Impact of 17β-Estradiol on Natural Water's Heterotrophic Nitrifying Bacteria

Ziyi Dong, Changhao Xiao, Weihua Zeng, and Jinbo Zhao

***Abstract*—In this research, bottom water samples were collected from nature water. After cultivating and selecting, bacteria which could use $(NH_4)_2SO4$ as the only nitrogen source had been selected. The bacteria in different cultures with different concentration of 17β-estradiol (E2) were cultivated, and every group's concentration of $N\text{-}NH_4^+$, $N\text{-}NO_3^-$ and OD600 were measured. The result shows that compare with the control group, in which no E2 was added, the growth of heterotrophic nitrifying bacteria had been promoted when the concentration of E2 was in range of 1-100 ng/L. In addition, heterotrophic nitrifying bacteria's growing speed has a positive correlation between the E2's concentration. However, low concentration of E2 (like 0.1 ng/L), could inhibit the growth of heterotrophic nitrifying bacteria. Considering the impact of E2 on heterotrophic nitrifying bacteria, it is necessary to intensify the detection to E2 in the future.**

***Index Terms*—17β-estradiol, nitration, heterotrophic nitrifying bacteria.**

I. Introduction

Environmental estrogens (EEs) are estrogen analogues, and estrogen-like chemical substances those are distributed in the natural environment and can interfere or harm the endocrine function of the body. Common and major environmental estrogens include estrone (E1), estradiol (E2), estriol (E3), bisphenol A (BPA), and so on [1]. In recent years, with the development of China's economy and industry, the natural environment of the environment estrogen shows a wide distribution, the concentration of the trend. There are reports that the total concentration of estrogen in river water bodies could reach more than 100 ng/L [2]. For E2, it can be detected in many rivers in china, the concentration is generally 0-3ng/L [3], concentrated distribution of 0-1ng/L, individual up to 30ng/L or more [4].

Existing research on environmental estrogen has focused on the effects of environmental estrogen on the physiological function of humans and animals, and there is ample evidence that EEs can cause abnormalities in the endocrine system, nervous system, and immune system [5]. For a long time, it has been thought that estrogen only has an effect on higher organisms with its receptors, so the research involving microorganisms focuses on the use of microorganisms to degrade environmental estrogen, and there are few studies on the impact of estrogen on microorganisms. But there have also been reports in recent years show that estrogen can also have a significant effect on microbes. In this regard 17β-estradiol is studied more environmental estrogen. The effects of E2 on methane bacteria in anaerobic sediments were studied and a certain concentration of E2 was found to affect the rate of methane and carbon dioxide production in sediments [6]. It has also been reported that E2 can also inhibit the growth of methane-producing bacteria while being degraded [7]. There are also reports that E2 inhibits the denitrification of microbes, but increases the proportion of N2O in the product. [8] It is concluded that the effect of environmental estrogen on microorganisms is present, but the mechanism of the action and the effect of all aspects are not known. In addition, heterogeneous nitrification bacteria have many advantages and high nitrification speed, and have become a hot research topic for biological nitrification in recent years. [9] In natural waters, ammonia nitrogen can be used or oxidized by the nitrification of heterotrophic nitrifying bacteria, thus moving closer to a relatively reasonable level. High concentration of ammonia nitrogen in water is one of the main causes of water eutrophication. [10] If the nitrification or proliferation of heterogeneous nitrification bacteria is abnormal, it will have an adverse effect on the nitrogen cycle of natural water bodies. Based on the current situation of this study, this study obtained the heterotrophic nitrifying bacterial flora from natural water bodies, explored the effects of E2 on its growth condition and nitrification, and obtained preliminary results.

II. Methods

The North Moat, which located in Beijing, China, is a part of the water system of the North Canal, which basically covers the entire Beijing City North Second Ring Road. The Water System of the North Canal is an important water system in Beijing, where 70% of the population lives. Although the water quality of the North Canal system has improved in recent years, most of the water in the urban area of Beijing is in Chinese poor V-type standard, ammonia nitrogen concentration of 10mg/L. [11] This experimental sample was taken on October 20, 2019, at the North Moat in Beijing, at Tanxishengjing Park (116.3692 °E, 39.9486 °N) near the Water Tank, and obtained 50mL of the bottom water, with a measured sampling depth of about 59.5cm. Put the taken water samples in centrifuge tubes, refrigerated them in ice packs and transported them to the refrigerator, temporarily stored them at 4 ℃, and carried out training work on the same day.

Manuscript received May 7, 2020; revised November 22, 2020. This work was supported by the Major Science and Technology Program for Water Pollution Control and Treatment (No. 2018ZX07111003).

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doi: 10.18178/ijesd.2021.12.1.1312

1) Enrichment culture: the water sample was shake well, at room temperature activated 24h, then added the water sample 5mL to the enrichment culture liquid, at 25 ℃, low light, 160 rpm concussion culture for 24h. Rich culture formula: 10g per liter with trypsin, 5g of yeast extract, 5g NaCl.
2) Preliminary screening: took the rich culture fluid 5mL, added 100mL BM culture fluid, in 25 ℃, 160 rpm concussion culture 24h. OD600≥1.00 indicates that the culture concentration is enough, otherwise needs to continue culture. The BM culture liquid is equivalent to the amino nitrogen concentration of 100 mg/L of ammonium sulfate as the only nitrogen source of the culture. BM culture solution is: per liter containing (NH4) $_2SO_4$ 0.472g, sodium butyrate 5.62g, K_2HPO_4 0.147g, NaCl 0.125g, $MgSO_4$ 0.125g, $FeSO_4$ 2.5mg, $MnSO_4$ 2.5mg. [12]
3) After the culture ending, take 5mL culture fluid to 100mL BM culture fluid, repeat step (2) in the culture method 2 times, get heterotrophic nitrifying bacteria flora.
4) Save: the resulting bacteria liquid and glycerin 1:1 volume mixed, at -20 ℃ low temperature preservation.

Configured the BM culture fluid, took 5mL of glycerin-preserved bacteria to add in 100mL of BM culture solution, and immediately put the glycerin-preserved bacteria back into storage. The above BM culture fluid was treated at 25 ℃ to be treated with low light culture of 24h, and the experimental bacteria liquid was obtained.

Divided tapered bottles into 5 groups, and added 100mL BM culture fluid into each bottle. The purchased 17 β-estradiol (purity 99%, RUIBIO) was removed from the storage environment of 4℃, a series of concentrations of solutions were immediately configured, 1mL solution was dripped into the BM culture fluid of each group, and the BM culture fluid of the E2 concentration, which was finally used in the experiment. Added 1mL bacteria liquid to each bottle of culture, and set aside in a thermostatic incubator at 25 ℃. In 1d, 2d, 3d, 5d, 7d, respectively, the ammonia nitrogen concentration, nitrous nitrogen concentration, OD600 value of the culture fluid were determined. Reference to the State Environmental Protection Administration "Water and Waste Monitoring Analysis Method" (4th ed.), [13] using the method of Nessler's Reagents spectrophotometer to determine NH^{4+}-N, N- (1-nixiamine) - ethyl diamine photometric method to determine NO_3^--N, the method of UV spectrophotometry to determine OD600.

Average the two measured data for each set and graph and analyze it through Origin 2017 software.

III. Result and Analysis

A. Effects of E2's Concentration on the Use of Ammonia Nitrogen by Nitrification Bacteria

Ammonia nitrogen is one of the common indicators of nutrients in water, which is directly related to water eutrophication. Tenfold gradient was set, then added E2 of 0, 0.1, 1, 100ng/L to the culture fluid and get the results below.

NH_4^+ is the only initial nitrogen source in the solution, and its consumption can partly reflect the growth of bacteria. After 2d, the control group without E2 consumed 44.3% of ammonia nitrogen, whereas the E2 concentration of 1 ng/L group consumed 95.6% of ammonia nitrogen, and the E2-10 ng/L group also consumed 79.5%. E2-100 ng/L group was slightly higher than the control group, at 53.7%. In contrast, the E2-0.1ng/L group consumed only 25.9%. Other data and overall trends are shown in Fig. 1.

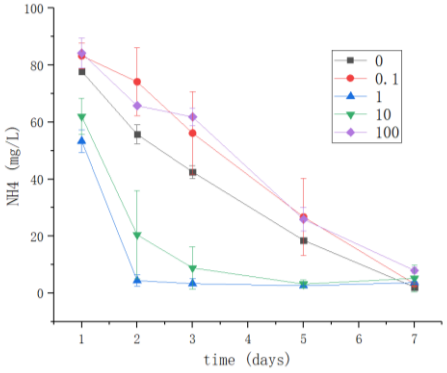

Fig. 1. Ammonia nitrogen concentration changes over time.

It can be found that, compare with the control group of E2-0, the NH_4^+-N using was faster with three groups of E2-1 ng/L and 10 ng/L. It shows that the utilization capacity of nitrification bacteria to ammonia nitrogen increased when E2 is 1 and 10 ng/L, and the utilization capacity of nitrification bacteria to ammonia nitrogen is reduced when E2 is 0.1 ng/L and 10 ng/L.

The impact of ammonia nitrogen utilization is also estimated. Since a group of ammonia nitrogen consumed by 95% at 48h, only the first 48h data were selected to get the figure below.

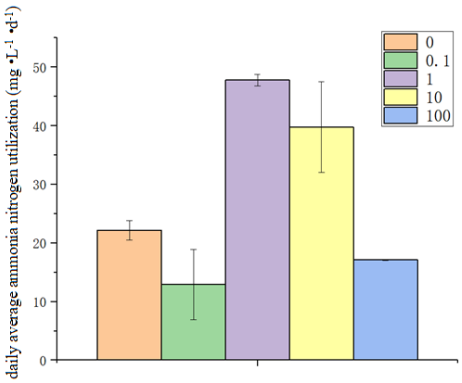

Fig. 2. Degradation rate of ammonia nitrogen in each group for the first 48h (mg•L^{-1}•d^{-1}).

As can be seen from Fig. 2, adding 0.1 ng/L concentration of E2 can reduce the daily average ammonia nitrogen utilization rate of 48h by 41.6%. Therefore, the inhibition effect of ammonia nitrogen utilization of heterotrophic nitrifying bacteria at E2 concentration of 0.1ng/L was significant. Correspondingly, the addition of 1, 10 ng/L E2 will be the use of ammonia nitrogen by heterogeneous nitrification bacteria to produce a certain role in promoting.

In the above, when the E2 concentration is 0-1 ng/L, the small concentration change will also have a significant effect on heterotrophic nitrifying bacteria.

As can be seen from Fig. 3, when the E2 concentration is 10-100ng/L, the change of E2 concentration will not make a

significant change in the trend of ammonia nitrogen utilization of heterotrophic nitrifying bacteria, and the utilization of ammonia nitrogen varies slightly between the control groups. So compare with E2 of 0-1 ng/L, in the 10-100 ng/L concentration segments, the effect of E2 concentration change on heterotrophic nitrifying bacteria was less.

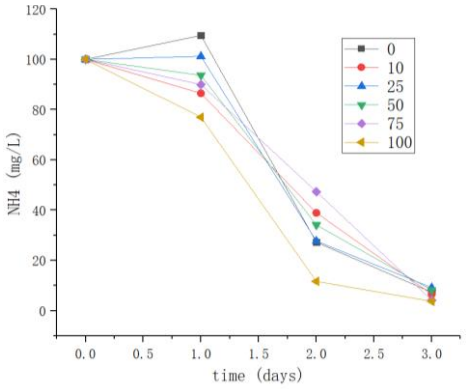

Fig. 3. Effect of 10-100ng/L E2 on ammonia nitrogen utilization, got by same bacteria sample and experimental method.

B. Effects of Different E2 Concentrations on Nitrification Products

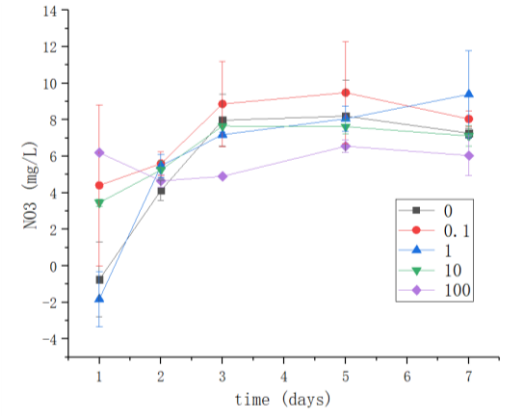

Fig. 4. Nitrogen concentration changes over time.

In BM culture fluids, no $NO_3^-$is initially contained, so all NO_3^- comes from the nitrification of bacteria. Fig. 3 shows that after the start of culturing 24h, the concentration of NO_3^- in two groups of E2 with concentrations of 0 and 1 ng/L was lower, whereas the other groups were at 4-8 mg/L. Subsequently, the overall variation of the nitrogen concentration in each group was not significant, and eventually stabilized at 7-10 mg/L.

Although nitrification bacteria may produce N_2O, NO_3^- or directly assimilate nitrogen sources during nitrification, so the nitrogen concentration of nitrification cannot accurately measure nitrification, the overall trend of NO_3^--N growth in Fig. 3 is about the same. The data shows that the E2 concentration at 0-100ng/L had little effect on the general trend of nitrous oxide production by heterogeneous nitrification bacteria.

C. Effects of E2 Concentrations on OD600

OD600 is the absorbent value of liquid at a wavelength of 600nm, it is positively correlated with the concentration of bacteria in the liquid, in this experiment, no non-life suspension or lactose was contained in the culture system, so OD600 can be used to reflect the total concentration of bacteria in the culture fluid.

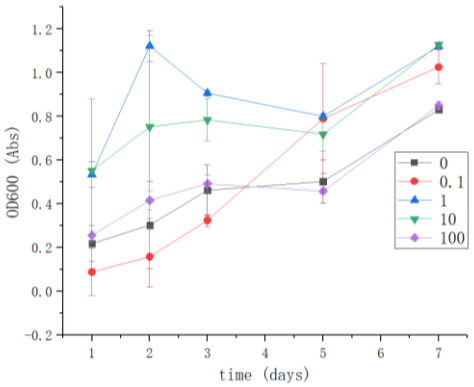

Fig. 5. OD600 changes over time.

As can be noted from Fig. 5, compared to the control group without E2, after 72h, E2 of 0.1ng/L group's OD600 is 29.8% lower, and E2 of 100ng/L is basically the same. But E2 at 1ng/L and 10ng/L two groups are higher, with 96.1% and 69.7%. After 7d, E2 concentrations 0.1, 1, 10, 100ng/L were 23.6%, 34.9%, 35.9% and 7.7% higher than the control group's OD600.

In Fig. 5, the OD600 values in two groups with E2 concentrations of 1 ng/L and 10 ng/L were always higher than in the control group, whereas the E2 of 0.1 ng/L group was lower for most of the previous period. In the first 72h, the three groups with 1, 10 and 100 ng/L of E2 had higher OD600 values than the control group. It is worth noting that OD600 value of 0.1 ng/L E2 group increased faster than the control group on the 7th day, indicating that compared to E2 to the use of ammonia nitrogen inhibition of heterotrophic nitrifying bacteria, E2 to the proliferation of heterogeneous nitrification bacteria is limited and just in the first 3d of the shorter period, heterotrophic nitrifying bacteria seem to be somewhat "adapted" to E2.

In the previous ammonia nitrogen data, in all groups added E2, NH_4^+-N took advantage of two groups with E2 plus 1 ng/L and E2 to 10 ng/L, whereas E2 concentrations were relatively slow in groups with a concentration of 0.1 mg/L. NH_4^+ plus is the only initial nitrogen source in the solution, and its consumption can partly reflect the growth of bacteria. This is also verified on the OD600 data.

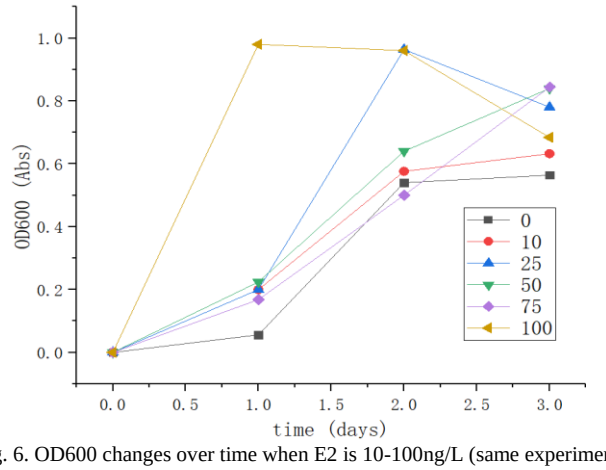

Fig. 6. OD600 changes over time when E2 is 10-100ng/L (same experiment in Fig. 3).

When E2 is 10-100ng/L, there is a more interesting phenomenon. In each group that E2 been added, OD600 value generally increased and then decreased or slowed down. If it is considered that: 1) the resources and time consumed by each bacterial division once are the same, with 0.05 units of resources consumed and during 1 unit of time each bacterial divided into nt; 2) when resources are exhausted in the culture medium, the bacteria will no longer divide; 3) in this experiment, it can be roughly considered that the amount of nutrients (s) in each group is the same, set as 100 units, and the initial number of bacteria (n_0) is the same, set as 1 unit, which can be obtained by using dev-c ++ programming simulation.

TABLE I: USING PROGRAMS TO SIMULATE BACTERIAL SPLIT RESULTS

n_t	g	n
1.5	18	985~1478
2	11	1024~2048
4	7	4096~10384

g: number of last generations *n*: number of last bacteria

Obviously, with limited nutrients, the faster bacteria divide, or the shorter the time it takes to divide each generation, the faster it will reach the peak and the peak of the total.

In Fig. 6, almost every experience group's OD600 values were greater than the control group, otherwise it was similar, indicating that E2 had a significant effect on the growth of heterotrophic nitrifying bacteria. If fitted to it:

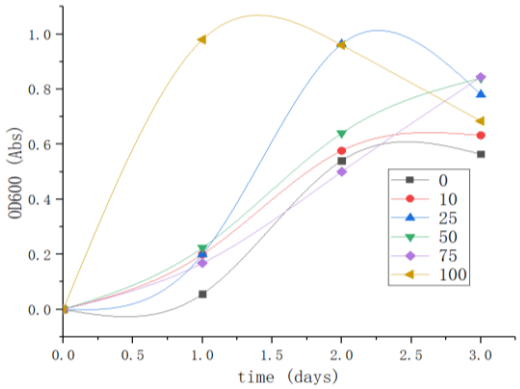

Fig. 7. OD600 for different E2 concentrations changes over time (fitting).

Select the peak of the fitted curve, map again with E2 concentration x-axis and fit linearly to get the following image. (The E2 of 75ng/L group cannot get a peak, the measured maximum is 0.85, so estimated peak to be 1.00, which has been marked in the figure.)

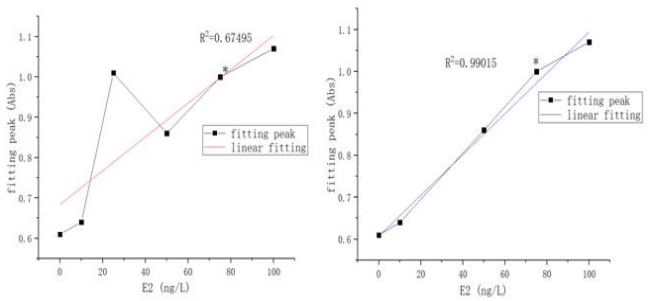

Fig. 8. peaks of OD600 and E2 concentration (left figure: all data; right figure: without E2=25ng/L group's data).

It can be seen that the maximum number of bacterial proliferation is approximately linearly positively correlated with E2 concentration. Among them, E2 of 25ng/L group has a large deviation. If it is screened out for linear fitting again, a very ideal linear relationship can be obtained.

Similar phenomena have been observed in other reports, says the growth of vibrio in the water body has a good linear relationship with E2 concentration in 0-1ng/L, [14] which is similar to the conclusion obtained in this paper. It is speculated that E2 has a catalytic effect on the growth of many microbes in water, whereas different microbes are differently sensitive to E2, and vibrio may be more sensitive to E2 than the whole heterotrophic nitrifying bacterial population in the water body.

D. E2's Influence to Nitrifying Bacteria, Base on the OD600 and Ammonia

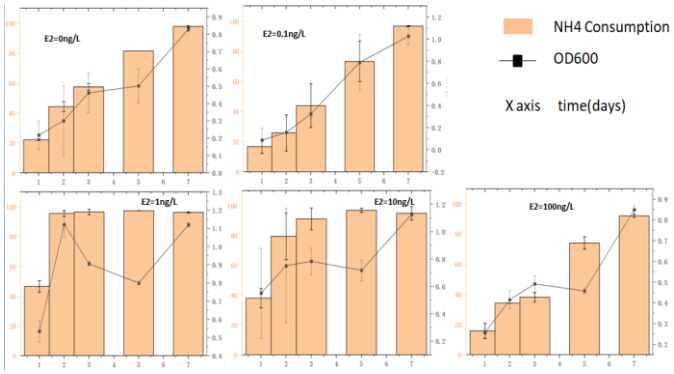

Fig. 9. OD600 (Abs) and NH4 consumption (%) of all groups.

In both groups with higher NH_4^+ utilization rates of 1 ng/L and 10 ng/L, there was a decrease of OD600 been observed, and group with 100ng/L E2 had similar phenomenon. Considering these two groups had more than 90% of consumption at the turning points, it may be because of bacteria's excessive proliferation at earlier stages, which made the concentrate of ammonia decrease sharply but the number of bacteria was big, resulting in that some of bacteria died for lack of ammonia in medium stage, and finally back to the equilibrium point.

The result of NH_4-N and OD600 measures shows that E2 have the effect of "low concentration of E2 could inhibit bacteria, medium concentration of E2 could promote bacteria and high concentrate of E2 have lower promoting effect". This has also been reported in other study. According to Lin's research, one kind of composite E2 degradation bacteria grown better when the E2 was 5mg/L and 10mg/L, compared with that when E2 was 1mg/L and 20mg/L. [15] So it can be assumed that E2 have the same effect to the proliferation and NH_4^+ using of nitrifying bacteria. For the group of heterotrophic nitrifying bacteria in natural water, the turning points of E2's effect might be between 0.1-1ng/L.

IV. CONCLUSION

This article has proved that the concentration of E2 in the range of 0-100ng/L has a significant effect on the use of ammonia nitrogen by heterogeneous nitrification bacteria in natural water bodies over a period of one week. The detail influence as shown in that: compared with the control group without E2, heterotrophic nitrifying bacteria was inhibited when E2's concentration was 0.1 ng/L, and its daily N-NH_4^+

consumption speed had a 41.6% reduction. Heterotrophic nitrifying bacteria was promoted when E2's concentration was 1 ng/L, 10ng/L or 100 ng/L, and its daily N-NH_4^+ consumption speed increased by 88.8%, 79.6% and 21.4%. But the influence of E2 to heterotrophic nitrifying bacteria wasn't so significant.

In addition, over the one-week study period, the concentration of E2 in the range of 0-100ng/L has a certain effect on the proliferation of heterogeneous nitrification bacteria in natural water bodies, which could be described as "low concentration of E2 inhibited it in a short time but promoted it later, medium or high concentration of E2 promoted it". As shown in that: compared with the control group without E2, after 72 hours, group with 0.1ng/L of E2's OD600 was 29.8% lower, but 1ng/L, 10ng/Land 100ng/L, these three groups, their OD600 were 96.1%, 69.7%, 29.5% higher than the control group. However, after 7 days, 0.1, 1, 10, 100ng/L's groups'OD600 were all higher than the control group, which were 23.6%, 34.9%, 35.9%, 5.4% higher.

When E2's concentration was in the range of 10-100 ng/L, it's concentration influenced the N-NH_4^+ consumption speed by nature water's heterotrophic nitrifying bacteria, but the overall influence wasn't huge. But in this range, heterotrophic nitrifying bacteria's proliferation speed showed positive correlation with E2's concentration, and the OD600 even showed linear relation to it(R^2=0.67495).

E2 had been widely detected in Chinese natural water [16]. E2, as one of the environmental estrogens, could not only influence human body and harm various kinds of animals, but lead to nitrifying bacteria growing abnormally, which results in the declining of nature water's ammonia self metabolic ability, and the aggravating of it's eutrophication. So the monitoring of E2 in the nature water should be intensified in the future.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

. This program was done under the guide of Dr. Jinbo Zhao. The samples collecting and experiment was done by Ziyi Dong and Changhao Xiao. Data analysis was done by Ziyi Dong. The paper was written by Ziyi Dong and Changhao Xiao. Dr. Jinbo Zhao and Prof. Weihua Zeng provided experimental equipments, materials and site for the program.

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