Short Communication

Removal of microbial biofilm on Water Hyacinth plants roots by ultrasonic treatment

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The capacity of floating aquatic plants to purify sewage effluents drops rapidly as a result of biofouling processes that occur on the plant roots. This is due to the high concentration of microorganisms (MO) present in the wastewater. The ability of US to remove MO from the roots was studied using commercially available sonicators at intensity levels ranging from 2.7 W/cm² to 81.4 W/cm² (corresponding power levels ranging from 75 W to 500 W) while varying application periods between 5 and 60 min. The results show that MO can be removed effectively (up to 98%) by exposing the Eichhornia crassipes roots to US for 5 min at the intensity level of 64.5 W/cm². The efficiency of the wastewater treatment increased with exposure time and power input. The study proved that the US treatment is effective in removing MO that otherwise adhere to the roots, by more than two orders of magnitude.

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1. Introduction

Aquatic plants, such as Water Hyacinth (Eichhornia crassipes), Pennywort (Hydrocotyle umbellate), and duckweeds (Lemna sp.) are known to be effective in single pass wastewater treatment processes [1]. The major characteristics of such plants are their extensive root system and rapid growth rate. In the process of their growth, the plants consume pollutants such as ammonia, phosphates, and nitrates [2,3]. Furthermore, the extensive root system of the plants with large surface area provides an attractive biological substrate for adhesion of MO that is abundantly present in the wastewater. These MO are believed to have a major role in the treatment process. This biofouling of plant roots decreases the water cleaning efficiency by such aquatic plants.

In recent years, the use of ultrasound irradiation for the destruction of organic pollutants in fresh and waste waters has received increasing attention. The process comprises cyclic formation of microbubbles, where their growth and subsequent collapse occur in short time intervals with release of intense and focused energy [4].

Successful removal of a wide range of organic pollutants from relatively dilute aqueous solution using sonochemistry has been reported, and it appears that the applications of this novel means of reaction in environmental remediation and pollution prevention is unlimited [4–8]. Currently ultrasound technology is used in the food industry for degassing of liquids, cleaning of process lines and welding of packages.

It is hypothesized that, ultrasonic treatment can reduce the number of MO attached to plants roots. Ultrasound, in its most basic definition, refers to pressure waves at frequencies of 20 kHz or higher [9,10]. Sonochemical degradation in an aqueous phase involves several reaction pathways and zones such as pyrolysis inside the bubble, and/or at the bubble–liquid interface, and/or at the liquid bulk [11]. Ultrasound could cause physical disruption and chemical reaction within the material to which it is applied [12].

Sonication processes that occurred in various media have been studied by several researchers [13–17]. It was shown that ultrasound treatment should induce additional microbial inactivation.

The effectivity of US treatment on reduction viability of bacteria (Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa), fungus (Trichophyton mentagrophytes), and viruses (Feline herpesvirus type 1 and Feline calicivirus) using frequency in the range of 20–26 kHz have been reported by various workers [18–20]. The mechanism of microbial extermination involves thinning of cell membranes, localized heating and production of free radicals as the result of cavitation [10,21].

Previous investigations, conducted in our laboratory, demonstrated that E. crassipes plants were most effective in cleaning the sewage during the first 4–5 days [22]. These plants which were initially grown on fresh water, substantially improved the water quality by decreasing its chemical oxygen demand (COD), biochemical oxygen demand (BOD), total suspended solids (TSS) and turbidity.
In longer time spans, the relative change in these parameters was minor. Advanced water management of aquatic plant treatment systems requires understanding the behavior of MO during ultrasonic treatment. The scarcity of data concerning the effect of ultrasonic treatment on aquatic plant roots in sewage, calls for the conduction and analysis of related tests.

This study aims at estimation of low frequency US treatment efficiency to remove MO from the roots under laboratory conditions. The time needed for removal of MO attached to plants roots is not clear.

2. Materials and methods

The investigation was carried using E. crassipes plants that were grown in a fresh water pond. Three experimental sets were performed:

Set 1: In the first part of this set, the kinetics of MO accumulation on aquatic plant roots, during 13 days, were recorded. This was done with three 6 L containers that were used to grow two plants in each container. These plants were initially supported by fresh water. Prior to immersion of the plants in the containers, their roots were thoroughly washed under a tap water stream. Initially, at t = 0, all containers were filled with 6 L of fresh water which was used to support the plants for 2 h. Then, periodically (on day 0, 1, 3, 4, 6, 7, 8, 9 and 13) 3 L of water were withdrawn, and 3 L of row sewage were added to each container, so as to set the mixture volume back to the 6 L operating level. The total volume of added row sewage was 27 L. Each container was supplied with 0.3 L/min aeration, applied for 10–12 h/day. In order to determine accumulation of MO on the plant roots, three root samples were taken on day 0 (before sewage addition), 3 and 10 of the experiment. The sampling procedure was as follows: a 1 cm root sample was cut off each tested plant and carefully immersed for 1–2 s in 20 ml of sterile 0.85% NaCl solution. The purpose was to remove loose non-adherent MO. The sample (with the adherent microbial biofilm) was then inserted into a tube that contained 10 ml of sterile solution (0.85% NaCl + 0.1% Tween 80). Tween 80 was added as surfactant in order to improve the removal of MO cells from the root surface, thereby providing good dispersion of these cells in the suspending media (i.e. to avoid formation of microbial aggregates). Adherent MO were removed from the root surface by shaking with a mechanical shaker Vortex-2 (Scientific Industries Inc., USA) for 60 s, after which the root was withdrawn. The remaining solution was shaken for 40 s, and then tested for its MO content. Counting of MO (heterotrophic aerobic bacteria – HAB) was performed by the heterotrophic plate count (HPC) method described in Standard Methods [23]. The plating was done in triplicates. Total count of HAB was determined by this method. The MO were cultivated on Petri dishes, with R2A agar (Difco), in incubator at 37 °C for 24–48 h. It is important to note that the initial MO concentration was determined on samples of pre-cleaned roots prior to their placement in the experimental containers. All experimental procedures (sample collections and handling) were performed according to Standard Methods [23].

In the second part of Set 1, the extent of adherent MO removal from roots, exposed to wastewater, was determined by simple manual washing in fresh water. In these experiments three 6 L containers were planted with E. crassipes (2 plants per container). In the first 13 days wastewater was added to all containers and the number of MO was determined according to procedure described in part 1 of the set. Then, in day 14 of their exposure to sewage, the roots from containers 2 and 3 were thoroughly washed by manual shaking in sterile solution (0.85% NaCl + 0.1% Tween 80). To this end each plant was successively washed for 1 min, in three retorts containing 1.4 L of fresh suspending solution. Plants from container 1 were not washed and used as control. At this stage, the number of MO accumulated on the plant roots from container 1 was compared with that on the rinsed plant roots from containers 2 and 3. MO counting on tested roots was performed as described above.

Unwashed plants from container 1 and rinsed plants from container 2 were returned for 1 day to the start conditions of the test (fresh water). Plants from container 3 were returned to the same water medium they were taken from. On day 14 of exposure to sewage, 3 L of water from each container were replaced by 3 L of raw sewage, and recording of BOD and COD was continued. The same procedure was repeated on the 15th exposure day.

The purpose of experiments carried out in Sets 2 and 3 was to estimate the potential of US treatment for reducing the number of MO attached to E. crassipes plant roots, as compared to that of the mechanical procedure performed in Set 1.

Set 2: In this case, the efficiency of MO removal from the roots by low frequency US, as a function of treatment time, at constant power input, was determined. Ultrasonic device, Bransonic-220, operated at 20 kHz and 0.2 kW was used to this end. Fig. 1a shows a schematic description of the first experimental setup that is based on an US container. The container was built of thin metal sheets without absorptive lining. This results in a reflective thin metal envelop and free water surface. The absorption coefficient of the surface area was taken as 0.1. Because of the strong acoustic reflections more acoustic energy is delivered to the specimen, and the acoustic intensity distribution in the container, relatively far from the bottom is evenly distributed. The steady state of the acoustic power injection into the system leads to a balanced acoustic field, and the supplied acoustic power equals that absorbed by the system.

Plant roots, exposed to the wastewater for 13 days in container 2 (see set 1), were selected for the experiments. Due to size limitation of the available US cell, only a section of the root system could be treated. Consequently, the US treatment was applied to 1 cm root samples, which were cut in triplicates from the central part of the root. The plant roots were sonicated by the Branson-220 device. Each experiment was repeated three times. Immediately after it was cut, each root sample was immersed in 20 ml of sterile solution that was used to gently wash it for 1–2 s (see set 1). Control untreated samples were transferred to a tube with 10 ml of sterile solution for MO count. Washed root samples were transferred to the US cell (glass tube) with 60 ml of the same sterile solution. The tube with the root section, was then transferred to the metallic ultrasound tank (30 × 23 cm²) filled with tap water to a level of 5 cm. The transducers at the bottom of this tank generated tone bursts, probably due to cavitation, and the frequency of the burst could be tuned to 20 kHz. The test tube was suspended on a tripod, 1 cm clear of the bottom, and 7 and 14 cm from the walls.

The sonicator was operated in the continuous mode at about 30% of its maximum output setting and was typically delivering about 10% of its rated power to the sample [24]. Calorimetry [25] was performed to measure the power output of ultrasound, which was 75 ± 0.5 W for experiments in this set. The intensity from the probe tip based on calorimetry measurements was 2.7 W/cm².

The sound pressure levels are given in logarithmic scale, using decibel (dB) units, with a specific definition that follows. Estimates of the sound pressure levels in the container water, for sound radiated through the container bottom, were done by using a formulation borrowed from “room acoustics” theory. Since the problem involves short ultrasonic waves, we assumed multitude of acoustic reflections that distributes the sound field evenly far from the acoustic source (bottom of the container).
Let $\alpha$ be the absorption coefficient of sound at the enveloping surface of the liquid in the ultrasound tank. The sound pressure level $L_p$ far from the source is given, following Urick [26] as

$$
L_p = \frac{S \alpha}{1 - \alpha} + 10 \log_{10} \left( \frac{P}{R_c} \right) \text{ dB},
$$

where $R_c$ denotes the “room constant”, which is an expression that accounts for the absorption of sound at the enveloping surface of the liquid, $P$ is the source power in Watts, $S$ is the enveloping area of the liquid in the ultrasound tank in square meters; $R_c = 10^{-12}$ W is the reference level of $P$. For $\alpha = 0.1$, $S = 0.191$ m$^2$ and $P = 200$ W, the result of $L_p$ is 165.8 dB.

The sound pressure level can be found also by using power intensity derivation from calorimetry measurements. The result is $2.7\ W/cm^2$ or $2.7 \times 10^4 \ W/m^2$, and in dB

$$
L_p \approx 10 \log_{10} \left( \frac{I}{I_0} \right) = 10 \log_{10} \left( \frac{2.7 \times 10^4}{10^{-12}} \right) = 164.3 \text{ dB},
$$

where $I$ denotes acoustic intensity in W/m$^2$ obtained by the calorimetric measurement; $I_0 = 10^{-12}$ W/m$^2$ being the reference level for $I$, is equal $R_c$.

The resulting 164.3 dB of the calorimetric experiment is in good agreement with results calculated by the “room acoustics” theory, which is 165.8 dB, an error of 0.9%.

During exposure to US, the tube was covered with aluminum foil. The ultrasonic treatment time was 10, 15, 30 and 60 min. Immediately, after the US treatment, the root sample was transferred to a tube with 10 ml of sterile solution for MO count. The solution temperature in the tube was measured at the end of each US treatment.

Set 3: High-intensity ultrasound was applied using VCX 500 (Sonic and Materials Inc.) equipment, comprised of an electric generator and transducer. The power supply converted the standard 50 Hz AC to 20 kHz, with a maximum ultrasound power output of 500 W. Fig. 1b shows details of the second experimental setup.

In this experimental series, the influence of US intensity on the treatment efficiency was studied. The US treatment was carried out in a glass container containing 150 ml of sterile solution, in which the 1 cm root sample was immersed. In contrast to set 2, here the ultrasound tip was introduced directly into the sample containing US cell. In order to avoid overheating due to application of high US power, the cell was placed in an ice bath. Following the immersion of the tip in the tested solution, it was activated for 5 min at different power levels. The corresponding energy input ranged between 11,264 and 23,841 J. The experimental procedures were the same as specified in set 2. Here, power output of ultrasound, which was between 57.3 and 108.3 ± 0.5 W for experiments in this set as compared to 75 ± 0.5 W in set 2. The intensity from the probe tip based on calorimetry measurements was between 43.2 and 81.4 W/cm$^2$. The resulting theoretical sound pressure level was 182 dB, as compared with the 165.8 dB in set 2. A range of 176.3–178.1 dB was obtained from the calorimetric test and by using Eq. (2) These experimental results are in good agreement with the calculated theoretical result, 182 dB, giving an error between 2.1% and 3.2%. All experiments in the study were made in triplicates, and temperature changes during the ultrasound treatments were recorded.

Note that, the efficiency of the sonication treatment was assessed by counting the number of MO attached to a single root sample (root fragment 1 cm long that was cut off from a tested plant). In this article the number of MO detected by the HPC meth-

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**Fig. 1.** (a) Scheme of the US experiment with Bransonic-220 and (b) scheme of the US experiment with Ultrasonic Processor-VCX Series.
od on tested root samples are given in CFU (colony forming units) [23] per single root sample.

3. Results

Set 1: Fig. 2a and b demonstrates SEM micrograph of a tested *E. crassipes* root section that was grown in sewage, before exposure to US. Strong accumulation of MO (biofouling) on the root’s surface is clearly seen. Fig. 3 depicts the kinetics of MO attachment to plant roots that were exposed to a system with periodic sewage addition.

It can be seen that the number of MO counted on these roots, increased with the level of sewage dosage. The main increase in the number of MO that was attached to roots was observed during the first three exposure days: close to two orders of magnitude (Fig. 3). During the following exposure (from day 4 to 13) the number of adhering MO increased by one order of magnitude on a single root sample. In fresh water (control), the corresponding number of MO counted on each root sample during all experiment, ranged between 9.2 and 13 × 10^5 CFU (colony forming units). Note that in Fig 3 control data is not shown. It was found, that manual washing with sterile fresh water of roots that were exposed for 13 days to wastewater, decrease the number of attached MO by an order of magnitude, from (5.1 ± 3) × 10^8 to (5.4 ± 2) × 10^7 CFU per root sample. Such rinsing and removal of adhering MO, was demonstrated to enhance the water purification capacity of the roots. This was demonstrated by the substantial improvement, for example, of the BOD (removal 80.6%).

Set 2: The experimental results are presented in Table 1. Data shows that 99.2% of accumulated MO may be removed from the plant roots, when exposed to US. At constant US intensity (2.7 W/cm²), the number of MO remaining on the root, decreased with treatment time in the US bath. Initial number of bacteria, on each root, was taken as 100%. An US exposure time of 30–60 min resulted in more than 98% removal of the microorganisms from Water Hyacinth roots. At least 20 min of US treatment is needed for 90% MO removal. It is important to note, that the maximum MO removal achieved in these experiments was 99.2%. However, the residual number of MO on the plant roots was still too high, relative to the pre-sewage stage. The results of temperature measurements carried out in the US cell, at the end of each experiment, are presented in Table 1.

Set 3: Fig. 4 and Table 2 summarize the experimental results. As can be seen in the figure, the higher the power input, the higher the level of MO removal from *E. crassipes* roots. Setting the power at 57.3 and 74.3 ± 0.5 W, (intensity 43.2 and 56.0 W/cm²) decreased the number of MO on the plant roots by one order of magnitude, with removal efficiencies of 74.1–92.1%.

Curve fitting by a power function provides a good fit to the number of residual bacteria in this test. High values of the correlation coefficient squared R² were obtained for both power (R² = 0.9979) exponential (R² = 0.9854) fitting models.

At power 85.6 ± 0.5 W (intensity 64.5 W/cm²) the number of MO observed on plant roots decreased by close to two orders of

Fig. 2. (a) SEM image of the *Eichhornia crassipes* root before exposure to US, reference scale – 500 μm and (b) magnified SEM image with visible MO, reference scale – 2.5 μm.

Fig. 3. Average number of MO on plant roots that were exposed to periodic sewage addition. Each arrow points towards the relevant axis of the plot. The left vertical axis shows CFU (colony forming units) per single root sample. The right vertical axis and bars shows the total sewage dosage during experiment.
The study proved that the US treatment is effective in decreasing MO that otherwise adhere to the roots, by more than two orders of magnitude. The US treatment is expected to have a stronger effect on enhancing the root’s purification capacity, as compared to that obtained by manual washing. The ability of US treated aquatic plant to purify wastewater, needs further research in order to establish the extent to which the US enhances the root activity, and under what conditions.

Our results of ultrasound treatment, for removal of MO from roots, seem to be a combination of complex effects which were influenced by many factors including temperature, power input, kind and structures of MO. These results agree with those of [18–20,27], who demonstrated the effect of ultrasound on the rate of MO removal in other (excluding aquatic plant and sewage) studied systems. Results reported by Child et al. and Azadniv et al. [28,29] show reductions in growth rate of plant roots exposed to US. This should be taken in consideration when plants are subjected to ultrasound treatment. We believe that cavitation has a major damaging effect. The appearance of bubble provided evidence of the cavitation effect. In this context, results reported by Pagan et al. [30] demonstrate that temperatures up to 50 °C did not have any significant effect on ultrasonic inactivation.

Laboratory tests of US systems can be used for scale up to field systems. For example, the use of aquatic plants for sewage treatment can be enhanced by applying US in order to clean their roots. The cleaning operation can be done in special containers with ultrasonic “Sono-Des” units (produced by Bensonic Ltd.) in short cycles of plant treatment. The treatment can be on line or off line in special cleaning units external to the sewage processing system.

In conclusion, this study suggests additional ways to enhance the efficiency of wastewater treatment processes by aquatic plants. This involves control of MO population in order to refresh and enhance its performance, i.e. in conjunction with their growth on the plants roots. The results indicate that US may be an additional tool for control of the MO on roots system. The role of US, in enhancing the MO population removal, from plant roots, was demonstrated. This is one of the most significant results of this work, as no related data is available elsewhere. Further work is required in order to establish the extent to which the US enhances the root activity, and under what conditions.

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