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The Effects of Microalgal Diet With Enrichment of Fermented Organic Matters (Tofu Waste, Rice Bran and Fish Meal) on Growth and Reproduction of *Diaphanosoma brachyurum*

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Abstract. *Diaphanosoma brachyurum* is one of cladocerans that potentially used as live food organism for larval rearing in fish and shrimp hatcheries. In the culture of *D. brachyurum*, it usually uses live food from phytoplankton cells, but researches on the diet enrichment from fermented organic matters has not been widely informed. This study was aimed to investigate the different effects among different microalgal diets and a combination of the best microalgal diet with fermented organic matters on growth and reproduction of *D. brachyurum*. Two experiments were carried out in this study and those designs applied Completely Randomized Design (CRD). The first experiment, the different microalgal diets divided into four treatments with four replication and those were *C. vulgaris* (A); *C. calcitrans* (B); *N. oculata* (C); and *T. chuii* (D). The second experiment, the combination of the best microalgal diet and fermented organic matters (FOM) divided into five treatments with three replication and those were 100% *T. chuii* (A); 75% *T. chuii* and 25% FOM (B); 50% *T. chuii* and 50% FOM (C); 25% *T. chuii* and 75% FOM (D); and 100% FOM (E). The results showed that *T. chuii* has significant different ($P < 0.05$) than another algal cells, which as the best algal diet based on its total density ($25,875 \pm 1,142 \text{ ind.mL}^{-1}$); population growth rate ($0,163 \pm 0,022 \text{ d}^{-1}$); and egg production ($3,446 \pm 0,363 \text{ eggs.ind}^{-1}$) was better than *N. oculata* ($P < 0.05$). The results of the second experiment showed that with 50%:50% combination of *T. chuii* and FOM was confirmed as the best treatment ($P < 0.05$) based on its total density ($38,183 \pm 3,595 \text{ ind.mL}^{-1}$); population growth rate ($0,82 \pm 0,005 \text{ d}^{-1}$); and egg production ($2,418 \pm 0,031 \text{ eggs.ind}^{-1}$).

1. Introduction

As live food organism, *Diaphanosoma brachyurum* is a potential cladoceran which can be used for larval rearing in hatchery due to its benefits. A few studies have shown the application of *Diaphanosoma* sp. for growing Asian Sea Bass (*Lates calcarifer*) larvae [1] and post-larval shrimp (*Litopenaeus vannamei*) [2]. According to Hagiwara et al [3], *Diaphanosoma* sp. is able to reproduce parthenogenetically, having high toleration on a wide range of water salinity and its possibility for growing rapidly under appropriate diet and environment condition. According to Sipaub-Tavares and Bachion [4], cladoceran culture offers the possibility of obtaining a large number of individuals quickly under an appropriate condition of temperature, food and water quality, due to parthenogenetic reproduction of this organism.



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D. brachyurum should be constantly available in high number in order to fulfill the needs of aquatic organisms which it can be supported by giving a high-nutritional source for *D. brachyurum*. There is still a lack of information about the ideal food source which can be increasing the number of *D. brachyurum* in aquaculture activity. The ideal microalgal diet with fermented organic matter enrichment can be a solutive method to realize it as it seems to more practical to applicate. According to Hagiwara et al [3], beside phytoplankton, organic matter can be an alternative source to increase the population of zooplankton. According to Rajthilak et al [5], a combination of phytoplankton which enriches by fermented organic matter is able to increase the number of zooplankton and its nutritional value.

This research aimed to know the effect of different microalgal diet which enrich by fermented organic matters on growth and reproduction of *D. brachyurum* and to know the ideal kind of single microalgal diet and ideal dose of the combination of single microalgal diet and fermented organic matters which able to increase *D. brachyurum* production.

2. Research Method

This research was conducted in the Laboratory of Life Food Organism BBPBAP Jepara, Central Java, Indonesia in 2016 until 2017. This research was divided into two experimental categories, Experiment I was for *D. brachyurum* given with different microalgal diet and Experiment II was for *D. brachyurum* given with microalgal diet and fermented organic matters enrichment. This research applied experimental laboratories with Completely Randomized Design (CRD) in each treatment.

2.1. *Diaphanosoma* culture preparation

D. brachyurum stock for this research was obtained from BBPBAP Jepara. Adult *D. brachyurum* selection was conducted by isolating them one by one with a pipette and moved into petridish. Adults were observed under the microscope. After being isolated, the adults were cultured with an initial density of approximately 400 ind.L⁻¹ and fed by 1×10^6 ind.mL⁻¹ of *C. vulgaris* which that feeding with feeding dose based on [6]. This step was conducted to increase the stock of *D. brachyurum* that would be used for the research, especially for Experiment I.

D. brachyurum culture on this research was conducted with 50 mL volume flask bottle fulfilled by 20 mL of sterilized seawater. *D. brachyurum* adults were isolated from stocks by took them one by one with pipette and observed under a microscope then they put into bottle flask. The initial density of *D. brachyurum* was 1 ind.mL⁻¹ and culture in controlled environment which its water salinity was 25 ‰, 25 °C of temperature and pH 7. The culture environment was under 24-h lightning period without aeration. Water exchange was daily conducted by replacing it with 30% of new water. The culture was conducted under 24-h lightning period with 1500 – 1800 lux of light intensity.

2.2. Experiment I

This experiment consisted of four treatments with four replication. There were four dietary treatments, *Chlorella vulgaris* (A); *Chaetoceros calcitrans* (B); *Nannochloropsis oculata*(C); and *Tetraselmis chuii*(D). Each of those microalgae was cultured in 3 L volume of sterilized Erlenmeyer flask filled with 2 L of sterilized seawater. *C. vulgaris* and *N. oculata* were cultured with Walne Medium, then *C. calcitrans* and *T. chuii* were cultured with Guillard Medium. Microalgae were cultured at 25 °C temperature, 20 to 30 ‰ water salinity, pH 7, with a 24-h lightning period (approximately 1800 lux) with continuous aeration.

The volume of inoculant was 10% of the total of culture media [7]. Cultured microalga was given to *D. brachyurum* at the time of exponential phase as its high nutritional level [8]. The stock density of microalgae (cell mL⁻¹) was calculated each day by took microalgae sample and counted under a microscope with a haemocytometer (Improved Neubauer volume 0,0025 mm³). The feeding method used *ad libitum*. The number of microalgae which given based on the feed requirement of *D. brachyurum*, which it was 0,03 mg [6]. Dry weight of each microalgae was 12 pg.cell⁻¹ for *C.*

vulgaris[9]; 11,3 pg.cell⁻¹ for *C. calcitrans* [10]; 6,1 pg.cell⁻¹ for *N. Oculata* [9]; and 269 pg.cell⁻¹ for *T. chuii*.

The calculation formula of a number of microalgae (cells) which given to *D. brachyurum* was based on Lee et al [9], which The Number of Microalgae (cells) = Feed Weight (mg)/Microalgae Dry Weight (mg.cell⁻¹). Microalgae needed to be centrifuged before given to *D. brachyurum*. The feeding is given by micropipette. The quantity of given microalga depended on *D. brachyurum* density.

Table 1. Microalgal Diet Requirement for Individual *D. brachyurum* (cell.ind⁻¹)

Treatment	Number of Microalgal Diet ($\times 10^4$ cell)			
	<i>C. vulgaris</i>	<i>C. calcitrans</i>	<i>N. oculata</i>	<i>T. chuii</i>
A	250	-	-	-
B	-	265,48	-	-
C	-	-	491,80	-
D	-	-	-	11,15

2.3. Experiment II

This experiment consisted of five treatments with three replication. There were 100% microalgae (A); 75% microalgae with 25% fermented organic matters (B); 50% microalgae with 50 % fermented organic matters (C); 25% microalgae with 75% fermented organic matters (D); and 100% fermented organic matters. Microalgae that used in this experiment was *T. chuii* (based on Experiment I result) and its organic matters were tofu waste, rice bran, and fish meal. *T. chuii* was cultured the same way as Experiment I. Those organic matters should be refining them by sieve in order to get its similar size. Fermentation of organic matters was conducted by activating 1 mL of EM4 probiotic with 1 mL molasses and mixed with 100 mL water. EM4 probiotics contained *Lactobacillus casei* (minimum $2,0 \times 10^6$ cell. mL⁻¹) and *Saccharomyces cerevisiae* (minimum $3,5 \times 10^5$ cell. mL⁻¹). Each organic matter (tofu waste, rice bran and fish meal) was mixed with percentage dose 35 %: 35 %: 30% [10]. Those matters were mixed in 450 mL plastic jar volume and the activated probiotics were sprayed to the organic matters. The jar was tightly closed in order to make fermentation process for approximately 4 days. After that, the fermented organic matters saved in low temperature and then weighed for 0,2 g. They were mixed into 50 mL of 25 ppt sterilized sea water.

The feeding calculation of *T. chuii* was based on a formula from Lee et al [9] and fermented organic matters calculation was only based on *D. brachyurum* feeding requirement (0,03 mg). The dose of fermented organic matters in milligrams (mg) converted into milliliter (mL) as its essence that given to *D. brachyurum*. The fermented organic matters should be homogenized with water by shaking it first. The feeding method was conducted by micropipette.

Table 2. Microalgal Diet (cell.ind⁻¹) and Fermented Organic Matters (mg.ind⁻¹) Requirement for Individual *D. brachyurum*

Treatment	Microalgal Diet (<i>T. chuii</i>) ($\times 10^4$ cell)	Microalgal Diet (<i>T. chuii</i>) ($\times 10^{-2}$ mg)	Fermented Organic Matters ($\times 10^{-2}$ mg)
A	11,15	3	-
B	8,36	2,25	0,75
C	5,57	1,5	1,5
D	2,78	0,75	2,25
E	-	-	3

2.4. Measuring variables

Measuring variables of this research were divided into 2 categories, main and secondary variables. Main variables were total density (ind.mL⁻¹), population growth rate (d⁻¹) and egg production (eggs.ind⁻¹). Secondary variables were the density of *D. brachyurum* stages (ind.mL⁻¹), such as neonate, juvenile, adult, egg-laying adult, an adult with embryo and neonate production within the pouch.

Total density of *D. brachyurum* (ind.mL⁻¹) was the accumulation of total individuals of *D. brachyurum* in 20 mL culture media with formula Total Density = N/V (N: total individuals (ind); V: culture media volume (mL)). The number of each individual stages is also calculated with the same method.

Population growth rate (r) calculation is based on *D. brachyurum* density in exponential phase by using formula from Lavens and Sorgeloos [11], which is $r = (\ln N_t - \ln N_0)/t$. According to [12], N_0 is initial density, N_t is number density in t (cultivating time).

According to Dumont et al [13], egg production = $\sum s \times e / \sum n$, which it is a number of brood pouch, e is the average number of eggs per brood pouch and n is the total number of the egg-laying individual. Egg production is calculated from the total sample of egg-laying adult *D. brachyurum* which only has one brood pouch

Neonate production within pouch calculation is assumed with egg production calculation, which s is assumed as the number of the pouch, e is assumed as the number of neonate and n is assumed as the number of its adult.

2.5. Statistical analysis

The main data were analyzed by One-Way Analysis of Variance (ANOVA) to determine the effect of the treatments on the growth and reproduction of *D. brachyurum*. Duncan Test was conducted when the treatments had a significant effect. All data analysis were conducted by using SPSS 16.

3. Result and discussion

3.1. The result of experiment I

The result of Experiment I showed that *T. chuii* (Treatment D) had the highest number of total density of *D. brachyurum* (25,875±1,142 ind.mL⁻¹) in last day of culture and the lowest value was in Treatment C (*N. oculata*) (Table 3). Treatment D showed that the *D. brachyurum* growth was still in the exponential phase, instead of in other treatments (Fig. 1). Treatment D was high significantly different ($P<0,05$). The highest value of population growth performance was also in Treatment D (0,163±0,002 d⁻¹) with its lowest value was in Treatment B (*C. calcitrans*) (Fig. 2). Treatment D was high significantly different ($P<0,05$) on its growth rate value. It was bit contradictive with egg production value, which the highest number of egg production was in Treatment C where *N. oculata* produced 3,446±0,363eggs.ind⁻¹ and the lowest production was in Treatment C (*T. chuii*) (Fig. 3). The treatment was significantly different ($P<0,05$) except with Treatment A (*C. vulgaris*) ($P>0,05$).

Table 3. Value of Total Density in Day-20, Population Growth Rate, Egg Production, and Neonate Production within Pouch (Experiment I)

No.	Variable	Treatment			
		A	B	C	D
1.	Total Density (ind.mL ⁻¹)	1,925±0,104 ^b	0,025±0,029 ^c	0,013±0,025 ^c	25,875±1,142 ^a
	a. Adult	0,388±0,111	0,013±0,025	0,000±0,000	7,325±1,981
	b. Egg-laying Adult	0,663±0,307	0,013±0,025	0,000±0,000	4,050±2,401
	c. Adult with Embryo	0,313±0,048	0,000±0,000	0,000±0,000	8,975±1,569
	d. Juvenile	0,213±0,149	0,000±0,000	0,000±0,000	2,513±0,638
	e. Neonate	0,350±0,071	0,000±0,000	0,000±0,000	3,013±1,582
2.	Population Growth Rate (d ⁻¹)	0,108±0,002 ^c	0,052±0,005 ^d	0,127±0,004 ^b	0,163±0,002 ^a
3.	Egg Production (eggs.ind ⁻¹)	2,755±0,412 ^{ab}	3,104±0,705 ^b	3,446±0,363 ^a	2,242±0,089 ^b
4.	Neonate in pouch	2,747±0,561	2,722±0,896	2,536±1,838	2,817±0,309

(neonate.ind⁻¹)

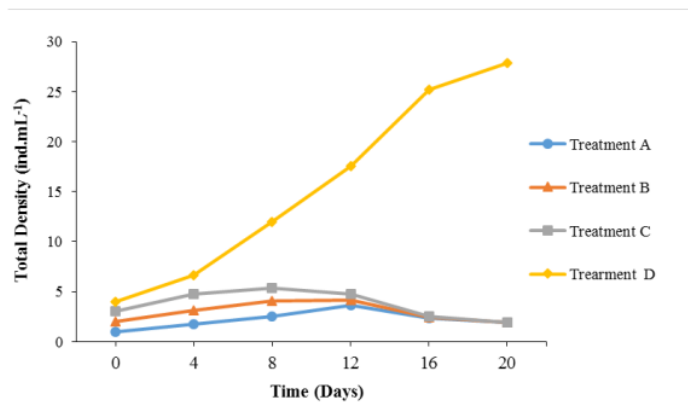


Fig. 1. Total Density of *D. brachyurum*(Experiment I)

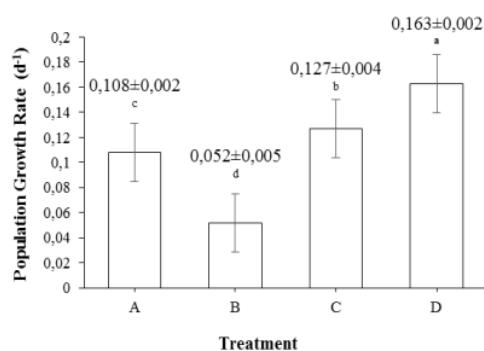


Fig. 2. Population Growth Rate of *D. brachyurum* brachyurum(Experiment I)

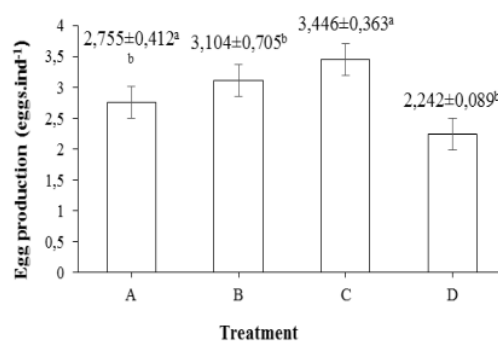


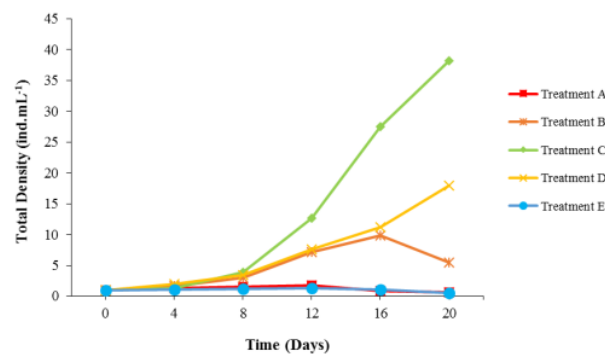
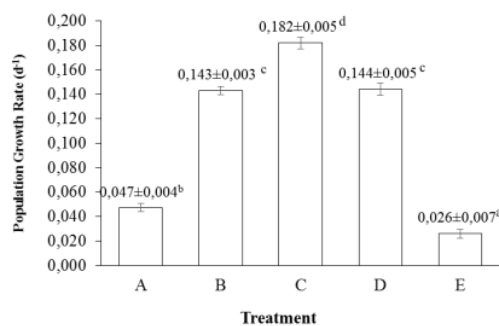
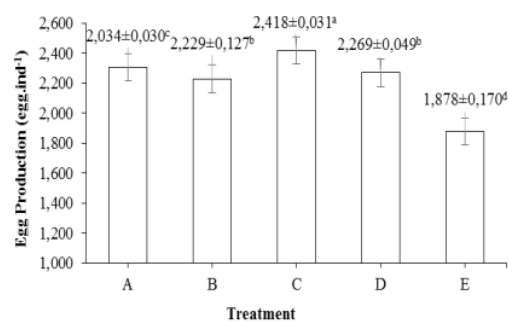
Fig. 3. Egg Production of *D. brachyurum* (Experiment I)

3.2. The result of experiment II

The result of Experiment I showed that *T. chuii*(Treatment C) had the highest number of total density of *D. brachyurum* (38,183±3,595 ind.mL⁻¹) in last day of culture and the lowest value was in Treatment E (100% fermented organic matters) (Table 4). The total density in Treatment C and D showed its exponential phase (Fig. 4.). Treatment C was high significantly different ($P<0,05$). The highest value of population growth performance was also in Treatment C (0,182±0,005 d⁻¹) with its lowest value was in Treatment E (Fig. 5). Treatment C was high significantly different ($P<0,05$) on its growth rate value. In egg production value, the highest number of egg production was also in Treatment C where *T. chuii* produced 2,418±0,031eggs.ind⁻¹ and the lowest production was also in Treatment E (Fig. 6). The treatment was significantly different ($P<0,05$).

Table 4. Values of Total Density in Day-20, Population Growth Rate, Egg Production and Neonate Production within Pouch (Experiment II)

No.	Variable	Treatment				
		A	B	C	D	E
1.	Total Density (ind.mL ⁻¹)	0,600±0,050 ^d	5,450±0,265 ^c	38,183±3,595 ^a	17,967±1,823 ^b	0,517±0,029 ^d
	a. Adult	0,233±0,029	1,533±0,058	14,600±0,953	7,117±0,666	0,467±0,029
	b. Egg-laying Adult	0,150±0,000	1,267±0,115	14,600±0,953	1,100±0,450	0,000±0,000
	c. Adult with Embryo	0,100±0,000	1,833±0,189	9,350±1,800	0,050±0,087	0,050±0,000
	d. Juvenile	0,067±0,029	0,533±0,058	4,600±0,805	6,950±0,747	0,000±0,000
	e. Neonate	0,050±0,000	0,283±0,029	4,933±0,465	2,750±0,200	0,000±0,000
2.	Population Growth Performance (d ⁻¹)	0,047±0,004 ^c	0,143±0,003 ^b	0,182±0,005 ^a	0,144±0,005 ^b	0,026±0,007 ^d
3.	Egg Production (egg.ind ⁻¹)	2,034±0,030 ^c	2,229±0,127 ^b	2,418±0,031 ^a	2,269±0,049 ^b	1,878±0,170 ^d
4.	Neonate in Pouch (neonate.ind ⁻¹)	2,286±0,126	2,391±0,037	2,457±0,051	2,382±0,174	2,001±0,046

**Fig. 4.** Total Density of *D. brachyurum*(Experiment II)**Fig. 5.** Population Growth Rate of *D. brachyurum*(Experiment 2)**Fig. 6.** Population Growth Rate of *D. brachyurum*(Experiment 2)

3.3. Discussion

According to the results in Experiment I and II, it can be suggested that *T. chuii* is an ideal single microalgal diet for *D. brachyurum* as it able to support its growth and reproduction. Combination of *T. chuii* and fermented organic matters also can be suggested as an ideal diet for *D. brachyurum* enrichment. *D. brachyurum* which fed by single *T. chuii* showed its highest value in every measuring variables (number of total density and population growth rate), except egg production as *N. oculata* could support *D. brachyurum* to obtain highest egg production, according to Experiment I results. In Experiment II, a combination of *T. chuii* and fermented organic matters showed its highest value in every main measuring variables. The type of diet, characteristics and nutritional value suggest as such important roles on growth and reproduction of *D. brachyurum*. According to Zamora-Tero et al [14], zooplankton population density is affected by dietary type, size, and nutritional value. Cheng et al [15] also told that especially for microalgal diet, the flagellate on phytoplankton can attract zooplankton to prey.

Several studies have been suggested for the effect of microalgal diet and organic matters on zooplankton growth and reproduction. *T. chuii* has morphological characteristic (flagellated and thin cell wall) and high nutritional value which supports *D. brachyurum* growth and performance. Thys et al [16] suggested that flagellated algae tend to have a thin cell wall and easy to lysis. According to Lavens and Sorgeloos [11], *T. chuii* contains of α -chlorophyll (3,83 pg.cell⁻¹), protein (83,4 pg.cell⁻¹), lipid (45,7 pg.cell⁻¹) and carbohydrate (32,5 pg.cell⁻¹). Persson [17] suggested that EPA and PUFA respectively have a role for cladoceran somatic growth and reproduction. According to Taipale et al [18], somatic growth and reproduction of zooplankton affected by lipid and protein of the diet, especially ω -3 dan ω -6 PUFA and amino acid. Non-essential biomolecule such as carbohydrate also has a role as an energy source for zooplankton, which its protein can effectively be used for somatic growth and reproduction of zooplankton.

As the result of Experiment I, it can be suggested that the nutritional value of *T. chuii* is proportional for *D. brachyurum*, especially in its total density and growth rate. According to egg production value in Experiment I, the nutritional value of *N. oculata* might have a greater impact than *T. chuii*. According to Suminto [19], *N. oculata* has a high value of lipid, such as n-3 HUFA 42,7%; EPA 30,5%; and DHA 12,2 %. EPA and DHA can enhance fecundity and egg development of zooplankton [9].

In Experiment II, a combination of 50% *T. chuii* and 50 % fermented organic matters can be suggested as an ideal dosage of both diets for *D. brachyurum* growth and reproduction performance. The addition of fermented organic matters conducts more nutrition source. Bacteria can be an additional diet for *D. brachyurum*. According to Nwachi [20], fermentation of organic matter increases the number of bacteria colon [21], it was shown that feeding on bacteria, in combination with algae, supports a long series of parthenogenetic generations of *D. magna*; in contrast, cultures failed if fed either on bacteria or on sterile algae.

D. brachyurum is suggested utilizing nutrition from probiotic bacteria to grow and reproduce. Bacteria can simplify the digestive system in *D. brachyurum* which diet nutrition can be easily and effectively digested. Probiotic bacteria utilize the nutrition from organic matters to grow and replicate so that the crude protein content in organic matters increases. According to Arief et al [22], probiotic bacteria is against pathogen bacteria, it makes the diet nutrition easily digested. Putri et al [23] show during the fermentation process, the number of bacteria colony are able to increase crude protein value of organic matters as they are a single-celled protein. Anggraeny et al [24] suggested that carbohydrate utilized by bacteria for growth. Bacteria is a single-celled protein which contains 31 – 51 % protein.

As the result of Experiment, I and Experiment II, both *T. chuii* and fermented organic matters can be taken into consideration as the ideal single microalgal diet nor the ideal combination diet when evaluating the dietary type when culturing *D. brachyurum*. However, the combination of both diets (50% *T. chuii* and 50% fermented organic matters) to be applied for developing and supporting growth and reproduction of *D. brachyurum* in the culture.

4. Conclusion

The microalgae diet of *Tetraselmis chuii* provides the best growth and reproduction which includes total density, growth rate and neonite production of *D. brachyurum* compared to other types of phytoplankton.

The combination of 50% *Tetraselmis chuii* cells and 50% fermented organic matters given results that was suitable to support the growth and reproduction of *D. brachyurum*.

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