doi: 10.17221/8523-CJAS

Egg surface decontamination with bronopol increases larval survival of Nile tilapia, *Oreochromis niloticus*

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ABSTRACT: Intensive tilapia egg incubation techniques create favourable conditions for microbial proliferation which often leads to massive mortalities of fish larvae. The effects of Nile tilapia (*Oreochromis niloticus*) eggs exposure to bronopol on decontamination of their bacterial surface and survival of larvae were observed. Immersion treatments of fertilized eggs were applied at 10, 25, 50, 100, and 250 mg/l of bronopol for 10, 20, and 30 min. This treatment substantially reduced the number of bacteria on Nile tilapia eggs. The greatest reduction in bacterial numbers (1.58×10^3 colony forming units/g of egg) was observed at the maximum treatment dosage, i.e. 250 mg/l for 30 min, but this was not significant when compared with treatments of 100–250 mg/l bronopol for 10–30 min. Treatments of 50–250 mg/l bronopol provided better larval survival (89.33–94.67%) than those of < 50 mg/l ($P < 0.05$). The larval survival obtained from the Nile tilapia eggs disinfected with 10 and 25 mg/l bronopol for 10–30 min was similar to that of negative controls (79.33–80.97%). Additional *in vitro* test was performed to determine the inhibitory potency of bronopol against bacteria cultured from fertilized Nile tilapia eggs. Twenty-two bacterial isolates (*Aeromonas hydrophila* ($n = 12$), *Staphylococcus aureus* ($n = 6$), *Escherichia coli* ($n = 2$), and *Micrococcus* spp. ($n = 2$)) responded to minimum inhibitory concentrations of bronopol ranging from 64 to 128 µg/ml. The study demonstrates that the immersion treatment of Nile tilapia fertilized eggs with 250 mg/l for 10–30 min, the concentration proven effective against bacteria cultured from fertilized eggs, significantly reduced bacterial load and improved larval survival.

Keywords: bacteria; desinfectant; egg disinfection; tilapia egg

INTRODUCTION

The dramatically expanding commercial aquaculture of Nile tilapia (*Oreochromis niloticus*) significantly influences global freshwater fish production (ca. 3.58 million metric tons in 2011 – www.thefishsite.com/reports/?d=2563#sthash.bqF0zmA6.pdf). Reproduction of Nile tilapia takes place in captivity where numbers of male and female broodstocks spawn naturally in a mating tank. After natural spawning, the fertilized eggs are collected and transferred to a hatchery unit for intensive artificial egg incubation. Bacterial infections are suggested to be a major cause of both egg losses and occurrence of deformed fish larvae (Harboe et al. 1994), however these are not attributed to specific obligate pathogenic bacteria, but rather to proliferation of opportunistic bacteria in the environment of intensive egg incubation (Skjermo and Vadstein 1999). The most common bacterial flora in hatcheries colonizing the surface of fish eggs are reportedly *Flavobacterium* sp., *Aeromonas* sp., *Pseudomonas* sp., and *Vibrio* sp. (Madsen et al. 2005; Austin 2006). The egg is colonized by bacteria because its surface represents a highly favourable substrate for bacterial growth and the extra-oral incubation raising embryos of mouthbreeding tilapia can lead to deficiency of secreting bactericidal agents derived from maternal mucus (Hansen and Falk-Petersen 2001). Major approaches that have contributed to the control of bacterial proliferation on fish eggs include UV

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or ozonated treatment of hatchery water (Grotmol et al. 2003) and application of chemicals suitable for surface disinfection of fish eggs (Atanasov et al. 2011). A wide range of chemicals were reported as egg disinfectants for the routine use in fish hatcheries, e.g. benzalkonium chloride (Douillet and Holt 1994), glutaraldehyde (Salvesen and Vadstein 1995), formalin (Stuart et al. 2010), hydrogen peroxide (Rach et al. 1998; Rasowo et al. 2007), iodine compounds (Salvesen and Vadstein 1995; El-Dakour et al. 2013), and chlorine-based disinfectants such as sodium hypochlorite (Harboe et al. 1994) and bronopol (Birkbeck et al. 2006; Oono et al. 2007). These disinfectants proved to be safe for different species of fish eggs, while highly toxic to aquatic pathogens. Currently, in Thailand, only potassium permanganate and 5% hypertonic saline are commonly used for the control of bacterial or fungal infections during incubation in tilapia hatcheries.

Bronopol (2-bromo-2-nitro-1,3 propanediol) is a wide-spectrum fungicide and bacteriocide (Saito and Onoda 1974). It acts against the cell walls of fungi and membranes of Gram-positive and Gram-negative bacteria with catalytic oxidation and production of active oxygen species such as superoxide and peroxide (Shepherd et al. 1988). Bronopol has been widely used as a preservative in the pharmaceutical and cosmetic industries for decades (Saito and Onoda 1974). The European Agency for Evaluation of Medicinal Products (EMEA) classifies bronopol as a safe chemical for aquaculture, and Pyceze solution (50% w/v bronopol, Novartis Animal Health, Camberley, UK) has been commercialized for the treatment and control of fungal *Saprolegnia* sp. infections in farmed rainbow trout, *Oncorhynchus mykiss*, and Atlantic salmon, *Salmo salar* kept in fresh water, and rainbow trout and Atlantic salmon fertilized eggs (www.vmd.gov.uk/espcsite/Documents/131969.doc). Specific applications of bronopol for a fungicidal effect were reported in freshwater salmonid eggs at a daily dosage of 50 mg/l, 30 min, 15 consecutive days (Branson 2002), control infections caused by *Flavobacterium* sp. in rainbow trout fry, such as anemic syndrome and bacterial gill disease at 20 mg/l bronopol for 30 min once daily, for up to 14 consecutive days (Sudova et al. 2007), and control of aquatic fungi and ciliated protozoa in striped catfish, *Pangasianodon hypophthalmus*, fingerlings at a daily dosage of 10 mg/l, 2 h, 5 consecutive days (Piamsomboon et al. 2013). The bronopol application for egg disinfection has been reported in many cultured fish species such as cod, *Gadus morhua*, haddock, *Melanogrammus aeglefinus* (Treasurer et al. 2005), halibut, *Hippoglossus hippoglosus* (Birkbeck et al. 2006), rainbow trout (Oono et al. 2007) and bluefin sea bream, *Sparidentex hasta* (El-Dakour et al. 2013), however, its application on Nile tilapia eggs has not been reported.

With respect to the described effectiveness of bronopol against microbial contamination in fish eggs, and the necessity to control bacterial colonization on eggs during the incubation in the intensive hatching system, this study was designed to evaluate *in vitro* and *in vivo* efficacy of bronopol against different bacteria which commonly colonize or spoil Nile tilapia fertilized eggs. The results can be applied in the hatchery routine management to decontaminate microflora on eggs and increase survival to hatch of Nile tilapia eggs.

MATERIAL AND METHODS

Bacterial isolation. Fertilized Nile tilapia eggs entering the hatchery were collected for bacteriological procedures. The egg samples were rinsed and homogenized with sterile normal saline solution (NSS). Bacterial isolates were cultured from a homogenate of 1 g eggs in 1 ml sterile NSS, plated onto Tryptic Soy Agar (TSA; Oxoid, Basingstoke, UK) and incubated at 30°C for 24 h. Identification of bacteria was performed using the API system (BioMerieux, Marcy l'Etoile, France) with a modification of the incubating temperature to 30°C for suitable growth of the aquatic bacteria. The identified isolates were stored in maintenance broth containing 20% glycerol and supplemented with 10% fetal bovine serum at –70°C until further applications.

Determination of minimum inhibitory concentration. A commercial bronopol product Antizol® (Virbac Asia Pacific Co. Ltd., Bangkok, Thailand), containing 50% bronopol w/v, was used in this study. The concentration of bronopol applied in the test was nominally determined based on an active ingredient, and a fresh solution of bronopol was prepared for each assay.

The minimum inhibitory concentration (MIC) procedures were conducted using an agar dilution method in accordance with recommendations provided by the Clinical and Laboratory Standards Institute (CLSI 2012). Mueller Hinton Agar (MHA; Oxoid), to which serial two-fold dilutions of the

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bronopol substance (2–2048 µg/ml) were added, was inoculated with a standardized inoculum of the tested bacteria (approximately 10^4 colony forming units (CFU)/spot) on the surface of the MHA. After a 18-hour incubation, the MIC was indicated as the lowest concentration of bronopol with no visible bacterial growth. Two reference strains, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922, were included in the procedure as control bacteria.

Efficacy of bronopol on disinfection of Nile tilapia eggs. Nile tilapia eggs used for the trials were obtained from the female breeders maintained in a commercial tilapia farm in Chachoengsoa province, Thailand, in May 2014. Fertilized eggs at a hatching stage (68–72 h post fertilization) (Fujimura and Okada 2007) were collected from the mouth of female breeders at the mating tank, weighed, and surface disinfected with bronopol following the experimental design before the incubation. Nile tilapia fertilized eggs were treated with each of five concentrations of bronopol (10, 25, 50, 100, and 250 mg/l) for 10, 20, and 30 min exposure time, and freshwater was an immersion control, three replicates per treatment. Each immersion treatment was performed in a 2-litre egg collecting bucket containing approximately 33.5 g of eggs (3590 ± 90 eggs) with appropriate aeration. After the immersion treatment, eggs were placed in the egg hatching jar (diameter 12 cm, length 30 cm) supplied with running water at a rate of 5 l/min. The water parameters were: mean temperature 29 ± 2°C, pH 6.5–7.5, dissolved oxygen 7.0–7.5 mg/l, nitrite and ammonia < 0.2 mg/l. Eggs were incubated in hatching jars until they hatched and then the newly hatched fry were maintained in incubation trays (8 × 24 × 30 cm^3) until yolks were completely absorbed.

The efficacy of bronopol to minimize bacterial colonization on tilapia egg was determined on the total bacterial count following the treatment. Approximately 1 g of eggs from each immersion treatment was collected for bacterial plating as described. Total viable bacteria were counted following 24 h incubation. The bacterial numbers in the hatchery water (CFU/ml) were also examined using spread plate method.

Safety evaluation of bronopol treatment for Nile tilapia egg disinfection. The safety of bronopol for Nile tilapia fertilized eggs was examined at different treatment dosages. The development of Nile tilapia fertilized eggs was monitored till the first day of larvae feeding (approximately 5–7 days post-hatch). Percentage survival was determined as the number of surviving larvae at the first feeding compared to the initial number of hatching eggs. Twenty eggs from each treatment were randomly collected with pipette for daily observation on embryonic development using stereomicroscopic examination.

Statistical analysis. Differences in the number of bacterial colonies and larval survival to the first feeding were compared using the analysis of variance (ANOVA). Fisher's Least Significant Difference (LSD) test was used to determine significant differences between the treatment groups at 0.05 level of probability ($P \leq 0.05$).

RESULTS

The bacteria isolated from Nile tilapia fertilized eggs were identified as *Aeromonas hydrophila* (12 isolates), *S. aureus* (6 isolates), *E. coli* (2 isolates), and *Micrococcus* spp. (2 isolates). The MIC values for bronopol against these 22 bacterial isolates were 64–128 µg/ml (Table 1).

The results of the bacterial count for each treatment of Nile tilapia eggs are shown in Figure 1. Bacterial numbers in fertilized egg samples immersed in fresh running water (controls) were 1888.33×10^3 to 1925.67×10^3 CFU/g per egg, and the total bacterial numbers counted at 10, 20, and 30 min immersion control were not significantly different. Nile tilapia fertilized eggs disinfected with bronopol (10–250 mg/l) exhibited significantly reduced bacterial counts and the higher the concentrations of bronopol, the higher degree of reduction was found. However, it was observed

Table 1. Minimum inhibitory concentration (MIC) of bronopol against *Staphylococcus aureus*, *Escherichia coli,* and bacteria isolated from Nile tilapia (*Oreochromis niloticus*) eggs

Bacteria	Source	MIC (µg/ml)
Staphylococcus aureus	ATCC 29213	128
Escherichia coli	ATCC 25922	128
Aeromonas hydrophila (n = 12)	tilapia eggs	64–128
Escherichia coli (n = 2)	tilapia eggs	64
Staphylococcus aureus (n = 6)	tilapia eggs	128
Micrococcus spp. (n = 2)	tilapia eggs	64–128

ATCC = American type culture collection

doi: 10.17221/8523-CJAS

Table 2. Percentage survival of Nile tilapia (*Oreochromis niloticus*) larvae following disinfection with bronopol (mean ± standard deviation)

Exposure time (min)	Concentration (mg/l)	Survival (%)
10	0	80.97 ± 2.83^{ab}
	10	82.37 ± 1.75^{ab}
	25	84.60 ± 3.84^{bd}
	50	83.93 ± 3.52^{ab}
	100	89.33 ± 1.53^{cd}
	250	92.80 ± 4.51^{ce}
20	0	80.67 ± 3.21^{ab}
	10	80.20 ± 1.45^{ab}
	25	82.37 ± 3.60^{ab}
	50	91.60 ± 3.14^{ce}
	100	90.67 ± 1.15^{ce}
	250	94.67 ± 4.51^{e}
30	0	79.33 ± 4.51^{a}
	10	81.07 ± 1.36^{ab}
	25	79.33 ± 1.53^{a}
	50	92.67 ± 3.21^{ce}
	100	90.73 ± 3.11^{ce}
	250	93.00 ± 2.65^{ce}

$^{a–e}$means within a column not sharing common superscripts differ significantly ($P < 0.05$)

that the reduction of bacterial loads was most efficient at the ≥ 100 mg/l bronopol treatment. The 100 and 250 mg/l bronopol treatments gave no difference in bacterial counts, regardless of various exposure times (10, 20, and 30 min). Total viable bacterial count of the water supplying the incubation system was 1.57 ± 0.38 × 10^3 CFU/ml.

The survival of Nile tilapia embryos was improved when the egg of hatching stage was treated with bronopol at concentrations of 50 mg/l for 20–30 min or 100–250 mg/l for 10–30 min. The mean percentage survival of tilapia larvae till their first feeding and data on the preceding bronopol treatment dosages are shown in Table 2. The highest survival (92.80–94.67%) was observed in the groups treated with 250 mg/l bronopol for 10–30 min, however, the mean survival of 100 and 250 mg/l bronopol treatments with different contact time (10–30 min) was not significantly different. Malformations of Nile tilapia embryos were not observed in any treatments and negative control groups. The disinfected eggs of all treatments, up to 250 mg/l bronopol for 30 min, showed successful development of Nile tilapia embryos, like in controls.

DISCUSSION

Bacterial species isolated from Nile tilapia eggs in our study (*A. hydrophila*, *S. aureus*, *E. coli*, and *Micrococcus* spp.) were also reported in Mozambique tilapia (*O. mossambicus*) eggs from the artificial incubation systems (Subasinghe and Sommerville 1985). In our study, *A. hydrophila* was the most frequently found isolate. This bacterium is recognized as an opportunistic pathogen that can be commonly found in hatchery water causing a substantial damage to the hatchery production of many freshwater fish species under artificial incubation conditions (Hanninen et al. 1997). The occurrence and severity of bacterial outbreaks during the hatching stages are dependent on the water source, water temperature, organic load, and incubation period (Starliper et al. 2015).

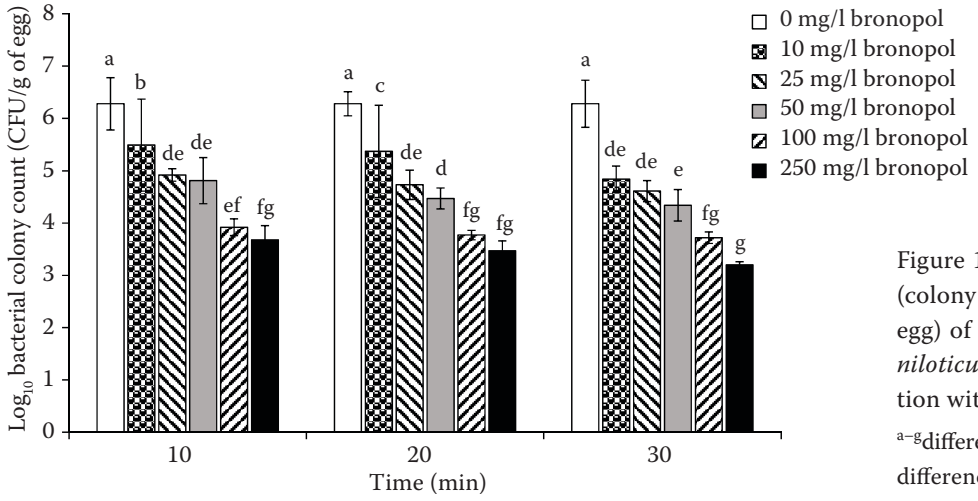

Figure 1. Bacterial colony counts (colony forming units (CFU)/g of egg) of Nile tilapia (*Oreochromis niloticus*) eggs following disinfection with bronopol

$^{a–g}$different letter means a significant difference ($P < 0.05$)

doi: 10.17221/8523-CJAS

Our findings of both Gram-positive and Gram-negative bacteria in Nile tilapia eggs point to the occurrence of various types of bacterial flora in the hatchery water, emphasizing the importance of egg disinfection in the hatchery husbandry.

The bacterial colony counts of non-treated Nile tilapia eggs (approximately 1.9×10^6 CFU/g) were acceptable within the ranges of bacteria colonized on the surface of salmonid eggs (10^3–10^6 CFU/g) (Austin 2006), however the viable counts in freshwater tilapia eggs taken in this study were higher than those in marine fish eggs. Treasurer et al. (2005) reported the number of 3100 to 38 000 CFU/ml of egg in cod and haddock, while the colony counts in California yellowtail, white seabass, and California halibut were 16.7, 110.0, and 233.3 CFU/ml of egg, respectively (Stuart et al. 2010). In our study, the bacterial counts (1.57×10^3 CFU/ml) in the hatchery water were comparable to those in the rearing system of California halibut (10^4 CFU/ml) (Stuart et al. 2010) and of Mozambique tilapia (6.25×10^3 CFU/ml) (Subasinghe and Sommerville 1985). The relatively high numbers of bacteria colonized on the surface of Nile tilapia eggs may be attributed to the rapid growth of the waterborne pathogens in temperate water temperature, particularly in the hatchery facility that is in the tropical region as Thailand, and the gelatinous matrix surrounding the eggs may be an appropriate substrate for bacterial proliferation.

Bacterial isolates colonized on Nile tilapia eggs were susceptible to a confined MIC range of bronopol (64–128 µg/ml), and these values were similar to the susceptible level of the ATCC strains, *S. aureus* ATCC 29213 (128 µg/ml) and *E. coli* ATCC 25922 (128 µg/ml). In this study, there was no significant difference of MIC values among Gram-positive and Gram-negative bacteria. The similar growth-inhibitory potency of bronopol against Gram-positive and Gram-negative bacteria observed in our study is in agreement with Bryce et al. (1978). However, these bacterial isolates from Nile tilapia eggs are less susceptible to bronopol when compared with other bacteria (i.e. *Moritella viscosa*, *Photobacterium phosphoreum*, *Tenacibacter ovoluticus*, *V. anguillarum*) from halibut and cod eggs (1–64 µg/ml) (Birkbeck et al. 2006). Apart from bronopol disinfection, other methods have been used for elimination of egg early embryo pathogens, e.g. the ultraviolet irradiation of water supply during egg incubation and yolk absorption successfully eliminated recurrent motile aeromonad septicemia among muskellunge (*Esox masquinongy*) fry (Calesante et al. 1981). Wright and Snow (1975) found that acriflavine (500–700 mg/l for 15 min) or iodine (100–150 mg/l for 15 min) successfully disinfected eggs of largemouth bass, *Micropterus salmoides*.

It was demonstrated in our study that bacterial counts from eggs in control groups were considerably higher (approximately 1.9×10^6 CFU/g) than those of bronopol treatments (1.58–311.70×10^3 CFU/g). The number of bacteria on eggs decreased substantially when eggs were treated with higher dosages (increased concentration of bronopol or longer immersion period). A significant improvement of percentage larval survival (89–94%) was noted when the bacterial counts from eggs were less than 29.22×10^3 CFU/g which was achieved from the treatment of ≥ 50 mg/l bronopol. With the minimum concentration of 50 mg/l bronopol, the 20 and 30 min exposure time yielded better survivals than the 10-min immersion. However, the improved larval survivals with a high concentration of bronopol (100–250 mg/l) were similar by the exposure time of 10, 20, and 30 min. Results indicate that the concentration increased to 100 mg/l of bronopol significantly enhanced the survival of Nile tilapia, and longer immersion time did not support better result. However, we observed that increasing the exposure time in lower concentration treatment promoted the effect like the increasing concentration of bronopol. For example, the percentage survival of eggs treated with 50 mg/l for 30 min was not different from that of eggs treated with 250 mg/l for 10 min. This presumption was also suggested in a previous study of the iodophor disinfection, when the increasing of iodophor concentration (150 mg/l for 30 min) was as effective as, but less toxic than the increasing of contact time (50 mg/l for 90 min) to provide a higher larval survival of Atlantic salmon (Jodun and Millard 2001).

The chemical disinfectants used for surface decontamination may be highly toxic to fish eggs and less toxic agents are preferable. The sensitivity of eggs to disinfectants also depends on the development stages of the treated eggs. Red drum (*Sciaenops ocellatus*) embryos were more resistant to disinfectants (formalin, sodium hypochlorite, benzalkonium chloride, polyvinylpyrrolidone iodine, and hydrogen peroxide) during the early tail-free stage compared to early embryo and early tail-bud stage (Douillet and Holt 1994). Bronopol has been

doi: 10.17221/8523-CJAS

used to disinfect fish eggs with varying degrees of success. Treasurer et al. (2005) reported a significant reduction in bacterial numbers and a higher survival (83–89%) of cod eggs disinfected with 50 to 500 mg/l bronopol for 45 s, compared with the non-treatment survival (48%), while the above dosages were not effective for haddock eggs treatment. El-Dakour et al. (2013) suggested that bronopol acts adversely on the sensitive egg stage in marine species by dehydrating the eggs, thus resulting in lower survival of larvae. Treating bluefin sea bream eggs with different bronopol concentrations (50, 150, 250, 350, and 450 mg/l) for 4 min showed no significant variation in hatching rates (39–59.9%), while the hatching rate observed in the seawater control eggs was higher (77.2%), and the high levels of 350 and 450 mg/l bronopol induced adverse effect on larval survival. These reports showed that the success of bronopol disinfection in fish eggs may vary among fish species, life stage, rearing water, and the specific dosage should be determined for each culture species if possible (Peck et al. 2004). Our study examined toxicity of bronopol disinfectant on Nile tilapia hatching eggs collected from female breeders' mouths. It was found that the development of bronopol-treated eggs in hatching to early juvenile stages was similar to these stages of Nile tilapia embryos obtained from successful artificial fertilization (Fujimura and Okada 2007), indicating that the bronopol dosage up to 250 mg/l for 30 min immersion does not disturb the embryonic development of Nile tilapia. With the evidence of antibacterial potency and safe application of bronopol on Nile tilapia egg observed in the present study, bronopol 100 mg/l for 10 min-immersion was effective against bacterial colonization and the immersion up to 250 mg/l for 30 min allowed normal embryonic development. This observation may suggest that bronopol concentration ≤ 250 mg/l is the NOEC (no observed effect concentration) value for an application in Nile tilapia egg disinfection with 30 min immersion treatment.

CONCLUSION

The study demonstrated efficacy and safety of bronopol disinfection in Nile tilapia egg at hatching stage, the dosage of 100–250 mg/l bronopol with 10–30 min immersion provided an effective reduction in bacterial colonization on the egg surface and appropriate embryonic development and percentage larval survival (> 89%) were attained subsequently to the treatment. The maximum dosage examined in this study, 250 mg/l bronopol for 30 min, was proven to be safe for Nile tilapia egg at hatching stage and effective against different species of opportunistic bacteria colonizing the egg surface. The immersion period of 10–30 min is benefit for bronopol administration that can accommodate to the decontamination procedure during the movement of hatching egg to the incubation system, which may take 10–30 min depending on individual management. The immersion administration of 250 mg/l bronopol for 10–30 min is suggested for Nile tilapia hatchery to reduce bacterial contamination and increase larval survival during the hatchery process.

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Received: 2014–11–26
Accepted after corrections: 2015–02–04

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