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Rapid assessment of toxicity of chlorinated aqueous solution by dissolved oxygen depletion and optical density bioassays

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Abstract

Background: Chlorination of wastewater effluent with high levels of residual organic matter has been suspected to the production of toxic and hazardous disinfection by-products (DBPs) including trihalomethane (THM) compounds.

Methods: In this study, two rapid techniques including dissolved oxygen depletion (DOD) and optical density (OD) bioassays were used to evaluate the chloroform toxicity of aqueous solution. The activated sludge was collected from aeration tank of a full-scale municipal wastewater treatment plant and used as a biological inoculum. In order to achieve an active and stabilized mixed culture of bacteria, the test cultures were transferred to a fresh nutrient broth culture media every day. The influence of chloroform on DOD and OD bioassays was examined at chloroform initial concentrations of 10-1000 µg/L.

Results: It was revealed that the application of chloroform at concentrations of 100 and 1000 µg/L showed moderate and extreme toxicity, respectively, and reduced bacterial activity. The estimated chemical concentration with 50% inhibition of bacterial activity for DOD and OD bioassays was 457 and 961 µg/L, respectively.

Conclusion: According to the results, the wastewater effluent should use bioassays in order to evaluate the effects of DBPs where the wastewater effluent is disinfected by chlorine compounds.

Keywords: Trihalomethanes, Chloroform, Disinfection, Wastewater, Biological assay, Oxygen, Inhibition

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Introduction

Chlorination is widely used for disinfection of water and wastewater around the world. During the chlorination process, chlorine reacts with natural organic matter and produces hazardous disinfection by-product (DBPs) (1, 2). One of the main groups of DBPs are trihalomethanes (THMs). The most well-known THMs include chloroform ($CHCl_3$), bromodichloromethane ($CHBrCl_2$), chlorodibromomethane ($CHBr_2Cl$), and bromoform ($CHBr_3$) (2,3). THMs, especially chloroform, have been more studied among all DBPs, because of its frequency (4).

Disinfected wastewater effluents have a negative effect on aquatic organisms as well as on the balance of the aquatic ecosystem and the receptor water body (5). Recent epidemiological studies have shown that THMs have adverse impacts such as sudden abortion, stillborn, and disorder in reproductive. Some researchers have reported that there is a correlation between THMs and colorectal and bladder cancers (4,6,7). Chloroform is a dominant THM that was identified by Rook in 1974 (8). It causes teratogenicity, hepatotoxicity, nephrotoxicity, and damage to the liver, kidneys, and central nervous system (8). Toxicity of chlorine itself is removed by dechlorination but this process does not remove DBPs (9). More than 600 DBPs have been identified but the toxicity of a few of them has been evaluated (10).

The chemical analyses just determine the components and concentration of contaminants that release into the environment but biological analyses can directly reflect the effect of contaminants on organisms (11). Bioassay has been used for assessment of environmental contaminant. Bioassay is a test in which living tissues, organism or a group of organisms are exposed with contaminants and the effects of chronic or acute toxicity on them are assessed (12).

There are several bioassay methods that evaluate various classes of toxicity including acute toxicity, cytotoxicity,

genotoxicity, estrogenic activity and so on (13). Fish, bacteria, crustaceans, and alga have been used in the bioassay methods (14,15). Most of the traditional acute toxicity tests were costly, time-consuming, and ethically questionable (15,16).

As bacteria are cheap to cultivate, grow rapidly, technically simple, require low space compared with fish bioassay, available and contain enzymatic and physiological processes, also found in larger organisms, they are good bioassay tools. Bacteria respond more than most other organisms to change their condition and sensitive to toxicity compound (17).

Many researchers studied the toxicity effects of different contaminants by bioassay. Residual chlorine in water affects the behavioral responses of *Daphnia magna* (18). Based on the toxicity categories generated by the United States Environmental Protection Agency, acute and chronic toxicity of some of the DBPs for *Daphnia Magna*, *Cyprinodont Variegatus*, and *Isochrysis Galbana* is slightly or non-toxic (19). The results obtained from SOS/Umu test showed that chlorination of drinking water can damage deoxyribonucleic acid (DNA) (20).

Chlorination of wastewater effluent had ecotoxicity effects but did not have a toxic impact on *Daphnia Magna* or any ecotoxicity by the THMs (21). To evaluate the toxicity of linden, mercury, and wastewater, dissolved oxygen depletion (DOD) and optical density (OD) bioassays were used. It was revealed that DOD and OD bioassays can evaluate the toxicity of the above-mentioned contaminants (17). Conventional techniques were precise but expensive, and need special equipment and trained personnel (16).

Due to the challenges of toxicity assessment, almost no studies have provided a complete toxicity assessment for chlorinated aqueous solution up to now. In this study, the toxicity of chloroform as an indicator of THMs was assessed using DOD and OD as rapid and user-friendly toxicity bioassays. Using these experimentally determined toxicity bioassays, the toxicity of chloroform on the mixed culture of bacteria was interpreted by AQ and AR indices.

Materials and Methods

Preparation of bacterial inoculum

The activated sludge was collected from aeration tank of a full-scale municipal wastewater treatment plant (Isfahan South WWTP, Isfahan, Iran) and was used as a biological inoculum. At first, approximately 1 ml of the activated sludge was transferred onto the prepared nutrient agar plates and incubated at 37°C for 24 h. Then, the grown colonies were suspended into 150 mL of nutrient broth culture and incubated at 37°C for 24 hours. After incubation, in order to provide the active and stabilized mixed culture of bacteria and keep the bacteria in the logarithmic growth phase, about 1 mL of the suspension of nutrient broth culture and bacteria were transferred to a fresh nutrient broth every day for two weeks. According to the previous study, *Pseudomonas*, *Micrococcus*, *Bacillus*, *Aeromonas*, and *Nitrobacter* spp., are the dominant species of bacteria in the activated sludge (22).

Toxicity assessment methods

In this study, two rapid techniques including DOD and OD bioassays were used to evaluate the chloroform toxicity of aqueous solution according to the methods described by Salama and Salem (17).

DOD experiment

Before experiments, the OD of the prepared bacteria inoculum was adjusted to 0.18 to 0.2 at a wavelength of 600 nm with dilution in distilled water. After preparation of synthetic chloroform solution with the initial concentration of 100 to 1000 µg/L, 50 ml of bacteria inoculum was mixed with 300 mL of test solution and transferred to sterile glass flasks. The amount of dissolved oxygen (DO) was monitored every 30 seconds with a dissolved oxygen sensor (model 5740 sc membrane, HACH, USA) until DO amount reached 50% of the initial DO amount.

OD experiment

The OD microbial bioassay was performed on synthetic wastewater containing chloroforms. The reduction of OD of the bacterial inoculum was measured using a spectrophotometer (2 OD spectrophotometer, Milton Roy Company). For this purpose, a serial solution of chloroform with concentrations of 10 to 1000 µg/L was prepared. The experiments were performed by adding 100 mL of chloroform solution to 250 mL sterile glass flasks containing 100 mL of sterile nutrient broth. Then, 0.1 mL of bacterial inoculum were suspended into the above-mentioned sterile glass flasks to obtain the OD of 0.18 to 0.2 at a wavelength of 600 nm, and then, incubated at 37°C for 24 hours. After incubation, the produced turbidity value in the blank and test solutions was measured at a wavelength of 600 nm.

Mathematics

In the DOD experiments, the activity quotient (*AQ*) was calculated according to Eq. (1).

$$AQ(\%) = \left(\frac{T_{50,B}}{T_{50,T}}\right) \times 100 \qquad (1)$$

Where, *AQ* is the activity quotient, $T_{50,B}$ is the time required for 50% reduction of DO in blank solution, and $T_{50,T}$ is the time required for 50% reduction of DO in test solution. The general guidelines for interpretation of AQ and evaluation of toxicity level are summarized in Table 1.

In addition, Eq. (2) was used to compute the reduction of bacterial activity in the OD experiments.

$$AR(\%) = \left(1 - \frac{A_T}{A_B}\right) \times 100 \qquad (2)$$

Table 1. Guidelines for interpretation of AQ

AQ Value (%)	Toxicity Degree
100	Non-toxic
80-94	Slightly toxic
50-79	Moderately toxic
<59	Extremely toxic

Where AR is the percentage of bacterial activity reduction, A_B is the bacterial absorption in blank solution, and A_T is the bacterial absorption in test solution.

Statistical analysis

To evaluate the effect of chloroform on the bacterial inoculum, the chemical concentration with 50% inhibition of bacterial activity (EC_{50}) indicator was used. The standard protocols were used to calculate the dose-response relationships and the obtained data were analyzed using Probit analysis by IBM SPSS statistics version 16 for windows to assess the EC_{50} concentration.

Results

Toxicity assessment by DOD technique

The obtained data on DO depletion during DOD experiments are illustrated in Figure 1.

As illustrated in Figure 1, the time duration for DO concentration depletion in blank test was 68 min. With adding chloroform and increasing its concentration, the time duration for DO concentration depletion was increased. When chloroform at concentration of 100 and 1000 µg/L was examined, time duration for DO concentration depletion expanded to 125 min and 416 min, respectively. This condition revealed that the duration of responses depending on toxicant concentration. The calculated AQ in terms of $T_{50,B}$ and $T_{50,T}$ are presented in Figure 2.

As seen in Figure 2, with increasing the chloroform concentration in test solution, the AQ value was reduced. The AQ of blank test was equal to 100%, indicating a non-toxic condition. When the experiment was conducted at chloroform concentrations of 100 and 1000 µg/L, the AQ values were obtained to be 54.4% and 16.3%, respectively. According to Table 1, the toxicity degree of chloroform at concentrations of 100 and 1000 µg/L were moderately and extremely toxic, respectively. The dose-response graphs for different concentrations of chloroform on the AQ values are shown in Figure 3.

As shown in Figure 3, with increasing the concentration of chloroform, the AQ value was reduced, and the EC_{50} concentration for chloroform by DOD technique was 457 µg/L.

Toxicity assessment by OD technique

The variation of OD during OD experiments is shown in Figure 4.

As seen in Figure 4, with increasing the chloroform concentration in OD experiments, the OD of bacteria was reduced. The higher and lower optical densities were related to blank and test solution of 1000 µg/L, respectively. The variation of AR values during OD experiments as the function of chloroform concentration is illustrated in Figure 5.

As illustrated in Figure 5, with increasing the chloroform concentration, the bacteria activity was reduced. When the initial chloroform concentration increased from 0 to 1000 µg/L, the OD of bacterial inoculum decreased from 52% to 25%. The application of chloroform at concentrations of 10 µg/L led to a decrease (15%) in the bacterial activity. The graphs of dose-response as the function of chloroform concentration are shown in Figure 6.

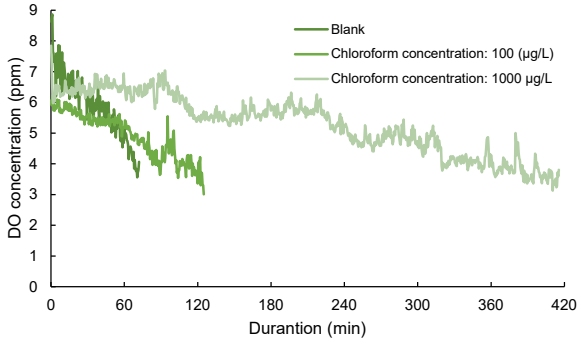

Figure 1. DO profile during DOD experiment.

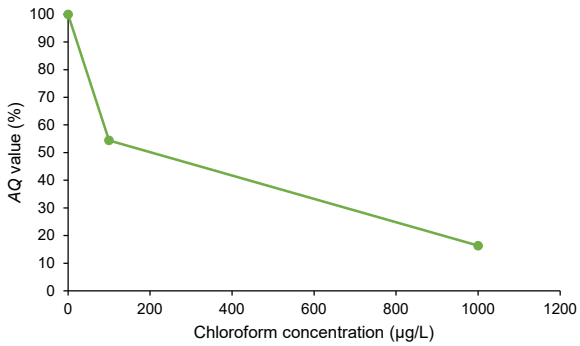

Figure 2. Activity quotients for chloroform during DOD experiment.

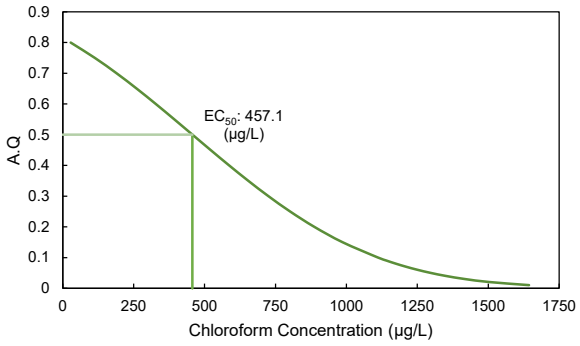

Figure 3. Dose-response curve of DOD.

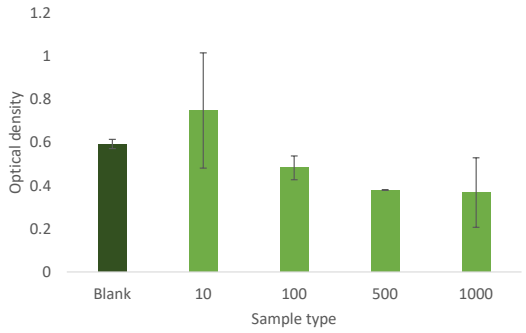

Figure 4. Variation of optical density during OD experiment.

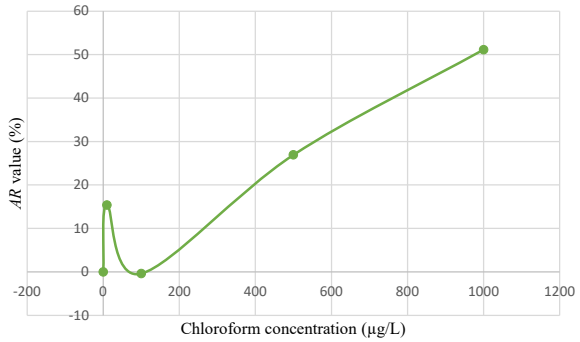

Figure 5. Activity reduction for chloroform during OD experiment.

According to Figure 6, the EC_{50} value of chloroform estimated for a bacterial consortium in OD technique was 961 µg/L.

Discussion

The findings demonstrated that with increasing the chloroform concentration, the time duration for DO concentration depletion in DOD method was increased (Figures 1 and 2). This condition revealed that the duration of responses depending on the toxicant concentration (17,23). The higher concentrations of chloroform caused lower depletion of DO (23). The application of higher concentrations of chloroform caused a significant reduction in the AQ value and noticeable toxicity for bacteria (24). According to the results of other studies, some pollutants allowed dissolved oxygen deplition and DOD methods to assay the toxicity of some heavy metals and trichloroacetic acid (23). The EC_{50} for chloroform by DOD technique was obtained to be 457 µg/L. Cao et al evaluated the toxicity of chlorinated effluents on some organisms and reported that chlorination evaluated acute toxicity and genotoxicity of wastewater and mortality of invertebrates. They also reported that the EC_{50} for chloroform on *Dafnia magna* was equel to 573.01 ppm (24), indicating that chloroform was more toxic for consortium of *D. magna*, and DOD method is sensitive enough to measure the toxicity of chloroform. In another study by Wu et al on the effects of THMs on Q67 luminescent bacterium, the EC_{50} was obtained to be 318 µg/L (25), confirming the toxicity of THMs.

During OD method, the higher concentrations of chloroform led to a decrease in the OD of bacteria, however, the variation of *AR* value of blank was slight. Recent studies have demonstrated that several pollutants had a toxic function for aquatic microorganisms and with increasing the concentration of contaminants, their activity reduced (15). The EC_{50} of chloroform for a consortium of bacteria in OD technique was 961 µg/L (Figure 6). Zare et al (22) evaluated the toxicity of industrial wastewater containing heavy metals on the bacterial consortium extracted from sequencing batch reactor (SBR) and reported that the EC_{50} and NOEC values were 6.43 and 1.35 ml/L, respectively, indicating that bacterial consortium was a suitable tool for bioassay.

Da Costa et al (26) evaluated the toxicity of secondary effluent disinfected with chlorine on aquatic organisms and found that effluent disinfected with chlorine was toxic for *Daphnia similis* and *Ceriodaphnia silvestrii* (cladocerans) and reported that the calculated EC_{50} values were 0.04 and 1.28 mg Cl/L, respectively (26). Based on the obtained results, the DOD toxicity assay was more sensitive than the OD toxicity assay so that it showed the toxicity effect at lower toxic concentrations, which is consistent with the results of other studies (17).

Recently researchers have found that chloroform decreases methylation that leads to the overexpression of proto-oncogene. Generally, it was suggested that THMs cause a decline in the methylation of the c-myc proto-oncogene, which lead to the uncontrolled expression of gene sequence (4). Direct contact by oral gavage with chloroform caused tumorigenesis (4) and hepatocellular tumor in mice; and in combination with inhalation, produced renal tumors in rats (7).

Chloroform did not cause mutations in bacteria and failed to induce chromosome damage or sister-chromatid exchanges in human lymphocytes. There is no evidence indicating that chloroform is capable of alkylating DNA (27). Researchers have shown that chloroform increased the proliferating cell nuclear antigen-labeling index and reduced the level of 5-methylcytosine in hepatic DNA in

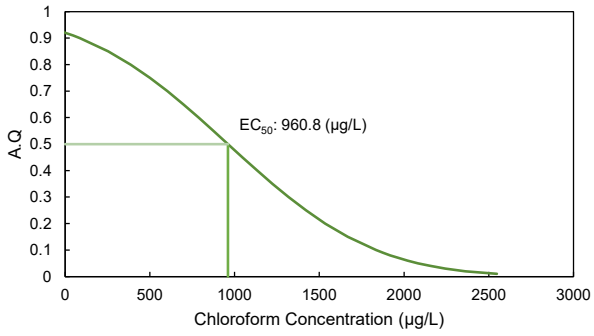

Figure 6. Curve of dose-response for OD experiments.

B6C3F1 mouse (28).

Conclusion

In the present study, the toxicity of chloroform as an indicator of THMs was assessed by DOD and OD as the rapid and user-friendly toxicity bioassays. In addition, the toxicity of chloroform on the mixed culture of bacteria was interpreted by the AQ and AR indices. According to the results, the expanded time for DO concentration depletion was observed by increasing the concentration of chloroform, which is consistent with dissimilar experimental results indicating that the OD of bacteria was decreased with increasing the chloroform concentration. The EC_{50} values estimated for DOD and OD bioassays were 457 and 961 µg/L, respectively. The application of chloroform at concentrations of 100 and 1000 µg/L showed moderate and extreme toxicities, respectively. Compared to OD bioassay, the DOD bioassay was more sensitive so that it could show the toxicity effect at lower toxic concentrations.

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Ethical issues

The authors certify that this manuscript is the original work of the authors, all data collected during the study are presented in this manuscript, and no data from the study has been or will be published elsewhere separately (Ethical code: IR.MUI.REC.1369.3.732).

Competing interests

The authors declare that they have no conflict of interests.

Authors' contributions

All authors contributed and participated in the data collection, analysis, and interpretation. Also, they critically reviewed, refined, and approved the manuscript.

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