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Evaluation of Juvenile Freshwater Mussel Sensitivity to Multiple Forms of Florpyrauxifen-Benzyl

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Abstract

ProcellaCOR® (active ingredient [ai], florpyrauxifen-benzyl) is an aquatic herbicide registered for use in 2018 for managing invasive and nuisance macrophyte species. Registration studies evaluating its acute toxicity revealed a favorable environmental profile; however, prior to this study, no information existed on the toxicity of florpyrauxifen-benzyl to native freshwater mussels (Family Unionidae), one of the most sensitive and imperiled faunal groups globally. We followed standard acute (96 h) toxicity test guidelines and exposed juvenile Fatmucket (*Lampsilis siliquoidea*) and Eastern Lampmussel (*Lampsilis radiata*) to the following formulations or compounds: ProcellaCOR SC and EC formulations, technical grade active ingredient (TGAI, florpyrauxifen-benzyl), and an analytical-grade sample of the weaker florpyrauxifen acid (FA). In all tests, the estimated median lethal concentrations to produce 50% mortality (LC50) were greater than the highest concentration tested of each formulation or compound. The no observable adverse effect concentrations (NOAEC, based on analytical recoveries measured at the highest concentration tested where no toxicity was observed) were TGAI = 26 µg/L, FA = 100,000 µg/L, ProcellaCOR® SC = 193 µg ai/L ProcellaCOR® EC = 585 µg ai/L and the NOAEC values for the registered commercial formulation products (ProcellaCOR® SC and ProcellaCOR® EC) were orders of magnitude greater (3.9× and 11.7×, respectively) than the maximum application rate (50 µg/L). Our results show that the herbicide formulations and compounds tested were not acutely toxic to juveniles of these two species of freshwater mussels, indicating minimal risk of short-term exposure from florpyrauxifen-benzyl applications in the environment for aquatic weed control. However, potential chronic or sublethal effects remain uncharacterized and warrant additional investigation.

Keywords Unionid · Toxicity · Herbicide · Florpyrauxifen-benzyl · ProcellaCOR® · Rinskor™

Nuisance and noxious aquatic vegetation is an increasing problem in natural resource conservation and management. Generally, aquatic plants are important components of aquatic ecosystems and provide many beneficial ecosystem services, including nutrient cycling, increased biodiversity, and improved water quality (Barko and James 1998; Srivastava et al. 2008; Thomaz and Cunha 2010). However, as a result of invasive species introductions and/or system nutrient imbalances, aquatic vegetation can also cause a host of negative ecosystem consequences (Chamier et al. 2012; Santos et al. 2011; Kuehne et al. 2016). Mitigating the impacts of invasive/nuisance vegetation through eradication and control measures often requires a multi-faceted approach incorporating mechanical, biological, and chemical management.

Over the past 50 years, aquatic herbicides have been used as effective options to slow the spread of invasive macrophyte species (Getsinger et al. 2008). Unfortunately, more recent research has identified complications potentially confounding future efficacy and management strategies with older registered herbicides. Michel et al. (2004) described the first selection-driven mutation inferring herbicidal (fluridone) resistance in the aquatic invasive, *Hydrilla verticillata*. Isolated populations of spotted duckweed (*Landoltia punctata*) have shown resistance to diquat (Koschnick and Haller 2006), and there have been reports of isolated hydrilla populations in central Florida resistant to endothall (Berger et al. 2011). Moody and Les (2002) provided molecular evidence for the existence of watermilfoil (*Myriophyllum spp.*) hybridization, surmising hybrid vigor may be a driver for the

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ubiquitousness of milfoil infestations. Furthermore, LaRue et al. (2013) found that the Eurasian watermilfoil (*M. spicatum*, non-native) and northern watermilfoil (*M. sibiricum*, native) hybrid exhibited greater growth rates and significantly reduced herbicide sensitivity when compared to the Eurasian watermilfoil genotype. These documented changes in response to herbicide management have had various levels of impact to operational management and in general, managers have been able to switch to other aquatic herbicides and integrated strategies through effective herbicide resistance management practices. However, in aggregate, they reinforce the management need for new herbicide technology to widen options for management and reduce risk of shifts in aquatic weed susceptibility.

The need for new herbicide tools for improved herbicide resistance management, and to steward an ever-increasing concern for non-target toxicity has led to the development of a new aquatic herbicide (ProcellaCOR®) intended to have greater selectivity, improved ecotoxicity, and reduced risk to human health. ProcellaCOR® was developed for aquatic weed control by SePRO Corporation (Carmel, Indiana USA) and was registered in 2018 as an aquatic herbicide by the US Environmental Protection Agency (US EPA) under the Federal Insecticide, Fungicide and Rodenticide Act. The active ingredient (ai) in ProcellaCOR® is florpyrauxifen-benzyl (log K_{ow} 5.5), a new synthetic auxin and member of the arylpicolinate class of herbicides discovered by Corteva Agriscience (Indianapolis, Indiana, USA; formerly Dow AgroSciences). Florpyrauxifen-benzyl is also identified as Rinskor™ and has use as a rice herbicide with several other developing uses for terrestrial weed control (Richardson et al. 2016; US EPA 2017; SePRO 2018a, b).

Recent research on the efficacy of florpyrauxifen-benzyl has indicated excellent activity on key aquatic weed species including hydrilla, water hyacinth, crested floating heart, Eurasian watermilfoil, and hybrid watermilfoil (Richardson et al. 2016; Netherland and Richardson 2016; Beets and Netherland 2018; Beets et al. 2019). For in-water application to submersed weeds, florpyrauxifen-benzyl has relatively short exposure requirements, ranging from 12 to 72 h, depending on species, stage of growth, and environmental conditions (SePRO 2018a, b). In addition, US EPA (2017) registration studies evaluating acute toxicity (48 and 96-h) of four freshwater invertebrate species (water flea [*Daphnia magna*], midge [*Chironomus riparius*], scud [*Gammarus pseudolimnaeus*], and snail [*Lymnaea stagnalis*]) using florpyrauxifen-benzyl and its now-registered aquatic formulations showed little or no toxicity. Tests were generally constrained by low solubility of the active ingredient, and a definitive median effective concentration (EC50) value was only determined for water flea using ProcellaCOR EC (alternatively identified as GF-3206) at 1320 µg ai/L (26× greater than maximum label use rate of ~ 50 µg ai/L or less). When compared to other current aquatic herbicides with similar use patterns, greater use rates (e.g., 1000–5000 µg ai/L), and a favorable environmental profile in baseline studies for registration, the aquatic herbicide florpyrauxifen-benzyl was considered reduced risk by US EPA (2017). Short exposure durations and reduced effective concentrations are not only beneficial to resource managers, but may also help to reduce toxicological hazard to non-target organisms, particularly in important high-biodiversity systems containing imperiled taxa such as freshwater mussels (Family Unionidae).

Unionids are experiencing significant worldwide declines and are among the most imperiled faunal groups in North America (Williams et al. 1993; Graf and Cummings 2007). Many researchers believe that these declines are attributed to their sensitivity to environmental pollution, poor water quality, and to other anthropogenic activities that adversely affect aquatic habitat (Williams et al. 1993; Williams and Neves 1995; Richter et al. 1997; Strayer et al. 2004; Cope et al. 2008; Archambault et al. 2014). Previous toxicological research has found freshwater mussels to be among the most sensitive aquatic organisms tested to a variety of environmental contaminants, such as nickel, zinc, chloride, copper (active ingredient in many algaecide/aquatic herbicide products) and ammonia (Keller and Zam 1991; Augspurger et al. 2003; Mummert et al. 2003; Wang et al. 2007; Cope et al. 2008; Wang et al. 2010). Currently, US EPA aquatic herbicide registration guidelines do not require freshwater mussel toxicity testing. However, freshwater mussels and their potential response to environmental contaminants are a growing concern in assessments of aquatic ecosystem health. Therefore, there is increasing interest in the need to assess potential responses of these organisms to aquatic herbicides used for invasive aquatic weed control (Getsinger et al. 2008; Archambault et al. 2015; Archambault and Cope 2016). The aim of this study was to provide initial sensitivity data for representative freshwater mussel species when acutely exposed to multiple ProcellaCOR formulations, the technical grade active ingredient florpyrauxifen-benzyl and a weaker acid of the herbicide (florpyrauxifen) that can be detected at low levels during degradation of the active ingredient.

Materials and Methods

Initial toxicity testing was conducted with the following test materials prior to product registration: ProcellaCOR SC formulation (suspension concentrate with 300 g ai per liter), ProcellaCOR EC formulation (emulsifiable concentrate with 25 g ai per liter), technical grade active ingredient (TGAI) florpyrauxifen-benzyl (Rinskor, XDE-848 BE, or SX-1552), and an analytical-grade sample of the weaker florpyrauxifen acid (FA) (XDE-848 or SX-1552A). Following product registration, additional toxicity tests were

conducted with the commercially available formulations, ProcellaCOR® SC (suspension concentrate with 300 g ai per liter), and ProcellaCOR® EC (emulsifiable concentrate with 25 g ai per liter). The two post-registration formulations were substantively identical as the pre-registration versions. The EC and SC formulations were obtained in liquid form, while the TGAI and FA were received as a powder. Stock solutions of each compound were prepared by adding to reconstituted hard water (ASTM 2013) and mixing on a stir plate until dissolution (20–30 min) at room temperature. Treatment concentrations bracketed the maximum use rate of 50 µg ai/L and were selected based on previous toxicity tests with non-target organisms. For the liquid formulations, concentrations were in threefold increases (0, 9, 27, 81, 243, 729, and 2187 µg ai/L), whereas treatment concentrations for FA increased by a factor of 10 (0, 1, 10, 100, 1000, 10,000, and 100,000 µg/L). Due to the low water solubility (15 µg/L) of TGAI, treatment concentrations (0, 5, 10, 25, 50, and 100 µg/L) were significantly reduced and dimethylformamide (DMF) was used in the preparation of a working stock, as a co-solvent, at a concentration of 0.25 mg/L. Co-solvents were not used for the two end-use products (EC and SC), which are formulated with emulsifying agents to increase functional solubility. Following ASTM (2007) protocol, a solvent control treatment was added to the TGAI test with a solvent (DMF) concentration equal to the greatest treatment concentration (0.025 mg/L).

Test exposure concentrations for the pre-registered formulations, TGAI, and FA in the initial study were verified from composite water samples taken at the initiation of the test and at the 48-h time-point. Water samples were preserved using formic acid to achieve a pH ≤ 4 and 5% methanol to limit potential TGAI association with glass of sample vials. The preserved samples were stored refrigerated (4°C) and shipped via overnight courier to EPL Bio Analytical Services (Niantic, Illinois, USA) for analysis with a UPLC-MSMS method (0.2 µg/L quantitation limit) modified from the analytical method used for registration studies (Huang and Walter 2015). Exposure concentration verification for a repeat study with ProcellaCOR® SC and ProcellaCOR®. EC were similarly preserved and delivered to SePRO's Research and Technology Campus (Whitakers, NC, USA) for HPLC analysis (1 µg/L quantitation limit).

Initial toxicity tests with the pre-registered formulations, TGAI, and FA were each performed with juvenile Fatmucket (*Lampsilis siliquoidea*) provided by the mussel culture laboratory at Missouri State University (Springfield, Missouri, USA). *Lampsilis siliquoidea* juveniles were propagated by infecting host-fish (*Micropterus salmoides*) with glochidia using standard propagation and culture methods (Barnhart 2006). *Lampsilis siliquoidea* is a common Interior Basin species widely distributed and stable in the Mississippi and Gulf drainages (NatureServe 2019) of the USA and has been used extensively in toxicological testing (Raimondo et al. 2016). Juvenile *L. siliquoidea* used for these experiments were 5–14 days old, with an average (±SD) shell length of 3.79 ± 0.51 mm.

Repeat toxicity tests with the registered SC and EC commercial formulations were performed with juvenile Eastern Lampmussel (*Lampsilis radiata*) provided by the Harrison Lake National Fish Hatchery (Charles City, Virginia, USA). *Lampsilis radiata* juveniles were propagated by infecting host-fish (*Micropterus salmoides*) with glochidia using standard propagation and culture methods (Barnhart 2006). *Lampsilis radiata* is an Atlantic Slope species widely distributed in freshwater systems along the Eastern Seaboard of the USA. *Lampsilis radiata* is generally considered secure; however, the conservation status for individual populations varies (NatureServe 2019). Juvenile *L. radiata* used for these experiments were 4–10 days old, with an average (± SD) shell length of 3.69 ± 0.24 mm. Both of these mussel species were selected for testing based on their wide geographic distribution and applicability to potential herbicide applications in surface waters of the Midwestern and Eastern regions of the USA, their relative ease of propagation and availability of juveniles for testing, and the availability of comparative toxicological data for the species and family. Moreover, the juvenile life stage was chosen for testing because previous research has shown that EC50s for juveniles were within a factor of 2 when compared to EC50s for glochidia (the larval stage) for 50% of paired tests across chemicals, and juveniles were more sensitive than glochidia by more than twofold for 33% of the comparisons made between life stages (Raimondo et al. 2016).

Mean temperature (range in parentheses) of juvenile mussels in culture water upon arrival to the laboratory was 18.6°C (13.5–22°C). Juveniles were acclimated to reconstituted hard water (ASTM 2013) and the test temperature of 20°C by placement into a 1:1 mixture of culture and reconstituted water for 2 h, allowing for a 2°C/h maximum rate of change, followed by a 1:3 ratio for an additional 2 h, and then 100% reconstituted water for 72 h prior to test initiation. Water-only static-renewal tests were conducted for 96 h with a ≥ 90% water and chemical renewal at 48 h (ASTM 2013). Survival was assessed at the 48 and 96 h exposure time-points by observing for foot movement or a heartbeat within a 5-minute period. For each test, control replicates (×3) contained 10 juveniles each, whereas all other treatment replicates (×3) contained 7 individuals (done to reduce the overall number of mussels needed for testing). Test acceptability is specified to be > 90% survival in the control treatment at 96 h (ASTM 2013); control survival in our tests averaged 93.9% at 96 h. All tests were conducted in light and temperature controlled incubators (Precision Model 818 Thermo Fisher, Marietta, Ohio, USA) held at 20°C and a light:dark cycle of 16:8 h.

Water chemistry analyses were performed at the 48-h time-point for each toxicity test. Mean (range in parentheses) water quality conditions during the experiments were as follows: 96.7 mg $CaCO_3$/L alkalinity (90–108 mg/L), 157.0 mg $CaCO_3$/L hardness (150–164 mg/L), 534.7 µS/cm conductivity (450–589 µS/cm), 8.4 pH (8.3–8.5), and 8.8 mg/L dissolved oxygen (8.2–9.7 mg/L); n = 6 determinations for alkalinity and hardness, n = 42 determinations for all other variables). Alkalinity and hardness were measured by titration following standard methods (APHA 1995) and all other water quality parameters were measured with a calibrated multi-probe system (YSI model 556 MPS, Yellow Springs Instruments, Yellow Springs, Ohio, USA).

The effect of each of the test materials on the survival of juvenile freshwater mussels was used to determine the no observable adverse effect concentration (NOAEC) and the median lethal concentration (LC50), both analyzed via the Trimmed Spearman-Karber method (Comprehensive Environmental Toxicity Information Software [CETIS], V1.8.0.12, Tidepool Scientific, LLC, McKinleyville, California, USA). The NOAEC represents the highest treatment concentration in which survival was not significantly different from the control. The LC50 was defined as the calculated toxicant concentration resulting in the mortality of 50% of exposed individuals within the specified time-point.

Results and Discussion

We found that LC50s could not be determined for any of the acute toxicity tests with ProcellaCOR materials due to the lack of mortality observed at the 48-h and 96-h assessment time-points. In fact, no mortality was measured during any of the 96 h exposures with juvenile *L. siliquoidea* and the mortality (3%) observed in juvenile toxicity tests with *L. radiata* occurred in the 9 µg/L (2 individuals), 27 µg/L (2 individuals), 243 µg/L (1 individual), and 2187 µg/L treatment (2 individuals) (overall survival, 97%). Therefore, none of the tested materials showed evidence of acute toxicity to freshwater mussels. Furthermore, the inability to elicit sufficient mortality required for the calculation of the median lethal concentration during acute toxicity tests indicates that the LC50s are greater than the highest treatment concentrations tested (Table 1). Moreover, compared to several other aquatic herbicides and algaecides that have been tested on juvenile unionid mussels, the ProcellaCOR formulations show the greatest margin of safety (Table 1). The NOAEC for each of the tests (ProcellaCOR SC, ProcellaCOR EC, TGAI, and FA in the initial study, and SC and EC in the repeat study) corresponded to the highest nominal treatment concentrations (2187, 2187, 100, 100,000, 2187, and 2187 µg/L, respectively). These results demonstrated that the pre-registration formulations and tests yielded the same results as the post-registration formulations and tests. However, in the analytical verification of treatment concentrations, we documented reduced recovery of florpyrauxifen-benzyl (9%–27%) versus target (nominal) concentrations for the unformulated TGAI and SC formulation in the initial study and with the two registered formulations in the repeat study (Table 2).

The under-recovery of active ingredient florpyrauxifen benzyl was likely due to lack of adequate solubility at higher test concentrations, physical properties of test materials, and low-level hydrolytic breakdown of active ingredient in TGAI and formulation tests. Considerably greater percent recoveries (87 and 75, respectively) of the lower treatment concentrations (5 and 25 µg/L) for the technical grade compound suggest saturation occurred near the 25 µg/L TGAI treatment (data not shown). This value is in general agreement with achieved concentrations in solution during other freshwater ecotoxicity studies of the compound for registration (40–60 µg/L range) and the estimated solubility of 15 µg/L at 20°C (US EPA 2017). With testing conducted under growth chamber lighting and at pH 8.4, low-level conversion of

Table 1 Sensitivity of juvenile freshwater mussels belonging to the genus *Lampsilis* to various commercially available aquatic herbicides and algaecides

HERBICIDE	AI	SPECIES	LC50 (µg/L)	MOS	CITATION
Sonar–PR®	Fluridone	*L. siliquoidea*	511 (309–843)	3.4	Archambault et al. 2015
Aquathol–K®	Endothall	*L. siliquoidea*	34,400 (29,300–40,500)	6.9	Archambault et al. 2015
Clearigate®	Copper	*L. cardium*	480 (401–575)	0.5	Popp et al. 2018
Clearigate®	Copper	*L. abrupta*	176 (149–207)	0.2	Popp et al. 2018
Nautique®	Copper	*L. abrupta*	3406 (2954–3928)	3.4	Popp et al. 2018
ProcellaCOR® SC	Florpyrauxifen-benzyl	*L. radiata*	> 193* (N/A)	> 3.9*	This Study
ProcellaCOR® EC	Florpyrauxifen-benzyl	*L. radiata*	> 585* (N/A)	> 11.7*	This Study

Results are based on nominal values except for the two ProcellaCOR formulations tested in this study, which are based on measured concentrations. Ninety-six hour LC50 values and 95% confidence intervals are presented in µg/L of active ingredient (ai). Margin of safety (MOS) was calculated by dividing LC50 values (based on nominal concentrations) by maximum label rates

*Corresponding nominal LC50 values for ProcellaCOR® SC and EC were > 2187 µg/L with a calculated MOS of > 43.7

Table 2 Comparison of measured recovery (0 h) and highest nominal treatment concentration for each of the six materials tested: (Pre-registration SC formulation, Pre-registration EC formulation, *TGAI* – technical grade active ingredient, *FA* – florpyrauxifen acid, ProcellaCOR® SC and ProcellaCOR® EC)

	SC	EC	TGAI	FA	SC®	EC®
Target	2187	2187	100	100,000	2187	2187
Measured	1023	1924	26	90,751	193	585*
% Recovery	47	88	26	91	9	27

Values are given in µg/L active ingredient for formulated compounds

*Measured value at 48 h was > 0 h value

TGAI to FA was observed in 48-h solution measurements and ranged from < 1% to 26% of target active concentrations. Registration studies documented potential hydrolysis of the active ingredient to FA at higher pH (hydrolytic half-life of 111 days at pH 7 and 1.23 days at pH 9 – US EPA 2017). The highest percentages of FA formation in these studies were observed for lower concentration tests of TGAI (25 µg/L and less) where presumably more active ingredient was in solution and perhaps had a greater tendency in this short exposure study for hydrolysis versus formulated materials. While ai recoveries of formulated products were generally poor at the higher concentrations, ProcellaCOR® EC recoveries at and below 243 µg/L ranged from 101% to 120%. However, ai recoveries from SC formulations were consistently less. The reduced recovery of the SC formulation may have been partly a result of the formulation's viscous nature and slight inaccuracies associated with pipetting very small volumes of viscous working stock solutions. In addition, recovery differences for high concentrations of the two formulations (2187 µg ai/L is 44 times maximum label use rate) may have been partly a function of different analytical testing methodology for the two studies (initial study—UPLC-MSMS; follow-up study—HPLC).

We report no evidence of acute toxicity to juvenile mussels of these two species for any of the compounds evaluated during this study. Our findings are similar to those of other freshwater invertebrates tested in which formulations of florpyrauxifen-benzyl showed little to no toxicity in acute tests; the 48-h LC50 for *Daphnia magna* was > 62.6 µg ai/L, the 48-h LC50 for *Chironomus riparius* was > 56.3 µg ai/L, the 96-h LC50 for *Gammarus pseudolimnaeus* was > 41.9 µg ai/L, and the 96-h LC50 for *Lymnaea stagnalis* was > 48.2 µg ai/L (US EPA 2017). The analytical under-recovery of florpyrauxifen-benzyl due to reduced solubility convoluted the determination of NOAEC values in our study. Therefore, we derived NOAEC values based on the analytical recoveries at the highest concentration tested where no toxicity was observed. Thus, NOAEC values for the tested materials are as follows: TGAI = 26 µg/L, FA = 100,000 µg/L, ProcellaCOR® SC = 193 µg ai/L (repeat study recovery) and ProcellaCOR® EC = 585 µg ai/L (repeat study recovery). Thus, the most conservative NOAEC estimates for each formulation (SC and EC) is based on the measured concentrations, as shown in Table 2. It is important to note that concentrations verified using analytical methodology quantified a solubility-limited target analyte (florpyrauxifen-benzyl) and may not be representative of other ingredients within the formulations. Directly applying these measured values of ai to other formulation ingredients is likely to misrepresent the potential toxicity of other ingredients in the formulation. Therefore, the potential effects of the non-active ingredients are better represented by the nominal values in this study because they incorporate the full formulation of active and non-active ingredients. It is important to consider these other ingredients because they are not always inert in biological activity. For example, Bringolf et al. (2007) found that *L. siliquoidea* glochidia were 5 times more sensitive to a surfactant (MON 0818) used in Roundup® formulations than the ai, glyphosate IPA salt.

While aquatic herbicides and algaecides are not used exclusively in lentic environments, their impacts on reservoir mussel populations may be consequential. Reservoirs can support unique mussel populations that are generally less surveyed and monitored in a quantitative manner than their lotic counterparts. Some lentic environments may contain greater mussel diversity than once thought (Ryan J. Heise, personal communication, Duke Energy, Huntersville, NC) and may even serve as refugia for adaptive lotic species facing extirpation elsewhere in their range. Therefore, applicators of registered technologies for weed or algae control in lakes and reservoirs should be cognizant of potential mussel populations in their treatment areas prior to any application (Cope et al. 2017). Additional research is needed on the role that reservoirs play in the conservation of mussel species (Pilger and Gido 2012). Therefore, protecting current populations by further characterizing the risks associated with aquatic herbicide/algaecide use should be a priority. When comparing mussel toxicity values for current use aquatic products, this new chemistry (florpyrauxifen-benzyl) offers promising safety margins (Table 1). Moreover, given the short exposure requirements (12–72 h) of ProcellaCOR®, these 96 h toxicity tests represent an environmentally

relevant exposure duration during application. However, our study only evaluated juvenile mortality of two unionid mussel species and we suggest that additional studies of other unionid life stages, other molluscs, other aquatic invertebrates, and potential sublethal effects may be prudent. Our results here, weighed with earlier research on herbicidal efficacy (Richardson et al. 2016; Netherland and Richardson 2016; Beets and Netherland 2018; Beets et al. 2019), support that ProcellaCOR® is an effective tool for managing invasive macrophytes while posing a lower risk to non-target species than other aquatic herbicides. Thus, ProcellaCOR® provides an option for natural resource professionals to manage invasive weeds in systems containing vulnerable invertebrate species without compromising efforts of conserving imperiled fauna, such as freshwater mussels.

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