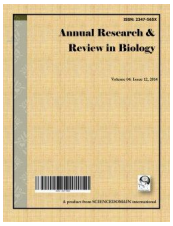

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Differential Response of Fresh Water Algae *Ankistrodesmus acicularis* and *Anabaena flos-aquae* to Dichlobenil Exposures

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Authors' contributions

This work was carried out in collaboration between both authors. Author AMAEA wrote the manuscript, performed the statistical analysis and managed the literature searches. Author MAED designed the study and wrote the protocol. Both authors read and author AMAEA approved the final manuscript.

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ABSTRACT

The massive use of herbicides may harm the growth of algae, which in turn might disturb the balance of the aquatic ecosystem. Herein the physiological as well as the biochemical responses of two fresh water algae namely *Ankistrodesmus acicularis* (Chlorophyta) and *Anabaena flos-aquae* (Cyanobacteria) to different concentrations of dichlobenil were assessed. Parameters including chlorophyll (a) Chl (a) content, EC_{50} values, carbohydrate and adenosine triphosphate (ATP) contents as well as the uptake of dichlobenil by both algal species were tested. The results indicated that a significant decrease in chlorophyll (a) content of both algal species to intermediate and high concentration of dichlobenil, whereas stimulative effect of low concentration of dichlobenil on Chl (a) content were recorded. Attainable results derived by probits analysis revealed that the EC_{50} values for both algae at 5th and 7th days approximate each other. Additionally, drastic decrease (98%) in ATP content was observed at 8.0 mg/L dichlobenil treatment in case of *Ankistrodesmus acicularis.* Furthermore, *Anabaena flos-aquae* cells activity was enhanced by the

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application of low and intermediate concentrations of dichlobenil (0.25-4.0 mg/L). Higher concentration level (16.0 mg/L) led to relative decrease in ATP content. *Ankistrodesmus acicularis* was found to be more sensitive than *Anabaena flos-aquae*. The uptake of dichlobenil was best ascribed to a pseudo-first order rate kinetic. The specific uptake rate constants of dichlobenil in case of *Ankistrodesmus acicularis* were relatively lower than the corresponding rates exhibited by *Anabaena flos-aquae.* In sum, the uptake of dichlobenil by algae may present environmental hazards and could affect the human health, through accumulation in the food chain.

Keywords: Dichlobenil; toxicity; green algae; cyanobacteria; physiological processes; uptake.

1. INTRODUCTION

Algae play an important role in the aquatic ecosystems, they are considered as the primary source for organic materials and oxygen on which fish and invertebrates are feed on [1]. Microalgae have been suggested as good candidates for fuel production [2]. Cyanobacteria are one of the biological agents that have been tested for the control of plant pathogenic fungi [3]. It produces some of biologically active compounds such as, phenols, terpenoids, and flavonoids [4].

Herbicides are used throughout the globe to control weeds, which is a part of agricultural management. They may enter the aquatic ecosystems and cause side effects on biological and functional properties. The intensive uses of herbicides may have impacts on non target organisms [5,6]. Because of their substantial uses, contamination of surface and groundwater with herbicide residues were often occur. Not only the parent herbicides pollute the groundwater; often stable degradation products are the cause of concern [7]. Because of the fast growth and short generation of microalgae, they can be used in ecotoxicological studies. Both parameters allow for assessment of acute and chronic toxicities and growth responses to pollutants, respectively [8].

Studies the toxic effects of herbicides on algae are essential for the assessment of the potential environmental impact of these compounds on aquatic ecosystems; however, the sensitivity of algae toward herbicides varies depending on the species and the analyte itself [9-11].

The aromatic nitrile "2,6-dichlorobenzonitrile" (dichlobenil) is a broad-spectrum herbicide, which has been applied to control weeds in nonagricultural areas, such as industrial sites, court yards, railway lines, motorways, car parks, in addition to forest plantations and fruit orchards. Furthermore, the analyte has been implemented in aquatic habitats in submerged aquatic weed control, particularly in association with aquaculture, such as fish hatcheries and in ponds under management of fish production [12].

In the topsoil, dichlobenil is rapidly metabolized to 2,6-dichlorobenzamide (BAM) [13]. With a high water solubility and low sorption affinity, the BAM can persist and easily leaches to ground water [14]. The dichlobenil and its degradation product BAM are among the most frequently encountered groundwater pollutants in Denmark, Sweden, Germany, and the Netherlands [7,15] in spite of the fact that its use has been discontinued in some of these countries. Dichlobenil was prohibited as an herbicide by the European Union in 2008 [16]. It is ranked among inhibitors of plant cell wall [17]. Due to its extensive uses in several terrestrial and aquatic habitats associated with the relatively high degree of persistence, volatility, and mobility, which may result in a substantial distribution together with its main metabolite to a broad suite of habitats and compartments. So far, very little information was reported regarding the potential effect of dichlobenile on algae. Thence, the aim of this work was to provide an overview about the adverse effect of dichlobenil on the growth of algae through investigating the chlorophyll (a) content, EC_{50}, and some physiological aspects of two fresh water algae; *Ankistrodesmus acicularis* (unicellular Chlorophyta) and *Anabaena flos-aquae* (filamentous Cyanobacteria) as well as their uptake to dichlobenil.

2. MATERIALS AND METHODS

Dichlobenil (97% purity) was purchased from Sigma-Aldrich (Germany). The stock and working solutions of dichlobenil were prepared in dimethyl sulfoxide (DMSO), because of its relatively low water solubility. DMSO was considered as a proper solvent in toxicity test [18]. The adverse effect of dichlobenil was carried out on two test organisms, namely *A. acicularis* (green alga) and *A. flos-aquae* (filamentous Cyanobacteria). The test organisms were isolated in pure culture from Nile River water and cultivated in standard algal

media, namely Algal Assay Procedure Bottle Test [19] for *A. acicularis*, while modified Watanabe media [20] for *A. flos-aquae.* The amount of algae inoculated in bioassay culture flasks was determined at the beginning of the experiments using chlorophyll (a) content.

The tested organisms were grown in fresh nutrient for 7 days prior to experiments, so that the organisms were in logarithmic phase when introduced to the toxicity test culture. The algal cultures were incubated at 24°C ± 2°C for *A. acicularis* while *A. flos aquae* was cultivated at 32°C ± 2°C under continuous white fluorescent light with intensity of 33.8 mE/m^2/s. The analyte concentrations were tested in the range of 1.0 to 8.0 mg/L for *A. acicularis* and .025 to 16.0 mg/L for *A. flos- aquae*, whereas the control flasks have the same amount of DMSO in the absence of dichlobenil. All the experiments were carried out in triplicate. The algal growth was determined by measuring the chlorophyll (a) Chl (a) content according to the Standard Methods [21]. The percent growth inhibition was calculated via comparing the Chl (a) of the tested concentration with control one. The percent inhibition was transformed into Probit to calculate the EC_{50} [22].

Algal biomass of each treatment was collected at maximum growth and used for total carbohydrate determination. Briefly, one mL of concentrated sulfuric acid was added to known weights from oven dried mass, left for 20 hour in an ice-bath. Subsequently, mass up the volume up to 7 mL using the concentrated acid [23]. The total carbohydrate content was measured as glucose according to Dubois et al. [24]. The ATP measurement took place using Backman liquid scintillation counter model L.S. 700 as given by Standard Methods [21].

Dichlobenil was extracted from the aqueous media by dichlomethane and its residues were determined by gas chromatography (GC), Hp 6890 (USA) equipped with electron capture detector (Ni^{63}) using capillary column HP 0.5. The column temperature was raised from 50 to 150°C with a rate of 10°C/min. The injector and the detector temperatures were 250°C and 280°C, respectively. Nitrogen was used as a carrier gas at a flow rate of 2 mL / min. The rate of dichlobenil uptake from solutions was assessed by Meites [25] in term of the rate of first order reaction, using the following equation:

$$\text{Log } C - \text{Log} C_0 = kt / 2.303$$

where C is the concentration at time t, C_0 is the initial concentration at t=0, and k is the specific reaction rate constant. A plot of the log C vs. time gives a straight line with a negative slope. Time required for 50% of the reaction to proceed is given by the equation: $t_{0.5}$=0.693/k,

where $t_{0.5}$ is the half-life time of the reaction

2.1 Statistical Analysis

Results were subjected to statistical analysis by Duncan's multiple range test [26]. Means followed with the same alphabetical in every column are not significant at 5% level.

3. RESULTS AND DISCUSSION

3.1 Changes in Chl (a) Content

Results given in Table 1 showed the changes in Chl (a) of *A. acicularis* and *A. flos-aquae* treated with different doses of dichlobenil. In case of *A. acicularis*, statistical analysis of Chl (a) values showed several arrangements of significant and non significant differences among the various treatments according to the applied doses of dichlobenil and the exposure time intervals. In case of 1.0 mg/L, the variation in Chl (a) content was not significant compared to the control by the end of 1st, 3rd and 10th days, however, the decrease in Chl (a) due to 1.0 mg/L was significant at the other time intervals. The intermediate and higher concentrations of dichlobenil (2, 4, and 8 mg/L) showed significant decreases compared to control and the variation in Chl (a) content between such treatment was also significant. In case of *A. flos- aquae,* the response in Chl (a) content varied according to the incubation period and the tested analyte concentration. At the first day of incubation, the algal cultures treated with low and intermediate concentrations of dichlobenile (0.25, 1, and 4 mg/L) did not exhibit a significant differences between their Chl (a) content and the control. Chl (a) content of the 16 mg/L dichlobenil treatment was significantly lower than the control. After the 2nd day of algal exposure to dichlobenil, there was a good significant relation between all treatments and control at all exposure periods, except for 0.25 mg/L treatment where there was insignificant differences from the control culture in the 3rd day of incubation. In general, the concentration of Chl (a) content tend to increase in the presence of low dichlobenil concentration levels. However, application of intermediate and high concentration levels significantly decrease

the Chl (a) content compared to the control culture. This trend was in line with the previous studies, which indicated a stimulative effect of low concentration of different herbicide on Chl (a) content [10,27].

Attainable results derived by probits analysis revealed that the effective concentration, which decreases Chl (a) content of both algal species to its half value, is a function of the exposure time interval. In case of *A. acicularis,* the EC_{50} values at 3, 5, and 7 days were 3.3, 2.4, and 2.3 mg/L, whereas the values were 3.4, 2.4, and 2.5 mg/L for *A. flos- aquae,* respectively. The EC_{50} values for both algae at 5^{th} and 7^{th} days approached each other. A previous report by Geyer et al. [28] found that the dichlobenil has a toxic effect on green algae (*Scenedesmus subspicatus*) with an EC50 of 2.7 mg/L at a 96 h growth study.

3.2 Variation in Carbohydrate and ATP Contents

The stress imposed on algal cells of *A. acicularis* and *A. flos- aquae* by different dichlobenil concentrations was reflected on carbohydrate and ATP contents. In general, application of dichlobenil resulted in significant decrease in carbohydrate content of both treated algal cultures. Differences in carbohydrate content of *A. acicularis* treated with 2, 4, and 8 mg/L were significant (Table 2).

However, at 1 mg/L dichlobenil treatment, carbohydrate content was not significantly different from the control culture. The same applies for variation in ATP content of previously given treatments were observed, as the concentration of dichlobenil increases the percent reduction in ATP content was increased till drastic value (98%) content was observed at 8 mg/L dichlobenil treatment. In case of *A. flos-aquae*, the variations in carbohydrate content between 0.25 and 1.0 mg/L were significant. However, the differences between 1.0 mg/L and control were insignificant, however, 0.25 mg/L dichlobenil concentration led to an increase in the accumulation of carbohydrate contents that was significant compared to the control (Table 2). Treatment of *A. flos aquae* with higher concentration levels (4 and 16 mg/L) resulted in significant decrease in carbohydrate content. ATP content, as a measure of cell vitality, showed that the *A. flos-aquae* cell activity was enhanced by the application of low and intermediate concentrations of dichlobenil (0.25-4.0 mg/L). Higher concentration level (16 mg/L) led to a relative decreases in ATP content, which coincide with the decreases in Chl (a) content (Table 1). This finding is in agreement with previous studies, which indicating that several organic pollutants and herbicides could affect certain physiological and biochemical processes in algae [29,30]. The decrease in total carbohydrate and ATP contents, attributed to dichlobenil exposure, is special concern since algae are primary producers of organic matter in aquatic environment which could affect higher levels in the food chain.

3.3 Uptake of Dichlobenil

Fellow up the dichlobenil concentration level dosed to both algal cultures revealed that the residual concentration progressively decreased as periods were extended. The uptake level of dichlobenil by both algal cells was relatively higher in the presence of low concentrations, where growth inhibition was rather limited (Fig. 1)

Table 1. Chlorophyll (a) content (µg/L) of the two different algal species after exposure to dichlobenil

Conc. of Dichlobenil (mg/L)	Chl (a) content over time (days)					
	1^{st}	3^{rd}	5^{th}	7^{th}	8^{th}	10^{th}
	Ankistrodesmus acicularis					
0	62.4^{c}	161.7^{d}	404.0^{e}	800.0^{e}	824.6^{e}	761.5^{d}
1	58.8^{c}	155.6^{d}	359.8^{d}	644.4^{d}	728.3^{d}	775.0^{d}
2	45.9^{b}	120.3^{c}	247.7^{c}	454.3^{c}	505.4^{c}	558.9^{c}
4	40.4^{b}	47.9^{b}	52.2^{b}	101.9^{b}	114.5^{b}	168.7^{b}
8	20.9^{a}	27.9^{a}	29.7^{a}	29.3^{a}	31.5^{a}	58.7^{a}
	Anabaena flos- aquae					
0	67.3^{b}	480.6^{d}	946.2^{d}	2180.5^{d}	2724.3^{d}	2512.0^{c}
0.25	61.8^{b}	437.8^{d}	1104.5^{e}	2451.7^{e}	3026.3^{e}	3444.4^{e}
1	56.7^{b}	329.9^{c}	704.2^{c}	1672.5^{c}	2207.8^{c}	2723.8^{d}
4	48.6^{b}	204.5^{b}	333.4^{b}	750.9^{b}	902.9^{b}	1106.4^{b}
16	24.3^{a}	58.2^{a}	89.3^{a}	226.2^{a}	264.8^{a}	325.8^{a}

Initial Chl (a) content = 32.8 µg/L

Table 2. Changes in carbohydrate and ATP contents of the two different algal species in the presence of dichlobenil at maximum growth

Dichlobenil Conc. (mg/L)	*Ankistrodesmus acicularis* Carbohydrate (mg/gd.wt)	ATP % of control	Dichlobenil Conc (mg/L)	*Anabaena flos- aquae* Carbohydrate (mg/gd.wt)	ATP % of control
0	139.8[d]	100	**00.0**	189[c]	100
1	128.1[d]	78	**0.25**	230[d]	185
2	98.4[c]	23	**1.0**	201[c]	125
4	43.5[b]	14	**4.0**	111[b]	102
8	12.9[a]	2	**16.0**	38.4[a]	95

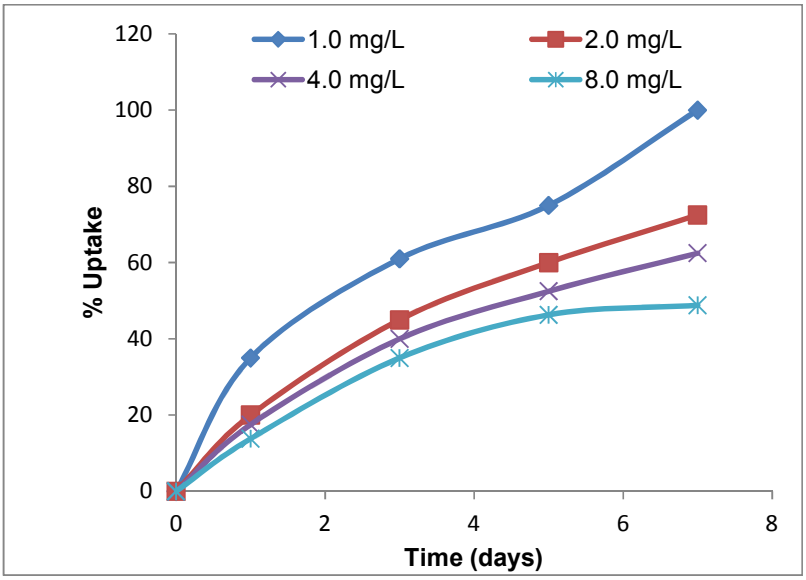

Fig. 1a. Percentage uptake of dichlobenil by *Ankistrodesmus acicularis*

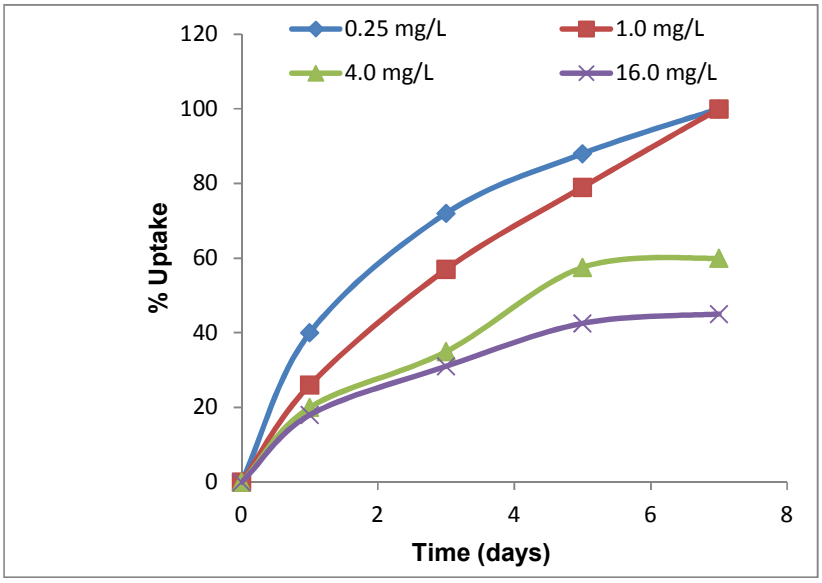

Fig. 1b. Percentage uptake of dichlobenil by *Anabaena flos- aquae*

Table 3. Kinetic data for the uptake of dichlobenil by the two different algal species

Dichlobenil Conc. (mg/L)	*Ankistrodesmus acicularis*		Dichlobenile Conc. (mg/L)	*Anabaena flos- aquae*	
	Specific rate constant (k/day)	Half life time $t_{0.5}$ (days)		Specific rate Constant (k/day)	Half life time $t_{0.5}$ (days)
1	0.64	1.10	**0.25**	2.5	0.28
2	0.51	1.36	**1.0**	1.27	0.55
4	0.38	1.82	**4.0**	0.36	1.93
8	0.25	2.77	**8.0**	0.20	3.50

The uptake of dichlobenil was found to proceed according to a pseudo-first order rate kinetic. The specific uptake rate constants in case of *A. acicularis* were relatively lower than the corresponding rates exhibited by *A. flos- aquae.* In addition, the specific rate constants were found to decrease as the concentrations were increased (Table 3).

This behavior is in accordance with the literature [31]. Meanwhile, the half- life time for a given concentrations of dichlobenil for both algal species are at variance. Such variation in the uptake rates and half- life time of dichlobenil for both algal species could have some implications on the aquatic ecosystem.

4. CONCLUSION

The results demonstrated that pollution with dichlobenil can effect on microalgae, which in turn affect on higher levels of food chain. The EC_{50} was used to evaluate the toxic effect of dichlobenil against *Ankistrodesmus acicularis* and *Anabaena flos- aquae.* The EC_{50} values for both algae at 5^{th} and 7^{th} days approached each other. The growth of the two algal species expressed as chlorophyll (a) content were significantly inhibited by the intermediate and high concentration levels of dichlobenil, meanwhile, stimulative effect was recorded for low concentration on Chl (a) content. Additionally, rapid and remarkable physiological responses occurred when the two algal species were exposed to dichlobenil. A drastic decrease (98%) in ATP content was observed at 8mg/L dichlobenil treatment in case of *A. acicularis.* Furthermore, *A. flos- aquae* cells activity was enhanced by the application of low and intermediate concentrations (0.25-4 mg/L). Higher concentration level (16 mg/L) led to relative decrease in ATP content. Based on the response in physiological and biochemical processes, *A. acicularis* was found to be more sensitive than *A.flos- aquae.* The uptake was found to proceed according to a pseudo-first order rate kinetic. The specific uptake rate constants of dichlobenile were relatively lower in *A. acicularis* than the corresponding rates exhibited by *A. flos- aquae.* The half- life time for a given concentrations in both algal species are different. Such variation in the uptake rates and half- life time of dichlobenil for both algal species could have some implications on the aquatic ecosystem. Persistent organic pollutants may have a toxic effect on algae as well as on human.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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