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Considerations on the Toxicity of Brilliant Blue FCF Aqueous Solutions before and after Ozonation

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Abstract: *The food dyes have a potential toxic effect on aquatic organisms which trigger the necessity of their removal from industrial or urban wastewaters. Many different removal methods were investigated for this purpose, but the ozonation and advanced oxidation processes (AOPs) were successfully applied in this field. However, the majority of studies emphasized that color removal by ozonation process did not report a complete mineralization of the dye and the resulted by-products may have a greater toxicity than the original compound. In this context, the paper presents a comparative ecotoxicity study of the dye Brilliant Blue FCF (BB FCF) before and after ozone treatment. The BB FCF toxic effect, before and after ozonation was investigated on crustacean (Daphnia magna), lethal or inhibitory concentrations for 50% of tested organisms (LC50 / EC50) were used to estimate the effect level. The dye showed no toxicity on crustacean (CL50/CE50>100mg/L) before ozonation. The ozonized solutions presented a high toxicity for crustaceans compared to initial dye due to the by-products occurrence.*

Keywords: *Brilliant Blue FCF, ozonized by-products, toxicity, aquatic life.*

1.Introduction

Synthetic dyes are widely used in many industries such as food, textiles, rubber, paper, plastics and in other daily used products. About 7.000 to 10.000 different commercial dyes and pigments are produced annually all around the world. Food manufacturers prefer synthetic dyes, because they produce a stronger color than natural colorings with a lower cost [1]. Brilliant Blue FCF (BB FCF) is a synthetic dye component of the triphenylmethane compounds with the molecular formula: C37-H36-N2-O9-S3.2Na [2]. The most commonly used synonyms in published literature are: Brilliant Blue FCF, Acid Blue 9, Alphazurine FG, E133, Erioglaucine disodium salt, FD&C; Blue no. 1, etc. [3]. These are used as food dyes in ice-cream, candies, drinks and other sweets.

Also it is often used in cosmetic, textile fields and in different research applications to detect the water infiltration in soil [4], for microbial fluorescent or blood cell staining [5, 6]. Sometimes, they are used in medicine for eye surgery and detecting lung aspiration in critically ill patients [7, 8]. In addition, BB FCF is an inhibitor of the Panx1 channels and it could be useful for the treatment of Crohn's, Acquired Immunodeficiency Syndrome (AIDS), melanoma, epilepsy, neurotrauma, inflammation, stroke and injuries to the central nervous system [9, 10].

Before 1993, the BB FCF dye has been forbidden in eleven European countries but after EU foundation this has been certified as a safe food additive [11]. The number of BB FCF in Alimentarius Codex (updated 2019) is E133. In 2017 based on a rat's intoxication study, the acceptable daily intake (ADI) for human was established to 0-6 mg/kg body weight [12, 13].

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In the medicinal uses, BB FCF dye is absorbed only 5% and 95% is excreted. Nowadays there is an ongoing controversy regarding the safety of this artificial food dye [14]. Numerous studies reported toxic effects on animals or human such as: convulsion, tumors of gastrointestinal origin and lymphoma in rodents [8]; increase of hepatic enzymes and bilirubin level in animal model [14]; oxidation of thyroid peroxidase forming aromatic amines (carcinogens) [15]; purinergic signaling (implicated in several cellular functions: proliferation of neural stem cells, vascular reactivity, apoptosis and cytokine secretion, mediating the effects of neural activity during development, neurodegeneration, inflammation, neuropathic pain and cancer) [10, 16]; attention deficit and hyperactivity in children [11]; cumulative absorption through lingual mucosa and through skin [17]. An alert of toxicity was launched by scientists and FDA regarding the BB FCF absorption and its lethal toxicity in three critically ill patients [18].

The dyes chemicals industry is under Regulation (EC) No 1907/2006 (REACH) [19] and must meet the criteria concerning the human and animal safety using the standardized methodologies for testing. The impact assessment of chemicals on the aquatic environment is described in the Regulation (EU) No 286/2011 [20] amending Regulation (EC) No 1272/2008, for the purposes of its adaptation to classification, labelling and packaging of chemical substances/mixtures legislation to Global Harmonized System of United Nations (GHS).

It has been estimated that about 10-15% of all the industrial dyes are released into the wastewater effluents [18, 21] during the dyeing process. Small amounts of dye can affect aquatic life and the entire food chain either by direct chemical / biological effect or by reducing the light transmission [15, 21]. Unfortunately, the present technology of WWTP cannot solve this dye issue and new wastewater treatment technologies are needed to improve the dye removal amount and subsequently to improve the water quality of the final effluents [22 - 24]. Various methods for BB FCF wastewater purification have been developed: physical methods - ultra sonication, ultrafiltration, micro- and nanofiltration or photocatalytic degradation, coagulation - flocculation, sorption, ion exchange membranes, electrochemical processes; chemical methods - oxidative processes (electrochemical oxidation, ozonation, advanced oxidation processes - AOPs), precipitation, complexation; biological methods – aerobic/ anaerobic degradation, use of fungi, algae and microbial fuel cells [14, 21, 25, 26].

The AOPs generating powerful oxidants have emerged as an important and cheaper class of technologies for the removal of organic contaminants from wastewater, and for remediation of organic contaminants in polluted soil and groundwater. These oxidation methods use oxidants like $K_2S_2O_8$, $KBrO_3$, KIO_4, Fenton's reagent, photo Fenton, H_2O_2 and ozone [22, 27].

Ozone may oxidize the organic and inorganic compounds from waste waters [27] but the resulted effluents has been shown to increase the general toxicity of the water for the rainbow trout larvae and reduce the immune responses in rainbow trout [22, 27-30].

Related to the known structure of the BB FCF dye (Figure 1), a concern is the potential formation of toxic ozonolysis by-products (Table 1). These by-products would require subsequent post-ozonation treatment for their removal [26, 31-34].

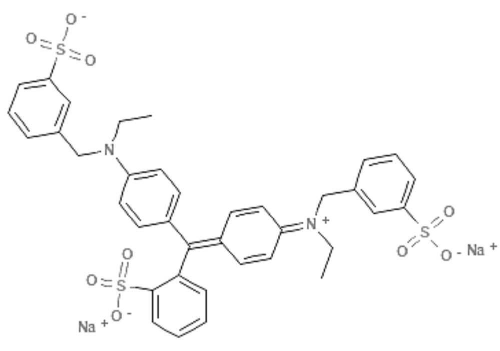

Figure 1. The Brilliant Blue molecule [35]

The paper goal was to evaluate the BB FCF ecotoxicity, specifically on aquatic life, before and after ozonation process. Acute aquatic toxicity [32] is normally determined using a fish model (testing 96-hours, LC50), a crustacean species (testing for 48-hours, EC50) and/or an algal species (testing for 72 or 96-hours, EC50. These species cover a range of trophic levels and taxa and are considered as surrogate for all aquatic organisms [20].

Our study was focused on the effect of BB FCF on planktonic crustaceans (*Daphnia magna*). The efficiency of ozonation process for color removal was evaluate based on the aquatic organism safety.

Table 1. BB FCF ozonation process and its by-products [28, 31-34, 36]

BB FCF oxidation type	Matrix	By-products
Electrochemical oxidation	Aqueous Solution of NaX	Ethanol, Acetaldehyde, Acetic acid, glyoxylic acid, formic acid
Ozone-based discoloration	Aqueous solution	N-(2-hydroxypropyl) benzene sulfonamide; 5-Sulfooxymethylfurfural; 2-Sulfobenzoic acid; 2-formylbenzenesulfonic acid
Ozone-based discoloration (simple mass-transfer, non-adjusted temp/pH)	Waste fruit leather	Carbonyl compounds (hexanal, ethanol, benzaldehyde, 2-furfural)
Ozone-based discoloration (simple mass-transfer, non-adjusted temp/pH)	Wastewater (ozonized fruit leather) solution / suspension)	Ethanal, propanal, butanal, pentanal, hexanal, octanal, acetone, 2-heptanone, 2-Furfural, benzaldehyde
AOP	Wastewater containing food dyes	Aromatic amines, phenolic compounds

2.Materials and methods

2.1Chemicals and equipment's

The dye BB FCF, analytical grade (CAS 3844-45-9), was purchased from Sigma Aldrich. The 1000 mg/L concentration of the dye stock solution was prepared in distilled water. Ozonation process was performed on solutions concentrations (prepared from stock solution) in the range of 1 – 50 mg/L BB FCF. An OZONOSAN Alpha-Plus Generator with medical oxygen was use. The continuous measurement of ozone concentration (1 – 130 mg O_3/L gaseous mixture – g. m) was assured by UV-Photometer that accessorized the generator. Under a constant oxygen flow rate, the ozone dose was constant. The batch experiments of 100mL were carried out using HEILDORPH VIBRAMAX 100 orbital shaker. H_2SO_4 0.1M was use to adjusted the solutions pH. An ORION 290A pH-meter was used for pH monitoring. The BB FCF final concentrations in the aqueous samples were detected using a CINTRA 404 UV/VIS Spectrometer. The maximum absorption wavelength of 630 nm was chosen to be used in the quantitative discoloration of BB FCF.

The laboratory BB FCF toxicity tests with invertebrates (crustaceans as *Daphnia*) were performed using reagents and growth media supplied by MicroBiotests Belgium.

2.2 Ozonation process

The experiment of the dye discoloration efficiency using ozonation in term of yield (% R_{BB}) was performed at ambient temperature using various concentrations of BB FCF, in range of 1 - 50 mg/L. All solutions were treated with the same dose of ozone (200 mg/L g.m.) for a contact time of 300 seconds, under continuously stirring (200 rpm). The experiment was fulfilled mostly at *p*H of BB FCF solution (*p*H = 7.05), excepted those where the influence of pH was evaluated when the tests were done at a *p*H = 4.03. Ozonation of dye samples was carried in glass recipients. Every time a volume of 100 ml of dye solution with different concentrations was added into the glass recipient. Ozone gas dose was supplied, followed by aeration for 5 min to remove residual ozone.

To evaluate the dye removal efficiency, the following formula was applied:

$$\%R_{BB} = (1-C_{iBB}/C_{tBB}) \times 100 \quad (1)$$

where

C_{iBB} = the initial dye concentration at time (t) = 0

C_{tBB} = the dye concentration at time (t).

2.3 Ecotoxicity test

The laboratory toxicity tests were executed using dye solutions prepared into a mineral medium, in the concentration range of 0.1-100 mg/L (starting to a stock solution of 1000 mg dye dissolved in 1000 mL distillated water), to estimate the median effective concentration (EC50) expected to produce a certain effect of BB FCF in 50% of test organisms, in a given population under a defined set of conditions. BB FCF - effluents obtained after ozonation process (range of 1 – 50 mg/L) were tested under various physical and chemical work conditions. The ozonized solutions were tested undiluted and 50% vol. diluted, with or without adjusted pH using 1 M NaOH or HCl. The median effective concentration (EC50) was estimated.

The *Daphnia magna* were directly exposed to the BB FCF solutions in a test batch within a continuous period of exposure of 48hrs, in accordance with OECD test 202 [37], Table 2.

The test conditions (temperature, pH and dissolved oxygen) were periodically monitored using a multiparameter WTW tip Multi 350i. The tests were performed in replicates for each tested concentration and control to ensure statistically relevant results.

Table 2. Toxicity test

Species	Test	Type of test	Endpoint effect	Test period / incubation
Daphnia magna	OECD 202 DAPTOXKIT F	Static, acute	Mortality / immobilization, LC50 / EC50	24-48h, 20°C

3.Results and discussions

3.1 Ozonation treatment of BB FCF

Color removal (% R_{BB}) in presence of various experimental conditions was around 96-99% for all BB FCF concentrations using similar ozone treatment in a neutral or acid pH media (Table 3). In case of BB FCF concentrations 1, 2.5, 5 and 10 mg/L and acid pH (4.03), the % R_{BB} was ≥ 99.5% (Table 3) indicating a good discoloration of ozonized solutions [26]. The acute toxicity of all ozonized solutions was tested in various conditions, such as: pH correction (in the range of 6.5 – 8.5) or 50% vol. dilutions.

Table 3. Characterization of BB FCF solutions used in toxicity tests and % R_{BB}

BB FCF Concentration (mg/L)	Treatment conditions			% R_{BB}
	pH	O_3 Concentration (mg/L g. m,)	Contact Time (seconds)	
1	7.05	200	300	99.92
	4.03	200	300	99.94
2.5	7.05	200	300	99.81
	4.03	200	300	99.87
5	7.05	200	300	99.68
	4.03	200	300	99.74
10	7.05	200	300	98.80
	4.03	200	300	99.50
30	7.05	200	300	97.66
	4.03	200	300	98.94
50	7.05	200	300	96.82
	4.03	200	300	97.04

3.2 Toxicity of BB FCF dye

The experiments on conventional and alternative test batteries represents a reliable way of estimation the complex and significant effects of chemicals on the entire food chain [38- 40].

Microbiotests performed with *Daphnia* or green algae are being applied more and more often to evaluate the inhibitory effects of chemicals, the current trend in the field of ecotoxicology being the abandonment or reduced use of vertebrate aquatic organisms (fish) [19, 40, 41, 42].

Daphnia magna (*Cladocera)* are small aquatic crustaceans commonly called water fleas. *Daphnia* was selected for ecotoxicity testing because crustacean are the most sensible aquatic organisms to pollutants toxicity, particularly useful because of its short life and good and rapid reproduction. There are still at least two reasons why *Daphnia* was selected for dye toxicity testing: a) to avoid any errors of results in the algal growth inhibition test due to the blue color of BB FCF that may cause interference; b) fish tests supposed a large number of organisms and this is in contradiction with international recommendations concerning vertebrate use.

All the acute toxicity tests were performed for BB FCF dye solutions in concentration range 0.1-100 mg/L. The BB FCF aqueous solutions (from 0.1 to 100 mg/L) whiteout any treatment reveled nontoxic effects for planktonic crustaceans, having only minor lethal/inhibitory effects (5-20%) on *Daphnia magna* after 48h of incubation (Table 4). The EC50 >100 mg/l indicate non harmful impact of the dye on these organisms. Similar results on *Daphnia magna* were reported for BB FCF between 97 mg/L to >1000 mg/L by the international data bases for chemical registration such as Ecotoxicology knowledgebase (ECOTOX) [43] and other Environmental Organization [44].

According to Regulation on the classification categories for substances hazardous to the aquatic environment [20], the BB FCF is classified as nontoxic for crustacean species (*Daphnia*)(EC50>100 mg/L).

Table 4. Acute toxicity results of BB FCF on *Daphnia magna* after 48h of contact

Solutions whiteout treatment (mg/L BB FCF)	**Average of:**		**Acute effect (%)**	**Toxicity EC50 – mg/L / REACH classification**
	pH	Dissolved oxygen mgO_2/L		
100	7.60	8.62	20	EC50 >100 mg/L Non toxic
50	7.03	-	20	
20	7.35	-	15	
10	7.35	-	10	
5	7.42	-	10	
1	7.38	-	5	
0.1	7.32	-	0	
Control (dilution media)	7.14	8.91	0	

3.3 Toxicity of BB FCF ozonized solutions

Daphnia toxicity tests were performed for BB FCF solutions treated with ozone in different conditions (ozone treated solutions pH 7; ozone treated solutions with acidification *p*H 4; ozone treated solutions *p*H 4 with *p*H correction and 50% vol. diluted ozone treated solutions with *p*H correction, to correspond to toxicity test conditions).

Figure 2 presented the results of acute toxicity of BB FCF ozonized solutions on *Daphnia*. The data shown an increased toxicity in case of ozonized samples comparing with the untreated BB FCF solutions. The toxicity results of ozonized samples were 5-10 fold higher than untreated samples. The ozone treatment corroborated with the acidification process improved the blue color of BB FCF removal, but increased their toxicity due to the high oxidation that can lead to by-products, potentially more toxic that the initial compound. The ozonized and acidified solutions at pH 4 reveled a maximum toxicity (100%) due to acid pH effect on organisms living. Similar results were also reported in other studies which highlighted an efficient discoloration of the dye and a by-products formation [45-46]. A greater ecotoxicity against *Artemia salina* was observed after ozonation of BB FCF solutions leading to the conclusion that the occurred by-products are more toxic that their precursors itself [31].

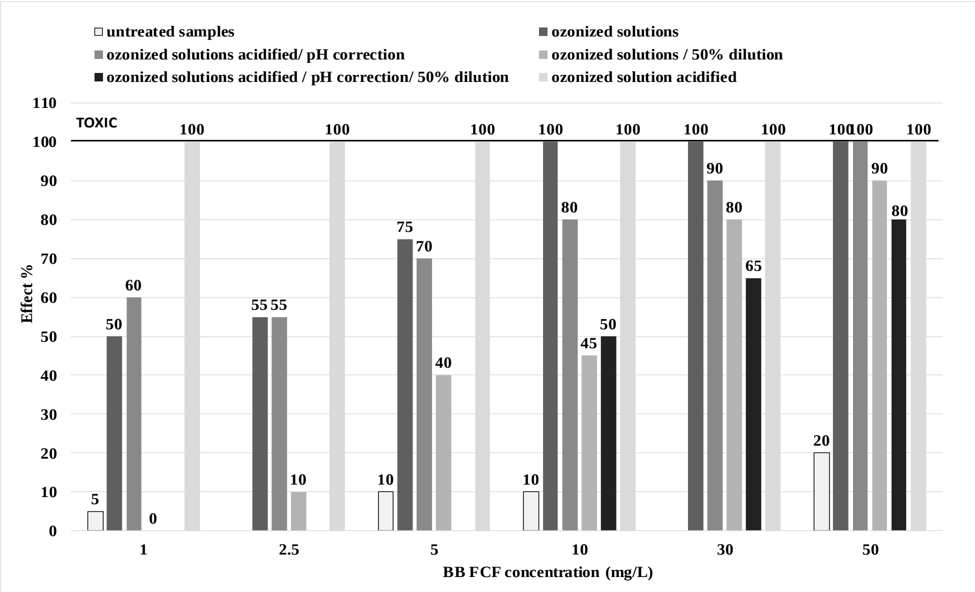

Figure 2. Acute toxicity of ozonized BB FCF solutions on planktonic crustaceans

The toxicity of ozonized samples decreased when they were 50% diluted, but even so their toxicity remained higher than untreated solutions (case of 5, 10, 30 and 50 mg/L BB FCF). The same trend of toxicity was also observed in case of ozonized and acidified solutions with pH correction and 50% vol. diluted.

BB FCF ozonized solutions and 50% vol. diluted reveled inhibitory /lethal effects between 0% and 100% on *Daphnia*, the level of toxicity being lower than undiluted samples. The median effective concentration – EC50(48h) of ozonized and 50% vol. diluted samples was estimated at 4.8 mg/L BB FCF (Figure 3). This value indicated that BB FCF solutions resulting after ozone treatment have had acute toxicity effects, with possible long lasting harmful effects to aquatic life.

Our previous studies shown significant toxic effect (80% mortality) of BB FCF solutions in concentration of 5 mg/L and 10 mg/L ozonized (200 mg O_3/L), under stirring condition (200 rpm) and acidified (pH 4) [26]. The toxicity effects decreased to 45-50%, after diluting these solutions in proportion of 50% with distilled water. This fact leads us to conclusion that the toxic effects can be diminished when the ozonized dye solutions do not exceed the concentration of 10 mg/L and are diluted with water in proportion of 50% (1:1 v/v), in a pH range of 6.5 – 8.5. Also, smaller concentration of BB FCF (≤ 5 mg/L) subject to ozone treatment and diluted 50% could cause much lower toxicity to aquatic organisms (10-40% mortality).

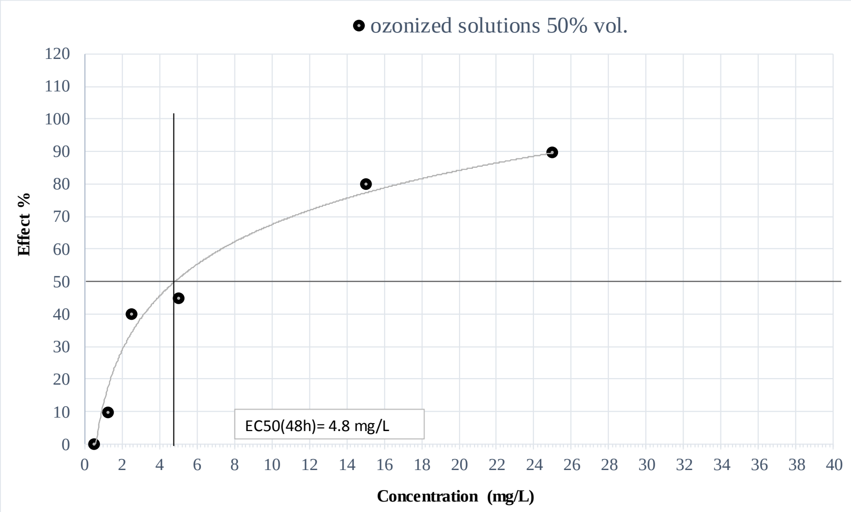

Figure 3. EC50(48h) estimation for BB FCF ozonized solutions 50% vol. diluted

Some studies on BB FCF degradation shown that color removal was not completed by the total mineralization of dye and the incidence of degradation compounds such as aromatic amines could be a problem for the living organisms [47]. Considering the Predicted Environmental Concentrations (PEC) estimated for BB FCF in surface water about <0.1 to 626 μg/L (in accordance with the economic sector – industrial, food, cosmetics) [44], the estimated toxicity level could be reduced about 100 times in the field. A PNEC (Predicted No Effect Concentration) value of 0.15 mg/l taking in to consideration 6 species of a taxonomic group [44].

4.Conclusions

The paper presented a comparative ecotoxicity study of the BB FCF dye on *Daphnia magna*, before and after the ozone treatment process, meant to eliminate the blue color. The dye at various aqueous solutions induced no toxicity / inhibitory effects on crustacean species (EC50 >100 mg/L).

The 200 mg O_3/L ozone treated solutions of BB FCF highlighted color efficiency removed more than 90%, but very significant toxic effects on *Daphnia magna* was observed (perhaps due to the fact that by-products were more toxic than initial compound).

In order to discharge dye solutions without toxic effects on aquatic life, the ozone treatment of the BB FCF in concentration ≤10mg/L must be supplemented by other processes such as pH adjustment and aqueous dilutions greater than 50% vol.

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