Development of a facile and high-throughput bioluminescence assay using *Vibrio fischeri* to determine the chronic toxicity of contaminated samples

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Abstract

Chronic toxicity testing using the luminescent bacterium, *Vibrio fischeri*, has recently been demonstrated to be a suitable bioassay for water quality monitoring. The toxicity evaluation is typically based on determining the EC₅₀ at specific time points which may lead to overlooking the dynamic nature of luminescence response and limits insight to possible mechanisms of action of target compounds. This study investigated various approaches (standard, integral, and luminescence rate inhibition) to evaluate the chronic toxicity of three target compounds (atrazine, trimethoprim, and acetamiprid) and using a 96-well plate based method. The chronic toxicity assay and the methods used for EC₅₀ calculation provided in this work resulted in a high-throughput method of chronic toxicity testing and indicated lower EC₅₀ than the values provided by the standard short term methods, indicating higher toxicity. This study emphasizes the need for additional chronic toxicity testing to further evaluate the toxicity of compounds or unknown samples.

Keywords: chronic toxicity, contaminants of emerging concern, high-throughput assay, *Vibrio fischeri*

Introduction

The risk associated with contaminants of emerging concern (CECs) such as pesticides, biocides, pharmaceuticals and personal care products in the environment is a rising issue of global concern as these substances have been detected at concentrations of µg/L in the aquatic environment (Halling-Sørensen et al. 1998). These compounds are not generally removed efficiently during conventional wastewater treatment and are eventually discharged into receiving waters (Kümmerer 2003). To facilitate the development and operation of wastewater treatment technologies minimizing the environmental risk associated with the discharge of treated wastewater, fast, simple and reliable testing methods are required.

The marine luminescent bacterium *Vibrio fischeri* has already been commonly used in various biological testing protocols for investigation of acute toxic responses. The bioluminescent bacteria test according to EN ISO 11348 has been commercialized as *Microtox*® by Modern Water, and was applied to determine the acute toxicity of numerous contaminants (de García et al. 2016). Other commercialized assays based on this microorganism are *LUMISTox*, *ToxScreen* and *Tox Alert*. Short-term bioluminescence inhibition tests are useful tools for assessing the ecotoxicological impact of unspecific acting toxicants but their low sensitivity due to a short exposure time (5-30 min) and current test approach using cuvettes for standard luminometers limit their use. The standard *Microtox* protocol is simple and relatively fast, but has limited amount of sample processing and requires relatively expensive supplies (i.e. freeze-dried cultures and diluents), hence it remains as a low-medium throughput system. There are numerous reports on acute toxicity of CECs using *V. fischeri* but only a handful of reports investigated chronic effects

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using this test organism (Backhaus et al. 1997; Froehner et al. 2000; Froehner et al. 2002; Gmurek et al. 2015; Majewsky et al. 2014; Menz et al. 2013). The applicability of a chronic toxicity test using *V. fischeri* in a high-throughput manner has already been demonstrated (Froehner et al. 2002; Gmurek et al. 2015; Menz et al. 2013), but most studies have only looked at specific end-points (i.e. luminescence inhibition after a specific amount of incubation time), which ignores the dynamic nature of the response over the exposure period (usually 24 h). Time dependent toxicity responses can provide additional information on the possible mechanisms of action of substances and insights on potential delayed toxicity of substances.

In the present work, we revisited the use of *V. fischeri* to investigate chronic toxicity and considered various methods to calculate effective concentration at half-maximal response (EC₅₀) from data gathered over 20 h (period over which an healthy culture can be maintained), to replace the time point approach currently used. Time dependent analysis of the luminescence inhibition was also used to discuss potential mechanisms of action of target compounds. To our knowledge, the chronic effects of trimethoprim (TMP), atrazine (ATZ) and acetamiprid (ACD) on *V. fischeri* presented have not been reported elsewhere. The sensitive, low-cost and high-throughput bacterial assay procedure and proposed EC₅₀ calculation methods described in this work provides a strong ecotoxicological screening tool for monitoring the efficiency of treatment technologies, the earlier detection of the formation of potentially toxic transformation products as well as the preliminary screening of new chemicals.

Methods and Materials

Chemicals - Atrazine (ATZ, 99.1%) and trimethoprim (TMP, >98%) were purchased from Sigma-Aldrich. Acetamiprid (ACD, >98%) was purchased from Santa-Cruz Biotechnology. Chemicals used in the culture medium were purchased from Fisher Scientific: Sodium chloride, trypton, yeast extract; Sigma-Aldrich: potassium chloride and magnesium chloride; and EMD Chemicals: glycerol. Carrier solvents used to prepare the stock solutions of CECs were LC-MS grade methanol (MeOH) and dimethyl sulfoxide (DMSO) purchased from Fisher Scientific and Sigma-Aldrich, respectively. Stock solutions of ACD (1000 mg/L) and TMP (1000 mg/L) were prepared in MeOH and ATZ (5000 mg/L) was prepared in 10% DMSO in MeOH (v/v). All stocks were stored at -20 °C until time of experimentation.

Cultivation and long-term preservation of test organism - To culture the test organisms to be used for toxicity testing, the freeze-dried luminescent bacteria (*V. fischeri*, NRRL-B11177, ATCC 49387) were first rehydrated in 1 mL of seawater complete medium (SWCM). SWCM consisted of 20.0 g/L NaCl, 2.0 g/L MgCl₂•6H₂O and 0.3 g/L KCl. Rehydrated bacteria (0.5 mL) were then transferred into 100 mL of nutrient supplemented seawater medium (NSSWM) and incubated at 22 °C and 150 rpm for approximately 24 h (or until exponential growth phase was reached as determined by optical density at 600 nm). NSSWM consisted of SWCM supplemented with 5.0 g/L trypton, 5.0 g/L yeast extract and 3.0 g/L glycerol. Samples (10 mL) of this incubated liquid broth containing grown *V. fischeri* were then transferred into 15 mL polyurethane centrifuge tubes and centrifuged at 5000 rpm for 5 min. The supernatant was then decanted and the remaining bacteria pellets were re-suspended with 1 mL of 30% glycerol/NSSWM (v/v) and placed in 1.5 mL cyrovials to be frozen under liquid nitrogen and stored at -80 °C until time of experimentation.

Preparation of test organism for chronic toxicity testing - To perform chronic toxicity assays, a cyrovial containing the preserved *V. fischeri* cultures were thawed in ice water and a 250 µL

volume of this culture was transferred into 50 mL of growth media (NSSWM) and incubated at 22 °C and 150 rpm for approximately 10-12 h (or until an optical density (OD) of 1-1.5). To maintain a healthy culture exhibiting a stable luminescence response, a dilution was performed every 12 h using the following formula:

$$\text{Volume (mL) of bacterial culture to be added to 50 mL of NSSWM} = \frac{0.1}{OD_{600 \text{ nm}}} \quad (1)$$

This approach was used to obtain a standardized initial cell density in the growth media. Every 12 h of incubation, in addition to OD measurements, luminescence measurements were also performed by transferring 200 µL of samples into black opaque polystyrene Costar 96 microwell plates and measured by a Beckman-Coulter DTX 800 Multimode detector to ensure that bacteria are in their late exponential phase (LEPC).

Preparation of dilution and assay plates - Prior to exposing the bacteria to target contaminants for toxicity testing, a dilution plate was prepared (Fig. 1). Sample (200 µL) or stock solution of compounds was placed in well A1 in the dilution plate. Well A2 contained 140 µL and wells A3 to A12 contained 180 µL of the respective carrier solvent (MeOH or 10% DMSO/MeOH). A volume of 60 µL from A1 to A2 and a volume of 20 µL from A1 to A3 were transferred. Starting from A2, the even numbered wells were serially diluted by a factor of 10 and similarly the odd numbered wells were serially diluted by a factor of 10 starting from A3. Following the serial dilution, all wells in row A contained a final volume of 180 µL except A1 (120 µL). Finally, 10 µL from each well in the dilution plate (for ATZ) and 25 µL (for ACD and TMP) were transferred into the corresponding well in the black opaque assay plate (rows B, C and D). 10 or 25 µL of the respective carrier solvent was also transferred into all wells of row F in the assay plate as the solvent control (C1 in Fig. 1). Solvents were allowed to evaporate prior to the addition of *V. fischeri* culture.

Figure 1 Dilution and Assay plate preparation for chronic toxicity experimentation

To standardize the initial cell density in each well, optical density of LEPC was measured and the following formula was used to produce the Plate Ready Culture (PRC).

$$\text{Volume (mL) of LEPC to be added into 50 mL of NSSWM} = \frac{0.4}{OD_{600 \text{ nm}}} \quad (2)$$

This proposed approach provides a standardized initial cell density and a consistent initial luminescence response of around 1000 relative light units (RLU). Following the evaporation of the carrier solvents, 200 µL of PRC were added to the wells in the black opaque assay plate, the plate was covered with permeable film (Breathe-Easy sealing membrane) to allow oxygen diffusion and facilitate aerobic respiration of bacteria. The luminescence in each well was recorded over 20 h every 15 min (shaking for 15 seconds prior to each measurement to prevent settling of

the bacteria and to homogenize the mixture in each well). Wells in the rows B, C, D of assay plate contained 12-point serial dilution samples of target contaminants (S) in PRC; whereas row F was reserved for the evaporated solvent control in PRC (C1) and row G contained only the PRC (C2). Thus, each assay plate contained three sample replicates and two negative controls with 12 replicates. In the case of ATZ the assay plate contained 0.5%(v/v) of DMSO in all the sample and solvent control wells, for the remaining compounds no residual carrier solvent was left in the assay plate. For TMP and ACD, C1 and C2 showed no considerable difference in luminescence however for ATZ, presence of DMSO resulted in a higher luminescence than that of C2. For those reasons the luminescence of all samples were compared to that of the solvent control (C1). The chronic effects of DMSO at varying concentrations on the bioluminescence of *V. fischeri* are discussed later.

Data Analysis

The evolution of luminescence as a function of time for the solvent control (i.e. negative control) and for a representative sample (e.g. 25 mg/L of ATZ) are represented in **Fig. 2a**. **Fig. 2b-d** show the percent inhibition of luminescence (I) calculated using three different strategies. In all cases, the luminescence decreases rapidly over the initial 5 h since the bacteria need to reach a critical cell density before exhibiting luminescence (i.e. quorum sensing). Thus, luminescence values recorded during this time window were not taken into consideration when calculating percent inhibition. **Fig. 2b** depicts how the percent inhibition was determined for each time point independently, defined as *standard inhibition calculation*, and calculated using (Eq.3):

$$I_{std,t} (\%) = \frac{L_{c,t} - L_{s,t}}{L_{c,t}} \times 100 \quad (3)$$

where, t is time of measurement, $L_{c,t}$ is the mean of the luminescence values of the solvent controls after t hours, $L_{s,t}$ is the observed luminescence of a particular sample after t hours and $I_{std,t}$ is the standard inhibition percentage at a given time for a particular sample.

Fig. 2c illustrates how the percent inhibition was determined for each time point using the area under the curves, referred to as *integral inhibition calculation*, and calculated using (Eq.4):

$$I_{integral,t} (\%) = \frac{A_{c,t} - A_{s,t}}{A_{c,t}} \times 100 \quad (4)$$

where, similarly to (Eq. 3) t is time of measurement, $A_{c,t}$ is the area under the curve of the mean of the luminescence values of the solvent controls up to t hours, $A_{s,t}$ is the area under the curve of the observed luminescence of a particular sample up to t hours and $I_{integral,t}$ is the integral inhibition percentage up to a specific time for a particular sample. $A_{c,t}$ and $A_{s,t}$ were calculated using the trapezoid rule.

Finally, **Fig. 2d** shows how the initial rate of luminescence of a sample containing a toxicant differs from that of the solvent control. The percent inhibition using these initial rates is referred to here as *rate inhibition calculation* and was calculated using (Eq.5):

$$I_{rate} (\%) = \frac{m_c - m_s}{m_c} \times 100 \quad (5)$$

where, m_c is the initial rate of luminescence calculated from the slope of the mean of luminescence values of the solvent controls, m_s is the initial rate of luminescence calculated from the slope of the observed luminescence of a particular sample and I_{rate} is the inhibition of initial rate of

luminescence. The estimate of the slopes for any particular curve was determined using a *Matlab* code which performs the following analysis. For each two data points on the curve **Fig. 2c**, a value of a slope is calculated. Once 10 consecutive positive slopes are achieved the final value of this range is taken as the initial estimate of the slope value. This estimate is compared to all other previously calculated slopes and the lower bound was determined once a 50% decrease in the slope is detected and similarly the upper bound was determined once the slope has increased by 50%. Using this final range of data points, a liner regression analysis was run to finalize the estimation of the slope (i.e. initial rate of luminescence).

The effective concentration showing a 50% inhibition (EC_{50}) for all three methods proposed above were calculated from the log-logistic fit of the concentration-effect curve (Eq.6) of inhibition plotted against log concentration (mg/L) using Prism (GraphPad Software):

$$I(\%) = Bottom + \frac{(Top - Bottom)}{1 + 10^{(LogEC_{50} - LogC) \times Hill\ Slope}} \quad (6)$$

where, I is the percent inhibition, $Bottom$ is the minimum inhibition (set to 0%) Top is the maximum inhibition (set to 100%) C is toxicant concentration (mg/L) and EC_{50} is the effective concentration (mg/L) at $I=50\%$.

Using the standard (Eq.3) and integral inhibition (Eq.4) calculation methods, an EC_{50} can be calculated at different time points of growth, whereas the EC_{50} value calculated using the values obtained from rate inhibition calculation yields a global estimate of the EC_{50} . To assess if the best-fit $logEC_{50}$ values differed between data sets, the built-in function of Extra Sum of Squares F-test was used in the GraphPad Software. The null hypothesis was set to “ $LogEC_{50}$ value same for all data sets” and the alternate hypothesis was set to “ $LogEC_{50}$ different for each data set”. The null hypothesis was rejected if the p-value was less than 0.05.

Figure 2 a) Raw data showing how the luminescence varies with time during the chronic toxicity testing of ATZ (inset shows evolution of luminescence within the first 5 h of testing). Calculation of Percent Inhibition using b) standard method c) integral method and d) initial rate of luminescence inhibition

Results and Discussion

184 **Fig. 3, Fig. 4 and Fig. 5** summarizes the calculated EC_{50} values for ATZ, TMP and ACD using the
185 luminescence inhibition data calculated by the standard method, integral method and rate method,
186 respectively. These figures also present representative dose-response profiles at three different
187 time points of the chronic toxicity test (**Fig. 3 and Fig. 4**, Panels a, b, c) and the overall dose-
188 response curve for the rate method (**Fig. 5**).

189 Using the standard method, the EC_{50} values (5-18 h) calculated for ATZ lie between 3.0 –
190 30.4 mg/L ($0.9353 < R^2 < 0.9867$) and as exposure time increases the value of EC_{50} also increase
191 (**Fig. 3d**) suggesting that new generations of *V. fischeri* can adapt to the presence of ATZ and
192 develop a higher tolerance to this compound at later stages of growth. Over the same range, a
193 similar trend is also observed when the integral method is used (**Fig. 4d**) with EC_{50} values of in the
194 range of 5.8 – 16.9 mg/L ($0.9493 < R^2 < 0.9735$). Such an observation would not be possible if only
195 a pre-determined time point at a later stage (e.g. 16 h or 24 h) was used to calculate an EC_{50} value
196 for indication of chronic toxicity, as done in most studies (Backhaus et al. 1997; Froehner et al.
197 2000; Froehner et al. 2002; Gmurek et al. 2015; Majewsky et al. 2014; Menz et al. 2013). In the
198 case of ATZ, for both the standard and integral method, the statistical analysis resulted in rejection
199 of the null hypothesis ($p < 0.0001$) and a different EC_{50} value can be presented for each time point.

200 The best-fit dose-response curves constructed for TMP using the standard method, shows
201 steep Hill Slopes (**Fig. 3b**). Therefore, the calculated EC_{50} values were only estimates and it was
202 not possible to construct the corresponding 95% confidence intervals. Due to the uncertainty
203 associated with calculation of these EC_{50} values, no biological interpretation can be made about
204 the trend observed in **Fig. 3e**. However, the Hill Slopes of the best-fit dose-response curves
205 constructed for TMP using the integral method (**Fig. 4b**) are not as steep and allows the calculation
206 of EC_{50} values and the corresponding 95% confidence intervals in the 9-18 h window of the chronic
207 toxicity test (**Fig. 4e**). The EC_{50} values for TMP in this window lie between 1.1 – 1.5 mg/L
208 ($0.9029 < R^2 < 0.9896$). For the data presented in this case, the null hypothesis was not rejected
209 ($p=0.2567$) and a single EC_{50} of 1.33 mg/L can be used within this time range
210 ($1.254 < 95\% C.I. < 1.412$, $0.9027 < R^2 < 0.9821$). This indicates that the newer generations of bacteria
211 are not capable of developing a tolerance to this compound within the investigated time frame.

212 In the case of ACD, both the standard (**Fig. 3f**) and integral method (**Fig. 4f**) result in the
213 calculation of EC_{50} over a range of 7 – 23 h. For both cases, the null hypothesis could not be
214 rejected ($p > 0.9999$) which meant that the EC_{50} values statistically did not differ from each other
215 across the experimental time line, suggesting that for ACD, as for TMP, the EC_{50} was independent
216 of exposure time. EC_{50} values of 35.7 mg/L ($33.1 < 95\% C.I. < 38.6$, $0.857 < R^2 < 0.973$) and 38.5
217 mg/L ($35.8 < 95\% C.I. < 41.4$, $0.9092 < R^2 < 0.9818$) are hence reported for the standard method and
218 integral method, respectively. For tests done routinely, it can be concluded that an earlier endpoint
219 can be chosen to describe the long-term toxicity, allowing shortening of the time required to study
220 chronic effects of similar toxicants to *V. fischeri*.

Figure 3 Dose-response profiles of a) ATZ, b) TMP and c) ACD and evolution of EC_{50} over growth time for d) ATZ, e) TMP and f) ACD based on inhibition percentages calculated using the standard method. Dotted lines = 95% confidence interval.

The global EC_{50} values calculated using the rate of luminescence inhibition method (Fig. 5), are comparable to the EC_{50} values determined using the standard and integral methods. An advantage of the rate of luminescence inhibition method is that data points must be collected only up to 10 h, which shortens the duration of chronic bioluminescence toxicity test when compared to other published methods where inhibition endpoints were calculated at specific time points, mainly 16 or 24 h (Froehner et al. 2002; Kümmerer 2003; Majewsky et al. 2014).

In all cases the integral method allows smoothing of the response and dampening the variation observed. Generally, it led to higher R^2 values and slightly lower or equivalent EC_{50} when compared to the standard method. But as there is no biological significance associated with the integral method, the EC_{50} values calculated using this method should not be compared to the values obtained with the other two methods. However, it is worth mentioning that regardless of the calculation method, ranking of the investigated compounds in terms of increasing toxicity follow the same trend: $ACD < ATZ < TMP$.

Figure 4 Dose-response profiles of a) ATZ, b) TMP and c) ACD and evolution of EC_{50} over growth time for d) ATZ, e) TMP and f) ACD based on inhibition percentages calculated using the integral method. Dotted lines = 95% confidence interval.

Figure 5 Dose-response curves and EC_{50} values calculated using the rate method for TMP, ATZ and ACD

In the literature, EC_{50} values calculated using acute tests (5-30 min) were reported to be 89 – 150 mg/L (Polo et al. 2011), 176.7 mg/L (Park and Choi 2008) and 129 mg/L (Dell'Arciprete et al. 2009) for ATZ, TMP and ACD, respectively. The chronic toxicity assay and the methods used for EC_{50} calculation provided in this work resulted in lower EC_{50} (indicating higher toxicity) than the values provided by the standard short-term methods by approximately a factor of 15, 120 and 4 for ATZ, TMP and ACD, respectively. According to Backhaus et al. (1997), compounds with unspecific mode of action exert their effects after short incubation times. These compounds predominantly interfere with the cell membrane associated processes, causing disturbances of the energy status of the cell and/or cause severe structural damages. Therefore, if EC_{50} determined by acute toxicity of a compound is equivalent or smaller than the EC_{50} obtained from chronic toxicity for the same organism then this compound can be considered to exhibit a nonspecific mode of action. Conversely, if chronic EC_{50} is less than acute EC_{50} , then the compound under investigation interferes with the biosynthetic pathways of the exposed organism. Based on the results presented in this work, all three compounds have lower chronic EC_{50} values and thus can be considered to exhibit specific modes of action inhibiting the biosynthetic pathways of *V. fischeri*.

It is known that short-term bioassays with luminescent bacteria significantly underestimate the toxicity of especially antibacterial compounds (Backhaus et al. 1997). TMP is known to bind to dihydrofolate reductase enzyme and inhibits the reduction of dihydrofloric acid to tetrahydrofolic acid (THF). THF is an essential precursor in the thymidine synthesis pathway, consequently bacterial DNA synthesis is severely affected (Brogden et al. 1982). This interruption of bacterial DNA synthesis is the most likely reason why the significant inhibitory effects of TMP on *V. fischeri* can only be observed in the long-term luminescence bioassay.

ATZ is a herbicide which interrupts the photosynthetic electron transfer system in green cells and ACD is a member of the neonicotinoid group of insecticides with a mode of action that affects the central nervous system of insects. The effects of both biocides on non-target organisms such as bacteria were seldom investigated. The observed inhibition of bioluminescence during chronic toxicity testing of ATZ and ACD could be attributed to interruption of various biosynthetic mechanisms. Due to the demonstrated binding of ACD and ATZ to DNA (Zhang et al. 2015; Zhang et al. 2013), it is probable that this modification of DNA can hinder transcription and replication and hence impact the natural biosynthetic pathways of the organism. Organisms with

aerobic metabolism face constant risk from reactive oxygen species (ROS) via reduction of molecular oxygen to radical species. These radical species can lead to reduction in cell membrane integrity, enzyme inactivation, protein denaturation and DNA mutation. Both ACD and ATZ have been shown to induce oxidative stress to gram negative bacteria (Yao et al. 2006; Zhang et al. 2012). All these reported observations can help explain the increased sensitivity of the long-term assay towards ACD and ATZ, however detailed mechanistic studies should be performed in order to identify which biosynthetic pathway is severely impacted.

The effect of the presence of DMSO (%) on the luminescence inhibition using the standard calculation method can be seen in **Fig. 6** at different time points. Previous research reported that 0.5-2% DMSO (v/v) does not exhibit significant toxic response for short-term (5-30 min) *V. fischeri* bioassays (Hernando et al. 2007; Kim et al. 2007), which is in agreement with the plot of the response after 15 min of exposure (**Fig. 6a**). For chronic toxicity of DMSO, the only EC₅₀ value reported in literature is 2.3% determined by growth inhibition of *V. fischeri* using OD_{450nm} measurements after 6 h (Gellert 2000). Results obtained here for longer-term exposure (**Fig. 6b** and **Fig. 6c**), did not allow the determination of an EC₅₀ value considering that below a DMSO concentration of 0.075% luminescence inhibition was less than 10%, between 0.075%-2.5% DMSO the luminescence was increased (negative inhibition) and above a DMSO concentration of 2.5% almost complete inhibition was observed. This behaviour of low dose stimulatory response and high dose inhibitory response, called hormesis, has been observed during exposure of *V. fischeri* to sulfonamides and explained based on quorum sensing pathways (Deng et al. 2012; You et al. 2016). Unfortunately, considering the structural differences between DMSO and the compounds previously investigated it might not be possible to extrapolate these results to DMSO. For another luminescent bacterium, *V. harveyi*, 1.45 – 2.9% DMSO was shown to increase bioluminescence by 40% after 15 min of exposure (Mariscal et al. 2003). Similarly, 0.5%-18% of other solvents (acetone, methanol and ethanol) were shown to activate bioluminescence of *P. leiognathi* and *V. harveyi*, (Sukovataya and Tyulkova 2001). It is probable that high electron-donor capacity of DMSO can help stabilize the intermediates during bioluminescence reaction but the mechanisms of action needs further investigation.

Figure 6 Effect of DMSO concentration on luminescence inhibition (%) at different time points during the chronic toxicity test: a) 15 min b) 8 h c) 16 h

As a conclusion, the high-throughput chronic toxicity method described and EC₅₀ calculation methods presented in this work allow time dependent toxicity analysis of samples in an economical and timely fashion (within 10 h). Based on the demonstrated different sensitivity of the chronic bioluminescence assay, when compared to the acute toxicity results, toxicity screening studies should also include chronic toxicity tests to better evaluate the toxicity of compounds or residual toxicity of treated wastewater.

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