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EFFECT OF AROMATIC HYDROCARBONS ON *CHLORELLA VULGARIS* GROWTH

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Abstract

Currently, there are multiple reports of the use of microorganisms to remove hydrocarbons. However, few reports evaluate the effect of benzene, toluene, and xylene on microalgae growth. Benzene, Toluene, and Xylene are aromatic hydrocarbon mixtures, commonly known as BTX. For this work, the effect of different concentrations of benzene, toluene, and xylene on microalgal growth was evaluated, starting with an inoculum of 8 x10^6 cel/ml, after that, the microalgae were cultivated for 8 days, during which a cell count was performed, and the kinetic parameters of biomass growth were determined. The results have shown that the microalgae known as *C. vulgaris* had higher growth in the presence of Toluene at 0.50 mg/l, meanwhile, the benzene and xylene treatments in the concentrations used, were under the control treatment.

Key words: *Chlorella vulgaris*, Benzene, Toluene, Xylene.

Introduction

Chlorella vulgaris is a unicellular green freshwater alga, whose cells appear isolated, although they may eventually form aggregates. It is a coccoid-type algae characterized by having a spherical shape and a size ranging from 2 and 6 µm (Lv *et al*., 2010); it belongs to the Eukarya domain, of the kingdom Plantae, in the division of the Chlorophyta (Guiry, 2019). This microalga has been exposed to various aromatic hydrocarbons such as 4-nonylphenol; 4-octyl phenol, and β-estradiol (Gao & Tam, 2011; Perron & Juneau, 2011), regarding the nonylphenol bacterial culture, it was found that *C. vulgaris* possesses the capacity to degrade it without suffering the toxic effects up to 96 hours of exposition, indicating that the microalgae was capable of damage recovering for this compound on the first days of exposure. De Morais *et al.* (2014) evaluated the response of the cyanobacteria *Mycrocystis aeruginosa* and *C. vulgaris* to pentachlorophenol (PCF), funding that microalgae are insensible to concentrations of 0.02, 0.10 y 0.99 µg/l, however, over the concentration of 0. 99 µg/l toxic effects were reflected as the progressive diminution of the cell concentration with the increase of the PCF. Rodríguez *et al*., (2014) and Cándido & Lombardi (2016) reported the growth of *C. vulgaris* on distillery vinasses, which present a dark brown color and are rich in phenolic compounds. Candido & Lombardi (2016) observed that changes in the higher microalgae growth were at the lower concentration on the vinasses. It was discussed that the darker tone of the vinasse could have photosynthetic effects on *C. vulgaris* and its growth rate. Xiong *et al*., (2016) evaluated the biodegradation capacity of *C. vulgaris* per acclimatization with multiple concentrations of levofloxacin (LEV) with third-generation fluoroquinolone antibiotic (FQ) that was detected in aquatic environments. Levofloxacin has insignificant effects on the growth of *C. vulgaris* under 5 mg/l, and the microalgae could recover its growth rate with high LEV concentrations (even up to 300 mg/l).

Microalgae such as *C. vulgaris* have interesting metabolic properties like the capacity for fast growth, simplicity of nutritional requirements, tolerance to extreme conditions, ability to synthetic and secrete some metabolite, and the potential of genetic manipulation that confers it a biotechnological interest (Borowitzka, 2013). As previously mentioned, the microalgae used for the degradation of environmental pollutants is part of those biotechnological interests by being presented as a potential strategy for the remediation of a wide variety of environmental contaminants in air, water, and soil (Ahmad *et al*., 2013; Muñoz & Guieysse, 2006) like those generated by the oil industry (Subashchandrabose *et al*., 2013). The group of volatile aromatic hydrocarbons known as BTX, formed by benzene, toluene, and xylene originated and emitted into the atmosphere during different anthropogenic activities. Their presence is reported in air, soil, and water (Aburto & Ball, 2009; Shinohara *et al*., 2019; Hicklin *et al*., 2018 and Anjos *et al*., 2021) those compounds have been classified as genotoxic, mutagenic, and carcinogenic (Edokpolo *et al*., 2014 Chaiklieng, 2021).

This study aimed to evaluate the effect of the BTX compound on the growth of the microalga *Chlorella vulgaris* under laboratory conditions.

Material and Methods

Microalga sample: Samples of microalgae *Chlorella vulgaris*, strain GVG001 were utilized. The strain was obtained from the Continental Algae Laboratory of the National Autonomous University of Mexico (UNAM). The acquired culture presented a volume of 15 ml and a cellular concentration of 85,000 cells/ml. subsequently, the propagation of the culture was carried out in a 250 ml capacity Erlenmeyer flask in a Bold basal modified medium, with a light intensity of 3000 luxes and a photoperiod of 12/12 hours (light/dark).

Culture medium: The reactive utilized in the project were reactive grade. The culture medium used for the propagation and aromatic hydrocarbons tests were prepared from stock solutions, that presented the following composition (g/l): 25 of NaNO3; 2.5 of CaCl2*7H2O; 7.5 of MgSO4*7H2O; 7.5 de K2HPO4; 17.5 of KH2PO4; 2.4 of NaCl; 50 of EDTA*Na; 31 of KOH; 4.98 of FeSO4*7H2O; 11.4 of H3BO3; 1.8 ml/l of H2SO4 y 1 ml/l of the micronutrients solution. Micronutrients solution presented the following composition (µg/ml): 8.82 of ZnSO4*7H2O; 1.44 of MnCl2*4H2O; 0.71 of Na2MoO4*2H2O; 1.57 of CuSO4*5H2O and 0.35 of CoCl2*6H2O.

Pollutant samplings: The solvents benzene, toluene, and xylene (Meyer Mexico TM) were used with a purity of 99, 98, and 96% respectively. To get to the treatment concentration, pattern solutions were prepared initially in a concentration of 87.60 mg/l benzene, 87.6 mg/l of toluene, and 86.50 mg/l of xylene, in the Bold basal modified culture medium.

Design of the biological treatment: The microalga growth in the presence of pollutants was evaluated using growth curves, through a fully randomized blocks design as shown in (Table 1).

Table 1. Fully randomized blocks design with BTX in different concentrations.

	BTX Pollutans					
Control	**Benzene (mg/l)**		**Toluene (mg/l)**		**Xylenes (mg/l)**	
BBM	0.35	0.50	0.50	0.70	0.50	0.70

Initially, cultures of the cellular density of 8 x10^6 cell/ml were obtained in a 50 ml flask, in which different volumes of the pattern solutions of benzene, toluene, and xylene were added; after that, the flask was calibrated to 100 ml with culture medium to get the hydrocarbons concentration indicated in table 1. Then it proceeded to homogenize the sample and, employing a graduated pipette, volumes of 7 ml were taken and then transferred to the experimental units consisting of test tubes with a 12 ml volume. The opening of test tubes was covered with septa rubbers and reinforcement of screw caps to ensure hermetically closure. Every treatment was done in triplicate. For the pattern solution of benzene, 400 and 570 µl looking to obtain concentrations of 0.35 y 0.50 mg/l respectively. For the toluene treatment 577 and 807 µl of the corresponding pattern, solutions were added, to obtain a final concentration of 0.50 and 0.70 mg/l correspondingly. Finally, to obtain 0.50 and 0.70 mg/l concentrations of xylene, volume 578 and 809 µl of the pattern solution were used.

Measure of the microalgal growth: The method used to quantify the growth of microalgae *C. vulgaris* was through the change in the cellular density using a Neubauer camera. This method is based on the microscopic enumeration or accountability of individual cells on the microscope. Homogenic samples for measure were taken for the cultures in the experimental samples using an insulin syringe that was inserted in the rubber septa.

Scientific parameters: The kinetic parameters calculated were the speed of growth (µ), cellular productivity (Qx), and duplication time (td) from the cellular growth data. For the obtention of parameters, it was necessary to do the treatments with natural logarithms in their exponential phase, obtaining a pendant that corresponds with the value of µ; td was got calculating the equation 1 (Eq. 1) y Qx using the equation 2 (Eq. 2) (Lee, 2002).

$td = \ln 2/\mu$) (Eq.1)
$Qx = \Delta c/\Delta t$ (Eq. 2)

Being:
ΔC: The variation of the biomass concentration in cells/ml.
Δt: The evaluated time of growth.

Results and Discussion

Growth curves of *C. vulgaris* in benzene: (Fig. 1) shows cellular growth observed in the treatments with benzene to a concentration of 0.35 (B 0.35) and 0.50 (B 0.50) mg/l, along with the treatment without hydrocarbons (BBM). In BBM a steep phase of growth from day 0 to day 2, concerning the treatments with benzene was observed a small exponential growth phase from day 0 to day 3 was in both cases, besides a lower cellular density. Even if the cultures started with a similar cellular density, the ones that had benzene presented an inferior growth compared to the obtained with the control treatment. Due to the symmetrical structure of benzene, its dipole moments are equal to zero, meaning that the polarity of the individual bonds and the contributions of unshared electron pairs cancel out exactly. As a result, the molecule less reactive on its own (McMurry, J. 2012). According to Othman *et al*., (2023) the presence of difficult-to-degrade aromatic hydrocarbons significantly disrupts vital processes like photosynthesis. This disruption can trigger excessive production of reactive oxygen species (ROS), which be related to the structural and functional damage (Xiong *et al*., 2016 and Wang *et al*., 2018). For the benzene exposure experiments, this might induce oxidative stress, prevent cellular growth density and cause damage to the cellular organizes implicated in the assimilation of CO2 (Xiong *et al*., 2016 and Wang *et al*., 2018). According to Wiessner (1970) and Droop (1974), enzymatic deficiency is the principal reason for the lack of use of organic carbon sources, which could show, in this study, that *C. vulgaris* doesn't have the required enzymes to lead to degradation of benzene. Additional strategies could be employed such as first altering the stability of the benzene molecule and subsequently the use of microalgae, which could allow benzene degradation to take place.

Growth curves of *C. vulgaris* in toluene: The growth curves of the toluene treatments were performed in a concentration of 0.50 (T 0.50) and 0.70 (T 0.70) mg/L. As shown in (Fig. 2), it was observed that both treatments were superior to BBM, and treatment T 0.70 presented a phase of cellular death on the first day, the cellular culture was recovered on day 2, where the exponential growth phase began. The increase in microalgae growth may be caused by the consumption of toluene. According to the database KEGG, aerobiotic degradation of toluene in bacteria leads to the formation of phenolic compounds, which serves as a substrate in a metabolic process like the Krebs cycle, by ortho excisions or catalyzed meta by catechol-oxygenase enzymes in some microalgae such as *Isochrysis galbana*, (Wang *et al*., 2018). According to Concha *et al*. 2018, some hydrocarbons provoke instability in the cellular membrane which cause a higher transference of compounds such as nutrients and oxygen between the culture medium and the microorganisms, this item was reported for the microalga *Botryococcus braunii*, the instability can be caused by some solvents, explaining also the exponential growth obtained.

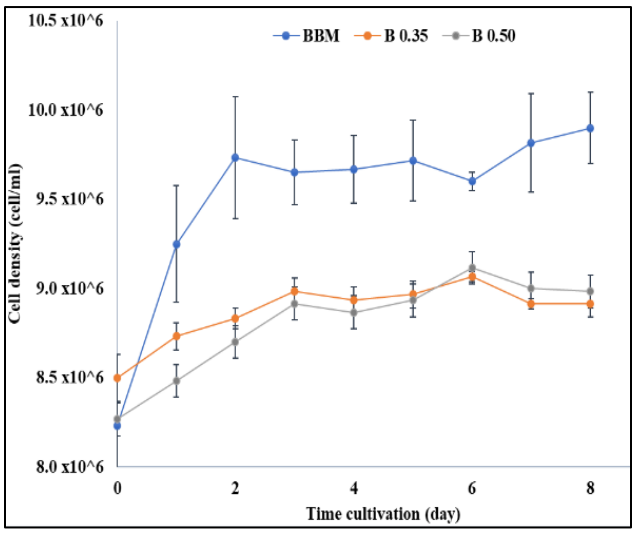

Fig. 1. Microalgae growth in benzene presence. B 0.35 corresponds to the concentration of 0.35 mg/L, while B 0.50 corresponds to the concentration of 0.50 mg/l benzene.

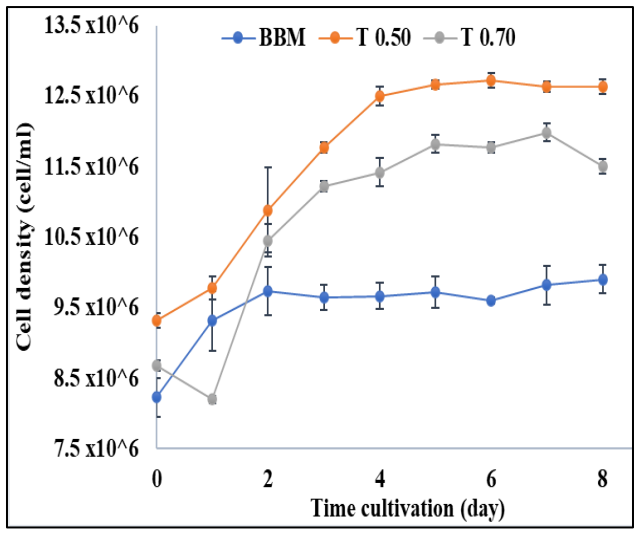

Fig. 2. Microalgae growth in toluene presence, T 0.50, corresponds to a concentration of 0.50 mg/L, while T 0.70 corresponds to the concentration of 0.70 mg/l of toluene.

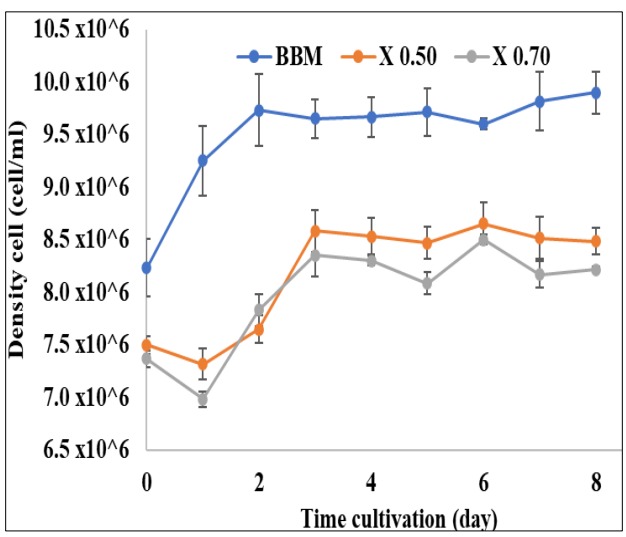

Fig. 3. Growth of microalga in presence of xylenes. X 0.50 corresponds to a concentration of 0.50 mg/L, while X 0.70 corresponds to the concentration of 0.70 mg/l of xylenes.

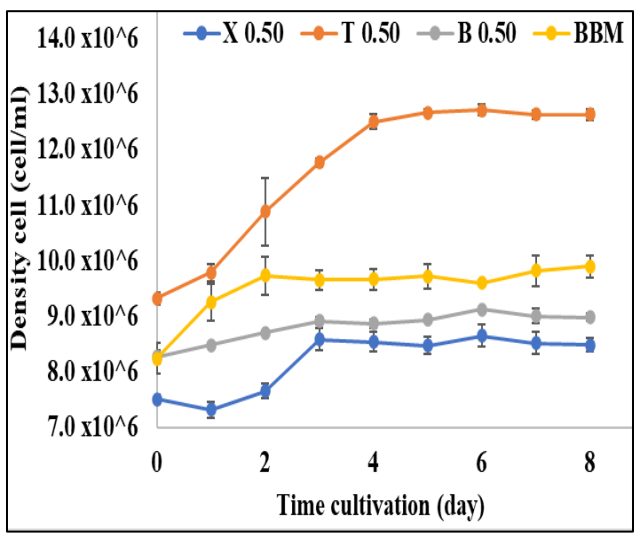

Fig. 4. Growth of the microalga in presence of BTX compounds, concentrations of 0.50 mg/l each.

Growth of Curve of *C. vulgaris* in xylenes: The growth curves observed in (Fig. 3) show that the xylene treatment to a concentration of 0.50 (X 0.50) and 0.70 (X 0.70) mg/l, it was found that the xylene treatments were inferior to the control treatment, which showed a phase of cellular death at day 1, followed by an exponential phase until day 3, and finally a stationary growth phase. The volatile aromatic hydrocarbons in high concentrations have a narcotic effect on the cell, in the microalga *Chlamydomonas reinhardtii* it is reported that they increase the fluency in the cellular membrane and inhibit the electrons transport that can be for changes in the thylakoidal membrane, followed by the loss of light collector pigments (Brack *et al*., 1998), this can provoke cellular lyses and the decrease of the microalgal growth. Duan *et al*. (2017) discuss that phenolic compounds can interfere with the formation of the chlorophyll-protein complex (LHC) that captures light in photosystem II (PS II) and its reduced photosynthetic efficiency and light absorption in microalgae, as also noted by Cho *et al*. (2016). As mentioned earlier, the degradation processes of aromatic hydrocarbons often generate reactive oxygen species (ROS), which can damage the cell membrane and lyse the cell (Cabiscol *et al*., 2000). Additionally, Gill and Tuteja (2010), point out that the accumulated ROS can further cause cell structure damage by attacking proteins, lipids, carbohydrates, and DNA due to its highly reactive and toxic nature.

Comparation of *C. vulgaris* growth on BTX: The following graphic shows the treatments that were made with benzene, toluene, and xylene separately at the concentration of 0.50 mg/l., it can be observed that the treatment with toluene is superior to the rest of the treatments, even the treatments with benzene and xylene are under the BBM treatment. The increase in the toluene growth suggests the capacity of microalgae to degrade this

compound and use it as a carbon source and energy. Semple and Cain (1995 and 1996) evaluated the phenol, cresol isomers, and xylenol isomers remotion (all aromatic hydrocarbons) and found that the number of substituents plays an essential role in the recalcitrance of those compounds, making them more susceptible to enzymatic attacks or that the action cannot be performed due to steric impediments of the molecule, founding easier to degrade p-cresol, following of o-cresol and finally from m-cresol. In (Fig. 4) it is observed that there is a higher growth of the microalga in presence of toluene than in xylene, which can be related to the number of substituents, besides the different positions that can cause some xylene isomers being more susceptible to the enzymatic attack.

Kinetic parameters: The kinetic parameters obtained from the treatment selecting the natural logarithm of the growth curves, in the exponential phase of each treatment are shown in (Table 2). It is significant to mention that each treatment shows different phases of exponential growth, while some of the curves showed a greater growth, others show poor microalgal growth. It is observed that the treatments with benzene and xylenes the higher growth speed (μ), were obtained with the control treatment, as well as the duplication time (td), and productivity (Qx). Regarding toluene treatments, the higher speed of growth (μ), duplication time (td) and productivity (Qx) were reached with the treatment of 0.70 mg/l (T0.70).

Table 2. Kinetic parameters of the growth curves in the exponential phase with the BTX treatments.

Treatment	μ (day $^{-1}$)	Td (day)	Qx (cell*day/ml)
BBM	0.0837	0.04185	208333
B 0.35	0.0177	0.00885	52083
B 0.50	0.0252	0.0126	89583
T 0.50	0.0772	0.0386	414583
T 0.70	0.1566	0.0783	352083
X 0.50	0.0798	0.0399	122916
X 0.70	0.0894	0.0447	106250

Conclusions

Microalga *Chlorella vulgaris* had the capacity of growing in a culture medium polluted with benzene, toluene, and xylene. There was a higher growth in the presence of toluene in concentrations of 0.50 y 0.70 mg/l, which suggests a possible metabolic path to use the compound as a carbon source.

The higher productivity (414583 cells a day/ml) was obtained with the toluene treatment at a concentration of 0.50 mg/l. The obtained results show that it is possible to use the microalga *C. vulgaris* as a remotion agent of aromatic compounds such as toluene.

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