloc without starter, (B) biofloc with molasses starter, (C) biofloc with tapioca starter, and (D) biofloc with sucrose starter. The floc was cultured in 100-L tanks with a salinity of 17 ppt. The tanks were stocked with *O. niloticus* with a size of 3.71±0.11 cm at a stocking density of 30 fish per tank. The fish were fed on a commercial diet two times a day at satiation for 40 days. The ammonia, nitrite, and nitrite concentrations were measured for an interval of 8 days.
Results: The study showed that the NH₃-N range was 0.02–0.07 mg L⁻¹ (mean, 0.03 ± 0.02 mg L⁻¹), NO₂-N range was 0.20–0.43 mg L⁻¹ (mean, 0.25 ± 0.12 mg L⁻¹), and NO₃-N range was 0.90–3.20 mg L⁻¹ (mean, 1.42 ± 1.19 mg L⁻¹).
Conclusion: Among the starters tested, molasses was found to be the best for biofloc in tilapia culture.

Keywords

Biofloc, Carbon, Molasses, Water Quality

Open Peer Review

Reviewer Status

| | Invited Reviewers | |
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Any reports and responses or comments on the article can be found at the end of the article.

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Author roles: **Putra I:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Resources, Validation; **Effendi I:** Conceptualization, Data Curation, Investigation, Methodology; **Lukistyowati I:** Conceptualization, Data Curation, Investigation, Methodology; **Tang UM:** Conceptualization, Data Curation, Methodology, Writing – Original Draft Preparation; **Fauzi M:** Conceptualization, Data Curation, Formal Analysis, Investigation; **Suharman I:** Data Curation, Formal Analysis, Investigation, Methodology; **Muchlisin ZA:** Supervision, Validation, Writing – Review & Editing

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REVISED Amendments from Version 2

In the introduction, we corrected MS articles according to the reviewers' suggestions; the statement "no information" was replaced by "limited information".

Any further responses from the reviewers can be found at the end of the article

Introduction

Water quality is a crucial factor in aquaculture systems. One important water quality parameter is nitrogen. In water, nitrogen can be found in the forms of ammonia, nitrite, and nitrate¹⁻⁵. Ammonia (NH₃-N) is produced from the breakdown of proteins from unconsumed feed, feces, and urine of fish. This compound will turn into nitrite (NO₂) when oxygen levels are poor⁶, which is toxic for fish⁷. By contrast, ammonia is changed into nitrate when the dissolved oxygen level is sufficient^{8,9}. Fish produces ammonia (inorganic N) through the osmoregulation process; feces and urine contribute about 10%–20% of total nitrogen¹⁰. The application of biofloc is one of the alternatives to overcome water quality problems especially in controlling total ammonia nitrogen in the aquaculture system¹¹⁻¹³.

Biofloc refers to the use of heterotrophs and autotrophs, which can convert organic waste into floc forms that can be utilized by fish as a food source^{4,11,14-16}. Biofloc technology is cheap, simple, and environmentally friendly^{13,17}. Several organisms, such as bacteria, plankton, fungi, and algae, and suspended particles exist in flocs^{18,19}. These organisms provide nutrition for cultured fish. However, the formation of biofloc needs a starter consisting of probiotics and a carbon source. Molasses, tapioca, and wheat flours are common starters in biofloc culture (African catfish, Nile tilapia, *Litopenaeus vannamei*)^{16,19-23}. Presently, limited information is available on the best starter for biofloc in the cultured system of red tilapia *Oreochromis niloticus*. Thus, the present study aimed to explore the best starter for biofloc in a red tilapia *O. niloticus* culture system.

Methods**Time and site**

The research was carried out for 40 days from February 2019 to March 2019 at the Aquaculture Technology Laboratory, Faculty of Fisheries and Marine, Riau University, Indonesia. The experiments were carried out within the ethical guidelines in animal research developed by NC3Rs. In Indonesia, no approval is required to conduct research on fish.

Experimental design

A completely randomized design with four levels of treatment and three replications was performed in this study; the tested treatment was four starters of biofloc, namely control without a starter (treatment A), biofloc with molasses starter concentration 0.48 gL⁻¹ (treatment B), biofloc with tapioca starter concentration 0.35 gL⁻¹ (treatment C), and biofloc with sucrose concentration 0.42 gL⁻¹ (treatment D).

The amount of carbon added is calculated based on the carbon content (C) in the ingredients and the nitrogen content in the feed given⁵ using the formula:

$$C/N = (\% \text{ C starter} \times \text{molecule weight of starter} + \% \text{ C feed} \times \text{feed weight}) / (\% \text{ N feed} \times \text{feed weight})$$

Based on this formula, to obtain a C/N ratio of 20: 1, a carbon source derived from molasses was 48 g with a C content of 37%²⁴, tapioca flour as much as 35 g with a C content of 50.3%²⁵, and sucrose 42 g with a C content of 42.3%²⁶.

Biofloc culture

Biofloc was cultured using 0.01 ml/L probiotic bacteria from Boster Multisel (*Bacillus* sp.)¹⁶, and the carbon source was from starters based on the tested treatments with the C/N ratio of 20:1. The starter was applied in cultivation media and probiotic bacteria administration. Probiotics and starter were applied into cultivation cultured media and left for 1 week, and the media were aerated continuously. The starters were added every week for maintaining the floc. Floc formation was indicated by green water and foam forming in the media.

The number of fish used in this study was 360 individuals and they were obtained from a hatchery in the Bengkalis Regency, Riau. The fish were 50 days old, 3.71±0.11 cm TL. As the sex of the fish cannot be identified morphologically, there is no separation of fish sex in this study. The fish was distributed into research tanks as the flocs are formed, 7 days after the starter was cultured in the tanks (total 12 tanks, 100L volume). The density of the fish was 30 fishes/100 L. Stocking of red tilapia larvae in the morning at 8 AM with a water temperature of 25°C. The fish were fed on commercial diet containing 38% crude protein, 5% crude lipid, and 6% crude fiber (PF1000, PT Matahari Sakti). Feeding was conducted *ad libitum*/satiation, 2 times/day (8 AM and 5 PM) for 40 days.

Measurement of nitrogen content

The ammonia (NH₃), nitrite (NO₂⁻), and nitrate (NO₃⁺) contents were measured by spectrophotometry. These parameters were measured every 8 days in each treatment for 40 days. Ammonia (NH₃), Nitrite (NO₂⁻) and Nitrate (NO₃⁺) were measured using spectrophotometer (Optima - SP300) at a wavelength of 630 nm²⁷. The measurement was conducted at 8:00 AM with three replications at 8-day intervals over 40 days (six timepoints).

Statistical analysis

The data were subjected to one-way analysis of variance followed by the post hoc Newman-Keuls test using SPSS 18.0 software. P<0.05 was considered to indicate a statistically significant difference.

Results

Over the six timepoints measured, the mean concentration of ammonia ranged from 0.02 mg L⁻¹ to 0.07 mg L⁻¹ (with an average value of 0.03 ± 0.02 mg L⁻¹), nitrite ranged from 0.20 mg L⁻¹ to 0.43 mg L⁻¹ (with an average value of 0.25 ± 0.12 mg L⁻¹), and nitrate ranged from 0.64 mgL⁻¹ to 3.20 mgL⁻¹ (with an average value of 1.42 ± 1.19 mg L⁻¹). The study revealed that the best concentrations of ammonia, nitrite, and nitrate were recorded in the biofloc starter of molasses. These values did not significantly differ from those obtained with the tapioca and sucrose starters, but they were significantly different from the control treatment without a starter (Table 1). Raw values for each replicate are available as *Underlying data*²⁸.

Table 1. Average values of ammonia nitrogen, nitrite, nitrate, turbidity, and carbon dioxide concentrations based on treatment. Data values are the mean and standard deviation. Mean values with different superscripts in the same row were significantly different ($p < 0.05$).

| Parameter | Unit | Treatment | | | |
|--------------------|-------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| | | Control (A) | Molasses (B) | Tapioca (C) | Sucrose (D) |
| NH ₃ -N | mgL ⁻¹ | 0.07 ± 0.05 ^b | 0.02 ± 0.01 ^a | 0.02 ± 0.01 ^a | 0.02 ± 0.01 ^a |
| NO ₂ -N | mgL ⁻¹ | 0.43 ± 0.25 ^b | 0.14 ± 0.09 ^a | 0.20 ± 0.12 ^a | 0.24 ± 0.14 ^a |
| NO ₃ -N | mgL ⁻¹ | 3.54 ± 3.21 ^b | 0.64 ± 0.46 ^a | 0.90 ± 0.96 ^a | 0.93 ± 1.12 ^a |

In the control treatment, the concentrations of ammonia, nitrite, and nitrate increased gradually with increasing experimental time. However, in the starter treatments, the ammonia concentration dropped during the first week of the experiment (day 8), became stagnant during the second and third weeks (day 16 to day 24), and increased again at day 32 of the experiment. However, the ammonia concentration decreased sharply at day 40 (Figure 1a). The nitrite concentration also decreased during the first week but increased slightly at day 16 and increased gradually until day 40 in treatments C (tapioca) and D (sucrose). By contrast, the nitrite concentration decreased at day 24, increased at day 32, and decreased at day 40 (Figure 1b). The nitrate concentration was relatively stagnant from day 1 to day 8. The nitrate concentration fluctuated during the experiment (Figure 1c). In general, the molasses starter (treatment B) yielded slightly better results than the other starters, but no significant difference was found between the treatments except the control. The data of the growth performance of the red tilapia fish *O. niloticus* has been published separately²⁹.

Discussion

The study revealed that the concentrations of ammonia, nitrite, and nitrate were significantly lower in the biofloc system using starters compared with those in the system without a starter (control). Biofloc has probiotic bacteria that can change ammonia to nontoxic materials (such as nitrate) that are useful for phytoplankton growth. Therefore, the ammonia and nitrate concentrations are low in the culture media^{19,21,30}. Biofloc does not necessarily only contain bacteria (for example, *Bacillus*), but is also composed of other useful microorganisms such as microalgae and zooplankton that are trapped by organic particles³¹. Algae and zooplankton can be used by cultured biota (tilapia) as natural food.

In general, the starters used in this study were carbohydrate compounds. However, the study showed that the molasses starter yielded slightly better results compared with the other starters. This finding indicated that molasses was the best carbon source for biofloc in the tilapia culture system. Molasses can provide a sufficient carbon level for heterotrophic bacteria that use this carbon as an energy source for growth^{6,19,21,23,32}. Molasses are a liquid byproduct from the sugar industry. This material has a total carbon content of around 37%²⁴. Therefore,

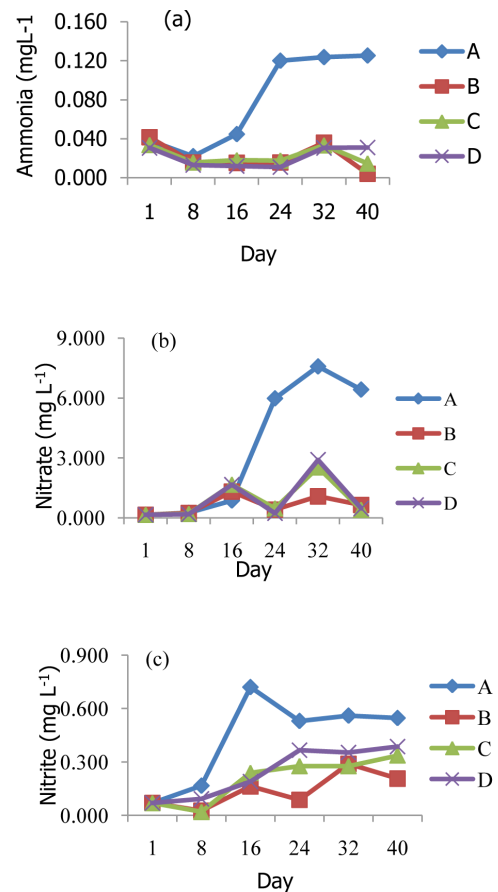


Figure 1. (a) Concentrations of ammonia, (b) nitrate, and (c) nitrite during 40 days of the experiment. A = control treatment, B = molasses starter, C = tapioca starter, and D = sucrose starter.

molasses are rapidly soluble in water and can be quickly absorbed by heterotrophic bacteria³. In terms of chemical structure, molasses are classified as a simple carbohydrate containing six C atoms (monosaccharides), while sucrose (treatment D) is a combination of two monosaccharides that contain 12 C atoms (sucrose). Tapioca is classified as a complex carbohydrate (60,000 C atoms) and is more slowly digested by bacteria than molasses^{25,33}.

Conclusion

Carbon source from molasses is effective in reducing concentrations of ammonia, nitrite, and nitrate in red tilapia culture with biofloc technology.

Data availability

Underlying data

Figshare: Effect of different biofloc starters on ammonia, nitrate, and nitrite concentrations in the cultured tilapia *Oreochromis niloticus* system. <https://doi.org/10.6084/m9.figshare.12027951>²⁸.

This project contains raw data for the concentration of ammonia, nitrate and nitrite in each tank at each timepoint.

Data are available under the terms of the [Creative Commons Attribution 4.0 International license \(CC-BY 4.0\)](#).

Acknowledgments

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Open Peer Review

Current Peer Review Status:  

Version 3

Reviewer Report 03 June 2020

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Muhammad Rizal Razman 

Research Centre for Sustainability Science and Governance (SGK), Institute for Environment and Development (LESTARI), Universiti Kebangsaan Malaysia (UKM), Bangi, Malaysia

The paper has been amended accordingly as suggested.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Agricultural and Biological Sciences, Environmental Science, Sustainability Science and Governance for Sustainability

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 03 June 2020

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Christopher Marlowe A. Caipang

University of San Agustin, Iloilo City, Philippines

The authors have addressed the suggestions/comments made on the manuscript.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Aquaculture, biotechnology, aquatic microbiology, fish health management

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 20 May 2020

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Christopher Marlowe A. Caipang

University of San Agustin, Iloilo City, Philippines

The authors have addressed the issues and suggestions in the earlier draft. However, I have notice that the statement in the revised version "Presently, no information is available on the best starter for biofloc in the cultured system of Nile tilapia *Oreochromis niloticus*." is still there and no additional information was given. The authors must indicate a thorough literature search if indeed no best starter is currently available.

If the authors can address this issue, then the manuscript can be considered to be accepted and indexed in the journal.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Aquaculture, biotechnology, aquatic microbiology, fish health management

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 11 May 2020

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Muhammad Rizal Razman 

Research Centre for Sustainability Science and Governance (SGK), Institute for Environment and Development (LESTARI), Universiti Kebangsaan Malaysia (UKM), Bangi, Malaysia

1. The work was clearly and accurately presented.
2. The work has cited the current literature, (36 %) of (2015-2020).
3. The study design was appropriate and the work was technically sound.
4. The work presented sufficient details of methods and analysis provided to allow replication by others.
5. The statistical analysis and its interpretation was appropriate.
6. The source of data underlying the results available to ensure full reproducible.
7. The authors should elaborate more for their conclusion. The conclusion should be reflecting the work that has been done. The conclusion given was too brief.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: (Agriculture and Biological Sciences, Environmental Science, Sustainability Science and Governance, Environment and Development)

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 01 May 2020

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Christopher Marlowe A. Caipang

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Summary: This ms aimed to determine the best starter of biofloc in a red tilapia *Oreochromis niloticus* culture system. Three biofloc starters (molasses, tapioca and sucrose) and control were tested in triplicate over a period of 40 days. Nitrate, nitrite and ammonia were monitored during the study. Based on the findings, molasses is considered to be the best starter for biofloc production among the starters that were tested.

Comments: The work presented the findings in a straightforward manner. However, the ms can be further improved following these suggestions:

1. Change the title to: Effect of different biofloc starters on ammonia, nitrate, and nitrite concentrations in the culture of tilapia *Oreochromis niloticus*.
2. The authors mentioned red tilapia in the Abstract but only tilapia in other portions of the text. Please be consistent.
3. In the Introduction, the authors wrote: "Molasses, tapioca, and wheat flours are common starters in biofloc culture". Please indicate to which species were these starters used.
4. The authors wrote: "Presently, no information is available on the best starter for biofloc in the cultured system of Nile tilapia *Oreochromis niloticus*". It is suggested that the authors conduct a thorough literature search, as there have been studies done on biofloc starters in tilapia. Please rectify this statement.
5. In the methods, please indicate how the biofloc was maintained in the study. The study only made mentioned the starter. In the 40 days, was there no addition of the starters to maintain the biofloc?
6. Please indicate water exchange rates? There was no water change in the control in 40 days, and the fish were quite big during stocking (approximately 3 g). With no water exchange in 40 days in the control and with the addition of feed, there is definitely a deterioration of water quality and lower fish survival. In the actual culture of tilapia, water exchange is done. For biofloc system, water exchange is minimal hence the results are compared in terms of water quality as well as savings in the cost of electricity or volume of water discharge.
7. Was feeding the same for the biofloc treatments and control? Please explain.
8. The authors should explain the water exchange and feeding management between the control and biofloc treatments and how these are controlled/managed relative to the study objectives.
9. Perform a correlation analysis on the water quality parameter over time. Related the values of the slope and intercept on the effects of water quality for each treatment.

10. ANOVA should also be done on the water quality per sampling point so that the readers will know when the differences became significant.
 11. Why did the authors conclude that molasses is the best starter? Based on the graph, all three starters had better water quality than the control. In addition, the differences in the water quality values (ammonia, nitrite, nitrate) among the biofloc treatments were not significantly different. It is suggested that authors perform additional statistical test to show that molasses is significantly better than the other 2 biofloc starters.
 12. The role of the probiotics in the study should be discussed. The authors should provide in the methods the initial counts of the probiotics that were applied initially.
- I hope that these suggestions will be considered by the authors when they revise the manuscript.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Aquaculture, biotechnology, aquatic microbiology, fish health management

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 04 May 2020

Iskandar Putra, Universitas Riau, Pekanbaru, Indonesia

1. Inquiry

Change the title to: Effect of different biofloc starters on ammonia, nitrate, and nitrite concentrations in the culture of tilapia *Oreochromis niloticus*.

Response: Thank you for your suggestion. I have already changed it

2. Inquiry

The authors mentioned red tilapia in the Abstract but only tilapia in other portions of the text. Please be consistent.

Response: Thank you for your suggestion. I have already changed it

3. Inquiry

In the Introduction, the authors wrote: "Molasses, tapioca, and wheat flours are common starters in biofloc culture". Please indicate to which species were these starters used.

Response: We added this information in Introduction

4. Inquiry

The authors wrote: "Presently, no information is available on the best starter for biofloc in the cultured system of Nile tilapia *Oreochromis niloticus*". It is suggested that the authors conduct a thorough literature search, as there have been studies done on biofloc starters in tilapia. Please rectify this statement.

Response: We have modified this statement

5. Inquiry

In the methods, please indicate how the biofloc was maintained in the study. The study only made mentioned the starter. In the 40 days, was there no addition of the starters to maintain the biofloc?

Response: The starters were added every week for maintaining the floc

6. Inquiry

Please indicate water exchange rates? There was no water change in the control in 40 days, and the fish were quite big during stocking (approximately 3 g). With no water exchange in 40 days in the control and with the addition of feed, there is definitely a deterioration of water quality and lower fish survival. In the actual culture of tilapia, water exchange is done. For biofloc system, water exchange is minimal hence the results are compared in terms of water quality as well as savings in the cost of electricity or volume of water discharge.

Response: There is no water exchange during conducting the experiment

7. Inquiry

Was feeding the same for the biofloc treatments and control? Please explain.

Response: Feeding applied for the biofloc treatments and control were same

8. Inquiry

The authors should explain the water exchange and feeding management between the control and biofloc treatments and how these are controlled/managed relative to the study objectives.

Response: There was no water exchange during the experiment and feeding was applied the same treatment for the biofloc treatments and control.

9. Inquiry

Perform a correlation analysis on the water quality parameter over time. Related the values of the

slope and intercept on the effects of water quality for each treatment.

Response: We did not perform a correlation analysis on the water quality parameter over time.

10. Inquiry

ANOVA should also be done on the water quality per sampling point so that the readers will know when the differences became significant.

Response: We calculated ANOVA of the water quality according to data at the end of the experiment

11. Inquiry

Why did the authors conclude that molasses is the best starter? Based on the graph, all three starters had better water quality than the control. In addition, the differences in the water quality values (ammonia, nitrite, nitrate) among the biofloc treatments were not significantly different. It is suggested that authors perform additional statistical test to show that molasses is significantly better than the other 2 biofloc starters.

Response: Based on the ANOVA, there was no significant difference of water quality among the starter treatments, but the molasses supplementation in the culture media showed the best water quality values compared to other starters

12. Inquiry

The role of the probiotics in the study should be discussed. The authors should provide in the methods the initial counts of the probiotics that were applied initially.

Response: Thank you for your suggestion. We added this information in methods

Competing Interests: No competing interests were disclosed.

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