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Inhibition of The Growth and Toxicity of Cyanobacterium *Chroococcus minutus* Using Extremely Low Frequency Electromagnetic Fields

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THE EFFECTS of extremely low-frequency electromagnetic fields (ELF-EMFs) on the growth and synthesis of microcystins (MCs) production of the harmful cyanobacterium *Chroococcus minutus* were examined by exposing cyanobacterial cells to various ELF-EMF frequencies (0.1–1Hz) for various exposure times (10–60min). *C. minutus* cultures exposed to ELF-EMF at a frequency of 0.5Hz for 40min had significantly fewer cells than the control. Under these conditions, intracellular and extracellular (MCs) and chlorophyll-a concentrations also decreased significantly. Within 48h, no extracellular MCs were detected in cultures exposed to ELF-EMF for 60min. This study offers an eco-friendly technique for removing harmful cyanobacterial toxins from water resources. By degrading extracellular MCs after cell lysis, ELF-EMF is non-destructive, non-reactive and safe for the environment. In addition, it prevents additional aquatic environmental pollution input.

Keywords: Cyanobacteria, Cyanotoxins, Electromagnetic fields, Water treatment.

Introduction

In the modern world, living organisms are exposed to non-thermal and extremely low-frequency electromagnetic fields (ELF-EMFs) from power lines, mobile devices, laptops and desktop computers (Chen et al., 2014). Thus, studying the effects of these magnetic waves on the biological functions of microorganisms is a major area of research interest. Numerous studies have examined the effects of electromagnetic fields (EMFs) on humans (Santini et al., 2009; Lekovic et al., 2020), bacteria (Fadel et al., 2014; Oncul et al., 2016), fungi (Gao et al., 2011) and cyanobacteria (Fadel et al., 2018; Mohamed et al., 2020a). The effects of electromagnetic fields on biological cells depend on several physical parameters, including frequency, intensity, exposure time, and the type of biological cell (Fojt et al., 2004; Segatore et al., 2012). Cyanobacteria are the most numerous, diverse, and widespread group of oxygen-evolving photosynthetic microorganisms (Stanier & Cohen–Bazire, 1977). Cyanobacteria are gram-negative bacteria that thrive in various environments, such as soil, freshwater, marine waters and hot springs (Seckbach & Oren, 2007). In aquatic habitats, cyanobacteria are frequently the primary source of nutrition for other organisms (Mohamed & Al-Shehri, 2013). Cyanobacterial blooms typically occur in aquatic ecosystems with high phosphorus and nitrogen concentrations (Mohamed, 2016a; Lu et al., 2019). Most cyanobacterial blooms frequently lead to the demise of most fish and other aquatic organisms (Mohamed et al., 2020b; Bláha et al., 2009). Multiple species of cyanobacteria produce potent toxins that pose a threat to humans and aquatic organisms (Neilan, 2013). According to a previous study, the toxic cyanobacterium *Merismopedia minima in* in drinking water was not eliminated by conventional water treatment methods and poses a threat to humans, animals and plants, particularly if they produce toxins (Bakr, 2022). *Chroococcus minutus* (Kützing) Nägeli is a unicellular cyanobacterium that produces microcystin (MCs) (Mohamed, 2016b; Mohamed et al. 2022). High *C. minutus* cell densities have been detected in Egyptian drinking water sources (such as the Nile River), resulting in a decline in water quality (Walter

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et al., 2018). The removal of *C. minutus* and other cyanobacteria from drinking water treatment plants should be conducted safely. Conventional methods for removing cyanobacteria in treatment plants (e.g., pre-oxidation, sedimentation coagulation, sand filtration, and chlorination) release intracellular toxins into the water through cell lysis (Walter et al., 2018). Previous studies found that ELF-EMF had different effects on different cyanobacterial species, with ELF-EMF strongly inhibiting the growth of *Anabaena circinalis and Cylindrospermopsis raciborskii* but having no effect on *Microcystis aeruginosa* (Fadel et al., 2018; Mohamed et al., 2020a). Consequently, this study investigated the effects of ELF-EMFs on *C. minutus* growth and MC production.

Materials and Methods

Exposure system

Square amplitude modulated waves were generated by an arbitrary function generator (BK Precision 4085 40MHz) as reported by Fadel et al. (2018). The carrier frequency wave was 10MHz with an amplitude of ± 10Vpp and a modulation depth of ± 2Vpp. Two parallel copper electrodes connected to the generator output were used to expose cyanobacterial cultures in sterilized tubes to square amplitude modulated waves. The temperature within the coil was maintained at 28±0.5°C.

Experimental setup

Tested cyanobacterial species

Chroococcus minutus strain used in this study was obtained from the microalgal culture collection of Botany and Microbiology Department, Faculty of Science, Sohag University, Sohag, Egypt. *C. minutus* was previously isolated from fish ponds in the Sohag region of Egypt and was reported to produce microcystin (Mohamed et al., 2020b). Subsequently, using a capillary pipette, a single cyanobacterial unit was transferred to screw-cap test tubes containing 2mL of liquid culture of BG-11 medium (Fayyad & Dwaish, 2016). In order to prevent the growth of eukaryotic organisms, 50µg/mL of filter-sterilized cycloheximide antibiotics were added to the medium. The tubes were kept in a climate-controlled chamber illuminated with 50µmol m^{-2} s^{-1} light and maintained at 28± 0.5°C.

Optimum resonance frequency determination

In order to determine the optimal resonance frequency for growth inhibition of *C. minutus*. 2.5mL of exponentially growing *C. minutus* cells were inoculated into 25mL glass test tubes containing BG-11 medium (final cell density= 3 × 10^4 cells mL^{-1}) and cultured for 24 h in the growth chamber conditions described in the preceding section. After incubation, the cyanobacterial cells were connected to the generator output at 100V m^{-1} and 0.1–1.0Hz for 20min. In addition, *C. minutus* cultures lacking an electromagnetic field were used as a control. Both the treated and control cultures were incubated under the same growth conditions for 48h.

Optimum exposure time determination

Additional experiments were conducted to determine the optimal exposure time of ELF-EMFs on *C. minutus* by exposing cyanobacterial cells to the electromagnetic field at the frequency that demonstrated the strongest inhibitory effect (0.5Hz) in the previous experiment for 10, 20, 40, and 60min. There were six replicate culture tubes for each treatment, three of which were incubated for 24h and the other three for 48h. For each incubation period, a control consisted of three culture tube replicates. The control and treated *C. minutus* cultures were incubated under the growth conditions described above. At the end of each incubation period, 2mL subsamples were collected under aseptic conditions for analyses of cell number, chlorophyll-a concentration and toxin concentration.Each experiment was carried out in triplicate.

Cell abundance determination

As a measure of *C. minutus* growth, cell density was measured using a Sedgewick Rafter chamber and a light microscope according to APHA guidelines (APHA, 1995). The total abundance of *C. minutus* in the samples was expressed number of cells $ml^{-1.}$

Chlorophyll-a determination

Using a 2mL sample aliquot filtered through a Whitman GF/C glass fiber filter, the chlorophyll-a concentrations of treated and control cultures were determined. Filters with attached cells were extracted in 80% methanol in the dark at 25°C for 24h. The metabolic solution was centrifuged at 10,000rpm for 15min, and the resulting color was measured by spectrophotometry at 663 and 644nm wavelengths. Subsequently, in accordance with Wettestein's findings (Wettestein, 1957), chlorophyll-a concentrations were calculated using the following formula.

Chlorophyll-a = 10.3 A_{663} − 0.918 A_{644}

Microcystin determination

In order to determine the MC concentrations within *C. minutus* cells (i.e., intracellular toxins) and MCs potentially released into the medium (i.e., extracellular toxins), 2mL aliquots of 48 day old cultures (i.e., control and ELF-treated cells) were filtered through GF/C filters. The filters containing retained cells were extracted in methanol (80%) for the determination of intracellular MCs, while the filtrate was used for the determination of extracellular MCs. The filters containing retained cells were extracted in 80% methanol for intracellular MCs. Next, the retained cell-containing filters were extracted in methanol (80%), centrifuged at 6,000g for 10min and the supernatants of each extract were combined to determine intracellular MCs. After removing the organic solvent, the aqueous fraction that remained was filtered through GF/C filter paper. According to Carmichael & An (1999), the filtered fraction was directly applied to preconditioned C18 cartridges for solid phase extraction (SPE). The MCs were determined using high-performance liquid chromatography (Agilent 1200) with a UV-photodiode array detector set to 238nm (Forensic Medicine and Clinical Toxicology Department, Faculty of Medicine, Sohag University, Sohag). Chromatographic separation was performed using a Zorbax Eclipse C18 column (150mm × 4.6mm, 5µm; USA) with 60% solution A (100%, v/v methanol) and 40% (v/v) solution B (0.05%, v/v aqueous trifluoroacetic acid). The detector resolution was set to 1.2m for 30min, and data from 200 to 300nm were collected (Mohamed & Al-Shehri, 2009).

Statistical analysis

C. minutus cultures treated with ELF-EMF and untreated controls were compared for differences in cell number, chlorophyll-a and MC concentrations using one-way ANOVA ($P< 0.05$) and Tukey's test using SPSS v.16.0.

Results

When exposed to 0.1–1.0Hz amplitude modulating frequencies, the growth curves of control and ELF-EMF-treated *C. minutus* cultures exhibited different responses. *C. minutus* cells exposed to a frequency of 0.5Hz exhibited the greatest growth inhibition ($P< 0.05$), as evidenced by significantly fewer cells compared to the control and other treatment frequencies (Fig. 1). After determining that 0.5Hz was the optimal frequency for *C. minutus* growth, the effect of exposure time (10, 20, 40, and 60min) was investigated. After ELF-EMF exposure and a 24h incubation period, a significant reduction in cell number was observed in the treated cultures relative to the control and this reduction varied significantly ($P< 0.05$) with exposure time (Fig. 2). After 24h of incubation, an increase in exposure time led to a significant decrease in the number of *C. minutus* cells. However, there was no complete inhibition of cyanobacterial growth at any exposure time tested. In contrast, after 48 h of incubation, ELF-EMF exposure inhibited *C. minutus* growth significantly more than after 24h ($P< 0.05$), and complete inhibition was achieved after 48 h in the 60min exposure treatment (Fig. 2).

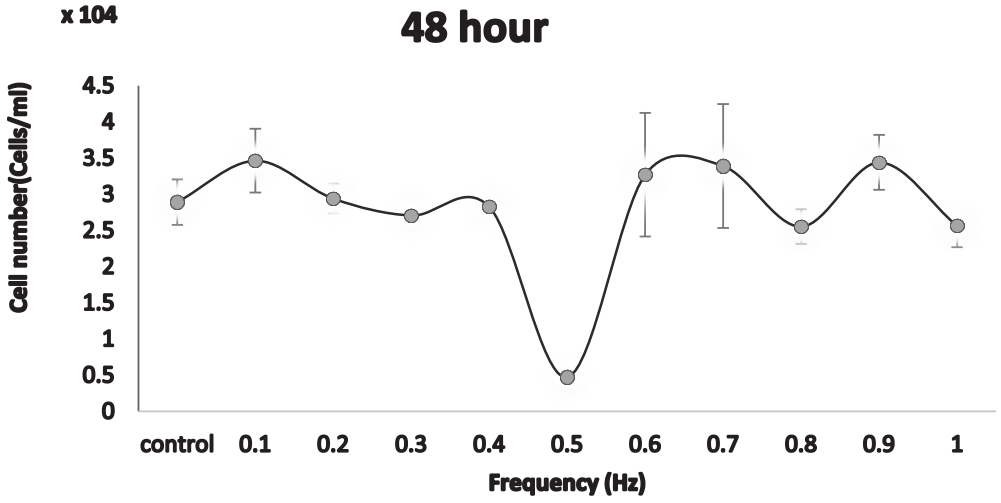

Fig. 1. Cell number of *C. minutus* exposed hours to square amplitude modulated waves at different frequencies (0.1–1.0Hz) for 30min periods and grown for 48h

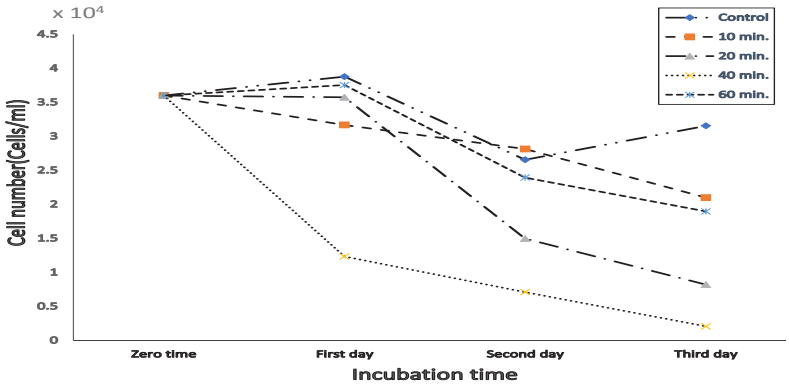

Fig. 2. Cell number of *C. minutus* cells exposed to 0.5Hz for varying exposure periods and grown for 24 and 48h

The concentrations of chlorophyll-a were also affected by ELF-EMF exposure at 0.5 Hz. After 24 and 48 h of incubation, the chlorophyll-a concentration of treated cells decreased significantly ($P< 0.05$) relative to the control (Fig. 3). The decrease was exposure time-dependent, with the chlorophyll-a content was reduced to zero in the 60-min exposure treatment after the 48-h incubation period (Fig. 3).

The intracellular and extracellular MC concentrations were also affected by exposure to 0.5-Hz ELF-EMF. The intracellular and extracellular MC concentrations of control cultures incubated for 48h were 22700.56 and 4550ng/mL, respectively, whereas these concentrations decreased dramatically in cultures exposed to ELF-EMFs for 40 and 60min. In cultures exposed for 40 min, the intracellular MC concentration was higher (260ng/mL) than in cultures exposed for 60 min (139ng/mL). Extracellular MCs were detectable in the medium of the 40 min exposure cultures (100ng/L) but not in that of the 60min exposure cultures (Table 1).

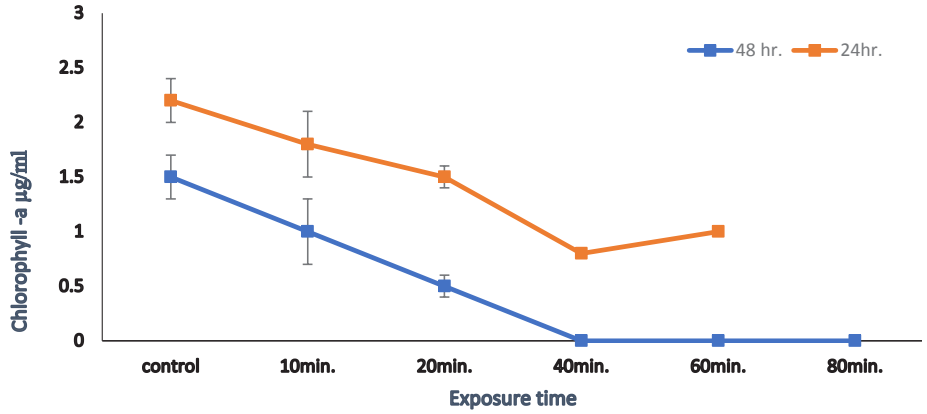

Fig. 3. Chlorophyll-a concentration of *C. minutus* exposed to 0.5Hz for different exposure periods and grown for 24 and 48h

TABLE 1. Intracellular and extracellular microcystin (MC) concentrations in control and treated cultures exposed to 0.5Hz for 40 and 60min, and for 48h

Samples	MC concentrations (ng mL^{-1})					
	Intracellular MCs			Extracellular MCs		
	MC-LR	MC-RR	Total	MC-LR	MC-RR	Total
40-min-exposure cultures						
Control cultures	2700	20000.56	22700.56	1230	3320	4550
Treated cultures	100	160	260	44	56	100
60-min-exposure cultures						
Control cultures	2700	20000.56	22700.56	1230	3320	4550
Treated cultures	40	99	139	0	0	0

Discussion

In this study, a novel method was used to inhibit the growth of the toxic cyano bacterium *C. minutus*. Typically, ELF-EMF (electrical signals) are produced by living cells during various vital functions (cell–cell communication) (Cifra, 2011). Consequently, this method of cell division inhibition relies on locating the ELF-EMFs that resonate with the bioelectric signals generated during cell division (Fadel et al., 2011). The resultant resonant interference can either promote growth (constructive interference) or inhibit growth (destructive interference) (Shckorbatov, 2014). This study demonstrated that square amplitude modulated waves at 0.5Hz inhibited the growth of *C. minutus.* The frequency of 0.5Hz is disruptive *C. minutus* resonance, proving that the measured data are growth indicators. ELF-EMF interactions with cyanobacterium cells can result in deviations from the normal passage of ions, which can be detrimental to the process (Fojt et al., 2004; Fadel et al., 2017). The obtained data is consistent with earlier study by Inhan-Garip et al. (2011) which demonstrated growth inhibition of some gram-negative and gram-positive bacteria using application of 0.5mT and 50Hz strength magnetology for 6h. Inhan-Garip et al. (2011) also found that ELF-EMF altered *Agrobacterium tumefaciens* growth parameters at 1Hz at 90min. In our study, the resonance frequency effects of 0.5Hz were investigated at various exposure times, with 60min of exposure causing the death of all *C. minutus* cells within 48h of incubation. Previous research on cyanobacteria determined that *Anabaena circinalis* and *Cylindrospermopsis raciborskii* were most susceptible to growth inhibition by ELF-EMFs at a resonance frequency of 0.7Hz (Chen et al., 2019). In addition, our research revealed that 0.5Hz ELF-EMF decreased the chlorophyll-a concentration in *C. minutus* cell cultures. These findings are consistent with those of Mohamed et al. (2020a), who discovered a decrease in chlorophyll-a concentration in *C. raciborskii* cells exposed to ELF-EMF. The decrease in chlorophyll-a content may be attributable to fewer cyanobacterial cells or chlorophyll-a inhibition. Additionally, Zeeshan & Prasad (2009) reported that the detrimental effect of ELF-EMF on photosynthetic pigments may be mediated by active oxygen. Numerous hypotheses have been proposed to explain the biological mechanisms underlying the effects of ELF-EMF on living cells. By altering the ionization level of water molecules, ELF-EMF induces the formation of free radicals (Fernie & Reynolds, 2005), which influence the synthesis of macromolecules and relevant metabolic processes (Cabiscol et al., 2000). It has also been hypothesized that ELF-EMF alters the permeability of the ionic channels in the cell membrane (Oncul et al., 2016), thereby altering ion transport into the cells and causing biological changes in the living organism. In this study, the exposure of *C. minutus* cells to ELF-EMF at 0.5Hz for 40min reduced intracellular and extracellular MC concentrations. These toxin concentrations became undetectable after 60min of exposure to ELF-EMF at 0.5Hz. This suggests that ELF-EMF caused cell lysis and the release of intracellular toxins into the culture medium, and the absence of extracellular MCs in the medium of ELF-EMF-exposed cultures indicates the ability of such EMFs to degrade extracellular MCs after they are released from cells. These results contradict the conclusion of our previous study, which demonstrated that ELF-EMF did not lyse *C. raciborskii* cells, thereby preventing the release of cytoplasmic contents and cylindrospermopsin toxin. These results support the hypothesis that different species of cyanobacteria react differently to ELF-EMF (Fadel et al., 2018; Mohamed et al., 2020a).

Conclusion

This study clearly demonstrated that 60-min exposure to 0.5 Hz ELF-EMF 60 min produced the optimal growth inhibition of the toxic cyanobacterium *C. minutus*. The reduction in cell number was accompanied by a subsequent decrease in chlorophyll-a concentration. Cyanobacterial cells were lysed by ELF-EMFs, resulting in the release of MC toxins into the culture medium. Interestingly, ELF-EMFs also degraded the released extracellular MCs. The simultaneous growth inhibition of cyanobacteria and degradation of intracellular and extracellular toxins by ELF-EMF represent advantages over conventional methods for removing cyanobacterial cells from drinking water, which result in cell lysis and the release of toxins without the removal of extracellular toxins in the water. Nevertheless, direct application of ELF-EMFs to eliminate harmful cyanobacteria and their toxins in drinking water treatment plants should be preceded by additional in situ studies.

Conflict of interest: The authors declare that they have no interest conflicts.

Authors' contributions: Conceptualization: Asmaa

bakr. and Asmaa hosny methodology, A.B.; software, A.B and Asmaa Hosny; investigation, Asmaa Bakr. and Asmaa Hosny resources, Asmaa Bakr, Asmaa Hosny and Prof Dr Zakaria writing—original draft preparation Asmaa Bakr. and Asmaa Hosny and Prof Dr Zakaria Attia writing—review and editing, All authors have read and agreed to the published version of the manuscript.

Ethics approval: Not applicable.

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تثبيط نمو وسمية البكتيريا الزرقاء *Chroococcus minutus* باستخدام مجال كهرومغناطيسي منخفض التردد

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تناولت هذه الدراسة واحدا من الموضوعات الهامة التي تتعلق بتأثير الموجات الكهرومغناطيسية ذات التردد المنخفض جدا علي السيانوبكتريا السامة التي توجد في مصادر مياه الشرب. هدفت الرسالة إلي تحقيق بعض المعارف مثل:(1) ميكانيكية تفاعل هذه الموجات مع الخليه البيولوجية، (2) مقدره هذه الموجات علي احداث تأثير تحفيزي أو تثبيطي لنمو الكائنات الدقيقه حقيقييه النواه أوبدائيه النواه باستخدام نموذج الرنين المغناطيسي الحيوي للتحكم في العمليات الأيضيه المختلفة (MBMR)، (3) أستخدام هذا النموذج في تثبيط نمو السيانوبكتريا الضارة وبالتالي التخلص من اضرارها بطريقه أكثر آمانا عن الطرق التقليدية الأخري المستخدمة في محطات تنقية المياه.ولتطبيق هذا النموذج تم إختيار نوع من السيانوبكتريا السامة المنتشرة بشكل كبير في مياه الشرب وهو طحلب الكروكوكاس مينيوتس. (1) تم إنماء خلايا السيانوبكتريا في وسط غذائي BG-11 حتي الوصول إلي الطور الأسي ،(2) تم تقسيم الخلايا النامية إلي 11 مجموعة، 10 مجموعات منها عرضت للموجات الكهرومغناطيسية معدلة السعة ذات التردد المنخفض جدا في مدي تردد (0.1–1 هرتز) عند شدة مجال كهربي ثابتة 100 فولت/ متر ودرجه حراره 30 ± 0.5 ℃ لمدة 30 دقيقة ومجموعة واحدة لم تعرض للموجات معدله السعه ذات التردد المنخفض جدا (كنترول).كما أجريت تجارب إضافية لتحديد أفضل زمن تعريض للموجات الكهرومغناطيسية يحدث عنده أقصى تثبيط لنمو الخلايا وذلك بتعريض خلايا السيانوبكتريا للموجات الكهرومغناطيسية عند أزمنة مختلفة (0، 20، 40، 60 دقيقة) عند تردد 0.5 هرتز(التردد الحادث عنده أقوي تثبط للنمو في التجربة السابقة). تم تحضين مجموعات مزارع السيانوبكتريا المعرضة للموجات الكهرومغناطيسية والغير معرضة (كنترول) في حضان مضيئ عند درجة حرارة 30 درجة مئوية وشدة أضاءة 50 $\mu mol.m^{-2}.s^{-1}$. أخذت عينات من المزارع يوميا تحت ظروف التعقيم لتقدير عدد الخلايا وتركيز الكلوروفيل وتقدير سم الميكروسيستين النتج بواسطه هذا الطحلب حدث تثبيط لنمو السيانوبكتريمالكروكوكاس مينيوتس عند التردد الرنيني 5ه هيرتز تناقصت أعداد خلايا الكروكوكاس مينيوتس في المزارع المعرضة للموجات الكهرومغناطيسية عند تردد 0.5 هرتز هرتزقل محتوي الكلوروفيلكما انخفضت MCs داخل الخلايا وخارجها بشكل ملحوظ عند 40 دقيقة من وقت التعرض وتردد الرنين 0.5 هرتز وفي النهاية توصي الدراسة باختبار تأثير هذا النوع من الموجات الكهرومغناطيسية على الأنواع الأخري من السيانوبكتريا والكائنات الدقيقة شائعة الانتشار في مصادر مياه الشرب. كما توصي الدراسة بتطبيق هذه الطريقة في محطات معالجة مياه الشرب وخاصة أثناء مرحلة تجلط وترسيب الخلايا coagulation and sedimentation .