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Toxicity Assessment of Bioluminescent Rapid Bioassays (*Vibrio fischeri*) on Selected DBPs

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ABSTRACT

For the past 30 years more than 600 different disinfection by-products (DBPs) have been reported with many unknown ones yet to be discovered. Bioluminescence rapid toxicity tests are suitable toxicity screening for DBPs that caused adverse health effects. Previously, IC_{50} study on specific DBPs have not been conducted. This study aims to characteristically identify the sensitivity of bioluminescent rapid bioassays on selected DBPs (chloroacetic acid, trichloroacetic acid, bromoacetic acid and iodoacetic acid) by measuring IC_{50} of *Vibrio fischeri* on these compounds. IC_{50} are determined through luminescence that was measured using a Beckman Counter DTX 800 multimode detector. The 30-minute IC_{50} of selected DBPs are as followed: CAA (865.4 mg/L), TCAA (1119 mg/L), BAA (59.67 mg/L) and IAA (15.6 mg/L). It was found that Bioluminescent Rapid Bioassays based on *Vibrio fischeri* showed promising sensitivity on the selected DBPs and are suitable as a screening tools for DBPs.

INTRODUCTION

Toxicity measurement of wastewater, sediments, and contaminated water bodies is a very important part of environmental pollution monitoring. It is an accepted assumption that the simple measurement of chemicals concentration or other parameters like dissolved oxygen, biological oxygen demand and chemical oxygen demand, with reference to established regulatory rules, will not give an accurate account of the environmental noxiousness [1]. Through these only the pollutant's nature are shown but fail to indicate any information on how it may affect living organisms biologically. Therefore, much attention has been paid to biological sensors or detectors that lead to the development of bioassay. Rapid bioassays with certain sensitivity can be used to evaluate of biological effects on toxicity and eco-toxicity and allow incorporation of toxicity parameters in the regulatory framework.

Crustaceans, fish and algae were traditionally used for aquatic toxicity measurement. Farre and Barcelo [2] had reviewed various bioassays and tests based on these organisms and found that those from plants and animals require longer exposure time and sample volume. The complications of standardization of organisms and the need of skilled operators and special equipment have become major disadvantages. Hence, cost-effective, rapid and reproducible toxicity measurements based on microorganisms have gained more popularity. Bacterial bioassays usually involved cellular respiration, cell population growth, consumption of substrate, ATP luminescence and bioluminescence inhibition assays. Bioluminescence rapid toxicity tests can be helpful as preliminary indicators of anthropogenic-induced biological effects in aquatic systems. Bacterial bioluminescence has many advantages such as rapid response usually between 15 to 30 minutes, low cost, improved reproducibility, does not required extra substrates, high sensitivity, and on-line measurability has been use as an indicator of toxicity replacing most classical monitoring toxicity bioassay of fish or crustacean by cutting-edge technology [3-6]. Bioluminescence rapid toxicity tests are suitable toxicity screening for disinfection by-products (DBPs) that have become a considerable concern on the chronic exposure to DBPs in drinking water especially when some studies indicated a causative relationship between exposure to chlorinated water and bladder cancer in humans and other adverse health effects [7,8].

The disinfection of drinking water is crucial to ensure public health. However, reactions of commonly used disinfectants, such as chlorine, monochloramine, chlorine dioxide, and ozone with natural organic matter will result in the formation of DBPs [9]. In the last 30 years over 600 different DBPs have been identified and discussed but there are still many unknown DBPs out there [10,11]. The formation of endless DBPs in water bodies has strengthen the need for exploring the uses of bioluminescence rapid toxicity tests as screening technologies as they react to mixture effects of DBPs including contributions from unidentified ones and take into consideration the interactive effects that potentially leads to antagonism and synergy of DBPs in a complex mixture. Thus, bioluminescence rapid test as a bioassay is risk scaled with more potent compounds contribute more to the bioassay response than less potent chemicals [12]. In addition, Kaiser [13] outlined the correlation of luminescence inhibition in a bacterium with effective indication of toxic effects on higher organisms.

The commonly used bioluminescence inhibition assay species included strains of *Vibrio fischeri, Vibrio harveyi, Photobacterium leiognathi* and *Pseudomonas fluorescens*. In the past few years, the *V. fischeri* has been used by several agencies to assess the impact of chemicals in the environment [14]. In previous work, study of *V. fischeri* on toxicant like heavy metals and pesticides were abundant but those that focus on DBPs were less. This study aims to study the sensitivity of *V. fischeri* on selected DBPs (CAA, TCAA, BAA and IAA).

MATERIALS AND METHODS

Preparation of media, bacterial culture and standard solutions

All analytical grade chemicals used were purchased either from Sigma (St. Louis, MO, USA), Fermentas (USA), Merck (Darmstadt, Germany). Luminescence medium contained the following ingredients in 1 liter deionized water: NaCl (10 g), peptone (10 g), glycerol (3ml), yeast extract (3g). The medium was adjusted to pH 6.5 before autoclaving. Agar (18g) was added to the media for luminescence agar. Minimal salt medium (MSM) contained the following ingredients in 1 liter deionized water (g/l): $Na_2HPO_4.7H_2O$ (12.8 g), KH_2PO_4 (3.1 g), NaCl (15 g), NH_4C1 (1 g), $MgSO_4$ (0.5 g), and glycerol (3 ml) [15]. The medium was adjusted to pH 6.5 prior to autoclaving at 121 °C, 115 kPa for 15 minutes. *V. Fisheri* (Microtox®, Strategic Diagnostics Inc) which were supplied in a standard freeze-dried (lyophilized) state, were reconstituted in a salt solution then streaked on luminescence agar and incubated at 15 ^oC at 12-36 hours. Colony with bioluminescence will be culture in broth media at 15 ^oC and 100rpm orbital shaker [16]. Bacteria were maintained for routine use on luminescence slant agar at 4 °C. Standard solutions of CAA, TCAA, BAA and IAA were prepared by gravimetric method by dissolving them in deionized water and stored in solvent-washed polypropylene containers. From these stock solutions, working solutions of final concentrations of DBPs from 0.1 to 2000 mg/L were prepared fresh before use

Measurement of luminescence

All the experiments were repeated at least three times. Percentage luminescence and percentage of inhibition can be calculated as followed [17]:

$$Luminescene\ (\%) = \frac{Luminescene\ of\ sample\ after\ t\ minutes}{Luminescene\ of\ control\ after\ t\ minutes} \times 100$$

$$Inhibition\ (\%) = 100 - Luminescene\ (\%)$$

Luminescence was measured using a Beckman Counter DTX 800 multimode detector 200 µL of samples were pipetted into 96 wells DTX microplates before the readings were taken. The unit used for luminescence was Relative Luminescence Unit (RLU) [18].

Calculations of IC_{50}

The concentration of the toxicant (ppm) which caused a 50% reduction in light after exposure for t minutes is designated as the IC_{50}. IC_{50} calculations are outlined Shukor et al [19,20]. IC_{50} were calculated using Graphpad Prism version 6.01. Values are means ± standard errors.

Effects of DBPs on bioluminescence production

Preparation of bacterial cells was done by inoculating 1 % (v/v) of bacterial culture (OD_{600} = 0.7- 0.8) into 100 ml of luminescence broth medium and grown at 15^oC on rotary shaker (100 rpm) for 12 hours. The cultures were then harvested and subjected to centrifugation (10,000 xg) for 5 minutes. The spent supernatant was discarded and biomass of the bacteria cell were recorded before substituted with MSM to produce bacterial stock solution which was stored at 4°C before uses. The bioluminescence inhibition study was carried out by testing *V. fischeri* at different concentrations of toxicants DBPs that showed inhibitive effect in the range of 0.001 to 1000 mg/l in 96 wells DTX microplates. Deionized water was used as control to replace the toxicants in this case the DBPs. Readings were taken after 5 minutes of incubation with toxicants and recorded as luminescence for time zero. Following another 5 minutes of incubation period at room temperature with toxicants, a reading was taken again as 5th minute and repeated every 5 minutes thereafter until the 30th minutes.

RESULT AND DISCUSSION

Effect of selected DBPs on *V. fischeri*

Other studies on *V. fisheri* that involved DBPs were done on real-time sample containing various DBPs but not specifically dedicated to a single DBP at a time. This study was conducted at temperature of 28 °C in order to be able to compare with other bioassays that were done at such temperature. It can be observed that there is a decrease in relative luminescence unit (RLU) across time with the exposure of selected DBPs (**Figs.** 1 – 4). The results were normalized with blank.

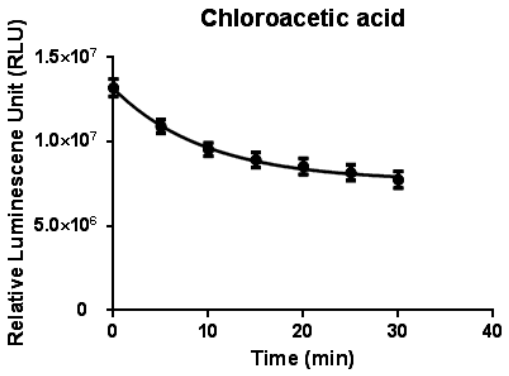

Fig. 1. Effect of CAA (100 ppm) on bioluminescence of *V. fischeri.*

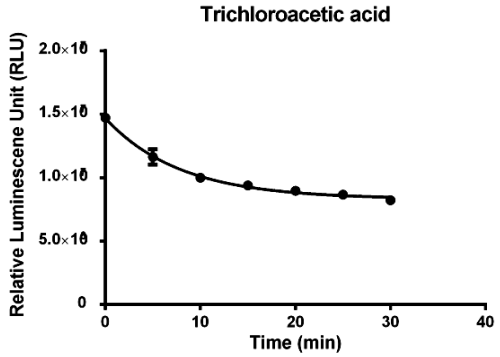

Fig. 2. Effect of TCAA (100 ppm) on bioluminescence of *V. fischeri.*

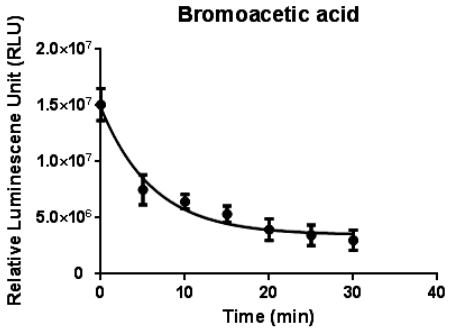

Fig. 3. Effect of BAA (100 ppm) on bioluminescence of *V. fischeri.*

The results performed for 30 minutes on the selected DBPs showed that toxicity towards *V.fisheri* was: IAA > BAA > CAA > TCAA. **Fig.** 5 shows the comparison of *V.fischeri* bioluminescence inhibition of selected DBPs of 100ppm at exposure time of 30 minutes. The LD_{50} of rat and mice also shown the same rank order of toxicity, with IAA being most severe while TCAA being least toxic (**Table** 1). IAA approximately two times more genotoxic than BAA is by recent study, the most genotoxic DBP in mammalian cells, with rank order for genotoxicity monohaloaceticacids follows: IAA > BAA > CAA [21].

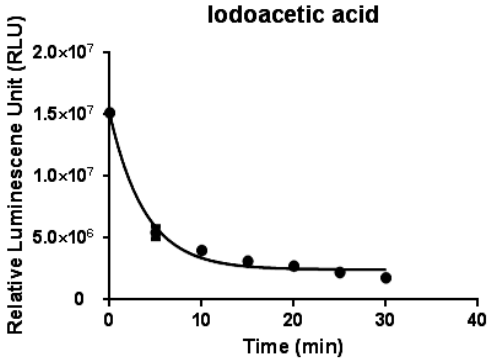

Fig. 4. Effect of IAA (100ppm) on bioluminescence of *V.fischeri.*

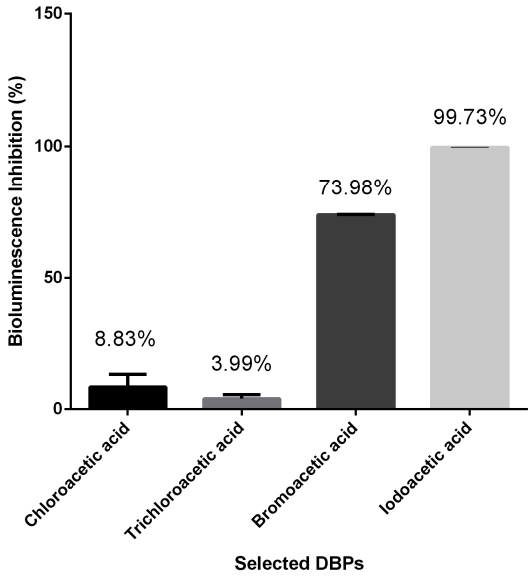

Fig. 5. Comparison of *V. fischeri* bioluminescence inhibition of selected DBPs.

Kaiser [13] has suggested that there are correlations of acute toxicity of *V. fischeri* with other aquatic species, i.e., fathead minnow, bluefill, catfish, goldfish, goldorfe, guppy, killifish, rainbow trout, sheepshead minnow, zebrafish, various Daphnia species, ciliate Tetrahymena pyriformis and various algae organisms and good correlations especially with mammalian (rat and mouse). However, EC_{50} was dependent strongly on the route of exposure which are oral, intravenous and intraperitoneal, the values of LD_{50} being least for the intravenous route. The *V. fischeri* EC_{50} values yielded the best correlation with intravenous LD_{50} data. Yang and Zhang [27] also showed the same rank order of toxicity of the selected DBPs using the marine polychaete *Platynereis dumerilii*.

Effect of the selected DBPs on *V.fischeri* also clearly suggested its sensitivity to the selected DBPs which are known to be organic chemicals. Abbondanzi et al. [28] highlight that *V. fischeri* show more sensitivity towards organic chemicals than *Pseudomonas fluorescens*. Both the organism yielded good sensitivity with metal ions.

Table 1. Non-human (mammals) toxicity values for CAA, TCAA, BAA and IAA.

DBPs	Test organism	Route Administration	ofLD_{50} (mg/kg)	Reference
CAA	Rat	Oral	76	22
	Mouse	Oral	255	22
TCAA	Rat	Oral	3200-5000	23
	Mouse	Oral	5640	23
	Dog	Oral	1590-2000	24
BAA	Rat	Oral	177	25
IAA	Mouse	Oral	83	26
	Rat	Intraperitoneal	75	26
	Dog	Intravenous	45	26

IC_{50} of *V. fischeri* on selected DBPs

The study was done using concentration of selected DBPs range of 0.001ppm to 2000 ppm. For both BAA and IAA, it was

observed that bioluminescence inhibition reach more than 99% after 800ppm whereas CAA and TCAA, the concentration range was extend till 2000ppm. Dose-Inhibition response curve was constructed using nonlinear regression models in GraphPad Prism 6.01. It was observed that the One Phase Decay model show highest correlation ($R^2 > 0.98$) (**Table** 1).

Table 1. Nonlinear regression curve to identify IC_{50} of the selected DBPs.

DBPs	Nonlinear Regression Model	Correlation coefficient	IC_{50} (ppm)
CAA	One Phase Decay	0.9872	865.4 ± 1.12
TCAA	One Phase Decay	0.9886	1119 ± 2.51
BAA	One Phase Decay	0.9902	59.67 ± 3.25
IAA	One Phase Decay	0.9832	15.52 ± 2.32

In term of rapid bioassay that based on reduction of luminescence, usually 30 and 15 minutes are chosen to identify IC_{50}. In this case, 30 minutes bacterial luminescence assay for BAA (**Fig.** 5), CAA (**Fig.** 7), TCAA (**Fig.** 8) and IAA (**Fig.** 6) showed better sensitivity. It can be observed that the 30 minutes of exposure time required little dosage or concentration to reduce its bioluminescence to 50% compared to 15 to 25 minutes exposure time. The results of bacterial luminescence assay performed for 30 min on the selected DBPs showed that the inhibition scale was: IAA > BAA > CAA > TCAA.

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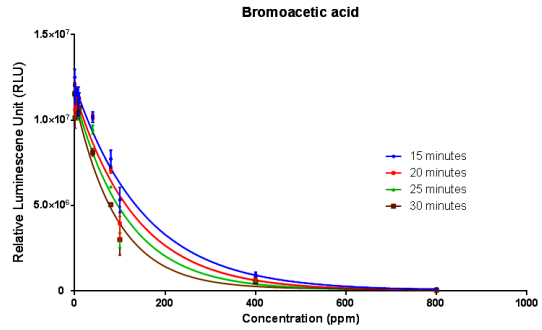

Fig. 5. Exposure time of 15, 20, 25 and 30 minutes of BAA with various concentrations on Luminescence of *V. fischeri.*

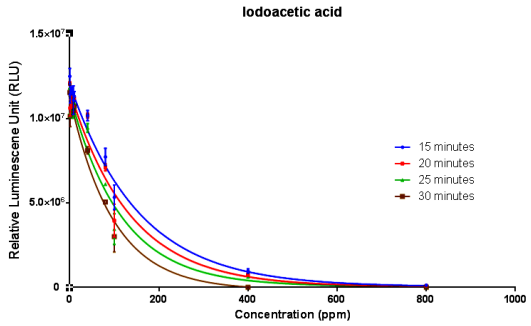

Fig. 6. Exposure time of 15, 20, 25 and 30 minutes of IAA with various concentrations on Luminescence of *V. fischeri.*

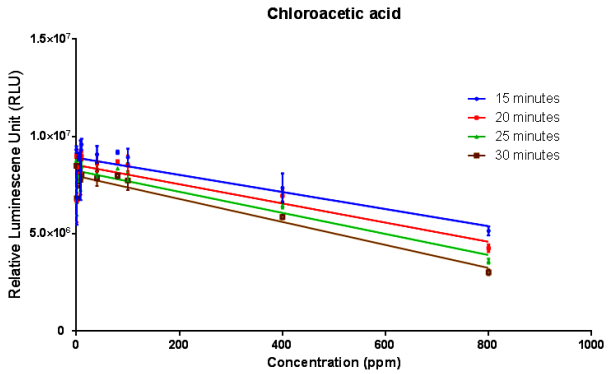

Fig. 7. Exposure time of 15, 20, 25 and 30 minutes of CAA with various concentrations on Luminescence of *V. fischeri.*

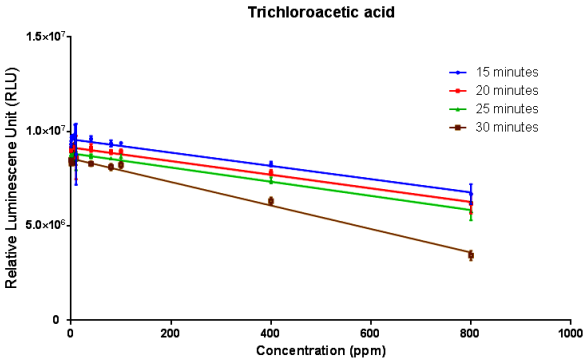

Fig. 8. Exposure time of 15, 20, 25 and 30 minutes of TCAA with various concentrations on Luminescence of *V. fischeri.*

The 30-minutes IC_{50} for TCAA were found to be 1119 ppm (1119 mg/l) which is more sensitive compared with EC_{50} of Daphnia magna at 2000 mg/L for 48 hours [29] and EC_{50} of *Xenopus laevis* (African clawed frog, embryo) at 1740 mg/L for 96 hours [30]. CAA (**Fig.** 9) however, showed 865.4 mg/L, in term of sensitivity it is less compare to 1.8 mg/L for 72 hours EC_{50} of Selenastrum capricornutum [31], 28 ug/L for 48 hour EC_{50} of Scenedesmus subspicatus [32] and 24 hours EC_{50} of *Daphnia magna* at 96 mg/L [33]. However, the use of *V. .fischeri* bioassay has shorter time advantage compared to others. Padrtova et al. [34] reported that among various species (algae, crustaceans, rotifers, bacteria and protozoan) algae and the bacterial bioluminescence assays showed the highest sensitivity in acute toxicity measurement for most of the samples. However, they stated that there are several limitations for the algal test included longer exposure time and higher species maintenance compared to rapid and economical luminescence inhibition assay. 30 minutes IC_{50} of BAAs (**Fig.** 10) showed 59.67 mg/L which was more sensitivity than 48 hours EC_{50} of *Scenedesmus subspicatus* (2.3 mg/L) [32]. The 30 minutes IC_{50} of IAA (**Fig.** 11) showed the highest sensitivity of 15.52 mg/L among the selected HAAs, IAAs currently are still considered new and unmonitored.

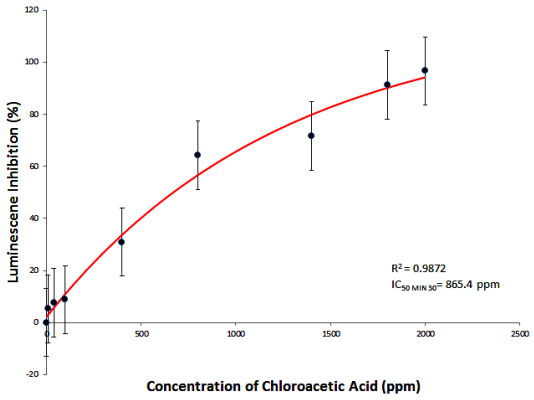

Fig. 9. Effect of Concentration of CAA on Luminescence Inhibition (%) of *V. fischeri.*

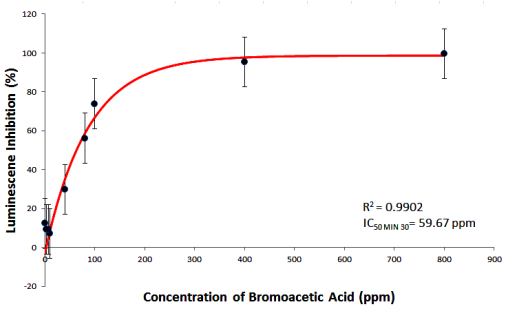

Fig. 10. Effect of concentration of BAA on luminescence inhibition (%) of *V. fischeri.*

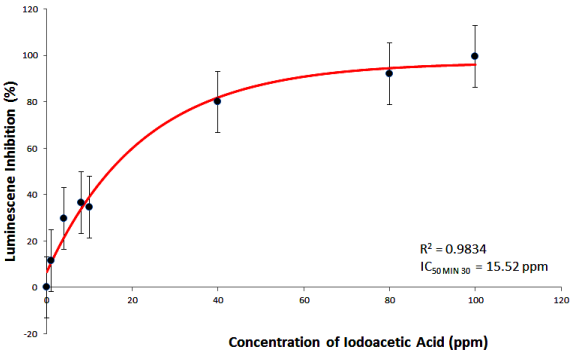

Fig. 11. Effect of concentration of IAA on luminescence inhibition (%) of *V. fischeri.*

In general, BAA, IAA, CAA and TCAA are listed in the category of haloacetic acids (HAAs), a range of chemicals with one to three halogens atom. HAAs are DBPs besides trihalomethanes produced during chlorination of water or use of ozone. HAA5, 5 HAAs are regulated by USEPA while there is currently no regulations for HAAs in drinking water for EU legislation. **Table** 2 shows the maximum contaminant level (MCL) for USEPA and guideline value for WHO. Nevertheless, 30-minute IC_{50} and other bioassays could not satisfy the guideline values of both USEPA and WHO. However, among these bioassays, the 30-minutes *V.fischeri* bioluminescence rapid assay is still the most sensitive bioassay towards selected DBPs.

Table 2. DBPs and guideline values in drinking water

DBPs	WHO (mg/L)	EPA (mg/L)
CAA	0.02	0.06
TCAA	0.2	0.06
BAA	0.02	0.06
IAA	N/A	N/A

CONCLUSION

V. fischeri bioassay had been tested for a variety of compounds. It is a rapid and reliable test of toxicity measurement with time and cost efficiency, as it has a short generation time and moderate cost of implementation. Sophisticated equipment can be omitted and this bioassay does require any much professional handling than any other simple bioassay available. The use of *V. fischeri* bioassay should be promoted as it can detect the level of toxicity of the water or compounds present in water in a short time. This technique can be an advantage to preliminary monitoring task. Studies on specific DBPs instead of mix DBPs with the use of bioluminescence bacteria regardless of which species are still insufficient. Thus, potential of bioluminescence bacteria in the term of DBPs monitoring have to be further explore and exploit. Further studies on the other single specific DBPs and their mixture should also be done to know more about the interactive effect between the specific DBPs, as our environmental water is usually filled with many different types of DBPs. This will help in the determination of the sensitivity of *V. fischeri* as an effective screening tool.

ACKNOWLEDGMENT

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