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Full Length Research Paper

Oxytetracycline residues in cultured gilthead sea bream (*Sparus aurata* L. 1758) tissues

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This research was carried out in order to determine residue depletion of oxytetracycline HCl in the muscle, skin, liver and spleen of healthy sea bream (*Sparus aurata*) which were stocked in floating net cage in Aegean Sea after oxytetracycline treatments (1st, 2nd, 3rd, 4th, 5th, 9th, 10th, 14th, 15th, 19th, 20th, 24th, 25th, 29th, 30th, 37th and 44th day). Oxytetracycline was given to fish once a day (75 mg/kg living weight) for 10 days by oxytetracycline supplemented pellet. A great variation was recorded in tissue residue of oxytetracycline depending on variation of feed intake. Oxytetracycline concentrations in muscle, skin, liver and spleen of healthy sea bream reduced under maximum residue limit, 24 days after treatment ceased. This is equal to 547.2 degree/days.

Key words: Oxytetracycline, gilthead sea bream, tissue residues.

INTRODUCTION

The rapid expansion of aquaculture industry has been followed by increasing bacterial disease problems. In the 1950s, oxytetracycline HCl (OTC) became the drug of choice for most bacterial fish diseases in Europe and the USA (Austin and Austin, 1987; Alderman, 1988). Although OTC is one of the most common antibiotics used in aquaculture, it has been licensed in Turkey for use in fish therapy since 2007. OTC is a broad-spectrum antibiotic widely used for the treatment of systemic bacterial infections (Björklund et al., 1991; Malvisi et al., 1996). It is administered to fish via artificial marine fish feed at the dose of 50 - 100 mg/kg body weight for 10 - 14 days (Salte and Liestol, 1983; Björklund, 1991; Austin and Austin, 1987).

The knowledge of pharmacokinetics, tissue distribution and residue depletion is important to improve therapy and minimize drug residues and the environmental impact of the drugs used in aquaculture (Malvisi et al., 1996). So far, pharmacokinetics and/or residue elimination of OTC have been reported in blue and channel catfishes (*Ichtalurus punctatus and Ichtalurus furcatus*) (Fribourgh, et al., 1969), African catfish (*Clarias gariepinus*) (Grondel et al., 1989), rainbow trout (*Oncorhynchus mykiss*) (Salte and Liestol, 1983; Grondel et al., 1989; Jacobsen, 1989; Björklund and Bylund, 1991), carp (*Cyprinus carpio*) (Grondel et al., 1987), hybrid striped bass (*Morone saatilis* male x *M. chrysops* female) (Xu and Rogers, 1993), ayu (*Plecoglossus altivelis*) (Uno, 1996), Atlantic salmon (*Salmo salar* L.) (Elema et al., 1996) and sea bass (*Dicentrarchus labrax*) (Malvisi et al., 1996; Rigos et al., 2002; Balta and Çağırgan, 2007). On the other hand, there are limited researches conducted on residue depletion of OTC on gilthead sea bream. Malvisi et al. (1996) examined tissue distribution and residue depletion after oral administration of OTC and Rigos et al. (2003) studied pharmacokinetics and bioavailability of OTC after a single dose.

The aim of this study is to determine residue of OTC by using bacteriological method in muscle, skin, liver and spleen tissues of sea bream following oral administration of drug under artificial feeding conditions.

MATERIALS AND METHODS

Experimental fish

Two hundred healthy gilthead sea bream (mean weight 290.05 ±

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43.81 g) were obtained from a private fish farm located in the Aegean Sea Shore of Turkey. The fish were divided into two groups (control and treatment groups), and placed in square floating cages (5*5*5 m). Fishes were fed at a rate of 1.5% biomass per day using commercial pellets (PINAR Marine Fish Feed, Izmir, Turkey; Pellet No: 4, contains: 88% dry matter, 46% crude protein, 3% crude fiber, 13% crude ash, 2.2% calcium, 1.5% phosphor, 12% crude fat).

The test fishes were acclimated under experimental conditions for 15 days, and all fishes were starved for 2 days before starting the experiment. Experimental group were fed for 10 days with OTC supplemented pellet and dosed at 75 mg/kg biomass per day and control group were fed with antibiotic-free pellets. Fishes in both groups were fed by hand once daily at the rate of 1.5% biomass per day during the treatment period. Water temperature was measured daily during this study. Recorded water temperatures varied between 20 and 26.5°C until the 44th day after the end of medication.

Preparation of medicated feed

To prepare the medicated feed, 50 g of oxytetracycline hydrochloride (Purity 98.7) and equal amount of starch were weighed, and mixed properly. Ten kilogram commercial pellet was measured and moistened by water spraying. All were mixed together for feeding and unused feeds were refrigerated for subsequent feedings.

Fish and tissue sampling

The first sampling was performed on the 1st day after treatment ceased (on the 11th day of treatment); following samples were taken on the 2nd, 3rd, 4th, 5th, 9th, 10th, 14th, 15th, 19th, 20th, 24th, 25th, 29th, 30th, 37th and 44th day. Five fish samples were taken randomly from control and experiment groups every sampling day. For residual analysis, 1 g muscle tissue was separated from the skin and bones, and 1 g skin tissue samples were taken from the dorso-lateral body area just posterior to the operculum. Spleen and liver were completely removed for residual analysis. Then, these samples were put in 5 ml polypropylene tubes and stored at -20°C until analyzed.

Test agar and preparation of microbial suspension

Antibiotic medium No: 2 Agar (Difco, 0270-14-4) was used for seeding bacteria. *Bacillus cereus* var. *mycoides* (ATCC-11778) were provided by Etlik Veterinary Control and Research Institute, Ankara, Turkey. Inhibition zones were measured with digital callipers (Mitutoyo). *B. cereus* var. *mycoides* (ATCC-11778) spores were prepared according to described method of Arret et al. (1971). Bacteria were inoculated in antibiotic medium no: 2 pen assay base agar (pH 5.95 ± 0.05) and incubated for 24 h at 37°C. The resulting growth from the nutrient surface was washed using 30 ml sterile water and poured in 50 ml centrifuge tube. Bacteria were held heat shock for 30 min at 70°C prior to centrifugation and the spore suspension were washed three times with 30 ml of sterile distilled water each time. Spore suspension was reconstituted with 30 ml of sterile distilled water and stored under refrigeration.

Preparation of OTC standard solutions and calculation of inhibition zones

The stock solution of antibiotic (1000 μgml^{-1}) standard was prepared by dissolving 101,317 mg OTC (British Pharmacopoeia, 1993) in 100 ml 0.1 N HCl. Fresh stock solutions of OTC were sterilized by filtration through a 0.45 µm disposable filter and stored under -20°C. The working solutions of OTC were prepared by serial dilutions with sterile 2% 0.1 M HCl containing methanol in the concentrations of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.1 μgml^{-1}. The dilute standard was used as negative control. Sterile antibiotic medium was poured into 8 ml sterile disposable plates. Six sterile porcelain beads (9 mm in diameter) were placed on the floor of plates at equal distance. A 0.4 ml suspension of the test organism, *B. cereus* var. *mycoides* (ATCC-11778) was added to each 100 ml antibiotic medium, 8 ml of the inoculated test medium was added to each plate. Then, porcelain beads were removed and 6 wells were bored in each plate. Each well was filled with 100 µl of standard solution. Each standard was studied as three parallel as in previous study (Balta and Çağırgan, 2007). The plates were incubated at 30°C for 18 h. The inhibition zones were measured with calipers and presented in Figure 1.

Preparation tissue samples and OTC analysis

The analysis of OTC was performed according to the procedure of Salte and Liestol (1983). Briefly, tissue samples (1 g) were placed in 1 ml 2% 0.1 M HCl containing methanol buffer. Weighed muscle and skin samples were homogenized (IKA T-8) at 8,000 rpm for 2 min and 15,000 rpm for 3 min, respectively. Weighed liver and spleen samples were homogenized (IKA T-8) at 8,000 rpm for 1 min. Homogenized muscle, skin, liver and spleen samples were centrifuged for 20 min at 15°C and 15.000 rpm, for 30 min at 30°C and 18.000 rpm respectively, and for 10 min at 15°C and 15.000 rpm by using refrigerated centrifuge (Hettich, EBA 12 R). The knife and shaft of the homogenizer was washed and cleaned with 2% 0.1 M HCl containing methanol buffer after each homogenization process. The homogenized fluid phase was concentrated under vacuum by using an evaporator and resuspended by adding 0.3 ml 0.1 M KH_2PO_4 buffer. The six wells (9 mm) on previosly prepared plates were filled with 100 µl of supernatant. All treatments were performed in triplicate. The plates were incubated at 30°C for 18 h. Inhibition zones were measured with calipers.

Withdrawal time calculation

The half-life ($t_{1/2}$) of the drug in fish tissues was calculated using $t_{1/2} = \ln 2/\beta$ (Baggot, 1977). The predicted withdrawal time for OTC from fish tissue was calculated using the statistical approach suggested by Salte and Liestol (1983). The method involves the linear regression from the natural logarithm of drug residue concentrations against time. The 95% confidence limits for linear equation were calculated using the method described by Steel and Torrie (1980).

RESULTS AND DISCUSSION

Mean OTC concentrations versus time recorded in muscle, skin, liver and spleen with scales are plotted in Figure 2 and reported in Table 1 with mean and ± standard errors and range values. There was a considerable variation of fish-to-fish in drug residue levels within each group at any sampling time, resulting in high standard deviations as reported in Table 1, and high-lighted in Figure 2 which shows the data obtained after medicated feed for skin, muscle, liver and spleen.

The first sampling was carried out on the 1st day after treatment ceased. The highest OTC concentration in muscle, skin, liver and spleen tissues of healthy sea bream

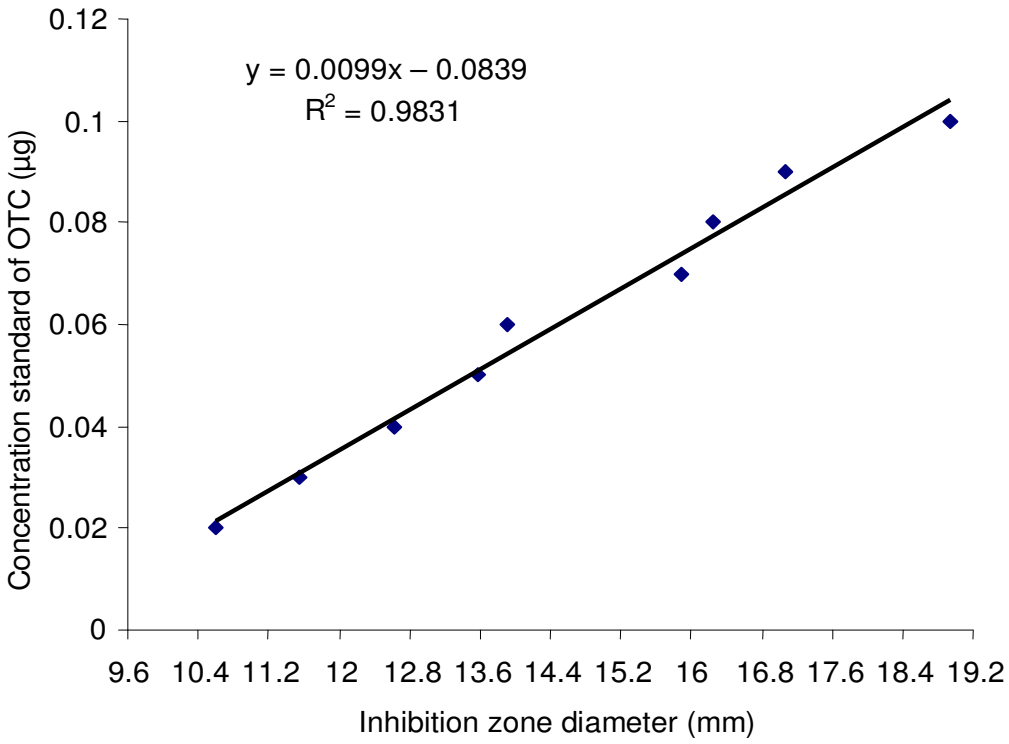

Figure 1. OTC standard and inhibition zones 2% 0.1 M HCl containing methanol (logarithmic regression).

was reached on the 2nd day after drug treatment. The results of the zones of inhibition produced by wells containing OTC in muscle, skin, liver and spleen of sea bream using standard curves generated from standard dilutions are shown in Table 1 and Figure 2.

The minimal muscle and skin tissue residue levels decreased under 0.1 µg/g on the 24th day after the end of the treatment. The minimal liver and spleen residue levels also decreased under 0.1 µg/g in the 19 and 14th day, respectively. Traces residue of muscle and skin tissues could be detected in the samples of 44th day. Besides, inhibition zones did not consist of in the liver and spleen tissue extracts on the same day.

Minimal residue levels in muscle and skin were determined in 0.1 µg/g. OTC concentrations in healthy sea bream declined in muscle (0.060 µg/g) and skin (0.049 µg/g) under maximum residue limit (MRL), 24 days after treatment ceased. This is equal to 547.2 degree/days.

The results showed that residue levels of OTC in the muscles, skins, liver and spleen of sea bream varied widely in samples of same day. A variation in drug intake is evident from the inter-subject differences in OTC tissue concentrations. The highest concentration measured in sea bream on the 2nd day was in liver, skin, spleen and muscles tissue, respectively. On the 24th day after the treatments, the mean OTC level in the tissue samples was measured as 0.049 µg/g in the skin tissue and 0.060 µg/g in the muscle tissue. Similar results were also reported by Malvisi et al. (1996) in sea bream. Malvisi et al. (1996) reported that oral administration at a dose of 75 mg/kg body weight OTC per day for 14 days at 19 - 28°C water temperature, resulted to high variation in muscle and skin tissues concentration of sea bream.

Since fishes are poikilothermic and water temperature affects fish metabolism, an increase in water temperature presumably would increase the rate of elimination of OTC residue. The results obtained from OTC residue studies on trout indicated that excretion time of OTC decreased at higher temperature (Salte and Liestol, 1983; Jacobsen, 1989; Xu and Rogers, 1993). The contradictions of these results were also reported by Fribourgh et al. (1969) from channel catfish and blue catfish. OTC residue studies on similar results were also found in the work of Salte and Liestol (1983). Different residue results were found in same individual cultured rainbow trout in fresh water (Salte and Liestol, 1983). This variation was mainly caused by the medicated feed intake, the stomach emptying rate, the absorption of drugs as well as the performance of the fish in terms of their metabolic and excretory capacity.

Haug and Hals (2000) claimed that the slower elimination of drugs in fish at low temperature compared to high temperatures may partly be due to the low production of bile and urine at low temperatures. Furthermore, Ellis et al. (1978) reported that the increase of the water temperature per °C generally results to an

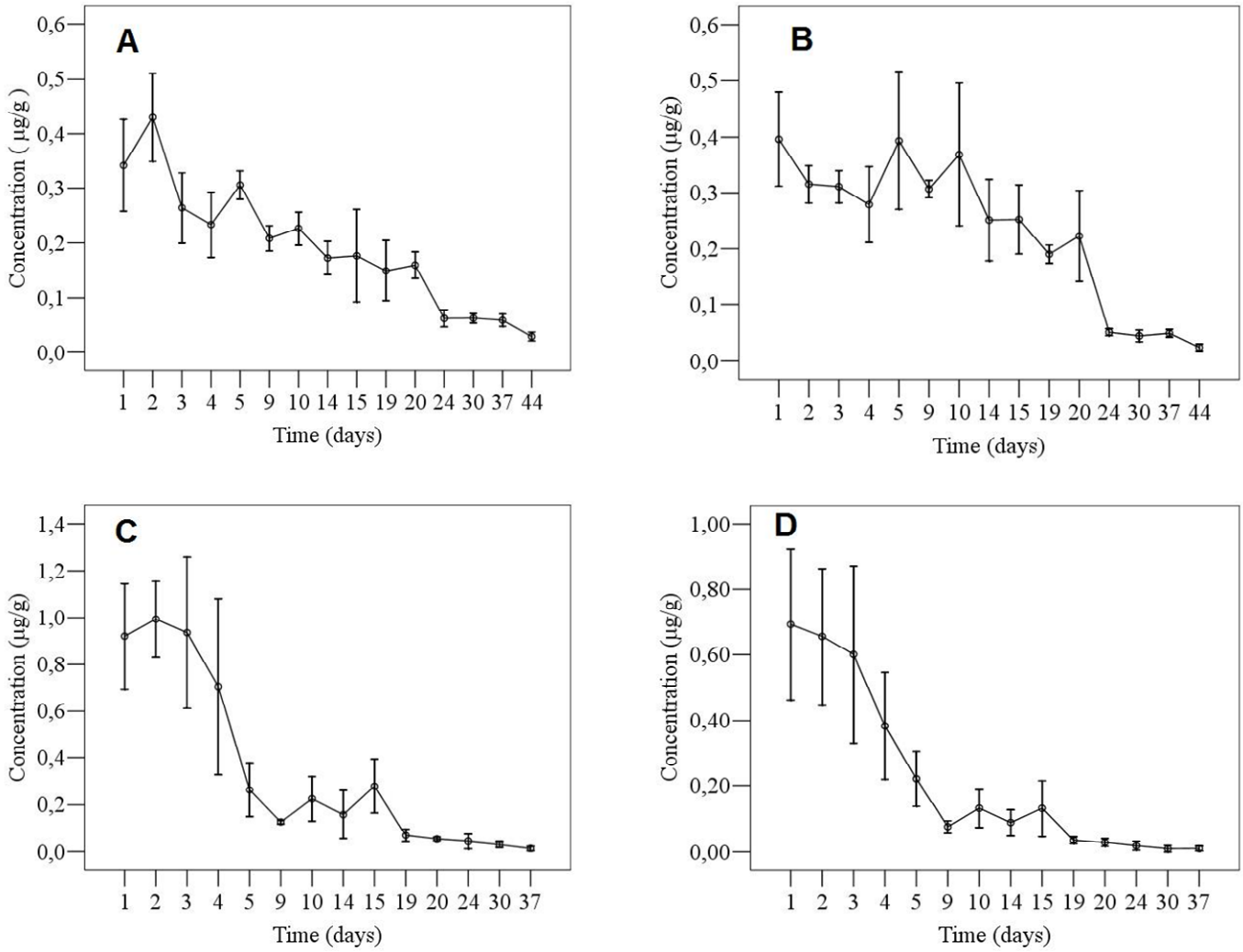

Figure 2. OTC concentrations in sea bream after treatment. (A) Muscle, (B) skin, (C) liver and (D) spleen.

increase of 10% in the metabolic and elimination rate of the fish. The results from the previous studies suggest that a 500 degree/day withdrawal time would cover the elimination of OTC (Björklund, 1991; Tennant, 1992; Kaya, 1994; Malvisi, 1997).

Our results showed that OTC residues in the muscle and skin of sea bream were higher than the MRL (0.1 µg/g) on the 20th day post dosing, but fell below the detection limit, which was less than MRL, on the 24th day after the last treatment of medicated feed at a dosage of 75 mg/kg body weight per day for 10 days at mean water tempe-rature of 22.8°C. Salte and Liestol (1983) recommended an OTC withdrawal time of 42 and 65 days for the muscle of rainbow trout acclimated to 9 and 7°C saltwater, respectively. Zhang and Li (2007) reported that OTC withdrawal period was affected by fish species and water temperature, and became shorter with higher water temperature.

The present study firstly deals with the microbiological agar-diffusion method residue profiles in sea bream. The results showed that absorption and elimination of OTC were slow. Long withdrawal period (at least 24 days) was suggested following oral administrations at a dose of 75 mg/kg body weight per day for 10 days at mean water temperature of 22.8°C for human consumption of sea bream. On the other hand, this study was carried out with healthy sea bream in field conditions, but repeating this study in infected sea bream in conditions of field can provide more reliable results. Uno (1996) reported significant differences in absorption of OTC between healthy and vibriosis-infected fish. The maximum OTC levels of infected fish tissue were lower than those in healthy fish.

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Table 1. OTC residue levels measured in muscle, skin, liver and spleen of sea bream after treatment (mean ± standard errors).

Day	Muscle (µg/g)	Skin (µg/g)	Liver (µg/g)	Spleen (µg/g)
1	0.342 ± 0.09 (0.238 - 0.466)	0.396 ± 0.09 (0.332 - 0.562)	0.841 ± 0.38 (0.288 - 1.325)	0.652 ± 0.32 (0.208 - 0.978)
2	0.428 ± 0.09 (0.350 - 0.564)	1.546 ± 0.51 (1.100 - 2.400)	1.144 ± 0.98(0.228 - 2.715)	0.671 ± 0.57 (0.164 - 1.642)
3	0.261 ± 0.07 (0.198 - 0.346)	0.311 ± 0.03 (0.268 - 0.348)	0.748 ± 0.50 (0.195 - 1.346)	0.537 ± 0.37 (0.132 - 0.975)
4	0.221 ± 0.06 (0.154 - 0.320)	0.277 ± 0.07 (0.184 - 0.380)	0.564 ± 0.50 (1.356 - 0.078)	0.370 ± 0.39 (0.958 - 0.035)
5	0.238 ± 0.09 (0.120 - 0.342)	0.350 ± 0.17 (0.155 - 0.580)	0.263 ± 0.13 (0.418 - 0.086)	0.194 ± 0.12 (0.376 - 0.045)
9	0.174 ± 0.04 (0.110 - 0.228)	0.307 ± 0.02 (0.220 - 0.326)	0.127 ± 0.01 (0.138 - 0.116)	0.076 ± 0.020 (0.096 - 0.042)
10	0.225 ± 0.03 (0.192 - 0.282)	0.349 ± 0.12 (0.228 - 0.530)	0.180 ± 0.13 (0.336 - 0.136)	0.105 ± 0.08 (0.216 - 0.083)
14	0.172 ± 0.03 (0.146 - 0.222)	0.240 ± 0.07 (0.166 - 0.330)	0.126 ± 0.11 (0.312 - 0.082)	0.07 ± 0.052 (0.134 - 0.042)
15	0.176 ± 0.09 (0.067 - 0.324)	0.252 ± 0.07 (0.193 - 0.342)	0.167 ± 0.17 (0.384 - 0.188)	0.079 ± 0.088 (0.213 - 0.078)
19	0.147 ± 0.05 (0.066 - 0.210)	0.188 ± 0.01 (0.165 - 0.212)	0.040 ± 0.04 (0.088 - 0.042)	0.022 ± 0.021 (0.046 - 0.028)
20	0.160 ± 0.02 (0.122 - 0.194)	0.223 ± 0.09 (0.138 - 0.338)	0.021 ± 0.03 (0.056 - 0.048)	0.012 ± 0.016 (0.035 - 0.023)
24	0.060 ± 0.01 (0.037 - 0.078)	0.049 ± 0.006 (0.046 - 0.062)	0.026 ± 0.03 (0.074 - 0.027)	0.011 ± 0.013 (0.032 - 0.012)
30	0.062 ± 0.01 (0.047 - 0.072)	0.045 ± 0.012 (0.030 - 0.062)	0.018 ± 0.02 (0.041 - 0.023)	0.006 ± 0.008 (0.017 - 0.012)
37	0.058 ± 0.01 (0.050 - 0.081)	0.048 ± 0.007 (0.042 - 0.060)	0.011 ± 0.01 (0.024 - 0.013)	0.008 ± 0.0079 (0.018 - 0.011)
44	0.029 ± 0.01 (0.021 - 0.035)	0.024 ± 0.004 (0.021 - 0.027)	-	-

Animals Research Group (VHAG-1333).

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