PRELIMINARY STUDY REGARDING THE INFLUENCE OF BFA ON SOME MICROORGANISMS

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Plastic is one of the most used materials now, its usefulness being indisputable. Packages and plastic waste represent a serious problem for the society, because of it reached an amount too huge for the natural environment and it affects life. Polycarbonate and epoxy plastics used for food, beverages, water packaging, several dental sealants and composites contain bisphenol A (BPA) as additives or monomers. The presence of this compound in environment determines long-term pollution and life perturbation, which represent an endocrine-disrupting for human.

In this paper, experiments were performed to examine the toxicity and antimicrobial effect of BFA with different concentrations on different types of microorganisms from soil, mesophilic bacteria, Streptococcus faecalis and fungi. It has been found that microorganisms mesophilic bacteria and Streptococcus faecalis from soil are affected by the presence of BFA. Lower BFA concentrations of the order of 10^{-4}mg / L have a greater influence on the populations of microorganisms, but after 70-90 days, they adapt and repopulate the environments. At concentrations of 1 mg / L of BFA, the disturbing action is maintained for a longer period, even if it is lower at the beginning, and constantly affects the populations of microorganisms. There are also microorganisms that are not affected by BFA and which can be potential its degraders.

Keywords: plastics, bisphenol A, microorganisms' exposure, toxicity, and antimicrobial activity

1. Introduction

Bisphenol A was first synthesized in 1891, it was produced by condensing two molecules of phenol with one molecule of acetone in the presence of HCl and ion exchange resin. The resin acts as a catalyst in the acidic media at temperatures between 60 and 80 ° C, at a molar ratio phenol:acetone ranging from 3: 1 to 10: 1.

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Following the condensation reaction, it results a mixture of p, p-BP (bisphenols) and its o, p-BP (bisphenols) isomer, as well as small amounts of impurities, including triphenyl and polyphenols, formed by the reaction of phenol with mesityl oxide. This is a self-condensing product of 3 molecules of acetone, followed by dehydration [1].

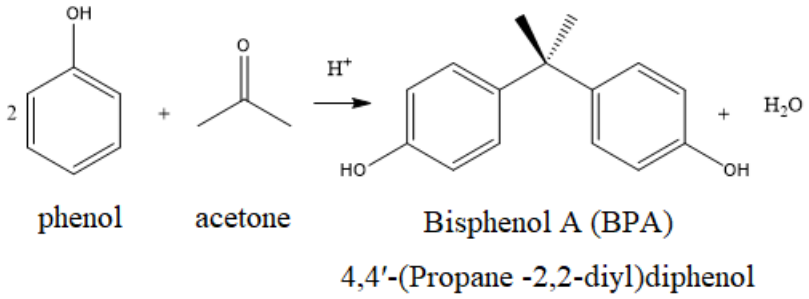
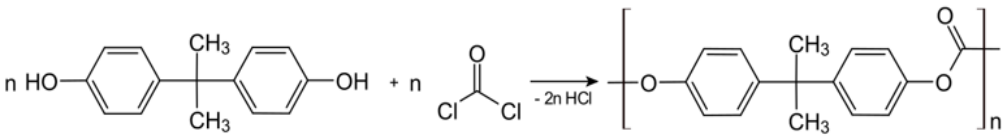


Fig. 1. Equation of the chemical reaction of BFA formation [1]

In 2022, BPA production will reach about 10 million tons, one of the largest until now [2]. Since 1940, it has been used in thermal packaging, plastic containers and, later on, in the industrial production of Digital Video Disc (DVD). It has attracted special attention through mass production, and because the plastic waste is released into the environment by human activity [3]. Nowadays, BPA is used to obtain polycarbonate plastics and epoxy resins, for their synthesis, using dimethylcarbonate [4] or phosgene [5]. Thus, the plastics obtained are characterized by hardness and clarity, being used in a wide range of consumer goods: sports equipment, water containers, DVDs, CDs. BFA-based epoxy resins are used in the manufacture of thermal paper, as coatings inside beverages and food cans, as well as for the drinking water supply network [6].



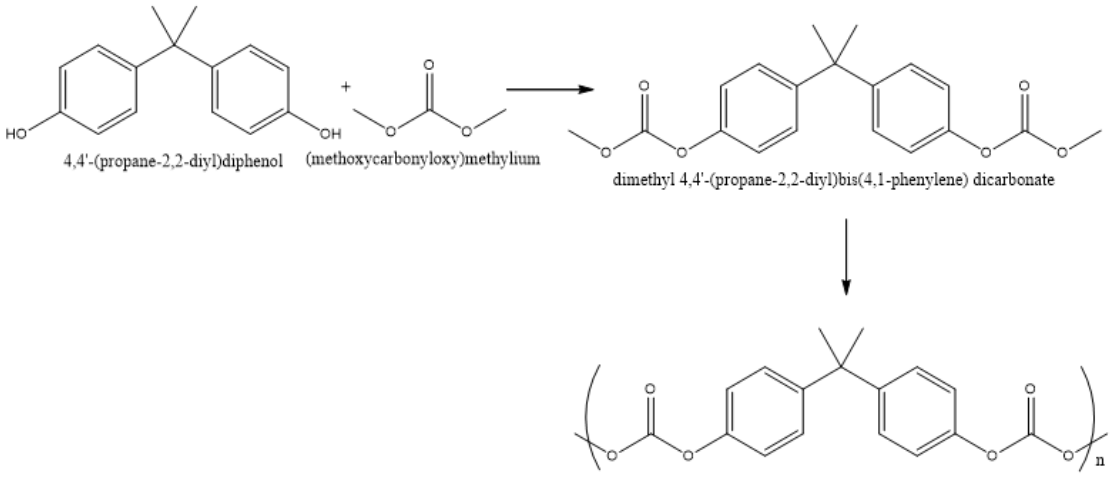


Fig. 2. Synthesis of polycarbonates [5]

Industrial products containing bisphenol are ubiquitous in the environment. Bisphenols tend to accumulate in aquatic organisms and to bio magnify, so the pollution they cause in surface waters has attracted considerable attention. In China, values of BFA concentrations of 96 ng /L were recorded from the analysis of surface water samples [7, 8, 9]. BFA toxicity is obvious for all microorganisms, being correlated with the concentration and time of BFA addition [10]. Bisphenols A disrupt the soil homeostasis and reduce the abundance of *Proteobacteria sp*. and *Acidobacteria sp.,* while increasing that of *Actinobacteria sp.* [11]. The mechanisms of bisphenol toxicity for microorganisms are limited. A posible way to actn is the binding of BFA by intercalation into bacterial membranes cells, blocking lipid synthesis [12].

BFA in the environment is decomposed mainly by a variety of microorganisms. More and more BFA-degrading bacteria have been isolated, identified, characterized and used for BFA treatment in sewage treatment plants. Bacterial strains that degrade BFA have been isolated from soils, sludge, rivers, sea water and even food samples. These bacterial strains are capable of growing on BFA, as the sole source of carbon and energy included gram-negative strains *Sphingomonas sp., Pseudomonas sp., Achromobacter sp., Novosphingobium sp., Nitrosomonas sp., Serratia sp., Bordetella sp., Alcaligenes sp., Pandoraea sp., Klebsiellasp. and Cupriavidus sp.* and gram-positive strains *Streptomyces sp. and Bacillus sp.* [13, 14]. Bacteria with high BPA biodegradability are limited. Many environmental factors such as: the amount of biomass, temperature, pH, metal ions, biological compounds, and oxygen affect the degradation efficiency of bacterial strains [15].

In this paper, we study the toxicity and antimicrobial activity of BFA with different concentrations, between 1 mg/L and 10^{-4} mg/L, on some microorganisms present in water and soil.

2. Materials and methods

Sterilised reagents and glass were used in the experiments.

2.1. Preparation of BFA solutions

For conducting the experiments, solutions with the following BFA concentration were prepared: 1 mg / L, 0.1 mg / L, 0.01 mg / L, 0.001 mg / L, 0.0001 mg / L. Preparation methods: in the first step, a stock solution of 1 mg BFA/ L was prepared. 0.10 g of BFA (Merk) was weighted and placed in a 100 mL volumetric flask. 30 mL of ethanol was added until the BFA was completely dissolved. Then, distilled water was added up to 100 mL. The working solutions with the concentrations: 0.1 mg / L, 0.01 mg / L, 0.001 mg / L, 0.0001 mg / L were prepared from the stock solution.

2.2. Inoculum preparation

To prepare the soil inoculum for bacteria and fungi, a sample of 2 g of dry soil was used, which was introduced into a sterile Erlenmeyer flask and diluted with 200 mL of distilled water. It was kept in contact for 2 hours under stirring, then the suspension was filtered. The resulting clear liquid was used as inoculum with dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}.

The inoculum for mesophilic bacteria and *Streptococcus faecalis* was obtained using a microbiological product grown on nutrient Agar at 37 °C and sodium azide broth, respectively, which was further diluted. Dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were performed. Sterile phosphate buffer solution was used for dilutions, and dilutions were performed according to the rule of successive dilutions.

2.3. Toxicity of BFA

Toxicity was tested on three categories of microorganisms: *Streptococcus faecalis,* mesophilic bacteria, soil bacteria and fungi. The antibacterial activity was tested as well, using *Streptococcus faecalis,* mesophilic bacteria, soil bacteria.

Samples inoculation was performed on sterile culture media specific for bacteria - simple Agar (GN) and for fungi potato starch Agar (GAC).

2 cm^3 of sterile distilled water were distributed in each sterile Petri dish, both for the sterilization control sample of simple Agar, and for potato starch, and

without BFA. 200Then, samples were obtained with the inoculum 1 cm^3 of the dilutions mentioned above, and BFA with concentrations of 0.1 mg / L,
0.01 mg / L, 0.001 mg / L, 0.0001 mg / L. The samples were duplicated for both soil bacteria, mesophilic bacteria, *S. faecalis* and fungi. Specific culture media, melted at temperatures below 50 °C, were distributed over samples of different concentrations in inoculum and BFA, and the method of incorporating the liquid sample into solid medium was used. After the culture media hardened, the resulting samples were incubated in Memmert incubator at 37 °C for 96 hours. The number of colonies-forming units, CFU / mL, were countered at different time moments. The experimental results are presented in Figs. 3-5.

2.4. Antimicrobial activity of BFA

The experiments were performed using the diffusometric antibiogram method, adapted to Mueller-Hinton culture. The Agar medium was seeded with bacteria. The 10 µL of BFA with different concentrations were contacted with discs of 6 mm diameter respectively, and these are placed multiple times on the surface of the environment.

The test substances, together with the appropriate controls and blanks, were aerobically incubated for 96 hours at 37 ^oC in the Memmert incubator. The reading was performed by examining the plates on the Colony Counter Stuart device. After the measurement on 2 diameters, the arithmetic means per test and the arithmetic mean on the type of extract were computed. The results were showed in Figs. 6-8**.**

3. Results and discussions

3.1. Toxicity of BFA

It can be seen that BFA has a toxic effect on *S faecalis* causing a decreasing of their number (see Fig. 3). For example, a concentration of 1 mg / L of BFA has a less toxic effect than a concentration of 10^{-4} mg / L. At a concentration of 1 mg / L the inhibitory effect is manifested for the first 48 hours, after which the number of CFUs increases to values comparable with BFA-free sample. This proves that, at high concentrations of BFA, *S. faecalis* adapts more easily and is not affected by its toxicity. At a concentration of 10^{-4} mg / L, BFA the populations of microorganisms are most affected, the adaptation period exceeds 80 hours, after which they suffer mutations that favor their adaptation to the environment and their multiplication.

Bacteria from the soil are influenced by the presence of BFA at concentrations of 1 mg / L (see Fig. 4). It is adapted to the new conditions after a

period of 96 hours, and their number increases to over 50 CFU, when the concentration of bacteria in a BFA-free environment reaches around 380 CFU.

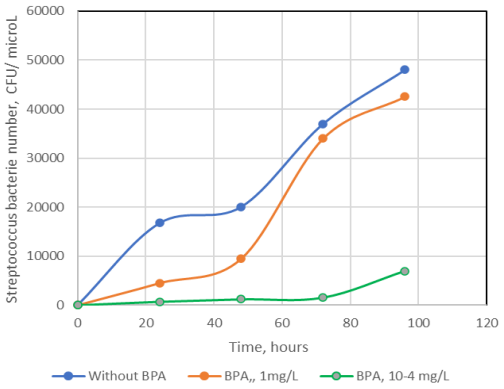


Fig. 3. Variation of number of CFU of *S. faecalis* in time at three concentrations of BFA: 0, 1 mg / L and 10^{-4} mg / L

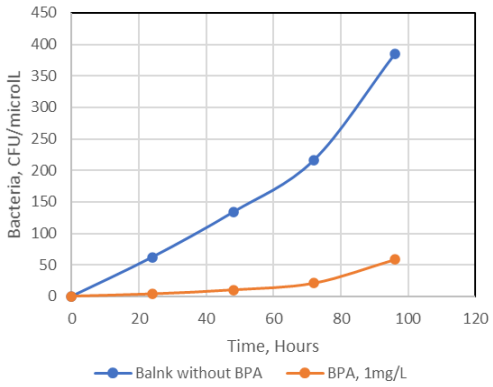


Fig. 4. Variation of CFU of mezophyl bacteria in time at two concentrations of BFA 0 and 1 mg / L.

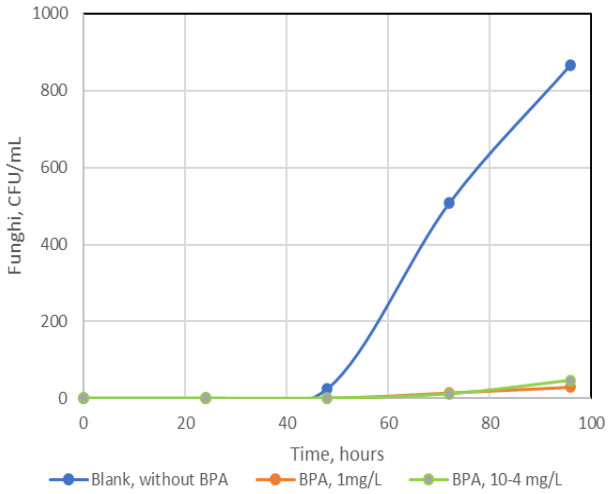


Fig. 5. Variation in time of number of fungi CFU at tree concentrations of BFA 0, 1 mg / L and 10^{-4} mg / L

Environmental fungi are influenced by the presence of BFA. Fungi appear on the medium of growth after 48 hours, both in the presence and in the absence of BFA. The presence of BFA inhibits the formation of fungi colonies, a concentration of 1 mg / L of BFA causes a toxicity higher than a concentration of 10^{-4} mg / L, but not significantly more important (See Fig. 5).

3.2. Antimicrobial activity

Antimicrobial activity of BFA on the microorganisms from soil, mesophilic bacteria and *S. faecalis* was studied. For describing the phenomena, Figs. 6, 7 and 8 show the variation of diameter of the inhibition zone in time.

From the analysis of Figs. 6 - 8, it can be observed that, in accordance with the conventional semi-quantitative interpretation, the diameter of inhibition zone is below 20 mm, the same microorganisms are sensitive to the effect of BFA.

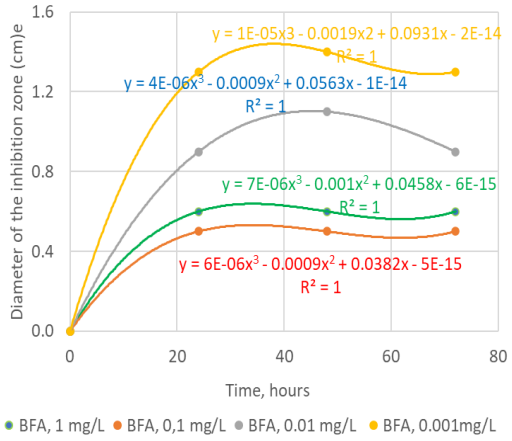


Fig. 6. The variation of diameters of inhibition zone for bacteria from soils

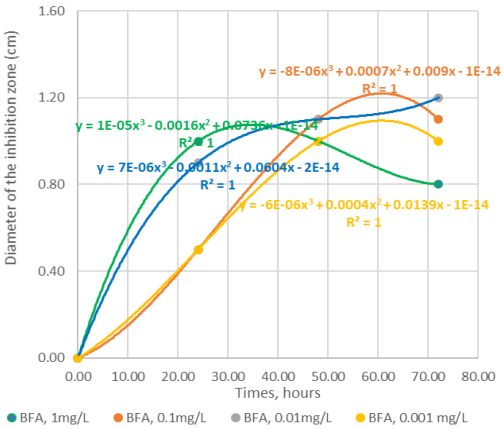


Fig. 7. The variation of diameters of inhibition zone for mesophilyc bacteria

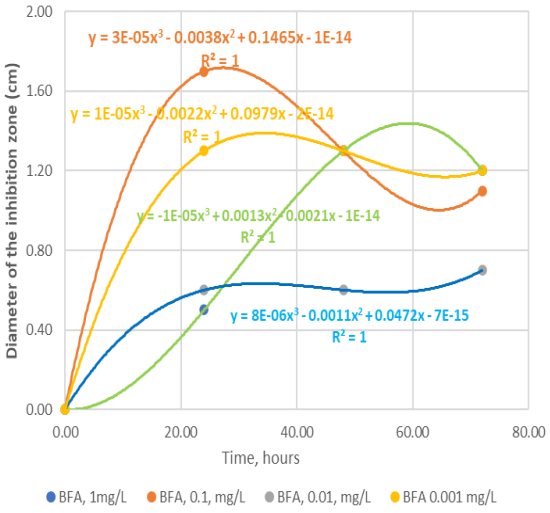


Fig. 8. The variation of diameters of inhibition zone for bacteria *sp S. faecalis*

The inhibition zone is not completely free of microorganisms, for them the BFA has have inhibition area. These colonies have been selected and the species of microorganisms will be identified in future studies.

The diameter of the inhibition zone increases with decreasing BFA concentrations for soil bacteria, the smallest diameter being characteristic of a concentration of 0.1 mg / L BFA (see Fig. 6), followed by that of 1 mg / L. For concentrations of 0.01 and 0.001 mg / L, the diameter of the inhibition zone for soil microorganisms tends to increase or remain constant over time. After 72 hours, it decreases, which shows that the microorganisms undergo genetic mutations that allow them to survive in the presence of BFA. For concentrations higher than 1 mg / L and 0.1 mg / L, the inhibition zone is constant, and the microorganisms are affected by BFA.

Mesophilic bacteria are affected by the presence of BFA(see Fig. 7). For concentrations of 1 mg / L, BFA the diameter of the inhibition zone increases constantly over time, the microorganisms being inhibited. At a concentration of 0.1 mg / L and 0.001 mg / L, the inhibition zone increases less than 1 mg / L and much faster after 48 hours, after which it remains constant. At concentrations of 0.01 mg / L, the diameter of inhibition increases in the first 48 hours, after which it decreases, fact which proves an adaptation of microorganisms to the presence of BFA. *S. faecalis* bacteria are also affected by BFA concentration, according to Fig. 8. The diameter of the inhibition zone shows maximum values at BFA concentrations of 0.1 mg / L, followed by 0.001 mg / L, 0.01 mg / L and 1 mg / L, respectively. Lower concentrations have more pronounced inhibitory effects in the first 24 hours, after which the inhibitory action decreases, the microorganisms adapt to BFA pollution and colonize the areas close to the disc. At a concentration of 1 mg / L of BFA, the size of the inhibition zone increases more slowly after 24 hours, but its inhibitory effect is maintained and even increases over time.

4. Conclusions

From the analysis of the experimental data, it can be seen that BFA has an inhibitory effect on microorganisms. The maximum inhibitory effect is obtained 24 hours after administration, after which some microorganisms develop resistance. The maximum inhibitory effect is obtained at a concentration of 0.01 mg/L BFA, for all three concentrations of microorganisms. The inhibitory effect is very small for microorganisms' concentrations of the order of 72×10^4 UFC / mL.

The inhibitory effect decreases over time, so that the number of microorganisms that are adapted to the effect of BFA has increased after 48 and 72 hours. The inhibition zone is not completely free of microorganisms, for them the BFA has not inhibition area.

Lower concentrations of BFA have a more pronounced antimicrobial action in the first 48 hours, after which it decreases due to genetic mutations and adaptation suffered by microorganisms. A high concentration of BFA, of the order of 1 mg / L, has a lower antimicrobial action, but which is maintained and even increases over time. BFA affects populations of microorganisms in water and soil, with consequences on the processes that take place at that level.

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