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Research Article

The Investigation of Ultrasonic Degradation and Toxicity Reduction of MC-RR sourced by Harmful Algal Bloom (HAB)

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Abstract

In recent years, the progress of studies on toxic cyanobacteria growth caused by the increase in harmful algal blooms (HABs) in aquatic environments requires the treatment of cyanobacterial toxins in drinking water resources. Traditional treatment processes in Drinking Water Treatment Plant (DWTP) for the treatment of cyanobacterial cells that cause problems such as taste, odor, etc. in water lead to cell lysis and release of cyanotoxins and also lead to the possibility of producing toxic by-products in water. These toxic by-products cannot be further removed by classical treatment processes. Moreover, even if these compounds are partially treated in the DWTPs, the toxicity of the by-products pose a threat to drinking waters. Therefore, there is a need to treat these compounds by Advanced Oxidation Processes (AOPs) that produce non-toxic by-products. Based on the context, the treatment of MC-RR, a cyanobacterial toxin compound commonly found in the aquatic environment, by ultrasonic oxidation process as an important AOPs, at low and high frequencies, was investigated and, the toxicity of the by-products after oxidation was evaluated in this study. According to results, it was determined that ultrasonic oxidation of MC-RR at a frequency of 578 kHz for 30 minutes complied with 2nd order kinetics and provided a concentration reduction of 10-12%. Initial concentrations of MC-RR (1.25 µg/L) were observed to reduce the viability of healthy mouse liver cells to approximately 85%. The cell viability either remained the same or increased by 5% compared to the initial concentration at the ultrasonic oxidation processes applied in this study. This result was accepted as an indication that the by-products obtained by ultrasonic oxidation were not more toxic than the initial concentration of MC-RR.

ZARARLI ALG ÇOĞALMASI KAYNAKLI MC-RR'NİN ULTRASONİK ARITIMININ VE TOKSİSİTE AZALTIMININ İNCELENMESİ

Anahtar Kelimeler
İleri Oksidasyon Prosesi
Ultrasonik Oksidasyon
MC-RR
Toksisite

Özet

Son yıllarda su ortamlarında zararlı alg çoğalmalarının artışı ile meydana gelen toksik siyanobakteri büyümesi konusundaki çalışmaların ilerlemesi, içme suyu teminindeki siyanobakteriyel toksinlerin arıtılmasını zorunlu kılmaktadır. Suda tat, koku vb. problemlere neden olan siyanobakteriyel hücrelerinin içme suyu arıtma tesislerinde geleneksel yöntemlerle arıtılması hücre parçalanmasına ve siyanotoksinlerin suya salımına sebep olmakta ve ayrıca toksik yan ürünler üretme olasılığına yol açmaktadır. Bu toksik yan ürünler ise klasik arıtma yöntemleriyle

giderilememektedir. Ayrıca bu bileşikler su ortamında kısmi olarak arıtılsalar bile meydana getirdikleri yan ürünlerin toksisitesi içme suyu için bir tehdit oluşturmaktadır. Bu nedenle bileşiklerin toksik olmayan yan ürünler üreten İleri Oksidasyon prosesleri (İOP) ile arıtılmasına ihtiyaç duyulmaktadır. Bu bağlamda bu çalışmada su ortamında yaygın olarak bulunan siyanobakteriyel toksin bileşiklerinden MC-RR'nin önemli bir İOP olan ultrasonik oksidasyon ile düşük ve yüksek frekanslarda arıtımı incelenerek oksidasyon sonrası ürünlerin toksisitesi değerlendirilmiştir. Çalışma sonunda, MC-RR'nin 30 dakika boyunca 578 kHz frekansta ultrasonik oksidasyonunun 2. mertebe kinetiğe uyum gösterdiği ve %10-12 oranında bir konsantrasyon azalması sağladığı belirlenmiştir. MC-RR'nin başlangıç konsantrasyonlarının (1,25 µg/L) sağlıklı fare karaciğer hücrelerinin canlılığını %85 civarına düşürdüğü gözlenmiştir. Çalışmada kullanılan ultrasonik sistemlerde hücre canlılıkları ya aynı kalmış ya da başlangıç konsantrasyonuna göre %5 daha artmıştır. Bu durum, ultrasonik oksidasyon ile elde edilen yan ürünlerin MC-RR'nin başlangıç konsantrasyona göre daha toksik olmadığının bir göstergesi olarak kabul edilmiştir.

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INTRODUCTION

In recent years, it is recently focused on micropollutants (MPs) that have newly emerged in the water environment in terms of both type and quantity, since their concentrations have reached measurable in DWTPs. As traditional processes in DWTPs are generally designed for basic parameters such as taste, odor, color, turbidity, dissolved oxygen, it is not possible to treat MPs that causes public health concern. Among these MPs, some toxic compounds caused by harmful algal blooms (HABs) have become frequently important in recent years due to pose hazard both the aquatic environment and public health [1] (He et al., 2016). Although HABs generally occur naturally, but the eutrophication problem has accelerated on a global scale due to increasing human activities. The main factors that transform algal blooms into harmful or even toxic products are classified as untreated urban wastewater, atmospheric deposition, groundwater runoff, and agriculture-livestock and aquaculture. The development of anoxic conditions in freshwater on a global scale, and the development of large algal blooms that lead to toxic or detrimental effects for ecosystems, human health, and even fisheries and/or recreational activities, are associated with strong correlations between total N, P loads and phytoplankton production [2] (Anderson et al. 2002).

The main groups known to cause HABs in freshwater environments and produce toxins are cyanobacteria, also known as blue-green algae. The most important species known to produce toxins among cyanobacteria species, Microcystis group algae, are responsible for eutrophication occurring globally and are considered as the biggest problem of DWTPs by causing taste and odor problems in drinking water. Many studies have shown that many toxic compounds are released into the aquatic environment by HABs and these compounds cannot be treated in DWTPs and can directly affect public health as well as ecosystems. These toxins are usually called cyanobacterial toxins (cyanotoxins) and it has been recommended that the ten-day exposure dose for school-age children and adults should not exceed 1.6 µg/L in drinking water for the most well-known and most researched compounds in the microcystin groups, which have been proven to have carcinogenic properties as well [3-5] (Funari and Testai, 2008; USEPA 2022; WHO 1998).

The cyanotoxins are structurally composed of various chemicals, the mechanisms behind their toxic effects are also different. Therefore, cyanotoxins should be examined in five different classes according to their action mechanisms on humans, animals and plants: hepatotoxins, neurotoxins, cytotoxins, tumor-forming and dermatotoxins/irritant toxins. Toxic cyanobacterial growths, which have been observed in eutrophic and hypereutrophic lakes, ponds and rivers worldwide since the late 1980s; as well as in aquatic environments such as reservoirs that are stagnant or very slowly mixed, have become the biggest problem of freshwater ecosystems all over the world [6,7] (Chen et al., 2006; Mishra et al., 2018). Eutrophication was first observed in the 1950s in Western European and North American lakes and reservoirs, and has since become widespread in some regions, causing deterioration in the aquatic environment, especially in drinking water resources. Recent studies have shown that 54% of lakes in the Asia-Pacific Region, 53% in Europe, 28% in Africa, and 48% and 41% in North America and South America, respectively, are eutrophic. Rising surface water temperatures due to the effects of

climate change and nutirent loads are accelerating eutrophication and related HABs in aquatic environments [8-9] (WHO 1999; Turner et al. 2018). WHO reports indicate that 60% of HABs contain toxins. For example, record temperatures of 2007 at Lake Taihu in China, the third largest lake in China as a source of drinking water for many major cities of more than 30 million people, have caused a significant increase in algae, which in turn released toxins into the water, making the drinking water toxic [10] (Wu et al. 2011).

The widespread presence of Cyanobacteria leads significant costs in DWTPs, since the various metabolites, toxins and taste and odor compounds they produce in water cause significant water quality problems. Methods such as disinfection with chlorine or ozone, active carbon adsorption and chemical oxidation are generally used for the treatment for these compounds and the quality of drinking water can be successfully increased. In most cases, taste and odor compounds or toxins are located inside the cells and 50% to 95% of these metabolites are released during classical treatment processes. Therefore, it is very important to remove intact cells with the traditional approach of coagulation, flocculation, sedimentation and filtration during treatment and this situation is often seen as the biggest obstacle to the treatment of these cells. However, with the optimization of the treatment plant, these obstacles can be overcome and intracellular metabolites can be purified by removing cyanobacterial cells. Although it is stated that traditional coagulation-flocculation processes do not disrupt the cell structure, cell lysis occurs in sewage sludge and the presence of cyanobacterial toxins is detected. These traditional approaches are also seen as ineffective for the treatment of extracellular cyanobacterial metabolites. Even when pre-oxidation with permanganate was added to traditional coagulation-flocculation processes, the risk of microcystin cell disintegration could not be reduced [1, 11] (Antoniou et al. 2014; He et al. 2016).

When sound waves produced by the application of power ultrasound in the range of 20 kHz - 10 MHz interact with molecules in the liquid medium, chemical reactions can be initiated, which is a special field in chemistry called sonochemistry. Cavitation bubbles created in the water medium by ultrasound undergo a series of mechanisms including formation, growth and explosion processes, which eventually result in a cavitational collapse. The growth of cavitation bubbles occurs by diffusion of vapor dissolved in water into the bubble. Therefore, a bubble can contain vaporized water molecules and dissolved gas molecules. When the bubble size reaches a radius of a few μm, it collapses in very short periods of time such as nanoseconds, and the collapsed bubbles create extreme conditions such as shock wave formation and transient high temperature (up to 5000 K) and high pressure (up to ~1800 atm). Then, hydrogen radicals (•H) and hydroxyl radicals (•OH) are formed in the solution from the sonochemical reaction of water molecules to further oxidize of the target organic compounds [12] (Mason et al., 2011). In addition to these radicals, organic molecules in solution can also form organic radicals with redox potential. A number of factors such as dissolved gas in the environment, ultrasonic power and frequency, temperature of the solution and type of solvent can affect the cavitation efficiency and the properties of the radical products formed in the bubbles [13-14]. In this study, the treatment of one of the most important cyanobacterial toxin compounds, MC-RR, which is the most frequently detected compounds in surface waters, was investigated with ultrasonic oxidation, as an advanced oxidation processes (AOPs), in a reactor with low and high frequencies applied. The toxicity analyses of the by-products after ultrasonic oxidation of MC-RR were also performed on a healthy mouse liver cell line (AML-12) by in vitro method.

MATERIAL AND METHOD

The oxidation of MC-RR was investigated in a laboratory environment using an ultrasonic system with low frequencies of 20 and 40 kHz and high frequencies of 578, 862, 1142 kHz, and the toxicity of the products formed after oxidation. The ultrasonic reactor has a 750 mL reaction volume and is shown in Figure 1. The glass reactor was kept in constant temperature and the temperature rising was prohibited via the double cell reactor wall during the oxidation. MC-RR was commercially provided in solid form and its stock amount was 50 μg (10007868 50 μg Cayman). The first dilution was made with 1 mL MeOH). The dilution solution was 3% MeOH [15] (Li et al., 2011) and ultrapure water was used in all experiments (Milli-Q). A total of 1000 ng/mL (1 ppm) with 5 mL volumes of MC-RR were prepared and used in oxidation experiments. Then, the initial concentration of MC-RR was diluted as 1,25 μg/L to conduct further experiments. Kinetic experiments were monitored in time with the function of the MC-RR concentrations. The MC-RR samples taken during the oxidation reaction was kept in +4 ^{o}C until they analyzed in LC-MS/MS.

Fig.1. The ultrasonic reactor with low and high frequencies.

MC-RR Analysis

The methods used for the analysis of microcystins should be economical, rapid and highly sensitive. Conventional methods for the sensitive detection of microcystins mainly include typical bioassays, protein phosphatase inhibition assays (PPIA), immunoassays, high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectroscopy (LC-MS). Therefore, liquid chromatographic (LC) coupled detection and separation with mass spectrometry (MS) is a robust and powerful method for the identification of a wide range of cyanotoxins because it provides detailed structural information for individual identification of cyanotoxins with very low LOD values ranging from 1 to 10 ng/L [16] (Jaramillo and O'Shea, 2019). Therefore, the concentrations of MC-RR were analyzed with LC-MS/MS (Agilent Technology 6460 Triple Quad LC/MC) device and the analyzes were performed at Atatürk University Central Laboratories (DAYTAM-Eastern Anatolia High Technology Application and Research Center) through service procurement. The samples were analyzed in 6 replicates (n>5) during method validation.

Toxicity Evaluation

AML12 cell culture (alpha mouse liver 12 cells) are healthy cells isolated from the normal liver (hepatocyte) of a 3-month-old mouse obtained from the American Type Culture Collection (ATCC) and are frequently used in toxicology studies in the literature [17] (Liu et al., 2024). The cell was delivered with a cold chain and stored in a vapor phase liquid nitrogen freezer until opened. The cell was stored and opened under the conditions specified on the ATCC website (https://www.atcc.org/products/crl-2254); grown in DAYTAM cell culture laboratories and made ready for toxicity experiments.

RESULT AND DISCUSSION

The Ultrasonic Oxidation of MC-RR

The degradability of MC-RR was performed in an ultrasonic system that have low frequencies of 20 kHz and 40 kHz and high frequencies of 578 kHz, 862 kHz and 1142 kHz. Toxicity tests were carried out using AML-12 healthy mouse liver cells by in vitro method. The initial concentration of MC-RR was 1.25 µg/L and the natural pH value was determined as 6.00±0.2. The initial experimental volume was 750 mL. Sampling times were 1, 3, 5, 10, 15, 20, 25 and 30 minutes. Oxidation efficiencies obtained at 0 and 30 minutes are shown in Fig. 2. The most effective frequency was defined as 578 kHz of ultrasonic frequency even though it had a low oxidation capacity for MC-RR.

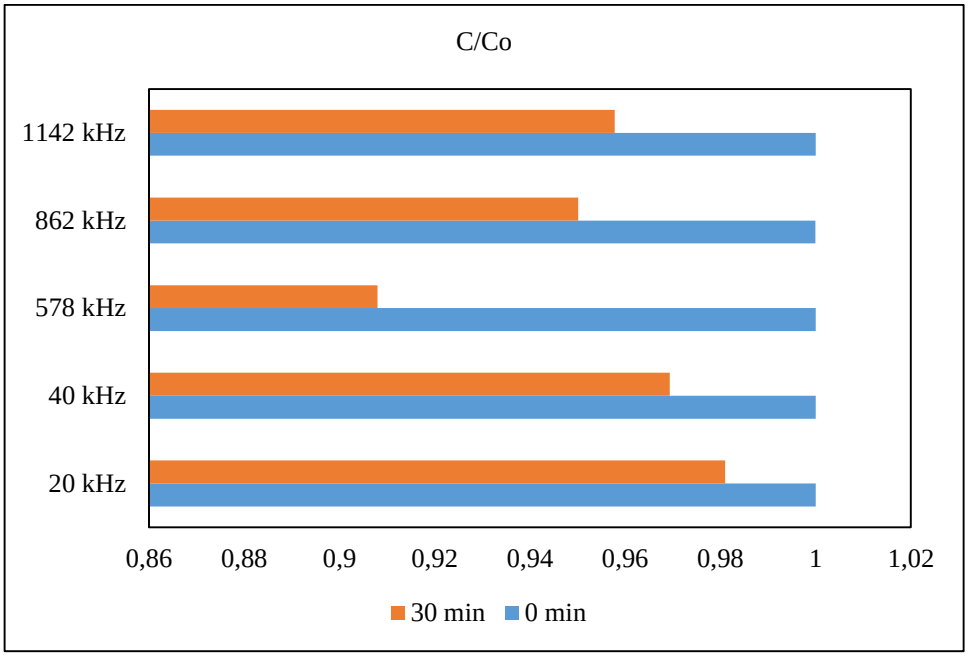

Fig.2. The ultrasonic oxidation of MC-RR with different frequencies (MC-RR=1.25 µg/L, pH=6.75, T=20 ºC, t=30 dak)

Fig. 3 shows the reaction kinetics of ultrasonic oxidation of MC-RR in the 578 kHz high frequency system. Accordingly, it can be said that high frequency ultrasonic oxidation is more effective on the degradation of MC-RR than low frequencies. Depending on the initial concentration, a degradation efficiency of 10-12% was recorded at the end of 30 min of oxidation for the frequency of 578 kHz. At other frequencies, oxidation of MC-RR was found to be either very low or negligible. When the reaction kinetics were examined, the degradation rate of MC-RR also complies with the 1st and 2nd order kinetics. However, it is also seen in the literature that the degradation rate of microcystins by hydroxyl radical is mostly 2nd order degradation [18-19] (Huber et al. 2003; He et al. 2015). In this study, the R^2 value for the 2nd order reaction rate constant was 0.7651 and it is little bit greater than the first order R^2 value of 0.7614.

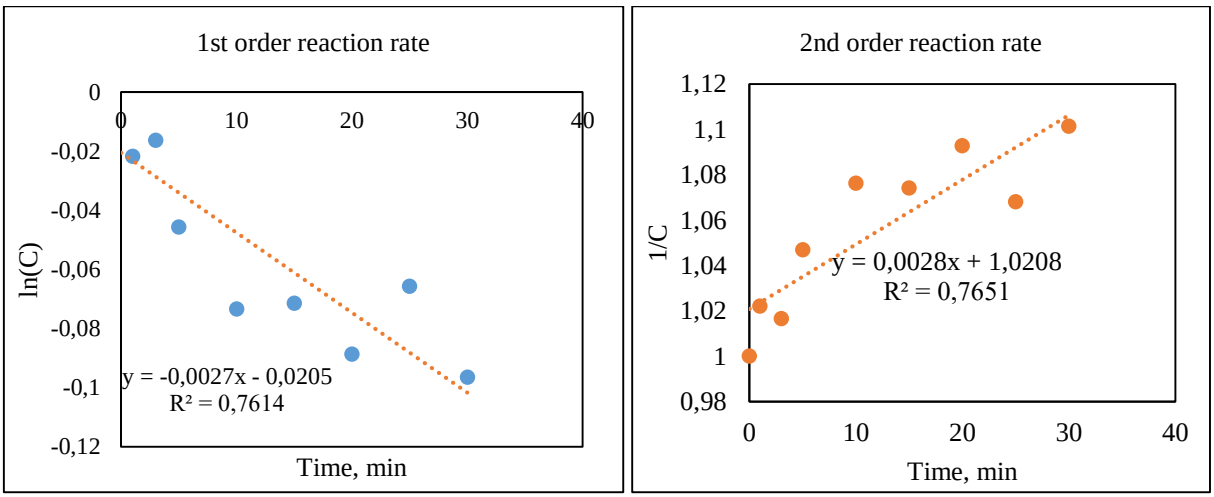

Fig.3. The concentration reduction of MC-RR by ultrasonic oxidation with 578 kHz frequency.

It can be mentioned that the oxidation efficiency of MC-RR as increased with increasing frequency with an optimum that of 578 kHz, after then it decreased. This effect is explained based on the literature as the "cushioning effect". Since the high power applied to the system at high frequencies will create more temperature

in the reactor, the vapor content of the solution increases, which has a cushioning effect on the collapsing bubble, increasing the resistance to the inward collapse movement of the cavitation bubble during the collapse phase. This reduces the released energy and then the number of free radicals decreases. This means less oxidation efficiency [20] (de Andrade et al. 2021). Therefore, increasing the power given to the system in ultrasonic oxidation has an increasing effect on the oxidation efficiency up to a certain point. For this reason, the oxidation efficiency decreases at frequencies above 578 kHz.

According to the results obtained from ultrasonic oxidation experiments, although low-frequency systems are not very effective in the degradation of MC-RR, 40 kHz provides relatively better oxidation efficiency. It is thought that this oxidation efficiency obtained under longer reaction times and experimental conditions such as pH of the system, initial MC-RR concentration, and the presence of other gases in the solution can be improved [14,21] (Eren 2012; Eren and O'Shea 2019). There are a limited number of studies in the literature on the ultrasonic oxidation of microcystins, and most of them are on the oxidation of MC-LR. The most important parameter affecting ultrasonic reactions is the frequency applied to the system by the ultrasonic power, and waves emitted at different frequencies cause further growth of the cavitation bubble and, as a result, higher pressure cavitation collapse [22-23] (Sivakumar et al. 2002; Servant et al. 2003). There are limited number of the studies on the ultrasonic oxidation of MC-RR in literature. Song et al. (2006) studied the ultrasonic oxidation of MC-RR at 640 kHz frequency at the initial concentrations of 4.0 μM and found a rapid degradation of 80−90% of the substrates being destroyed within 105 min with a pseudo-first order reaction rate of 0.53 ± 0.09 min^{-1} [24]. In another study, the degradation of MC-RR with a different AOPs method, ozonation, was conducted and 25.0% degradation efficiency was achieved while it was reached to 82.4% with 6 molar ratios of ozone. The study also evaluated the toxicity of the end-products revealed that the hepatotoxicity of MCs expressed as damage in mouse liver was greatly reduced or eliminated by the ozonation process [25] (Miao et al., 2010). The degradation of MC-RR was also conducted via UV/H_2O_2 process and it could be observed that the reaction was fast at the initial stage and 84% MC-RR could be removed within 30 min and 94.83% MC-RR removal occurred within 60 min. The results also showed that both of the pseudo-first-order and second-order kinetics was suitable for the degradation of MC-RR via photolysis process [26] (Qiao et al., 2005). The kinetic results of these studies are similar to the results obtained in our study, since the kinetic approach of MC-RR oxidation by AOP is compatible with both 1st and 2nd order reaction rates. However, the application of ultrasonic oxidation alone on the treatment of microcystins still has low removal efficiencies. Therefore, combining the processes with other AOPs can achieve better treatment efficiency, but toxicity research results are still needed.

The Toxicity Reduction of MC-RR After Ultrasonic Oxidation

AML-12 healthy cells were used in toxicity experiments seeded into 96-well plates with 5 wells each and 1 series containing a control cell group and kept in an incubator set at 37±2 ºC for 24 hours and grown by allowing them to stick to the plate. Each result was analyzed in five replicates and cell deaths were determined by taking the averages in these five wells. 200 microliters of the medium solution containing 60,000 cells were seeded into each well. In addition to the control group cell seeding, since MC-RR was dissolved in MeOH, one well series was seeded with MeOH solution and one well series was seeded with H_2O_2 solution to compare the cell deaths.
After the seeding, the AML-12 cells incubated for 24 hours. The oxidation product solutions of MC-RR at ultrasonic frequencies of 20 kHz, 40 kHz, 578 kHz, 862 kHz and 1142 kHz were cultured as 200 microliters and filtered through a 0.22 μm membrane filter against any contamination risk. A series of five wells was left as a control group of healthy cells; 1 mM H_2O_2 was added to one series of five wells and 3% MeOH was added to the other series of five wells as 200 microliters, respectively, and incubated for 48 hours to be checked every 24 hours. Then, each cell was photographed with a light microscope, MTT kit was applied (Fig. 4) and cell counting was performed. The effect of MC-RR on AML-12 cells is seen more clearly after staining and incubating with MTT kit. However, AML-12 cells applied with MTT kit were analyzed in ELISA at 570 nm for the percentage viability test.

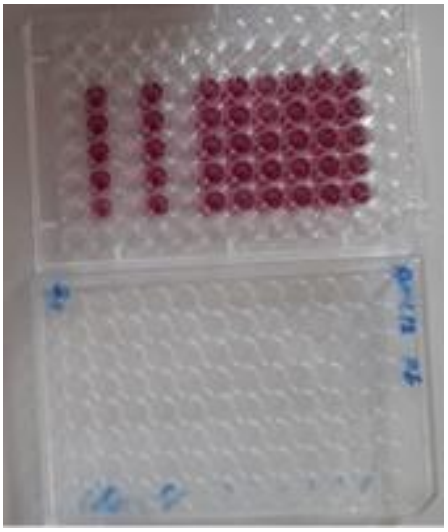

Fig.4. The Seeded AML-12 cells with MC-RR and stained with MTT.

Light microscope images of healthy control group AML-12 cells are also shown in Fig. 5. It was observed that the cells adhered and grew healthily in a total of five wells.

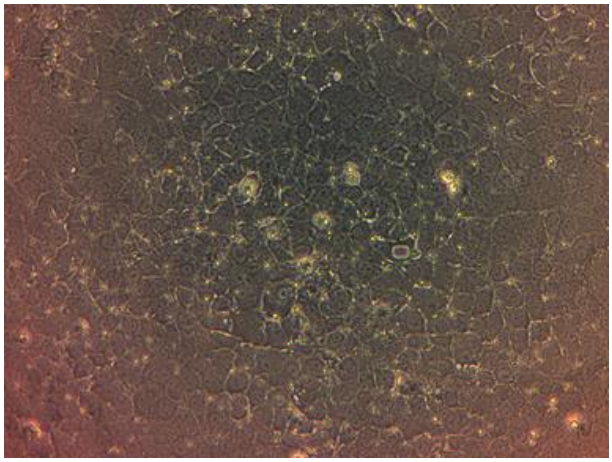

Fig.5. Control group of healthy AML-12 cells.

Accordingly, the oxidation by-products obtained at 30 minutes from the ultrasonic oxidation of MC-RR at all frequencies. The toxicity effects of 3% MeOH and 1 mM H_2O_2 solution on AML-12 cells were determined by light microscopy. H_2O_2 was applied for control purposes (Fig.6), and MeOH was applied to compare the effects on the cells since it is the active solvent of MC-RR (Fig.7).

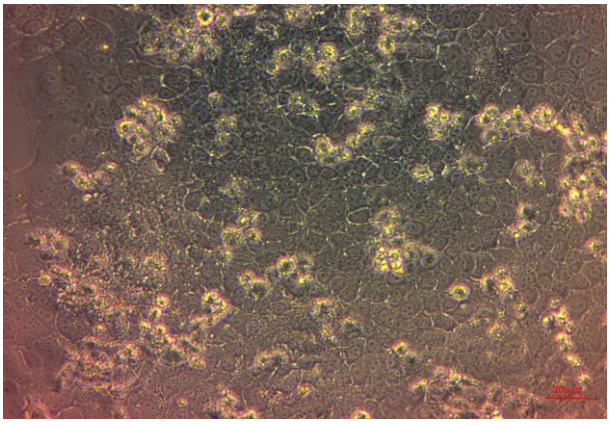

Fig.6. The effect of 1 mM H_2O_2 on AML-12.

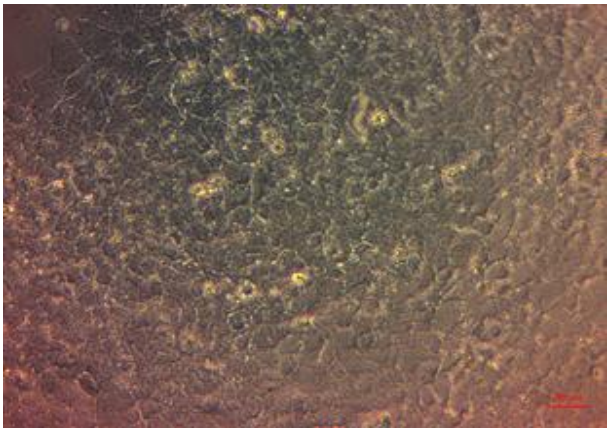

Fig.7. The effect of %3 of MeOH on AML-12.

When Fig.5 and Fig.6 are compared, that is, when healthy AML-12 cells are compared with AML-12 cells treated with 1 mM H_2O_2 solution, cell deaths are clearly seen. When Fig.5 and Fig.7 are compared; since it was observed that 3% MeOH used to dissolve MC-RR had no effect on cell activity, it was accepted that the only active component on AML-12 would be MC-RR solution. In order to express the results as numerical values and to determine the net deaths of the cells, a cell count test should be performed. For this purpose, AML-12 cells stained with MTT kit were determined by ELISA test at 570 nm wavelength by taking the control cell as a reference and the cell viability percentage was determined and the results are shown in Fig.8. The analysis of results was made in triplicate.

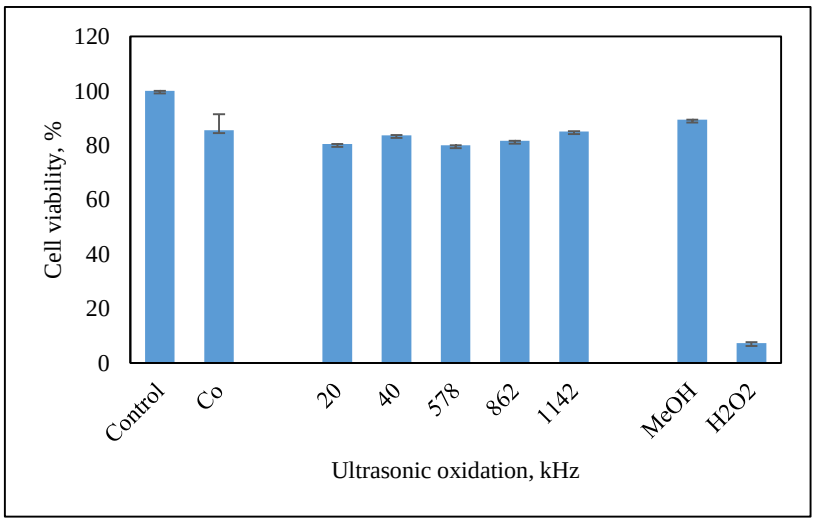

Fig.8. The toxicity of MC-RR by-products after ultrasonic oxidation with different frequency.

It can be easily understood that the MC-RR concentration reduction efficiency and cell viability values obtained from all ultrasonic frequencies. The toxicity effect of MC-RR at an initial concentration of 1.25 µg/L on AML-12 cell culture was such that it reduced cell viability from 100% to 85.6%. The toxicity effect of the solutions obtained by subjecting the initial 1.25 µg/L MC-RR solution to ultrasonic oxidation for 30 minutes separately at low frequencies of 20 kHz and 40 kHz and high frequencies of 578 kHz, 862 kHz and 1142 kHz for 30 minutes on AML-12 cell line remained the same in 1142 kHz oxidation and decreased very little in the others. Accordingly, it is observed that the oxidation by-products in all ultrasonic systems do not create a major toxic effect compared to the initial solution. In other words, ultrasonic oxidation products had an increasing toxic effect on AML-12 cells at rates varying between 5-6% compared to the initial solution. The toxicity of the by-products obtained by ultrasonic oxidation of MC-RR for 30 min is the same frequency as the initial MC-RR solution at 1142 kHz. However, the increase efficiency is also quite low. At the end of the study, it was tried to show that the ultrasonic oxidation by-products were not much more toxic than the initial solution with both cell images and cell viability test counts. However, in order to obtain more precise results, it is possible to compare the results obtained by increasing the reaction times required for oxidation and providing oxidation of MC-RR with other AOPs. Although there are not many studies in the literature on the oxidation of microcystins with AOPs and the investigation of their toxicity; no acute toxicity was observed in the toxicity analysis performed with Daphnia magna after the

Fenton process of the MC-LR compound [27] (Park et al. 2017). In the in-vivo tests performed on mice with solutions obtained from the ultrasonic oxidation of MC-LR at a frequency of 640 kHz, no observable side effects were observed at any of the doses apllied within 24 hours after injection [28] (Hudder et al. 2007). Ultrasonic oxidation process, which is effective in controlling a wide range of organic content of waters without the need for any chemicals other than electrical energy, is the most preferred treatment process within the AOPs. However, there are still a limited number of studies in the literature on ultrasonic oxidation of microcystins as well as the evaluating of the toxicity of post-oxidation products.

CONCLUSION

The recent increase in toxic cyanobacterial growth in aquatic environments and the increasing awareness of the toxins they release in the aquatic environment requires a risk management of cyanobacterial toxin problems in health, entertainment, agriculture, aquaculture and drinking water supply. For this purpose, it is necessary to monitor cyanobacteria in drinking water sources and to provide on-site measurements of cyanobacterial presence, primarily, for the effectiveness of cyanobacteria removal processes. Disinfection with chlorine and UV treatment alone is not very effective in treating extracellular algal toxins. Although AOP has been effective in treating cyanotoxins in recent years, some of its processes still cannot neutralize all cyanotoxins and cannot help reduce toxicity, especially due to the external oxidants used. Therefore, ultrasonic oxidation of MC-RR at different frequencies was investigated and the results obtained from oxidation in ultrasonic systems for 30 minutes showed that it had a treatment efficiency of at most 10-12%. Since the degradability of microcystin group compounds is generally low, it has been observed in the literature that similar groups have low oxidation efficiency. Toxicity tests performed with the AML-12 cell line showed that the starting solution of MC-RR had a lethality of close to 15% on healthy cells. At the end of ultrasonic oxidation, it was observed that the toxicity of the products obtained either remained the same or increased to a maximum of 20%. The fact that the toxicity of the degradation products of ultrasonic oxidation of MC-RR did not increase significantly makes ultrasonic oxidation seen as a promising technology for the degradation of microcystins. Since literature studies on ultrasonic oxidation of MC-RR are quite limited and there are no toxicity analyses with AML-12 cell line, the results obtained from this study constitute a starting point for future studies on both ultrasonic oxidation of MC-RR and its initial and post-treatment toxicity.

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