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Chlorpyrifos Tolerance, Utilization and Its Biodegradation by the cyanobacterium *Nostoc muscorum*

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Abstract

Chlorpyrifos (CPS) is a highly toxic insecticide to aquatic organisms and one of the commonly used organophosphorus (OP) insecticides in EGYPT that are implicated in dangerous environmental and human health disturbs. In the present study, a blue green alga *Nostoc muscorum* was used to remove CPS, from contaminated wastewater. Treatment of algal strains with different concentrations of CPS increase chlorophyll (a), total carbohydrate and protein content at lower concentrations (0.2 and 20 ppm) while the higher concentrations (50, 100 and 200 ppm) reduced them. The algal culture was further subjected to grow under P-limitation in absence and presence of CPS. The growth and phosphorus content under P- limitation registered a very poor level. When the P- restricted medium was supplemented with CPS, the algal growth and phosphorus content of cells were increased significantly. Treatment of the tested strain with various concentrations of CPS gave a remarkable response in osmolytes and antioxidant enzymes where phenols, free proline content, catalase, peroxidase and superoxide dismutase activities were extremely significantly enhanced with increasing the concentration of CPS. Results of GC-MS showed that *N. muscorum* has the ability to degrade CPS to compounds with lower toxicity and beneficial uses and CPS and its main toxic metabolite 3, 5, 6-trichloro-2-pyridinol (TCP) were not detected. Morphological changes such as pigmentations increase in the number of heterocysts and reduction the size of vegetative cells were observed. The ability to survive at high concentration of CPS and enhanced degradation make this isolate an ideal and effective candidate for its application in such harmful chemicals bioremediation.

Keywords: chlorpyrifos, *Nostoc muscorum*, Biodrgradation, Phosphorous limitation, antioxidant enzymes, bioremediation.

Introduction

Nowadays, the use of pesticides is a double-edged sword where the manipulation of insecticides in crop fields for selective control of pests in modern agriculture practice has led to dangerous environmental contamination resulting in greater loss of crop productivity, growth and development of many beneficial micro-organisms, phytoplankton's (Chen *et al*., 2016). In the same time there are more than 9,000 species of insects and pests and 50,000 species of plant b pathogens which cause 13-14 % damage of agricultural crops (Pimentel, 2009). The use of pesticides rejects loss of crops by 35% from 42%, hence the application of pesticides become essential in agricultural production system. Not only improper handling but also uncontrolled and extensive application of OP pesticides by farmers have led to the contamination of surface and ground water resulting in aquatic creatures survive in environment with sub lethal to deadly levels of pesticides (Streit and Khun, 1994). Pimentel *et al*., 1991 reported that just 0.1% of utilized pesticides get through the target pests, leaving the majority of the pesticides to enter and influence the environment. OP pesticides are responsible for around 3 million poisonings and around 200,000 deaths per year, hence they are regarded as a major global health risk by the World Health Organization (Isbister *et al*., 2007).

CPS (figure 1) is a novel broad-spectrum OP insecticide and extensively applied to control a variety of pests on agricultural and animal farms (Saulsbury *et al*., 2009).

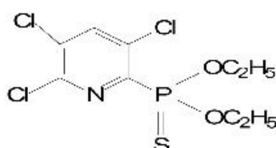


Figure 1. Molecular structure of CPS.

According to the (US-EPA, 2000), exposure to CPS and its metabolites has been associated with a diversity of nerve disturbs in humans and have high mammalian toxicity (Oliver *et al*., 1999 and Anderson *et al*., 2012). The decontamination of these insecticides from soil and aquatic systems is a hard task and as a result insecticides hold on these ecosystems for a long period of time. Ubiquities microorganisms in the environment can degrade and utilize the majority of OP compounds as a source of phosphorus or carbon or both (Bohnert and Richard, 1996). From these organisms, cyanobacteria are a diverse group of photosynthetic prokaryotes which contribute greatly to terrestrial as well as aquatic ecosystems Singh *et al*., 2016. They have the

capacity to decontaminate various pollutants, such as heavy metals (Ibrahim, 2011), pesticides (Ibrahim and Essa, 2010), organic pollutants (Sundaram, and Soumya 2011) and electronic wastes (Chatterjee and Abraham, 2017).

Therefore, this field of study is conducted to investigate the survival and tolerance of a blue green isolate with different concentrations of CPS, as well as assessing their efficiency for removing and recovering this pesticide from contaminated wastewater.

Material and Methods

Algal Strains

The algal strain (*N. muscorum*) was isolated from different water samples collected from Al-Fayoum Governorate, Egypt.

Chemicals

The OP insecticide used in this study is commercially available as PESTBAN, chemical name (O, O-diethyl O-3, 5, 6-trichloropyridin-2-ylphosphorothioate) was obtained from AGROCHEM company, Egypt (48% active ingredient).

Experimental Design

The selected algal isolate was batch-cultured in 500 mL Erlenmeyer flasks. Into each flask 200 mL of liquid culture of BG-11 medium (Rippka *et al*., 1979) was added. The initial inoculum was approximately 5×10^6 cell/mL. CPS was added to the culture medium to the final concentrations 0.2, 20, 50, 100 and 200 ppm. The culture flasks were kept under continuous illumination provided by daylight fluorescent tubes under light intensity of 3700 lux maintained constantly during the experiment. The flasks were incubated in a culture room 28°C ± 1 at under continuous shaking of 80 rpm. Samples were taken after every week intervals up to 7 weeks for the estimation of the growth in terms of chlorophyll (a).

After 3 weeks, 50 mL of algal cultures was filtrated by centrifugation at 1500 rpm for 20 minutes. The algal filtrate was used to determine CPS biodegradation products and soluble proteins in the culture medium, while algal cells used to estimate total carbohydrates, proteins, phenols, free proline and enzyme activities.

To obtain phosphorus-limited cultures, exponentially growing cells were inoculated into flasks containing medium with 1/10th of the original phosphorus concentration. The phosphorus-

limited cells were cultured in a medium without and with different concentrations of CPS and samples were taken for estimation of cell count, phosphorus content through the first 3 weeks.

Analytical Analysis

The algal culture was sonicated with Ultrasonic Homogenizer (Model: cp100, USA) to make them short fragments. Cell count was carried out using a standard haemocytometer under an Olympus BH-2 light microscope. Protein content of algal biomass was determined according to Bradford, 1976. For the determination of carbohydrate content in algal cells, the anthrone sulphuric acid method which was carried out by Fales, 1951 and adopted by Irigoyen *et al*., 1992 was used. The total phosphorus content in the algal biomass was measured by Inductively Coupled Plasma Emission Spectrometer (ICP) described by Parkinson and Allen, 1975. CPS biodegradation products were extracted from the culture medium according to Rasekhi *et al.,* 2014 and analyzed by Gas Chromatograpy Mass Spectrometry (GC-MS). Free proline content in the algal biomass was determined following the method of Bates *et al.,* 1973. Samples were prepared according to Kar and Mishra, 1976 for estimating enzyme activities where CAT activity was assayed according to the method of Chen *et al.,* 2000, POX activity by Bergmeyer, 1974 and SOD activity according to the method of Dhindsa *et al*., 1981.

Statistical Analysis

All statistical analyses were run by T-test. Data are average ±SD of three replicates.

Results

Impact of CPS on Growth of the Tested Algal Strain

Data recorded in Figure (2) indicated that the treatment of *N. muscurum* with various concentrations of CPS insecticide gave a remarkable response where lower concentrations (0.2 and 20 ppm) of CPS caused high significant increase (22%) in chlorophyll (a) content especially in 4,5th weeks of treatment. Moreover, the treatment with higher concentrations (50, 100 and 200 ppm) reduced chlorophyll (a) content (35.5%) in the first 4 weeks afterward chlorophyll (a) content of *N. muscorum* was increased significantly compared to control. The highest value (6.107µg/ml) of chlorophyll content was observed after 4 weeks at 0.2 ppm of CPS.

Effect of CPS on Carbohydrate and Protein Content of Algal Cells

Data present in Figures (3, 4) indicated that carbohydrate and protein content differed significantly from concentration to another. Treatment of *N. muscorum* with CPS caused high significant increase in carbohydrate and protein content (12-37%increase respectively) at low concentrations of CPS while at higher concentrations (16-7% decrease respectively) of the cells. Concerning with soluble protein in culture media adversely affected (28% decreases).

Impact of CPS on Free Proline and Phenol Content (µg/g dry weight) of *N. muscorum*

Data present in Figures (5, 6) exhibited that the free proline and phenol content of *N. muscorum* was extremely high significantly enhanced (2378- 23% increase respectively) with increasing the concentration of CPS.

Activities of Catalase, Peroxidase and Superoxide dismutase Enzymes of the *N. muscorum* under Various Concentrations of CPS

Data observed in Figure (7) indicated that by increasing the concentration of CPS, the activity of catalase(CAT), peroxidase(POX) and superoxide dismutase(SOD) enzymes were very high enhanced by (179, 260% and 129%) increment respectively.

The Ability of Algal Strain to Utilize CPS as Phosphorus Source

N. muscorum was grown under phosphorus limitation condition in absence and presence of CPS in order to inquire its ability to utilize CPS as a sole phosphorus source. In absence of CPS, the growth of cells was markedly dwindled under phosphorus limitation recording a decrement in the total cell count by 75 % compared with the unlimited cells (Table 1). In the other hand, the algal strains that were cultured under phosphorus limitation conditions and amended with CPS were able to grow and record a very high significant increase in the cell number (69%).

The ability of algal strains to use CPS as phosphate source was confirmed by analyzing the internal phosphorus content inside the algal biomass. Data in (Table1) revealed that the total phosphorus content of the cells that were cultured in media with P-limitation was very minor. When the limited culture was amended with CPS, the amounts of total phosphorus were increased to the same range spotted in the unlimited cells.

Identification of Degradation Products during CPS stress by (GC-MS)

The degradation products of CPS in filtrates of the algal cultures grown in BG 11 containing 20 mg/L of CPS were determined by (GC-MS). Data in table (2) showed that CPS and its main toxic metabolite TCP were not detected and compounds with lower toxicity were observed. No persistent accumulative metabolite was detected at the end of experiment.

Morphological Responses Observed Through Cultivation of *N. muscorum* Under CPS Stress

Significant morphological variations in cells of N2-fixing cyanobacterium *N. muscorum* in response to CPS were noticed in figure (8). More fragmentation, loss of some chlorophyll and distorted shaped cells also pigmentations, increase in the number of heterocysts and the reduction the size of vegetative cells as compared to control was observed.

Figure 2: Impact of Various concentrations of CPS insecticide on growth of *N. muscorum* in terms of chlorophyll (a) content.

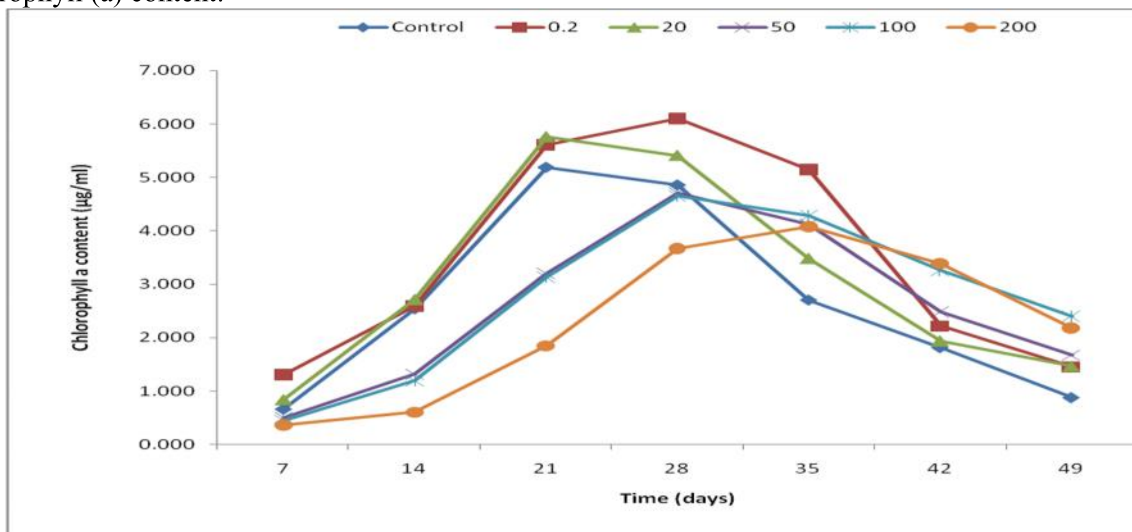
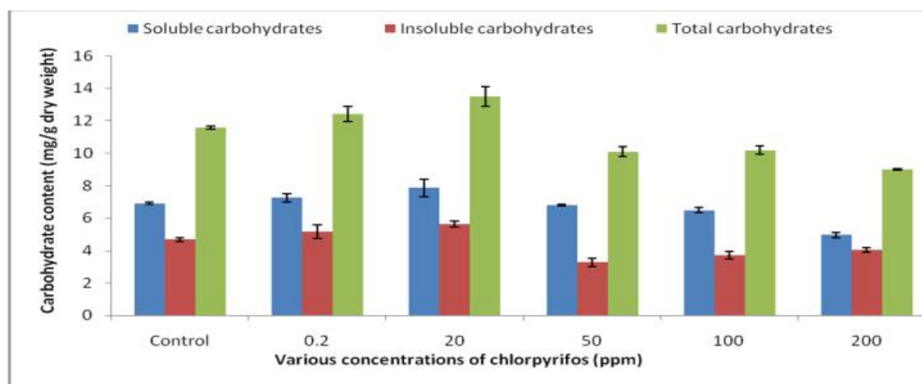
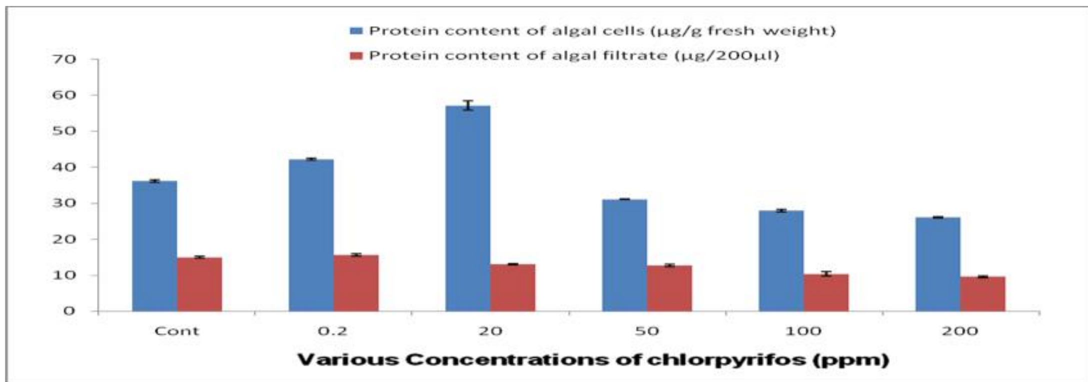


Figure 3: Impact of Various concentrations of CPS on carbohydrate content of algal biomass.



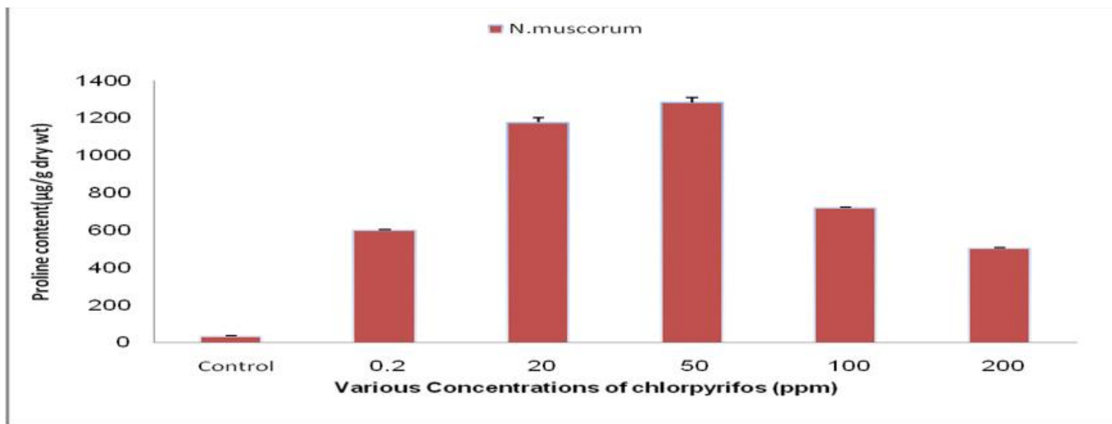
Data are means of three replicates and error bars represent the standard errors of the means.

Figure 4: Impact of various concentrations of CPS on protein content of algal biomass.



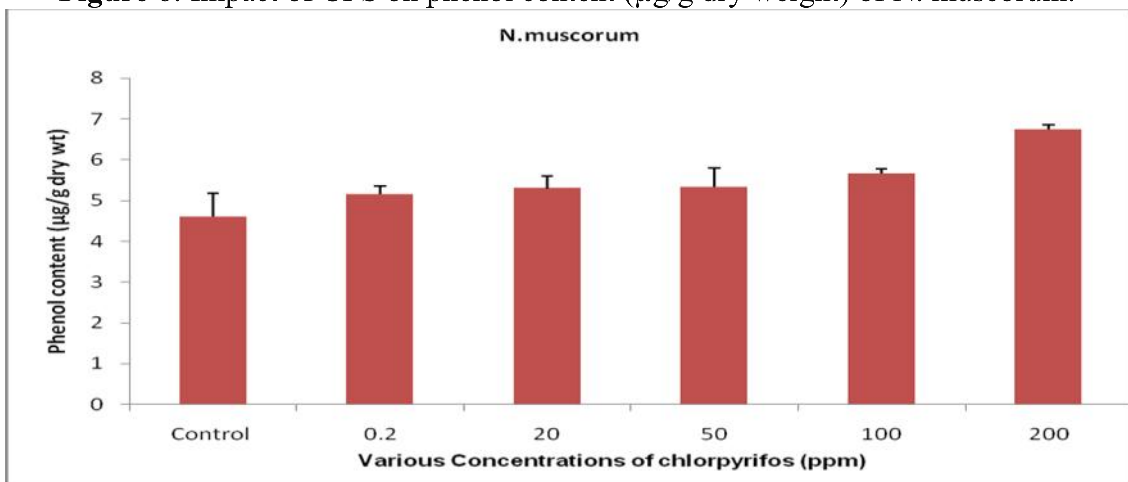
Data are means of three replicates and error bars represent the standard errors of the means.

Figure 5: Impact of CPS on free proline content (µg/g dry weight) of *N. muscorum*.



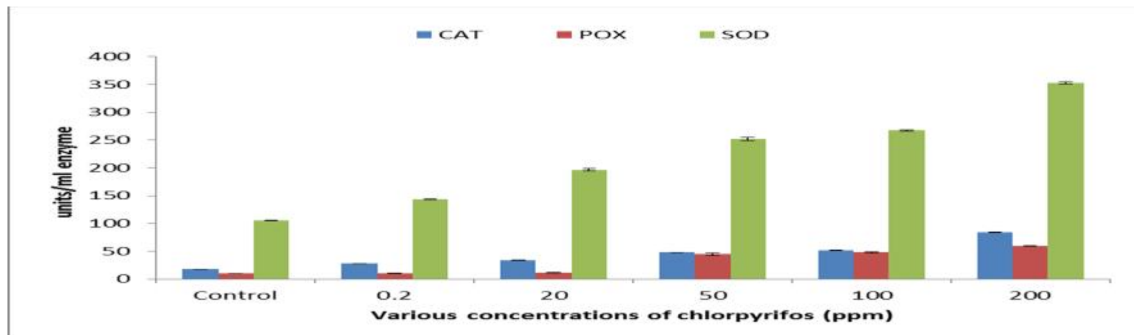
Data are means of three replicates and error bars represent the standard errors of the means.

Figure 6: Impact of CPS on phenol content (µg/g dry weight) of N. muscorum.



Data are means of three replicates and error bars represent the standard errors of the means.

Figure 7: Activities of catalase, peroxidase and superoxide dismutase enzymes of the N. muscorum under various concentrations of CPS.



Data are means of three replicates and error bars represent the standard errors of the means.

Table 1: The growth of algal strain, expressed as cell count and total phosphorus content of *N. muscorum* under unlimitation and p-limitation conditions with the addition of CPS.

Algal strains N. muscorum	**Cell growth** (No. of cells x10^4 /ml)	**Phosphorus content** (mg/g dry weight)
Unlimitation	1203±12	15.3± 0.22
P-limitation	285***↓^{a}± 5	6.3***↓^{a}± 0.13
P-limitation with different concentrations of CPS (ppm)		
0.2	816***↑^{b}± 5	7.8*↑^{b}± 0.08
20	504**↑^{b}± 21	14.4***↑^{b}± 0.02
50	536**↑^{b}± 12	13.2***↑^{b}± 0.06
100	405***↑^{b}± 6	14.6***↑^{b}± 0.06
200	327*↑ ^{b}± 2	33.4***↑^{b}± 0.08

Values are means of three replicates ± standard errors.

a Significant decrease compared with unlimitation condition .

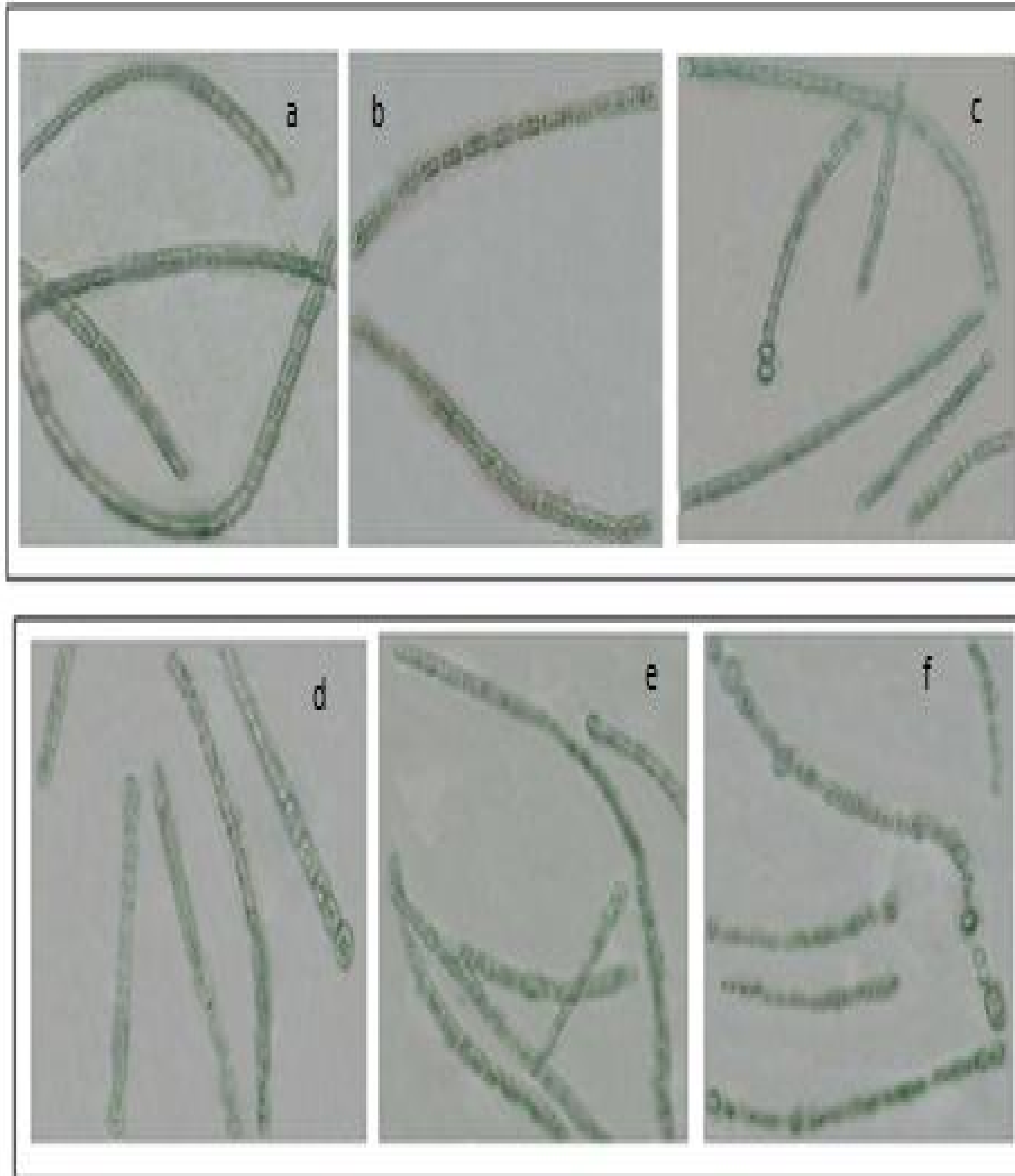
b Significant increase compared with P-limitation condition.

*↑ Significant increase at P < 0.05. **↑ High significant increase at p < 0.01.

***↑ Very high significant increase at P < 0.001.

***↓ Very high significant decrease at P < 0.001.

Figure 8: Morphological changes of *N.muscorum* under various concentrations of CPS.



Maintenance of axenic cultures of *N.muscorum* under (a) 0ppm (b) 0.2ppm (c)20 ppm (d) 50 ppm (e) 100ppm and (f) 200ppm of CPS stress.

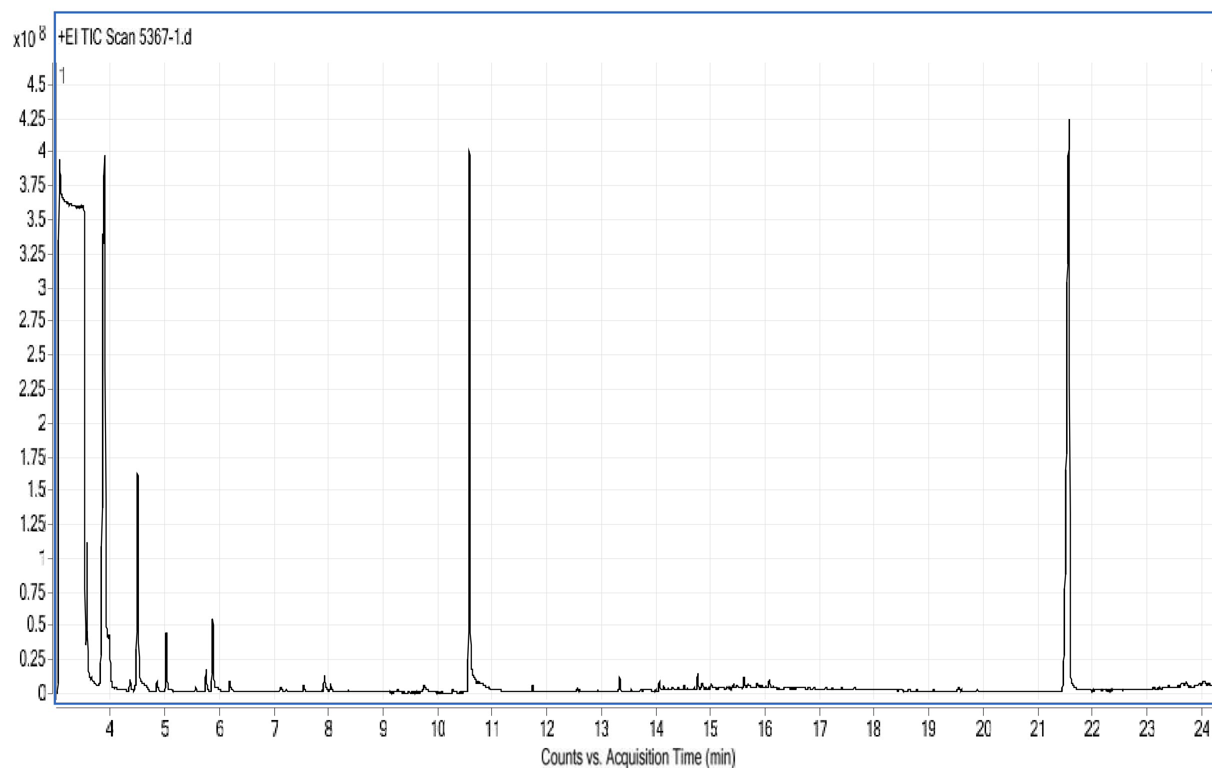
Table2: GC-MS biodegradation products of CPS.

No	Retention time	Name	Area	Peak area % of Nostoc
1	3.571	5-Methylhexahydro-1,3,5-triazine-2-thione	132759185	**2.769765**
2	3.888	Thiomorpholine	1261201805	**26.31255**
3	3.977	1,2,3-Thiadiazole-4-carboxamide	77668004.7	**1.620394**
4	4.365	2,3-Dimethylhexahydro-6H-pyrazolo[1,2-a][1,2,4,5]tetraazine	344722466	**7.191971**
		L-Cysteinesulfinic acid		
5	4.508	Picolinic acid, 6-methyl-	20561528.4	**0.428977**
6	4.856	Hydrouracil, 5,6-dihydroxy-5-methyl-	67714675.9	**1.412736**
		N(1)-[4-[4-Methoxyphenyl]-6-[trichloromethyl]-2-pyrimidinyl]-N(2),N(2)-		
7	5.03	Piperazine	13639561.7	**0.284563**
8	5.571	S,S-Bis[2-(diethylamino)ethyl] methylphosphonodithioate	13458720	**0.28079**
9	5.76	1,4-Dimethyl-pyridinium chloride	27709467.9	**0.578105**
10	5.882	3-Pyridinemethanol	102175763	**2.131701**
11	6.133	4-Pyridineethanesulfonic acid	4679185.13	**0.097622**
		Trichloroacetic acid, 2-phenylethyl ester		
12	6.197	3-Pyridinamine, N-methyl-2-nitro-	28261357.4	**0.589619**
13	6.936	Pyridine, 2-ethyl-	5452358.25	**0.113753**
		Thionicotinic acid		

14	7.04	Ethanone, 1-(4-pyridinyl)-	5138790.85	**0.107211**
15	7.559	4-Isopropylpyridine	14013153.2	**0.292357**
		Isonicotinylhydroxamic acid		
16	7.925	Pyridine, 4-tert-butyl-	25547631.6	**0.533002**
17	8.353	Metyridine	3742432.51	**0.078079**
18	9.754	Cyclohexanol, 2-[2-pyridyl]-	22021153.2	**0.459429**
19	10.298	3-Methy-2-benzothiazolinthion	6440583.67	**0.13437**
		1-Propyl-2-methyl-5-vinylpyrrole		
20	10.579	2-(2,3,3-Trimethyl-oxiran-2-yl)-pyridine	762815997	**15.91469**
21	11.752	N-[3-Methylaminopropyl]aziridine	7801906.88	**0.162772**
22	12.57	Acetyl turicine	9134917.49	**0.190582**
23	13.334	1,1-Bis[aziridyltrimethylamine]	15679039.1	**0.327113**
24	14.057	3-Chloro-6-[3-diethylaminopropylamino]pyridazine	10384415.9	**0.216651**
25	14.305	2-[3-Cyclohexylaminopropylamino]ethylthiophosphate	10032799.7	**0.209315**
26	14.726	N-[2-[1-Piperazyl]ethyl]-N'-[2-thiophosphatoethyl]-1,3-propanamine	12982558.6	**0.270856**
27	14.76	1,1,4-Trimethyl-3-pyrazalone	17876848.7	**0.372966**
28	14.855	3-Butyl-4-hydroxy-4,6,6-trimethylhexahydropyrimidin-2-thione	29419832.7	**0.613788**
29	15.032	1-Thiazol-2-yl-1H-pyrrole-2-carbaldehyde	20025910.3	**0.417802**
30	15.441	2-Chloro-1,3-bis(4-methylpiperazin-1-yl)-4-nitro-benzene	18729711.2	**0.390759**

31	15.615	2-Cyclohexene-1,4-dione, 5,6-dichloro-3-isopropyl-6-methyl-, 1-oxime, o-(4-nitrobenzoyl)-	18453296.6	**0.384993**
32	15.862	Dibenzo[ce]1,2-thiazin-9-ol, 2,10-dichloro-, 5,5-dioxide	15188535.5	**0.31688**
33	16.085	N'-[1-[4-Chlorophenyl]-1H-tetrazol-5-yl]-N,N-diethyl-1,3-propanediamine	14594392.8	**0.304484**
34	17.652	4-[γ-Diethylaminopropylamino]-5-[β,β-dimethyl-α-styryl]-6-chloropyrimidine	12125274.6	**0.252971**
35	19.549	Glucobrassicin	10348686.8	**0.215905**
36	21.54	Tetrazolo[5,1-a]phthalazine, 6-chloro-	1630654874	**34.02048**

Figure 9: Chart of GC-MS biodegradation products of CPS



Discussion

Concerning the toxic effect of pesticides, it is indispensable to get rid of these chemo-contaminants from the environment. Biological removal of chemo-pollutants becomes the method of choice, as microorganisms can utilize a diversity of xenobiotic compounds admitting pesticides for their growth and mineralize and detoxify them. The ministry of agriculture in Egypt agreed to apply this insecticide through 2014 as it was effective and cheap. Many scientists have isolated microorganisms from nature which have the capacity to degrade CPS and obtained good degradation yields (Thengodkar and Sivakami, 2010). Cells subjected to stresses have alterations in their metabolism in order to adapt with changes in their environment. The toxicity of pesticides may lead to induction of free radicals, and organisms may respond to this stress by stimulating antioxidant defense mechanism that includes enzymes such as SOD, CAT, and POD. Thus, the activity of antioxidant enzymes in the test organism grown in CPS was studied.

Our results showed that the growth of the examined strain was decreased as CPS concentration increased. In agreement with our results (He *et al*., 2013 and Kumar *et al*., 2016) reported that the effect of butachlor, acephate and imidacloprid on cyanobacterial and algal populations has been described to be low at low concentrations and thoroughly toxic at higher doses, thereby repressing their chlorophyll a content and imparting to a progressive decrease in growth.

The inhibitory effect of CPS could be due to the adsorption of this compound on the rich-lipid plasma membranes of the algal cells, thus, varying the membranes permeability (Rioboo *et al*., 2002) and diminishing photosynthetic activity.

Treatment of *N. muscorum* with CPS caused high significant increase in carbohydrate and protein content at low concentrations of CPS. In agreement with our results Manikar *et al*., 2013 and Battah et *al*., 2001) reported that the total carbohydrate and protein content of *A. variabilis* significantly increased following low dosess of malathion and thiobencarb respectively.

This increase in carbohydrate content may be due to the presence of some enzymes which can hydrolyse this OP compound and utilize CPS as nutrient sources (Xie *et al*., 2010). While the increment in protein synthesis suggested that CPS insecticide stimulated the synthesis of stress retarding proteins (Kumar *et al*., 2010).

High doses of CPS caused inhibitory impact on carbohydrate and protein content of the cells. Also, soluble protein in culture media adversely affected. A similar trend was also observrd in *A.*

fertilissima to chlorophenoxy herbicide (2, 4-D) (Kumar *et al*., 2010 and Salman *et al*., 2016). This response with different concentrations of CPS could be attributed to conversion of sugars into other metabolites and toxic action of this insecticide on the enzymatic reactions responsible for protein biosynthesis.

Level of proline content was highly accumulated with increasing CPS doses. A similar trend with was also observed in some cyanobacterial strains subjected to pesticides, salt stress and heavy metal (Salman *et al*., 2016, Fatma *et al*., 2007 and Sheeba *et al*., 2011and

Continuous increase of proline under CPS treatments suggesting its involvement in a free radical scavenging mechanism thereby has a protective role during stress. In consistent with Ashraf and Foolad (2007) who repoted that proline is an important antioxidant involved in the response to a variety of environmental stresses and acted as a signal or regulatory molecule that can activate multiple physiological and molecular responses.

Phenols formed during stress conditions, activate the various biochemical processes of the organisms. In consistent with our data (Kumar *et al*., 2010 and Kumar *et al*., 2012) reported an increase in the phenol content of *A. fertilissima, Aul.fertilissima* and *W.prolifica* was noticed in applied treatments of (2,4-D), Endosulfan and Tebuconazole. The increase in phenols content through CPS treatments suggested that phenols could be used as protectants to the organism during stress.

Algae contain several enzymatic and non-enzymatic antioxidant defense systems to maintain the concentration of ROS to protect cells from damage Noctor and Foyer, 1998). In agreement with our results Kumar *et al*., 2008 and Prasad *et al*., 2005, Manikar *et al*., 2013 and Galhano *et al.,* 2010 observed increased levels of all three of the enzyme activities in some cyanobacterial strains under test of endosulfan, malathion and bentazon pesticides suggesting the cyanobacterial stains try to accumulate more antioxidant enzymes so as to detoxify the various free radicals generated.

The increased activities of SOD, POD, and CAT in the test organism indicated that the CPS stress may have stimulated the generation of reactive oxygen species which were salvaged by elevated levels of these enzymes and assisted the organism to tolerate insecticide stress.

The growth and phosphorus content of *N. muscorum* under P-limitation recorded a very poor level. When the P-limited medium was supplemented with CPS, the algal growth and phosphorus content of cells were increased significantly. A similar trend was observed in the

growth of some cyanobacterial strains in phosphate deficient basal medium supplemented with anilofos, glyphosate indicated that herbicide and insecticide were used as a source of phosphorus (Singh *et al*., 2013 and Forlani *et al*., 2008). Our results indicated that the ability of tested strain of cyanobacteria to use CPS as a source of phosphorus when incorporated in growth medium in the absence of phosphate source.

The observed degradation products showed that CPS and its main toxic metabolite TCP were not detected and compounds with lower toxicity were noticed. Chen *et al.,* 2012 reported that a new fungal strain metabolized the supplemented CPS within 5 days. While Singh *et al.,* 2011 accounted that Synechocystis sp. strain PUPCCC 64 tolerated CPS up to 15 mg/L and major fraction of CPS was removed during the first day followed by slow uptake. The studied cyanobacterium could metabolize CPS producing a number of degradation products as evidenced by GC-MS chromatogram.

The observed morphological variations in *N. muscorum* cells exposed to CPS were agreed with Singh *et al.,* 2016 who reported that nearly 99% of cells of Synechocystis sp. PUPCCC 64 in response to Pretilachlor herbicide were lysed and pigments were released in medium. These morphological changes observed in the test organism may be due to the adaption of the cells to survive in the stressed environment or as a result of replacement of the central atom of chlorophyll, which results in the breakdown of photosynthesis (Neculita *et al* .,2005).

Conclusion

We have demonstrated in this study that *N. muscorum* strain tolerated CPS insecticide stress by increasing the level of antioxidant enzymes. Additionally, the algal strain has the capacity to utilize the pesticide and grow well in the medium supplemented with CPS as the sole phosphorus sources for growth, thus suggesting adaptation to oligotrophic environments. The ability to survive at high concentration of CPS, cheap easy culturing in the laboratory and enhanced degradation make this isolate an ideal candidate for its application in such harmful chemicals bioremediation. Hence, work in this regard should continue to characterize the genetic and enzymatic components responsible for the utilization of CPS and other OP pesticides of this strain in order to evaluate its efficiency for the bioremediation of these environmental pollutants.

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