IOP Conf. Series: Materials Science and Engineering **301** (2018) 012027 doi:10.1088/1757-899X/301/1/012027

Response of *Spirulina Platensis* to Sulfamethazine Contamination

Xiankuan Xu, Xiaohong Lu, Xiangjuan Ma and Huilong Xia*

School of Environmental Science and Engineering, Zhejiang Gongshang University, Hangzhou, China
Email: hlxia@zjgsu.edu.cn

Abstract. The interaction between a positive important biological energy source algae *Spirulina platensis* and an antimicrobial drug sulfamethazine was studied by a panel of bioassays. The result demonstrated that the acute toxicity of sulfadimidine on *Spirulina platensis* gradually increased with the increasing of the concentration of sulfadimidine. During the exposure, the toxicity of sulfamethazine on *Spirulina platensis* enlarged at first and reduced thereafter, and the summit was at 48 h, showing a typical "bell" curve. The EC_{50} in 48 h was 6.06 mg/L. *Spirulina platensis* was able to accumulate and degrade sulfamethazine simultaneously. The amounts of sulfamethazine accumulated and degraded by *Spirulina platensis* were 1.40 mg/kg and 0.64 mg/kg (dry weight) at 24 h, respectively. After 96 h of exposure, the accumulation decreased to 0.47 mg/kg, however, the degradation of sulfamethazine increased to 6.57 mg/kg. Due to biological activity, the removal rate of sulfamethazine was 5.2 times in the nutrient solution with *Spirulina platensis* as that of without *Spirulina platensis*. The results of this study implied that *Spirulina platensis* had a potential to remove sulfamethazine in the sulfamethazine contaminated aquatic ecosystems.

1. Introduction

Sulfonamides (SAs), as one kind of broad spectrum antimicrobial drug, play a major role in the livestock, aquaculture, and pharmaceuticals industries. While these drugs are widely used as specific therapeutics and prophylactics and growth promoters in animal feed [1], they also are poorly metabolized in animals. Hence, a high proportion of them are excreted unchanged in feces and urine. In the mean time, they are not completely degraded at ordinary sewage disposal plants [2]. It can thus be seen that they can be absolutely detected in the environment. Although environmental levels are usually very low, at ng/L in waters and mg/kg in soils and sediments, they may pose a hazard to human health due to their continued release and permanent presence in the environment [3]. Under low concentration exposure, SAs may result in the development of pathogenic bacteria drug-resistance, posing a potential problem for organisms [4].

Photosynthetic algae, exiting at the beginning of the food chain, can provide essential substances and energy in its simplest form for the survival of fish and invertebrates in most aquatic ecosystems, its species diversity and primary production immediately affect the function and structure of aquatic ecosystems. SAs can enter into the aquatic ecosystem through various channels and be accumulated by algae, from algae to the entire biosphere by the food chain, then interfere with specific biological systems. In the mean time, algae have the potential to accumulate and degrade organic contaminants [5], to a certain extent, changing the fate of organic contaminants [6]. The European Medicines Agency indicated that cyanobacterium was required for antimicrobial effect test [7]. Most studies aimed at understanding the interaction between blue algae and organic contaminant, less to the green algae.

Spirulina platensis, an edible filamentous cyanobacterium being mass-produced commercially in world as valuable food supplement, is a positive important biological energy source which can serve as an economic and constant supply source of biomass. The biomass of *Spirulina platensis* also showed a great potential to remove copper from aqueous media [8]. The major aim of the present study is to investigate the toxicity of sulfamethazine (Fig. 1), one of the most widely used SAs, towards *Spirulina platensis*, along with the feasibility of sulfamethazine removal by *Spirulina platensis* in contaminated water.

2. Materials and Methods

2.1. Algae Culture

Spirulina platensis was obtained from Xingxing Huo Er Company (Shandong, China). In brief, a 250 ml conical flask containing 100 ml of Zarrouk culture medium without $NaHCO_3$ was plugged with a silicone-foam plug and autoclaved at 121 ℃ for 30 min. After that, $NaHCO_3$ solution was sterilized by filtration (0.2 μm) and *Spirulina platensis* cells were added under bacteria-free operating environment. The conical flasks were placed in a growth chamber under the condition of a 12 h photoperiod at 100 μmol/(m^2/s) and 30 ℃/25 ℃(day/night). The conical flasks were conducted under static conditions and changed in position relative to each other 3 times per day to equalize irradiating light intensity.

2.2. Chemicals

Sulfamethazine (4-amino-N-(4, 6-dimethyl-2- pyrimidyl) benzenesulfonamide) was purchased from Accelerating Scientific and Industrial Development thereby Serving Humanity (98% purity). Citric acid, disodium hydrogen phosphate, EDTA, and acetone were purchased from Chengdu Kelong Chemical Reagent Company and were analytical grade. Methanol and acetonitrile, purchased from TEDIA Company, were chromatographically grade. Zarrouk culture medium was prepared for *Spirulina platensis* with analytical grade reagents [9]. The macronutrient solution had the following composition in g/L: $NaHCO_3$ 16.80, $NaNO_3$ 2.50, NaCl 1.0, and K2HPO4 0.50, K_2SO_4 1.0, Mg_2SO_4 7H_2O 0.20, $CaCl_2$ 2H_2O 0.04 and Na_2EDTA 0.08. The macronutrient solution was added with 1 ml/L of each micronutrient solution (A_5, A_6). Solution A_5 contained in g/L: H_3BO_3 2.86, $MnCl_2$ 4H_2O 1.8, $ZnSO_4$ 7H_2O 0.22, $CuSO_4$ 5H_2O 0.08 and MoO_3 0.01. Solution A_6 contained in mg/L: NH_4VO_3 22.9, $NiSO_4$ 7H_2O, 47.8, Na_2WO_4 17.9, Ti $(SO_4)_2$ 40.0 and Co $(NO_3)_2$ 6H_2O 4.4.

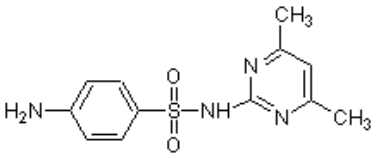

Figure 1. Chemical structure of sulfamethazine

2.3. Preparation of Sulfamethazine Solution

The sulfamethazine stock solution corresponding to 10000 mg/L of sulfamethazine was prepared by dissolving 1 g sulfamethazine in 1 mol/L NaOH solution and further diluted to 100 ml in a standard volumetric flask. Concentrations used in the definitive tests were based on results from range-finding tests.

2.4. Biomass Detection

After 20 days of cultivation, algal cells were harvested by centrifugation at 12000 rpm for 10 min. The biomass was extensively washed with distilled water, dried at 100 ℃ until the constant weight of sample reached.

2.5. Acute Toxic Experiments

In the algal growth-inhibition experiments, the range of initial concentrations of sulfadimidine prepared from stock solutions varied between 1-10 mg/L. All experiments were conducted in

triplicates. The chlorophyll content was measured at 24 h. 48 h, 72 h and 96 h after adding sulfadimidine to nutrient solution, respectively. The sample was centrifuged at 12000 rpm for 10 min to eliminate the supernatant. The biomass was thoroughly washed twice with distilled water to remove the residual growth medium and extracted with 5 ml of acetone (80%). After that, the mixture was stored at 4℃ in dark. The concentration of chlorophyll a was determined with UV-VIS spectrophotometer. chlorophyll a was quantified using the method described by Arnon [10] .
The test parameter considered the concentration found to inhibit 50% S*pirulina platensis* in 48 h as EC_{50}.

2.6. Sulfamethazine Accumulation and Degradation in Spirulina Platensis
To detect the accumulation and degradation of sulfamethazine, *Spirulina platensis* was treated with sulfamethazine at 0.2 mg/L for 0 h, 24 h, 48 h, 72 h, and 96 h, respectively. After treatment, *Spirulina platensis* was harvested, and sulfamethazine in the cells and nutrient solution was individually detected and quantified. Accumulation of sulfamethazine was calculated and expressed as mg/kg (DW) according to the method described by Ou et al [11]. Degradation of sulfamethazine was defined and calculated as follows:

$$C = (C_0\text{-}C_1\text{-}C_2)/M \quad (1)$$

Where C was the degradation in *Spirulina platensis*; C_0 was the quantity of sulfamethazine in the control (without *Spirulina platensis*); C_1 was the residual quantity of sulfamethazine in nutrient solution with *Spirulina platensis*; C_2 was the cellular accumulation of sulfamethazine; and M was the dry weight of *Spirulina platensis*.

The sample (20 ml) was divided into the supernatant and the biomass was obtained by centrifugation at 12000 rpm for 10 min. The supernatant (1 ml) was placed in clean centrifuge tubes (1.5 ml) and stored protected from light at 4℃. The biomass was thoroughly washed twice with distilled water and subjected to extract with methanol (1 ml) for 10 min on a shaker and for 20 minutes in ultrasonic bath. EDTA-Mcllvain buffer solution (40 ml) was added, the mixture was centrifuged to obtain the supernatant. The supernatant was filtered through a 0.45 μm membrane filter to eliminate the suspended matter, and applied on a Waters Oasis HLB column activated with 5 ml of methanol followed by 5 ml of distilled water. A wash step with 5 ml of methanol (5%) and with 5 ml of distilled water was applied after the supernatant loading. The SPE column was dried under vacuum for 10 min. And then it was eluted with 5 ml of methanol, collected in a 15 ml glass tube, and evaporated to dryness under a stream of nitrogen. 1 ml of methanol (40%) transferred into the glass tube was used to dissolve sulfamethazine, collected in clean 1.5 ml centrifuge tubes and stored protected from light at 4℃. It was worth noting that the solution must be filtered through a 0.22 μm membrane filter before injection.

Chromatographic separation was carried out on a C18 reverse phase column (4.6 mm×250 mm, 5 μm). Analytical column with mobile phase was consisted of a binary mixture of solvents A (80% acetonitrile) and B (20% distilled water with 0.01 mol/L of H_3PO_4). The detection wavelength was 270 nm. The flow rate was kept at 1 ml/min; injection volume 20 μl, the temperature of column was controlled at 25 ℃. The average fortified recovery sulfamethazine at 0.5 mg/L was 87.60%.

2.7. Statistic Treatment of Data
Experimental data were organized in Microsoft Excel Spreadsheet and the results were plotted in Origin 8. A logistic-regression model provided novel guidelines to predict toxicity of sulfamethazine on *Spirulina platensis* and estimated the EC_{50} values [12]. The SPSS statistical package was used. The difference was considered statistically significant when $P < 0.05$.

3. Results

3.1. Toxicity of Sulfamethazine on Spirulina Platensis
The experimental results showed that *Spirulina platensis* was inhibited by the addition of sulfamethazine and the inhibition effect increased with increasing concentrations of sulfamethazine

from 1 mg/L to 10 mg/L (Fig. 2). At the concentration of 4 mg/L sulfamethazine, the inhibition rates of chlorophyll a contents of *Spirulina platensis* after 24 h, 48 h, 72 h, and 96 h were 37.00%, 49.09%, 35.41%, and 16.13%, showing a typical "bell" curve. The 48 h - EC_{50}, based on the mean of chlorophyll a concentration of *Spirulina platensis*, was 6.06 mg/L.

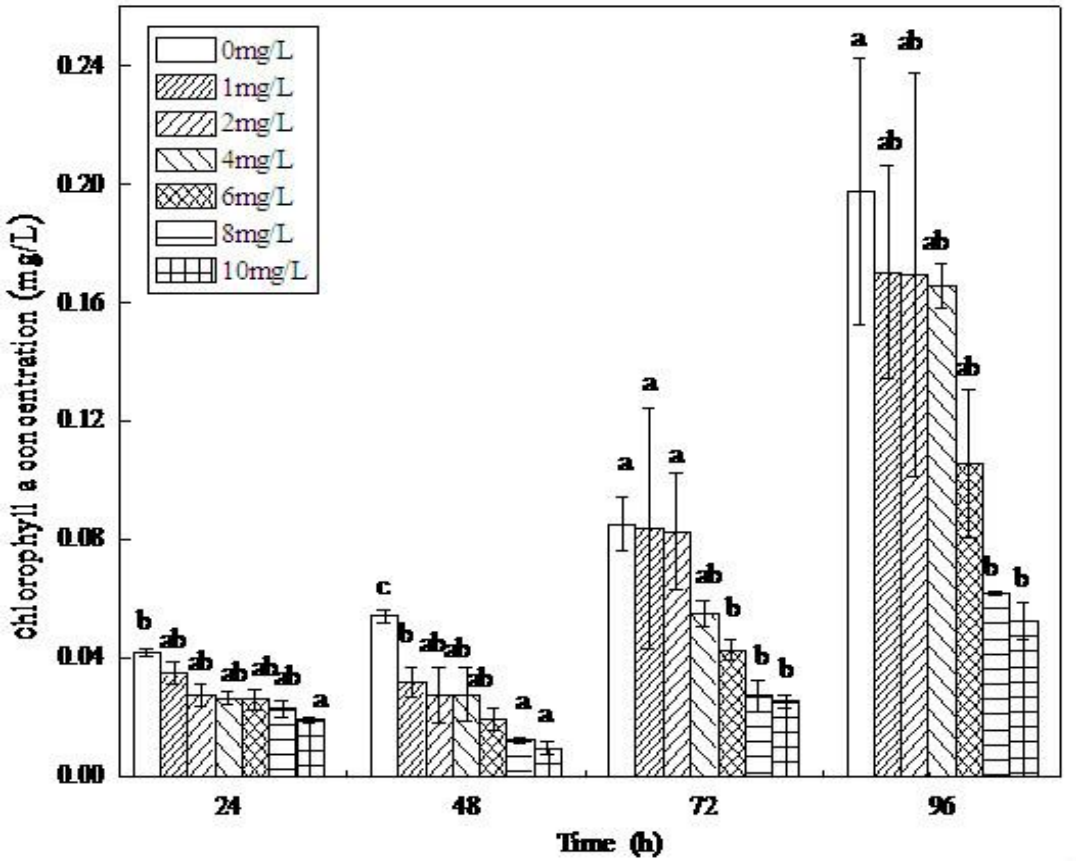

Figure 2. Content of chlorophyll a in *Spirulina platensis*

Spirulina platensis was treated with sulfamethazine at 0 mg/L, 1 mg/L, 2 mg/L, 4 mg/L, 6 mg/L, 8 mg/L, and 10 mg/L culture for 24 h, 48 h, 72 h, and 96 h, respectively. Algal growth was expressed by chlorophyll a content.

3.2. Accumulation and Degradation of Sulfamethazine by Spirulina platensis

Compared with the control (without *Spirulina platensis*), the residue of sulfamethazine in the nutrient solution with *Spirulina platensis* was always lower (Fig. 4), indicating that a portion of sulfamethazine in the nutrient solution was removed by *Spirulina platensis*. After 96 h, the removal rate of sulfamethazine in the nutrient solution with *Spirulina platensis* reached 2.85%. The removal rate constant of sulfamethazine with *Spirulina platensis* in the nutrient solution was 3.21×10^{-4} /h, and it was 6.17×10^{-5} /h in the absence of *Spirulina platensis*, implying that the removal rate of sulfamethazine was enhanced by over 4 times by *Spirulina platensis*.

Mainly two routes for *Spirulina platensis* to remove sulfamethazine at a certain extent in theory were namely accumulation and biodegradation. Accumulation of sulfamethazine in the first 24 h reached the maximum, and then gradually declined over the time (Table 1). Data from the experiment showed that the amount of accumulation was 1.40 mg/kg in 24 h and 0.47 mg/kg in 96 h of exposure. Similarly, the bioconcentration factor (BCF) was higher in 24 h and then declined from 24 h to 96 h. However, the degradation of sulfamethazine was progressively growing over the time, with the maximum value of degradation in 96 h (Fig. 4). In conclusion, the accumulation and degradation of sulfamethazine on *Spirulina platensis* could occur simultaneously.

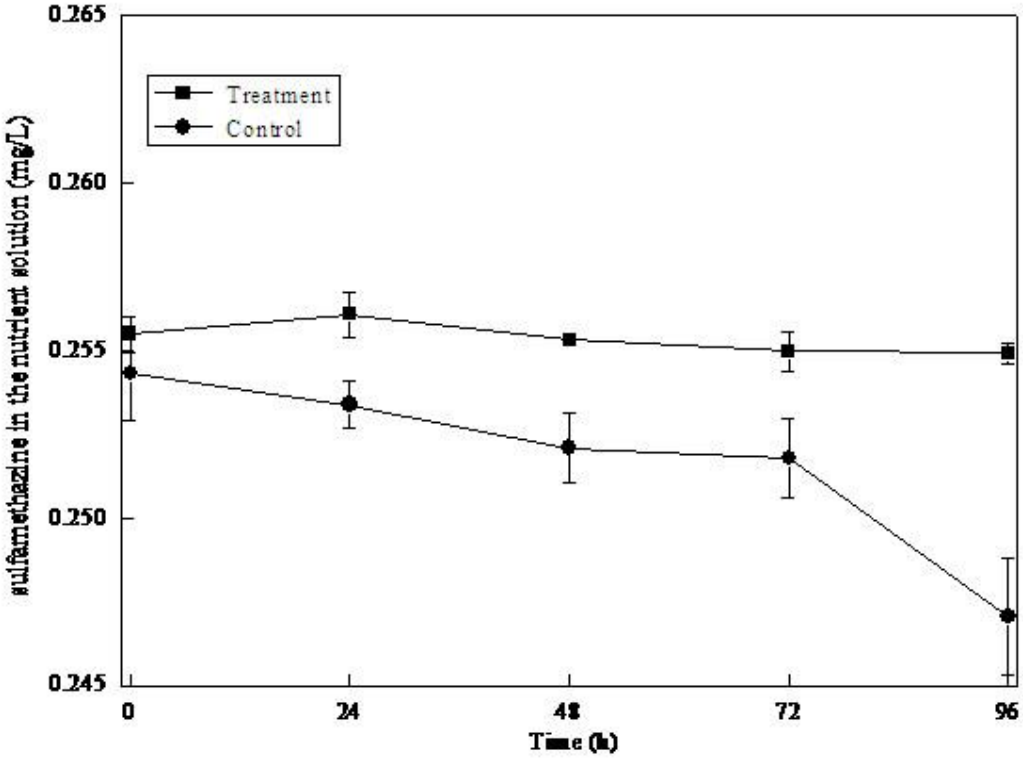

Figure 3. Residual sulfamethazine in the nutrient solution

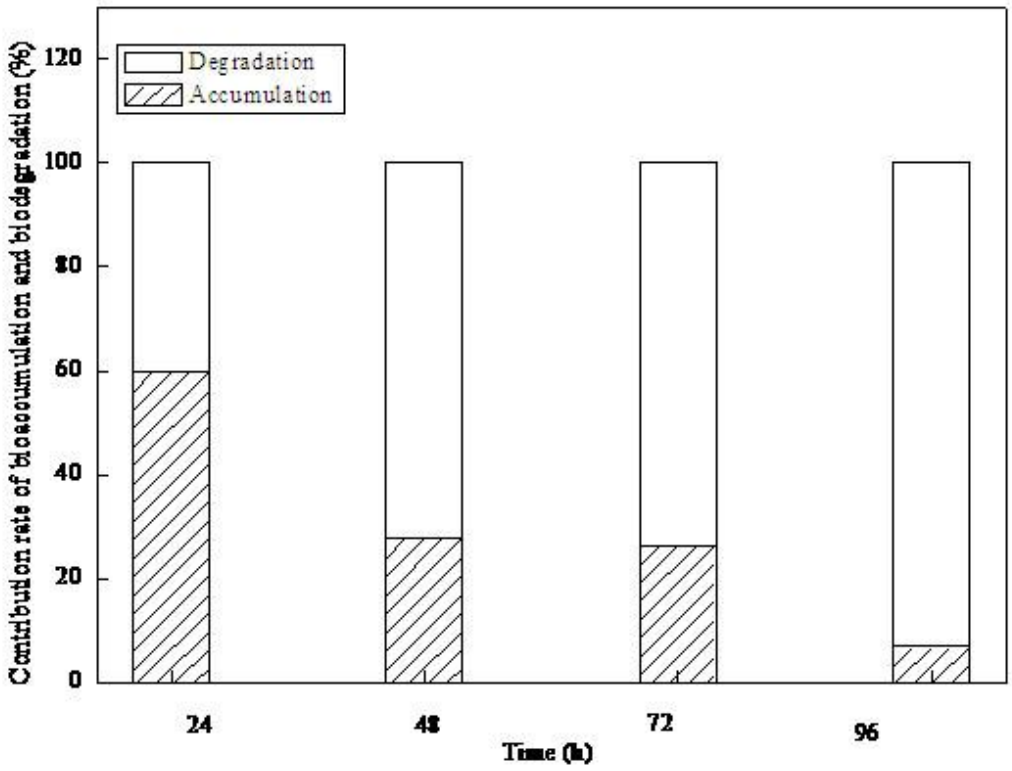

Figure 4. Contribution rates of bioaccumulation and biodegradation on the removal of sulfamethazine by Spirulina platensis

Table 1. Amount of sulfamethazine accumulated in Spirulina platensis and bioconcentration factor (BCF) when cultivated with 4 mg/L sulfamethazine

Time (h)	Accumulation (mg/kg)	Bioconcentration factor (BCF)
24	1.40	5.53
48	1.10	4.37
72	0.66	2.62
96	0.47	1.89

*Data shown were the means of three trials. BCF was the ratio of concentrations of the sulfamethazine in *Spirulina platensis* to that in the nutrient solution.

3.3. Discussion

In this experiment, chlorophyll a content was used as the index for *Spirulina platensis* biomass and corresponding sensitivity. The results indicated that sulfamethazine was toxic and had a negative effect on the growth of *Spirulina platensis*, in which the inhibition effect of sulfamethazine on *Spirulina platensis* was a positive correlation to concentration of exposed sulfamethazine solution. In case of sulfamethazine, the cells ceased growing, began to die and lyse, and thus the *Spirulina platensis* lose chlorophyll a. Similar result was observed in the dose-effect experiment of kanamycin on *Spirulina platensis* [13]. With the exposure time, however, the inhibition effect of sulfamethazine on *Spirulina platensis* decreased. Similarly, previous studies demonstrated that the toxicity of butachlor and bensulfuron-methyl decreased as the exposure time varied [14]. Analysis showed that long time of exposure to sulfamethazine activated removal capability possibly and allowed the *Spirulina platensis* to become more adaptive to the environmental stress. In addition, sulfamethazine might be degraded into harmless substances by *Spirulina platensis*.

The efficiency of microalgal species, namely, *Spirulina platensis* to remove the sulfamethazine was evaluated. The results revealed that *Spirulina platensis* might enhance the sulfamethazine removal via degradation and accumulation. Degradation might take place simultaneously in the process of its accumulation. Experimental data indicated that the degradation in 96^{th} h was much greater than that in 24^{th} h, and the difference between them was more than one order of magnitude. The lowest amount of degradation in 24 h was 0.64 mg/kg. Furthermore, the removal rate constant of sulfamethazine with *Spirulina platensis* was 5.2 times as that of without *Spirulina platensis*, which demonstrated that *Spirulina platensis* exhibited greater efficiency in the removal of sulfamethazine. The removal rate of sulfamethazine was 0.52%, 0.87%, 1.23%, and 2.85%, respectively after 24 h, 48 h, 72 h, and 96 h. Accordingly, the acute toxicity of sulfadimidine on the *Spirulina platensis* gradually decreased from 24 h to 96 h. The research result indicated that *Spirulina platensis* had the potential to degrade sulfamethazine and habitat the environment contaminated with sulfamethazine.

4. Acknowledgement

The authors would like to acknowledge the financial support from Zhejiang Provincial Natural Science Foundation of China (Y5090229）and the New Talents Program from the Science and Technology Department of Zhejiang Province (2012R408065).

5. References

[1] Sarmah A.K., Meyer M.T., Boxall A.B.A., 2006. Global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere. 65(5), 725-759.

[2] AI-Ahmad A. Daschner F.D. Kumerer K, 1999. Biodegradability of cefotiam, ciproxacin, meropenem, penicillin G and sulfamethoxazole and inhibition of waste water bacteria. Archives Environmental Contamination and Toxicology. 37(2): 158-163.

[3] Hernando M.D. Mezcua M., Fernandez-Alba A.R., Barcelo D., 2006. Environmental risk assessment of pharmaceutical residues in wastewater effluents. Surface waters and sediments. Talanta. 69(2), 334-342.

[4] Witte W., 1998. Medical consequences of antibiotic use in agriculture. Science. 279(5353), 996-997.
[5] Liu J.Q., Liu H.T.A., 1992. Study of the degradation of azo-byes by algae. Acta Hydrobiologica Sinica. 16(2), 133-143.
[6] Liu H., Shen T., Sun L., 2008. Accumulation and biodegradation of Dibuty1 Phthalate in *Chlorella vulgaris*. Journal of Agriculture Environment Science. 27(6), 239l-2395.
[7] Maul J.D., Schuler L.J., Belden J.B., Whiles M.R., Lydy M.J., 2006. Effects of the antibiotic ciprofloxacin on stream microbial communities and detritivorous macroinvertebrates. Environmental Toxicology and Chemistry. 25 (6). 1598-1606.
[8] AI-Homaidan A.A., AI-Houri H.J., AI-Hazzani A.A., EIgaaly G., Moubayed N. M. S., 2014. Biosorption of copper ions from aqueous solution by Spirulina *platensis* biomass. Arabian Journal of Chemistry. 7, 57-62.
[9] Zarrouk C., Contribution a l′etude d′une cyanophycee: influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de Spirulina maxima (Setch et Gardner) Geitler [D]. Paris, France: University of Paris. 1966.
[10] Arnon D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidases in Beta vulgaris. Plant Physiology. 24(1), 1-15.
[11] Ou X., Lei M., Wang X., 2003. Accumulation and degradation of novel oxime insecticide HNPC-A9908 by Chlorella pyrenoidosa. Environmental Science. 23(5), 475-479.
[12] Isnard P., Flammarion P., Roman G., 2001. Statistical analysis of regulatory ecotoxicity tests. Chemosphere. 45(4-5), 659-669.
[13] Cao J.X., Xu Z.F., Qiu G.H., 1999. Studies on the sensitivity of Spirulina platensis to antibiotics and herbicide: relationship with selectable markers for genetic transformation. Bioresource Technology. 70(1), 89-93
[14] He H., Se J., Lu S., 2011. Toxic Effects of Butachlor and Bensulfuron Methyl on Cyanobacterium *Spirulina platensis*. Journal of Agro-Environment Science. 30(6), 1070-1075.