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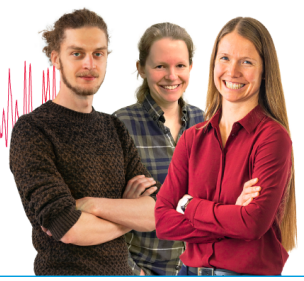

Toxicity Studies of *Bacillus thuringiensis* on Non-Target Organism *Daphnia* sp. (Diplostraca: *Daphniidae*)

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Abstract. Dengue hemorrhagic fever is caused by dengue viruses that infect female mosquitoes, mainly of the species *Aedes aegypti* and to a lesser extent *Aedes albopictus*. One example of a bioinsecticide is *Bacillus thuringiensis* israelensis (Bti), which is a naturally occurring soil bacterium that can effectively kill mosquito larvae present in water. MOSNON^{TM} is a biolarvicide product containing *B. thuringiensis*. The aims of this research were to analyze the effects of MOSNON^{TM} toward mortality and behavior of the non-target organism *Daphnia* sp. This study was conducted at the Laboratory of Ecology and Animal Diversity and Laboratory of Microbiology, Faculty of Mathematics and Natural Sciences, Brawijaya University. The toxicity test used 10 neonates of *Daphnia* sp. for each bottle that contained 250 mL MOSNON^{TM} solution. Toxicity test was divided into several concentrations of MOSNON^{TM}, they were 0, 5, 10, 15 and 20 ppm, and each experiment was repeated four times. Observations of *Daphnia* sp. mortality and behavior as well as abiotic factors (temperature, dissolved oxygen and pH) were conducted at exposure times of 0, 24, 48, 72 and 96 hours. Data were analyzed using ANOVA. The results showed that all concentrations of MOSNON had no effect on mortality or behavior of *Daphnia* sp. MOSNON^{TM} is a potential biolarvicide for mosquitoes and safe for non-target organisms (*Daphnia* sp.).

Keyword: Bioinsecticide, dengue hemorrhagic fever, MOSNON^{TM}

INTRODUCTION

Dengue hemorrhagic fever (DHF) is a dangerous disease caused by dengue viruses (DEN-1, DEN-2, DEN-3 and DEN-4). DHF can infect by vectors such as *Aedes aegypti* and *Aedes albopictus*.[1] DHF vectors can be controlled using chemical or biological larvicides. Chemical larvicides, such as temephos, have bad impacts on humans and the environment. Long exposure to temephos can cause larval resistance.[2] Temephos is also toxic to non-target organisms such as insects and some invertebrates.[3] Biological larvicides, such as *Bacillus thuringiensis*, have more advantages to control of DHF vectors because they have specific targets. *B. thuringiensis* has no effects on 125 families, 300 genera and 400 species of non-target organisms.[4]

Currently, *B. thuringiensis* application can be easily used in a product called MOSNON^{TM}. MOSNON^{TM} was produced by PT. Kyushu Medical Co., LTD, which is located in Hyakunen-kouen 1-1 Kurume, Fukuoka, Japan. MOSNON^{TM} is a bioloarvicide product containing 2% crystalline protein of *B. thuringiensis* serovar israelensis strain D142 at 10^9 CFU/g. And based on the official information from PT. Kyushu Medical Co., LTD., MOSNON^{TM}, it is safe for the environment.[5]

Research about safety testing of *B. thuringiensis* (MOSNON^{TM}) toward non-target organisms is still few, especially in Indonesia. One of the non-target organisms is *Daphnia* sp. (water flea). This is a small crustacean that has an ecological niche that is the same as mosquito larvae. In Indonesia, many ornamental fish breeders use *Daphnia* sp. as fish food. Ornamental fish usually live in aquariums that can be used by mosquitoes as breeding

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places. *Daphnia* sp. is often used as an organism for toxicity studies because they are sensitive to water pollution and also are an international standard organism used for toxicity studies.[6]

The aims of this research were to analyze effect of MOSNON^{TM} toward mortality and behavior of the non-target organism *Daphnia* sp.

EXPERIMENTAL DETAILS

Culture of *Daphnia* sp. in Laboratory

This study was conducted at the Laboratory of Ecology and Animal Diversity and Laboratory of Microbiology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, Indonesia. *Daphnia* sp. were bought from a fish breeder in Singosari, Malang. *Daphnia* sp. culture was conducted in the laboratory with three different aquariums for 15 days using ground water. *Spirulina platensis* powder was used for feeding *Daphnia* sp. every three days. Abiotic factors such as temperature, pH and dissolved oxygen (DO) were measured every 24 hours.

Acclimation of the Water Flea *Daphnia* sp.

Daphnia sp. that had eggs in a brood chamber were separated in a petri dish after 15 days of culture. Acclimatization of female *Daphnia* sp. took place over four days. *Daphnia* sp. were feeding once with *Spirulina platensis* powder. *Daphnia* sp. were observed every 24 hours to watch for hatching eggs. Newborn *Daphnia* sp. (neonates) were used for toxicity studies.[7]

Toxicity Test of *Bacillus thuringiensis* Toward Non-Target Organism *Daphnia* sp.

Toxicity test was conducted by randomized factorial design. Toxicity was tested in a jam bottle containing of 250 mL MOSNON^{TM} solution and 10 newborn *Daphnia* sp. MOSNON^{TM} concentrations were 0, 5, 10, 15 and 20 ppm.[8] Each concentration was repeated four times. Range of concentrations was based on the latest studies that said MOSNON^{TM} had an effective concentration for mosquito larvae of 2 ppm.[9] Mortality, behavior and abiotic factors (temperature, pH and DO) were observed at 24, 48, 72 and 96 hours.[10] Before counting mortality and observing behavior, MOSNON^{TM} solution was gently agitated for 15 second. Observation looked for abnormal behavior such as immobility, lethargy, circling and floating.

Data Analysis

Mortality data and abiotic factors both during culture and toxicity test were analyzed with descriptive statistical methods. Abiotic factors were also analyzed with ANOVA. One-way ANOVA was used for abiotic factors during culture and two-way ANOVA for abiotic factors during toxicity test. Behavior data were analyzed qualitatively.

RESULTS AND DISCUSSION

Abiotic Factors During Culture of *Daphnia* sp.

All abiotic factor data had a fluctuating pattern, present in figure 1. Those fluctuation patterns happened because laboratory conditions cannot be entirely maintained. *Daphnia* habitat temperature during culture was 18.00-22.83°C. Range of pH values were 7.36-8.70 and range of dissolved oxygen (DO) level was 1.00-1.35 mg/L. Based on the ANOVA analyses of temperature, pH and DO for each aquarium, every 24 hours have a different notation.

Daphnia sp. have a large temperature tolerance, but its optimum temperature is 18 to 22°C, and an acceptable pH is between 6.5 and 9.5 with the optimum being between 7.2 and 8.5. The DO varies from almost zero to super saturation with a minimum DO of 0.6 mg/L.[11,12]

Based on the universal standard, habitat temperature during culture should be in the range of 20 ± 2°C and range of pH should be 6.0 to 8.5. The DO during cultures should not be lower than 60% air saturation. Gentle aeration should be provided if necessary to maintain DO. High differences in abiotic factor values from these ranges can

cause population crash or the production of all males. Those unfavorable conditions also cause diapausing eggs.[6] A diapausing egg takes longer to hatch than normal eggs. This condition occurs when eggs in a brood chamber are surrounded by a membranous external wall called ephippia.[13] Diapausing eggs of *Daphnia* sp. are also used as a health criteria of the culture. A healthy culture will not present diapausing eggs.[6]

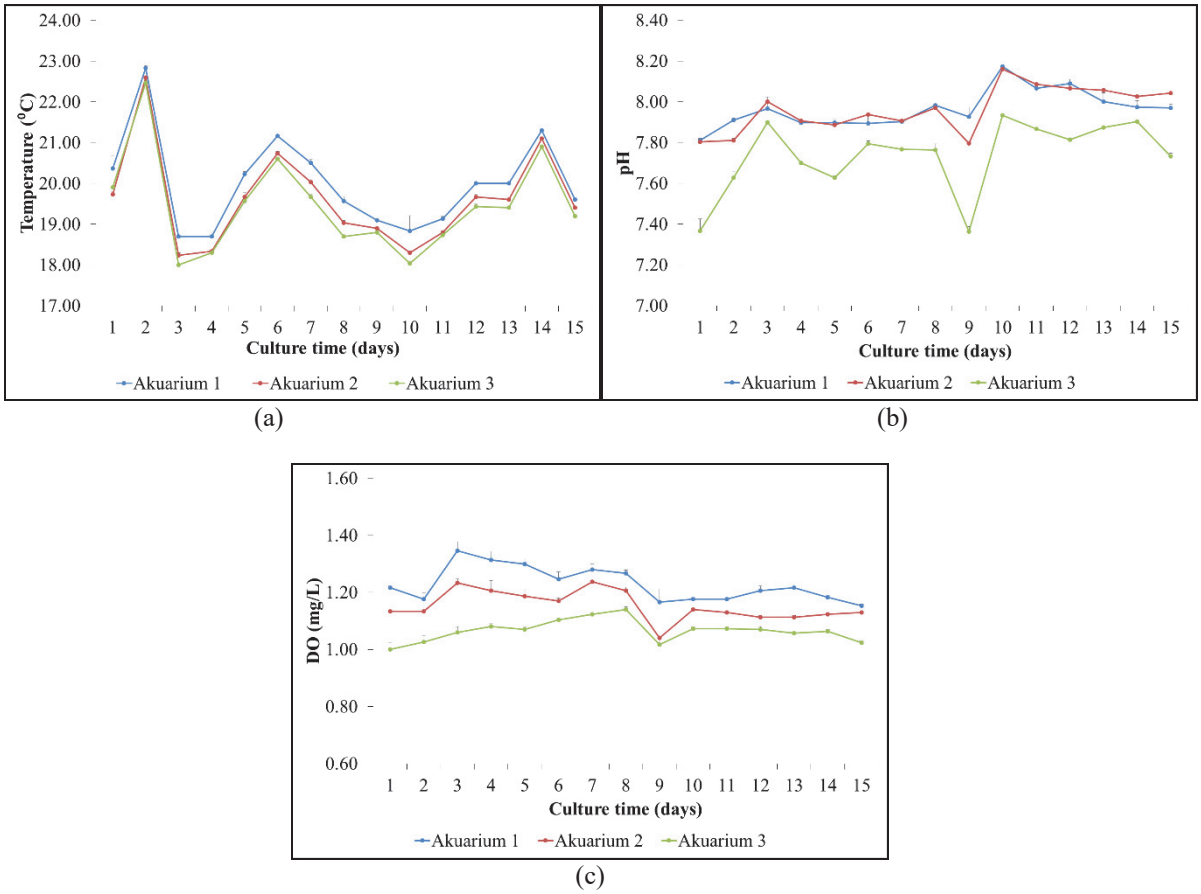


FIGURE 1. Abiotic factors during 15 days of culture of *Dapnia* sp in the different aquarium, (a) temperature, (b) pH, (c) DO.

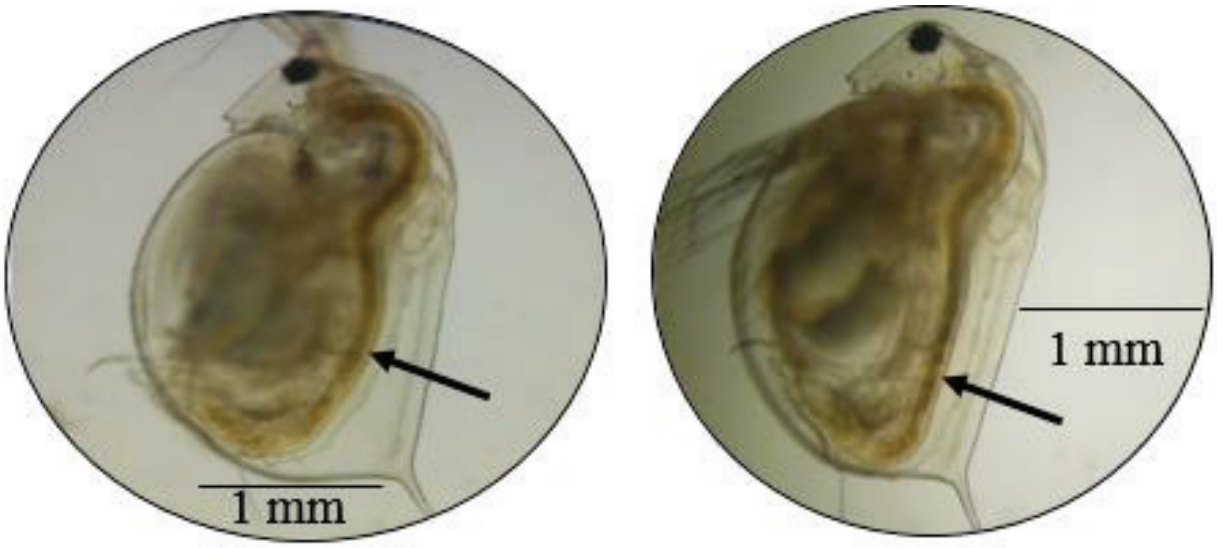
Mortality and Behavior of *Daphnia* sp. During Toxicity Test

All concentrations of MOSNON^{TM} had no negative effects toward *Daphnia* sp. (table 1) There was no mortality or abnormal behavior during the 96 hours exposure period. The digestive organ of *Daphnia* sp. did not have any injury at any concentration of MOSNON^{TM} based on microscopic observation.

TABLE 1. Percentage of mortality and behavior of *Daphnia* sp. during toxicity test

Five concentration of MOSNON^{TM} (ppm)	Exposure time (hours)	Mortality (%)	Abnormal behavior of *Daphnia* sp.			
			Immobility	Lethargy	Circling	Floating
0; 5; 10; 15; 20	0	0	–	–	–	–
0; 5; 10; 15; 20	24	0	–	–	–	–
0; 5; 10; 15; 20	48	0	–	–	–	–
0; 5; 10; 15; 20	72	0	–	–	–	–
0; 5; 10; 15; 20	96	0	–	–	–	–

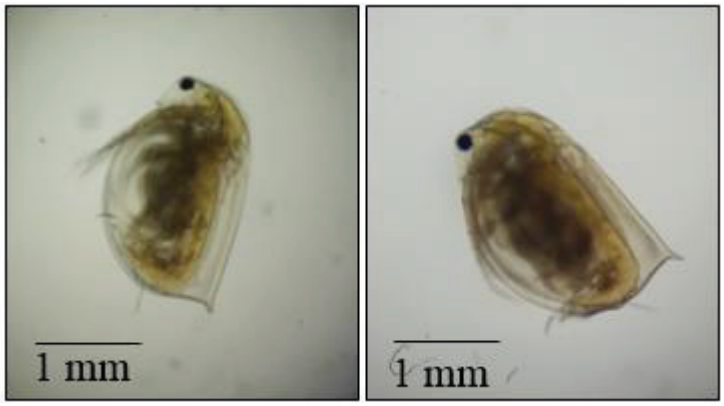
MOSNON^{TM} contains *B. thuringiensis* that produces a toxin called ICP (insecticidal crystal protein) and δ-endotoxin. Both toxins kill insects by entering the digestive track. The toxins can be activated by high pH (alkalinity) in the digestive track.[14] MOSNON^{TM} effectively kills mosquito larvae by making holes in their digestive track. Mosquito larvae have alkaline pH in their digestive track; therefore, *B. thuringiensis* toxin can be activated and cause lysis in the digestive tract and create pores in membranes Fig. 2. Based on the latest study, the effective concentration of MOSNON^{TM} for killing mosquito larvae (*Aedes aegypti*) was 2 ppm.[9] *B. thuringiensis* in MOSNON^{TM} did not kill *Daphnia* sp. because the toxin cannot be activated. The pH of *Daphnia* sp. digestive tract is 6.0 to 6.8 in the anterior part of the midgut and 6.6 to 7.2 in the posterior part. *Daphnia* sp. are also known as small-filter feeders that filter water to ingest their food. *Daphnia* sp. usually consume particles from around 1 μm up to 50 μm, although particles of up to 70 μm in diameter may be found in the gut content of large individuals Fig. 3. *Daphnia* sp. mainly eat small organisms such as bacteria, single cell algae, protista, detritus and yeast. Food passes through the gut by peristaltic contractions of the gut wall. The normal digestive track of *Daphnia* sp. is divided into three parts: the esophagus, the midgut and the hindgut. The peritrophic membrane contains the food and prevents it from entering the ceca. Epithelial cells do not have phagocytic particles but absorb molecules of food.[13]



(a) (b)

FIGURE 2. Digestive organs of *Daphnia* sp. have no effect, (a) before MOSNON^{TM} exposure, (b) after MOSNON^{TM} exposure.

(a) (b) (c)



(d) (e)

FIGURE 3. *Daphnia* sp. after 96 hours in MOSNON^{TM} exposure, (a) 0 ppm, (b) 5 ppm, (c) 10 ppm, (d) 15 ppm, (e) 20 ppm.

Daphnia sp. behavior did not turn abnormal based on results of observation after 15 seconds of gentle agitation of MOSNON^{TM} solution test. *Daphnia* sp. are a group that normally if after those agitations they are still active move to the opposite of water flow and they can swim to surface of the water figure 4. However, if there was abnormal behavior, *Daphnia* sp. will show immobility, lethargy, circling and floating figure 5. Immobility and lethargy show when *Daphnia* sp. just make very few movements, swimming through the water flow and located on the bottom of the bottle. Abnormal behavior signs of *Daphnia* sp. will be seen if *Daphnia* sp. are under the stress from the unfavorable environment. Observing *Daphnia* sp. behavior within the toxicity test was important to determine the validity of the data and the *Daphnia* sp. reaction towards the test solution. If as many as 10% of *Daphnia* sp. showed abnormal symptoms in the control treatment it can be stated that the data acquisition is invalid[6].

Observation results of *Daphnia* sp. did not show negative effects such as mortality and abnormal behavior even though tested with MOSNON^{TM} concentrations more than 2 ppm. The digestive organ system of *Daphnia* sp. also appeared normal. It can be said that MOSNON^{TM}, which contains *B. thuringiensis* inside, was safe for *Daphnia* sp. as a non-target organism.

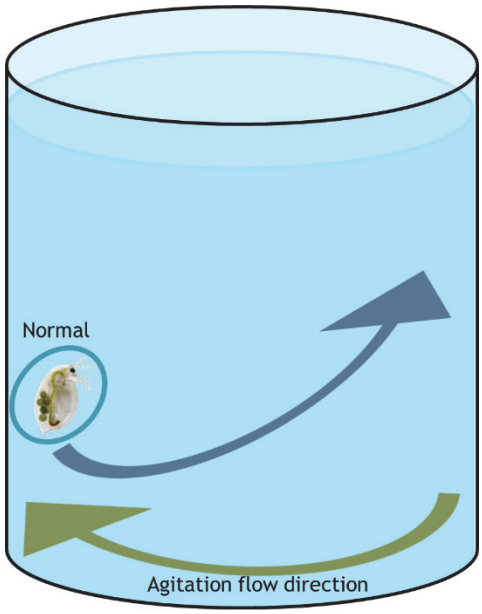


FIGURE 4. *Daphnia* sp. behavior for all MOSNON^{TM} concentration within all time exposure.

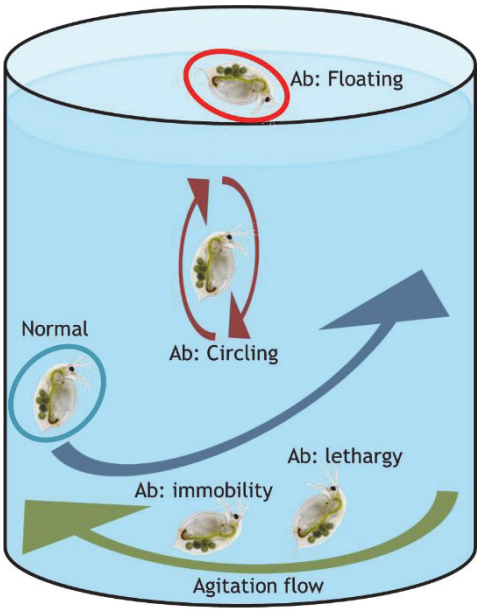
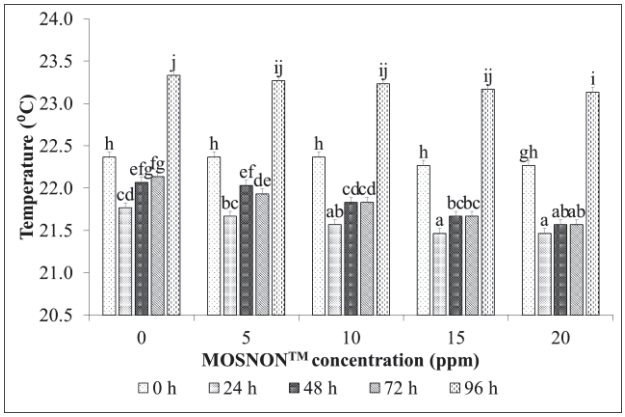


FIGURE 5. Abnormal behavior illustration of *Daphnia* sp. compared to normal behavior[6], Ab: abnormal.

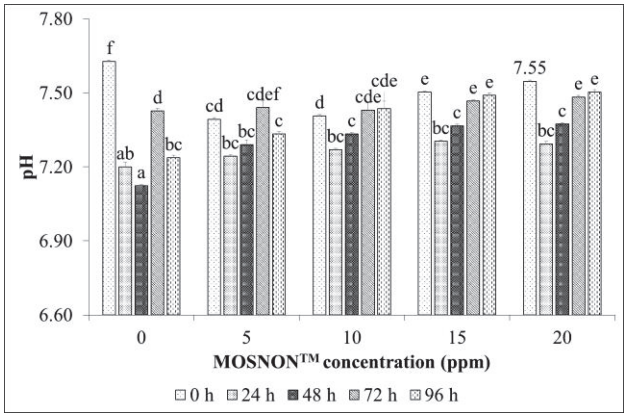
Measuring Abiotic Factors During Toxicity Test of *Daphnia* sp.

The results of abiotic factors analysis after 96 hours exposure showed a fluctuation pattern present in figure 6. The range of temperatures during the toxicity test was 21.5-23.3°C, pH range was 7.12-7.63 and DO range was 0.93-1.05 mg/L. Based on the ANOVA results, temperature, pH and DO for each concentration every 24 hours had a different notation. Those results mean that abiotic factors have an impact on mortality of *Daphia* sp., but there was no mortality in this research; therefore, although all abiotic factors have different notations, the ranges were still tolerant for *Daphnia* sp. to live.

Abiotic factors are an importance parameter for toxicity tests. Unfavorable abiotic factors can cause mortality to test organisms or can affect the test solution, either making it more or less toxic. Temperature, pH and DO of the test solutions should be checked during the test. Temperature during the test was in the range of 20 ± 2°C, pH within the range 6.0 to 8.5 and DO below 5.5 mg/L, but *Daphnia* sp. can still alive at DO 0.6 mg/L. *Daphnia* sp. can survive in an oxygen poor environment because of their ability to synthesize hemoglobin that is promoted by high temperatures and a high population density.[11]



(a)



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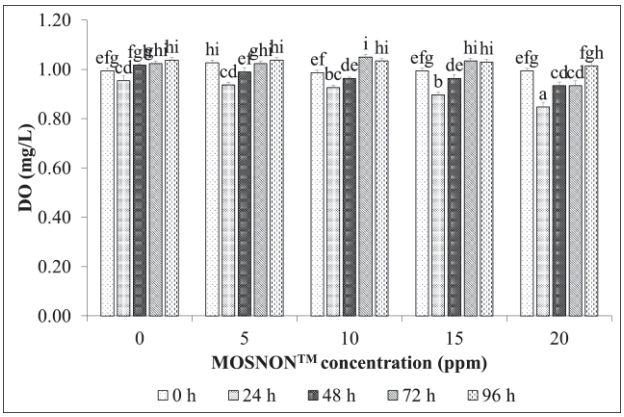

(c)

FIGURE 6. Abiotic factors during toxicity test, (a) temperature, (b) pH, (c) DO.

SUMMARY

The summary of this research is that *B. thuringiensis* in MOSNON^{TM} has no effect on the mortality or the behavior of the non-target organism *Daphnia* sp., and they have no abnormal symptoms during 96 hours of exposure using MOSNON^{TM}.

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