Bioremediation Potential of *Chlorella vulgaris* and *Nostoc paludosum* on azo Dyes with Analysis of Metabolite Changes

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Abstract:

Microalgae have been used widely in bioremediation processes to degrade or adsorb toxic dyes. Here, we evaluated the decolorization efficiency of *Chlorella vulgaris* and *Nostoc paludosum* against two toxic dyes, crystal violet (CV) and malachite green (MG). Furthermore, the effect of CV and MG dyes on the metabolic profiling of the studied algae has been investigated. The data showed that *C. vulgaris* was most efficient in decolorization of CV and MG: the highest percentage of decolorization was 93.55% in case of MG, while CV decolorization percentage was 62.98%. *N. paludosum* decolorized MG dye by 77.6%, and the decolorization percentage of CV was 35.1%. Metabolic profiling of *C. vulgaris* and *N. paludosum* were performed using NMR spectroscopy. Based on 1D and 2D NMR data, 43 compounds were identified in the polar extract of *C. vulgaris*, while 34 polar metabolites were successfully determined in *N. paludosum*. The identified compounds included carbohydrates, amino acids, organic acids, dipeptides, steroids and phenols. Statistical analysis was carried out to recognize the pattern of metabolite variation between control and dye treated samples. Principal component analysis (PCA) and hierarchical cluster analysis showed that samples treated with MG are clearly separated from the control in both types of algae. Based on heat map data, the level of carbohydrates and amino acids concentrations are strongly affected by bioremediation of MG dye compared with CV dye. In conclusion, the present study proved that CV and MG dyes are considered as stress factors and the studied algae species exert their bioremediation activity without the dyes being absorbed into the cells.

Key words: Azo dyes, Crystal violet, Microalgae, Metabolomics, Malachite green, NMR spectroscopy.

Introduction:

Algae are photosynthetic organisms that are distributed in different habitats. Algae have very diverse applications including use as fertilizer, fish feed, human food or food ingredients, production of biofuels, bioremediation, drug production and in other applications like filters or for obtaining minerals (1). Microalgae are rapid-growing microorganisms that live in complex habitats and are capable of tolerating harsh environmental conditions (2). Chemically, microalgae are rich with proteins, carbohydrates, vitamins and lipids (3). Moreover, novel bioactive metabolites extracted from microalgae, especially *Nostoc* and *Chlorella* have been reported (4, 5). The antimicrobial activities of *Nostoc* and *Chlorella* were also

reported (6), and they have been shown to exhibit antitumor, anticancer and anti-oxidant activities (7-9). Their uses as bio-fuel, bio-fertilizer and in drug productions have been widely documented (10). Malachite green, an N-methylated diaminotriphenylmethane dye, has been widely used as the most efficacious antifungal agent in the fish farming industry. Although malachite green is not approved by the United States Food and Drug Administration, its worldwide use in aquaculture will probably continue due to its relatively low cost and efficacy (11). Crystal violet (CV), a triphenylmethane dye, is extensively used in the textile, pharmaceutical, food, and cosmetic industries and it is toxic to aquatic and terrestrial

life (12, 13). In recent years, the use of microalgae in bioremediation of wastewater has attracted great interest because it can be applied in the treatment of textiles industry wastewater such as *C. vulgaris* which reduce the physicochemical properties and azo compounds in textile wastewater (14). *C. vulgaris* and *Sphaerocystis schroeteri* were used in the decolorization of two textile dyes: blue and green colored dyes at different concentrations of 1, 5, 10 and 20 mg/l (15). *Aphanocapsa elachista* and *C. vulgaris* degraded four types of the industrial dye effluents with highly decolorizing efficiency (16). Fresh green algae (*Desmodesmus* sp.) were used to evaluate the effects of immobilization and some cultural conditions (Incubation time and dye concentration) to decolorize methylene blue and malachite green dyes (17). Environmental metabolomics involves the application of metabolomics to characterize the interactions between living organisms and their environment (18). The aim of this work is to study the bioremediation efficiency of *C. vulgaris* and *N. paludosum* on malachite green and crystal violet dyes and to characterize the effect of these dyes on the algal metabolites.

Materials and methods:
Chemicals
Malachite green chloride (MG), Crystal violet (CV), Extraction solvents and NMR standard components were purchased from Sigma Aldrich (St. Louis, Mo., USA).

Algal strains
The two algal species *C. vulgaris* and *N. paludosum* were obtained from Algae Laboratory, Botany and Microbiology Department Faculty of Science, Helwan University, Cairo, Egypt.

Algae cultivation and dye treatment:
The tested algae were grown in 250 mL Erlenmeyer flasks, containing 100 mL BG11 medium. The cultures were incubated under light regime at 16/8 h light/dark photoperiod for 2 weeks, in photo chamber daylight 2000 lux at 26±2 °C. Stock solutions of 1g /100 ml distilled water of the tested dyes were prepared. The respective dye stock solutions were added separately to 250 ml Erlenmeyer flasks containing 100 ml modified BG11 medium at concentrations of 20 ppm.

Decolorization study
The batch decolorization experiments were performed under incubation condition as mentioned before. Dye removal was monitored by means of color after 2 h. The aqueous media including tested dyes were separated from biomass using filter paper and the residual dye concentration in the filtrates

was determined calorimetrically using a calibration curve prepared at the corresponding optimum wavelength (588 nm for CV and 619 nm for MG) with a spectrophotometer (JENWAY-605 UV/VIS) according to the procedures outlined in the standard methods (19). Six replicates for each treatment and control experiments were measured, and then decolorization was calculated as follows:

Decolorization (%) =A0 - At/ A0× 100

A0 absorbance at zero time.

At absorbance after time of experiment (2h)

Metabolomic analysis using NMR spectroscopy
1. Sample collection
Algal biomass were harvested and immersed directly in liquid nitrogen, six replicates from each treatment were used. Samples were kept at -80 ºC for 6 h, followed by lyophilization for 24 h.

2. NMR metabolite extraction
Twenty milligrams from each sample were used for the metabolites extraction. Metabolites were extracted according to water loss percentage (20, 21) using 2: 2: 1.8 v/v methanol: chloroform: water (22). The polar fraction was separated and evaporated under vacuum.

3. NMR sample preparation and data collection
The polar fractions were resuspended in 620 μl of NMR buffer (1mM TMSP (Internal standard, (3-(trimethylsilyl)-2, 2', 3, 3'-tetradeuteropropionic acid), 100 mM sodium phosphate buffer at pH 7.3 and 0.1% sodium azide, in 99.9 atom % D_2O).

The 1D and 2D data were collected at 700 MHz with a BrukerAvance^{TM} III spectrophoto meter. NMR data collection and processing were performed according to (23).

Metabolic profiling and statistical analysis of NMR data
Polar metabolites were determined by comparing the ^1H and ^{13}C-HSQC NMR data with the Chenomx NMR suite library of compounds (Chenomx Inc., Edmonton, Alberta, Canada) and Madison Metabolomics Consortium Database (MMCD) (http://mmcd.nmrfam.wisc.edu/). Statistical analysis was carried out using MetaboAnalyst 4.0 software (MetaboAnalyst 4.0 - a comprehensive server for metabolomic data analysis) according to the bucket tables created using AMIX software with 95% confidence intervals (24). The analysis was done with 0.5-10.0 ppm spectral region and with 0.01ppm bucket widths. Water region (4.733-4.833 ppm) were excluded.

Results and Discussion:
Microalgae have been used as human food for many decades and are considered as a key

source for biofuel and bio fertilizer production. Nowadays, using microalgae in bioremediation has attracted more attention. Table 1 shows the bioremediation efficiency of *C. vulgaris* and *N. paludosum* for CV and MG dyes. *C. vulgaris* was most efficient in decolorization of MG and CV, the highest percentage of decolorization was 93.55% in case of MG, while CV decolorization percentage was 62.98%. *Nostoc* decolorized MG dye by 77.6%, and decolorization percentage of CV was 35.1%. Our results showed a clear superiority of *C.vulgaris* over *N. paludosum* in the process of color removal of the two dyes, but the greatest effect was apparent on MG followed by CV, these results were consistent with that obtained by Hoballah and Salem (25), which showed that the green algae had a higher capacity for crystal violet decolorization than blue-green algae.

Decolorization percentage obtained by *C. vulgaris* may be affected by crystal violet dye concentration: in the present study *C. vulgaris* decolorizes CV (20 ppm) by 62.98%, while Hoballah and Salem, 2015 recorded 51.6 % decolorization percentage with 10 ppm after 2 h by *Chlorella* (25). On the other hand, malachite green concentration did not affect its efficiency and our results showed that *Chlorella vulgaris* decolorization percentage was 93.55% which is higher than the percentages obtained by Hoballah and Salem, 2015 (25) for the dye concentrations of 10 and 50 ppm.

Table 1. Decolorization efficiency of *C. vulgaris* and *N. paludosum* for Malachite green (MG) and Crystal violet dyes (CV)   (20 ppm)

	Absorbance at 619 nm for (MG)			Absorbance at 588nm for (CV)		
	Absorbance at 0 h)	Absorbance after 2 h	%Decolorization	Absorbance at 0 h)	Absorbance after 2 h	% Decolorization
C. vulgaris	0.314	0.022	92.9	0.166	0.046	72.3
	0.311	0.017	94.5	0.163	0.058	64.4
	0.312	0.019	93.9	0.164	0.059	64
	0.310	0.021	93.2	0.162	0.056	65.4
	0.313	0.022	92.9	0.165	0.076	53.9
	0.312	0.019	93.9	0.164	0.069	57.9
Mean	0.312	0.02	**93.55**	0.164	0.061	**62.98**
N. paludosum	0.36	0.083	76.9	0.213	0.151	29.1
	0.355	0.079	77.7	0.21	0.134	36.2
	0.353	0.075	78.7	0.215	0.131	39
	0.357	0.090	74.8	0.22	0.141	35.9
	0.361	0.076	78.9	0.218	0.138	36.7
	0.360	0.077	78.6	0.212	0.141	33.5
Mean	0.358	0.08	**77.6**	0.215	0.139	**35.1**

The species of algae also can affect the efficiency of decolorization. *N. paludosum* decolorizes CV by 35.1%, while Hoballah and Salem (25) showed that *Nostoc* sp. decolorizes CV by 22% in case of 10 and 50 ppm, while *N. carneum* gave decolorization efficiency 44.225% of dye Methyl Orange concentrations 20 mg L^{-1} (26).

The decolorization process of dyes by algae has been done by different mechanisms: enzymatic degradation by *Nostoc*, sp. *C. vulgaris* and *Scenedesmu*s sp. have a high ability to decolorize the two dyes so they might be used in wastewater treatment of fish farms containing these carcinogenic dyes as antifungal agents (25). Also Hussein *et al*. (27) found that the main mechanism of the removal of mono-azo dye, tectilon yellow 2G (TY2G), was bioconversion in the case of unacclimated *Chlorella vulgaris* and degradation in the case of acclimated (Algae growing in media with 50, 200 and 400 mg/l TY2G) algae. On the other hand Mostafa *et al.* (16) illustrated that *Aphanocaps aelachista* and *C. vulgaris* degraded the industrial dye effluents using azo reductase enzyme.

Exploring the metabolic profile of microalgae is an essential step for understanding the nutritional value as well as the biofuel and bio fertilizer importance of these organisms. Nuclear magnetic resonance based metabolomics is a rapid and reliable technique that offers high-throughput fingerprinting, and is used widely for metabolic analysis of different types of microorganisms (22).

In this study, polar metabolites were identified by comparing our 1D and 2D spectra with NMR databases (Chenomx NMR Suite library of compounds and Madison Metabolomics Consortium Database). Table 2 shows the identified compounds in the polar fraction of the studied algae. In *C*

vulgaris, 43 compounds were identified and include monosaccharides (e.g. Glucose, Fructose), disaccharides (Sucrose), essential and nonessential amino acids (e.g. Phenylalanine, Threonine, Alanine, and Arginine), dipeptide (Glycylproline), steroid (Cholate), phenols (Homovanillate, Ferulate), vitamin (Riboflavin) and organic compounds (e.g. 2-Hydroxyvalerate).

Table 2. List of metabolites identified by ¹H and ¹H - ¹³C HSQC NMR analyses of polar extracts C. vulgaris and N. paludosum.

|	Metabolite names	*Chlorella vulgaris*	*Nostoc paludosum*
1	2-Aminobutyrate	√	√
2	2-Hydroxybutyrate	√	√
3	2-Hydroxyvalerate	√	√
4	Acetate	√	√
5	Acetoacetate	√	-
6	Alanine	√	√
7	Arginine	-	√
8	Aspartate	√	√
9	Betaine	√	√
10	Cholate	√	-
11	Choline	√	√
12	*cis*-Aconitate	√	√
13	Ferulate	√	-
14	Formate	√	√
15	Fructose	√	√
16	Galactarate	-	√
17	Gluconate	√	√
18	Glucose	√	√
19	Glucose -1-P	√	-
20	Glucose -6-P	√	√
21	Glycylproline	√	√
22	Glucuronate	√	-
23	Glutamate	√	√
24	Glutamine	√	√
25	Glycine	√	√
26	Glycolate	√	-
27	Dimethylamine	√	-
28	Homoserine	√	√
29	Homovanillate	√	-
30	Isobutyrate	-	√
31	Isoleucine	√	√
32	Lactate	√	√
33	Leucine	-	√
34	O-phosphocholine	√	-
35	Proline	√	√
36	Pyroglutamate	√	√
37	Phenylalanine	√	√
38	Pyruvate	√	-
39	Riboflavine	√	-
40	Sucrose	√	√
41	Succinate	√	√
42	Tartrate	√	√
43	Threonate	√	-
44	Threonine	√	√
45	*trans*-4-Hydroxy-L-proline	√	-
46	*trans*-Aconitate	√	-
47	Trehalose	-	√
48	Valine	√	√

The presence of carbohydrates, amino acids, vitamins and phenols in genus *Chlorella* has been reported in other studies: *Chlorella* was a remarkable source of food supplements due to presence of polysaccharides and other compounds (28). The presence of essential and non-essential amino acids in other *Chlorella* species has been reported by Thorp and Bowes (29). *C. vulgaris* has been reported as an important source of many vitamins and phenols, which are essential for human health (30, 31)

In *N. paludosum*, 34 metabolites were identified and include 14.7% carbohydrates, 52.9% essential and non-essential amino acids, and 32% dipeptides and organic compounds. Chemical shifts for all identified metabolites in both algae have been listed in Table 1 in the supplementary materials. Here, we determined the presence of glucose, fructose, sucrose and trehalose in the polar fraction of *N. paludosum.* Similarly, *Nostoc commune* release a variety of polysaccharides into the culture medium during cell growth and these polysaccharides can be used for the preparation of biopolymeric films as mentioned by (32). *Nostoc* species are nitrogen fixing organisms, rich with essential and non-essential amino acids and peptides compounds (33).

Principal component analysis (PCA) was conducted to illustrate the correlation between the algal control and dye treated algae based on metabolite level. Pair wise score plots between *C. vulgaris* control and azo treated samples showed that, control samples were clearly separated from MG dye treatments with a total variance of 67.7%, while samples from control were overlapped with CV dye treated samples with a total variance of 71.4% (Fig. 1, A & B). Similarly, *N. paludosum* (Fig. 1 C & D) samples from control were completely separated from the samples treated with MG dye, while control treatment and CV treated samples were overlapped explaining total variances of 81.4% and 85.2% respectively.

By comparing the decolorization percentage of *C. vulgaris* and *N. paludosum* to MG and CV dyes (Table1) with PCA analysis data, the results showed that as the decolorization percentage increases, the metabolite variation between control and dye also increases. The decolorization percentage of *C. vulgaris* and *N. paludosum* to MG dye is higher than the decolorization percentage of CV dye, which correlates to non-overlapping (Control samples versus MG treated samples) and overlapping (Control samples versus CV treated samples) ovals in the score plots of Fig.1.
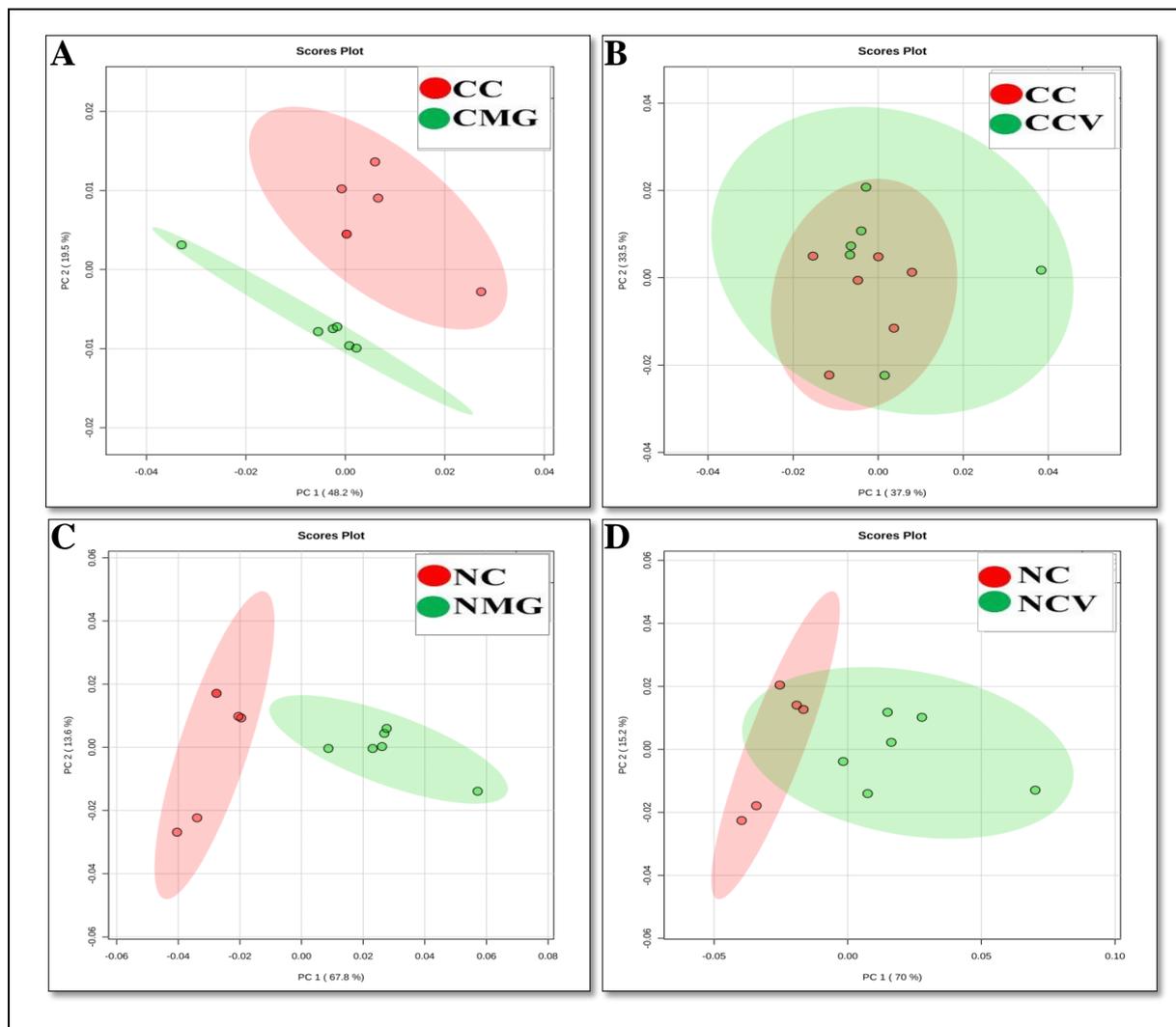
Figure 1. Pairwise principal component analysis (PCA) score plots between A) *C. vulgaris* **control (CC) versus** *Chlorella* **treated with Malachite green (CMG); (B)** *C. vulgaris* **control (CC) versus** *Chlorella* **treated with crystal violet (CCV); (C)** *N. paludosum* **control (NC) versus** *Nostoc* **treated with Malachite green (NMG); and (D)** *Nostoc* **control (NC) versus** *Nostoc* **treated with crystal violet (NCV). Colored ovals represent 95 % confidence intervals; each colored dot represents an individual sample.**

Hierarchical cluster analysis (HCA) (Fig. 2) showed two main clusters: control samples were clustered together in one group and MG treated samples were clustered in other group. Also, *N. paludosum* control samples were grouped together and separated from CV treated tissues (Fig. 2C) while samples from *C. vulgaris* control were integrated with CV treated samples.
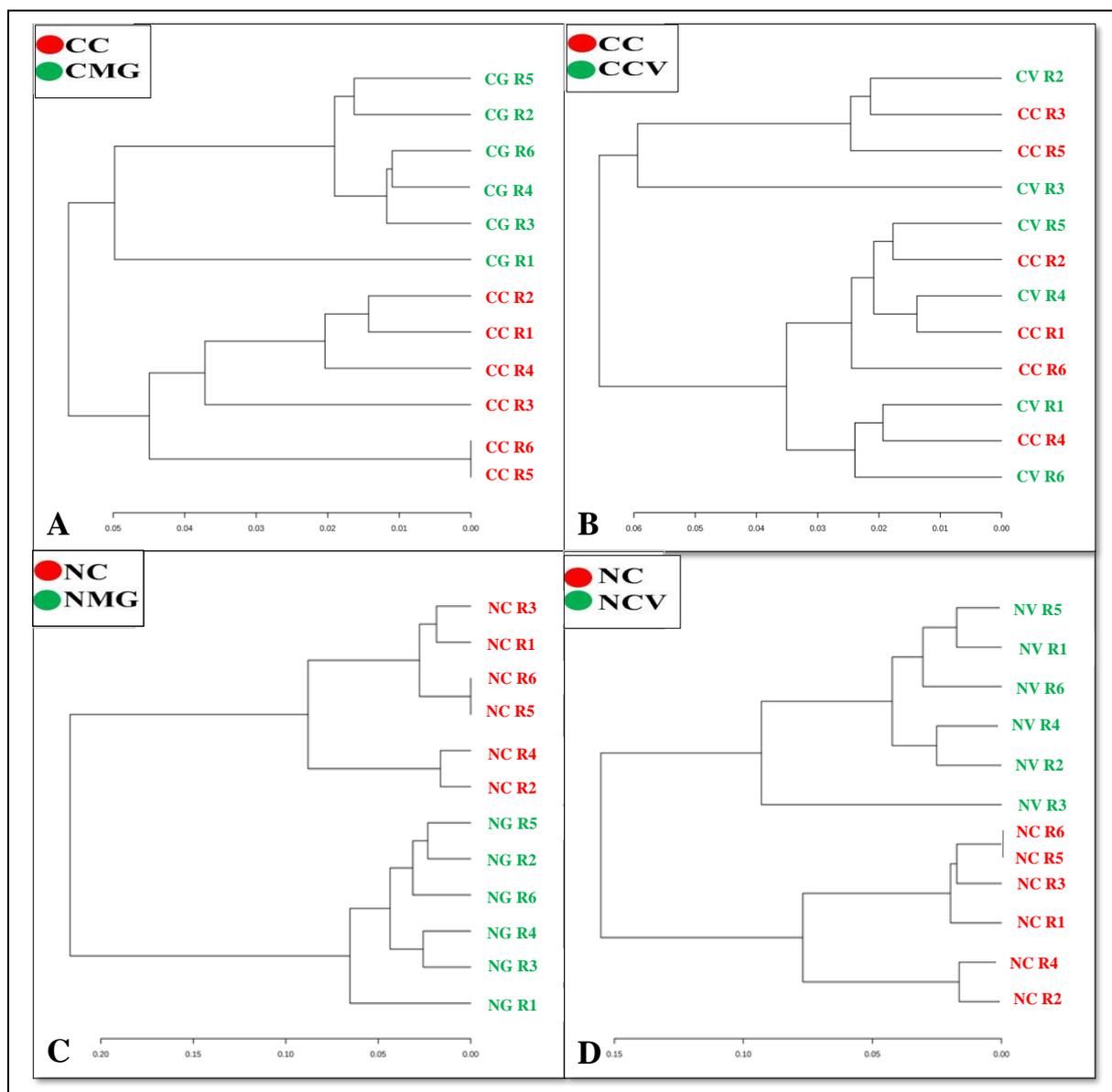
Figure 2. Hierarchical cluster analysis (HCA) showing correlations between A) *C. vulgaris* **control (CC) versus** *Chlorella* **treated with Malachite green (CMG); (B)** *Chlorella* **control (CC) versus chlorella treated with crystal violet (CCV); (C)** *Nostoc* **control (NC) versus** *N. paludosum* **treated with Malachite green (NMG); (D)** *Nostoc* **control (NC) versus** *Nostoc* **treated with crystal violet (NCV).**

Heat map correlation analyses were performed to identify the change in the metabolite concentrations based on dye treatments. In the case of *C. vulgaris*, the concentrations of monosaccharides (Glucose, Fructose, Glucose-1-P and Glucose-6-P) and amino acids betaine and valine were down regulated by using MG dye comparing with control and CV treated samples. In contrast, disaccharide (Sucrose) and amino acids (Aspartate, Threonine, Proline and Pyroglutamate) were up regulated by using MG dye. Using CV dye increased the concentration of amino acids aspartate and pyroglutamate compared with the control sample, but the level of these amino acids in MG treated sample is higher than CV treated samples (Fig. 3).
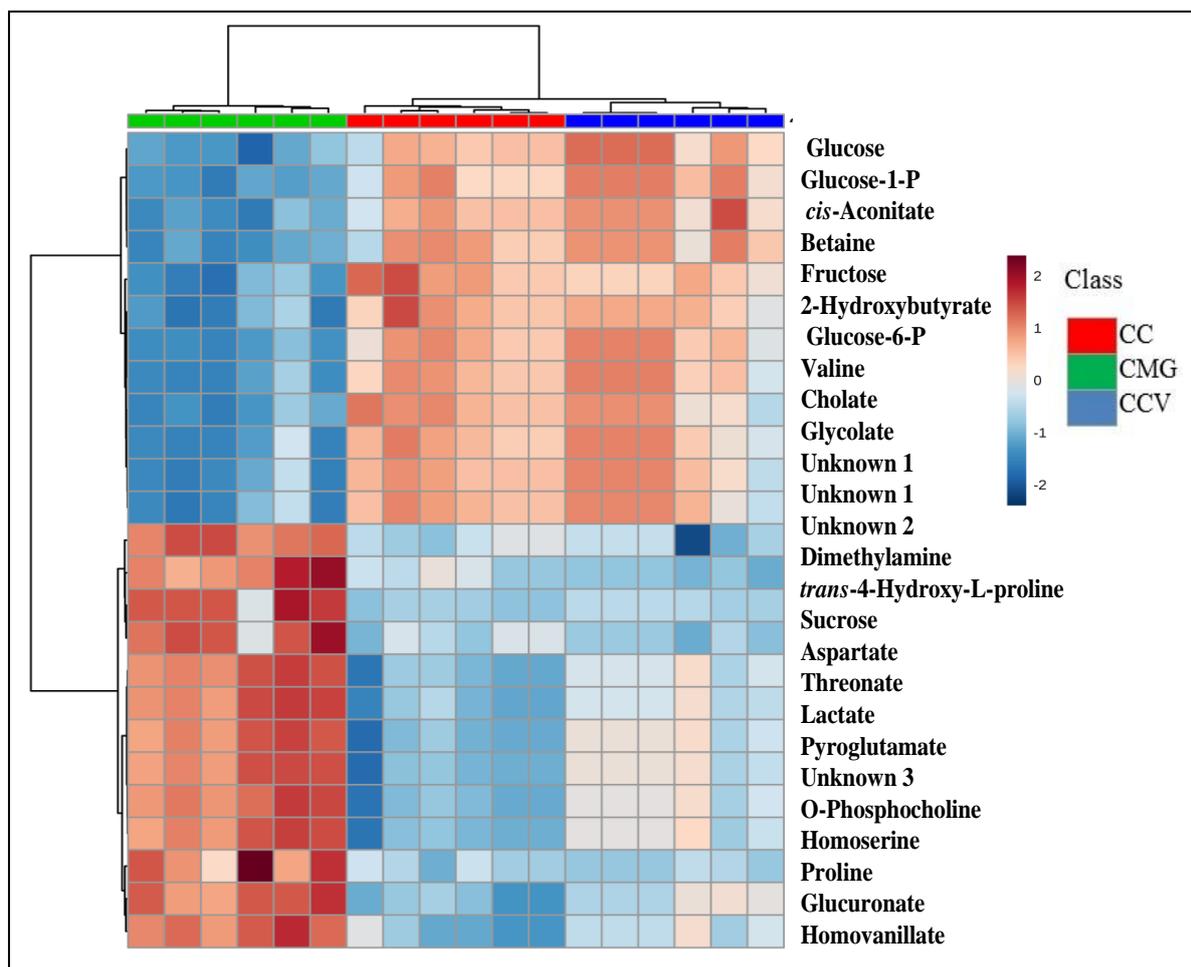
Figure 3. Heat map correlation analysis showing the effect of crystal violet (CV) and malachite green (MG) on the metabolites concentrations in the polar extract of *C. vulgaris*. Color scale is relative to the abundance of each compound. Each row represents a metabolite and each column represents a sample

An increase in the level of sucrose in the algal bioremediation process has been reported by Arora *et al.* (34). Sucrose metabolism is crucial in the diazotrophic growth of heterocystic strains of cyanobacteria, and also it can help in the glycogen synthesis in these strains (35). Proline is well known as an osmoprotectant: Mehta and Gaur (36) reported the accumulation of proline in *C. vulgaris* during stress conditions. Accumulation of pyroglutamate in green algae during stress conditions was reported by Wase *et al.* (37).

In *N. paludosum,* the concentrations of monosaccharides (Glucose, Fructose, and Glucose-1-P) were decreased by using MG and CV dyes. Also, the level of metabolites glutamine, threonine, pyruvate, glutamate, pyroglutamate, tartrate and choline decreased in dye treated samples compared with the control. On the other hand, sucrose and trehalose have been accumulated in MG treated samples (Fig. 4).
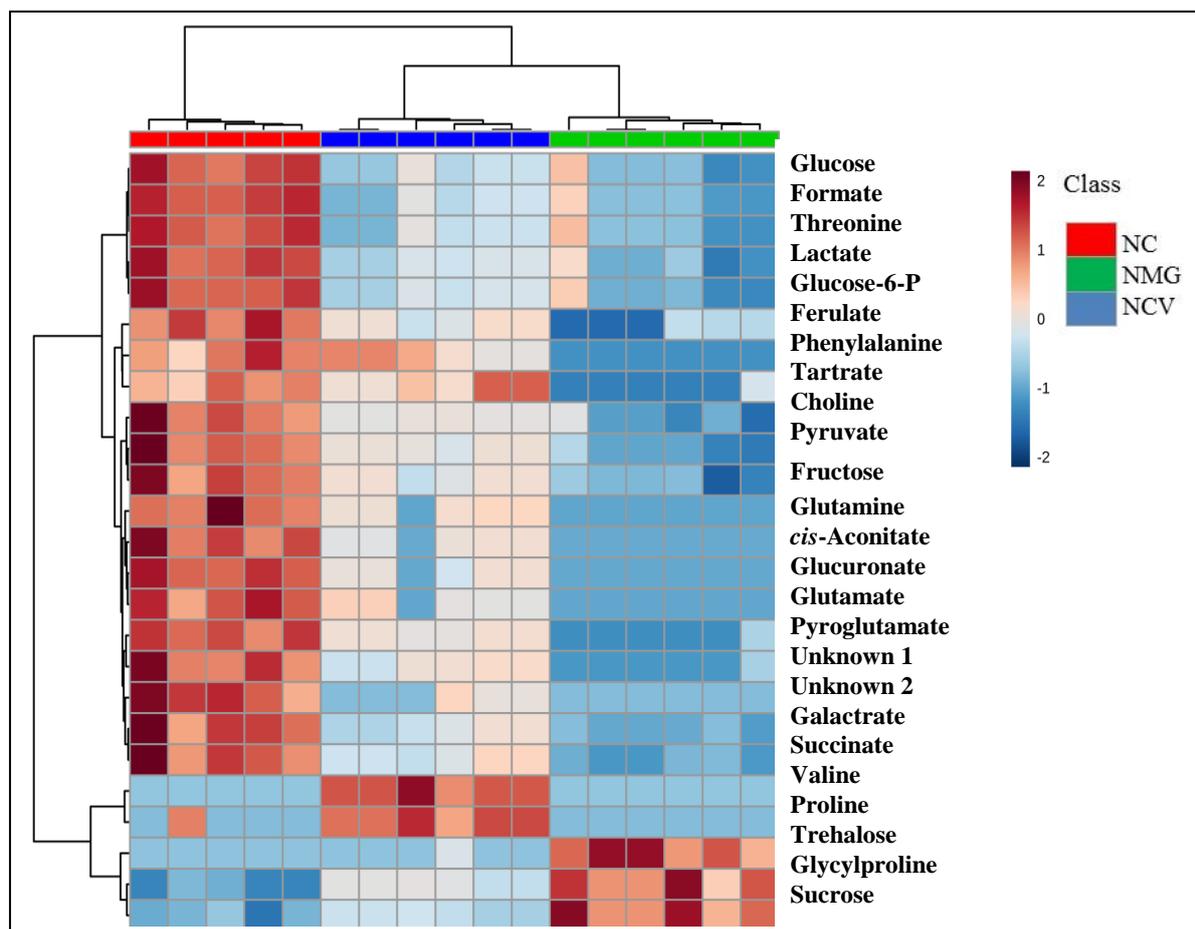
Figure 4. Heat map correlation analysis showing the effect of crystal violet (CV) and malachite green (MG) on the metabolite concentrations in the polar extract of *N. paludosum*. Color scale is relative to the abundance of each compound. Each row represents a metabolite and each column represents a sample

The decrease in sugar and amino acid concentrations in MG treated samples is higher than CV treated samples. Trehalose and sucrose play a role as compatible solutes during stress conditions because they protect the cell wall and cell proteins from damage (38, 39). The accumulation of sucrose and trehalose in cyanobactria during water stress has been reported (40). Proline was recorded to increase during stress conditions in *Anabaena variabilis* (41), also proline may confer a positive role to combat the effect of NaCl on the growth of *N. muscorum* (42)

Based on 1D and 2D NMR data, the changes in the algal metabolites were quantitative; that is, no new compounds were identified in the dye treated tissues in both *C. vulgaris* and *N. paludosum* compared with their controls. Furthermore, the dyes were not absorbed by the algal cells, and in fact, most metabolites that can be considered as stress factors for the algae. Our results have shown that the decolorization of Azo-dyes is due to enzymatic degradation mechanisms as mentioned by some studies (16, 25).

Authors' declaration:
- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in Claflin University.

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إمكانات المعالجة البيولوجية باستخدام طحلب *Chlorella vulgaris* و*Nostoc paludosum* على أصباغ الآزو مع تحليل التغيرات الأيضية

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الخلاصة:

استخدمت الطحالب المجهرية على نطاق واسع في عملية المعالجة البيولوجية لتحلل أو تكثيف الأصباغ السامة. شملت الدراسه الحالية تقييم كفاءة كلا من طحلب *C. vulgaris* و *N. paluodosum* فى إزالة اللون الخاص باثنين من الأصباغ السامة ؛ هما صبغه الكريستال البنفسجي (CV) و الملكيت الأخضر (MG) علاوة على ذلك فقد تم تحديد ملامح التمثيل الغذائي للنوعين. أيضا متابعة تأثيرالأصباغ على النمط الأيضي للطحالب التي تمت دراستها. أظهرت البيانات أن طحلب *C. vulgaris* كان أكثر فاعلية في إزالة تلوين MG و CV ، وكانت أعلى نسبة إزالة للون هى 93.55 ٪ في حالة MG ، بينما سجلت نسبة 62.98 ٪ لإزالة لون CV. اما فى حالة طحلب *N. paluodosum* كانت النسبة المئوية لإزالة لون MG هى 77.6 ٪ ، ونسبة إزالة اللون من CV كانت 35.1 ٪. تم عمل النمط الأيضي للطحلبين باستخدام التحليل الطيفي للرنين المغناطيسي النووي (NMR) استنادًا إلى بيانات 1 D و 2 D وتم تحديد 43 مركبًا في مستخلص طحلب *C. vulgaris* ، بينما تم تحديد 34 مركبا فى حالة طحلب *N. paluodosum* وشملت المركبات التي تم تحديدها الكربوهيدرات والأحماض الأمينية والأحماض العضوية و البيبتيدات الثنائية والفينولات. تم إجراء تحليلات إحصائية للتعرف على نمط تباين الأيض بين عينات مجموعه السيطره والطحالب المعالجة بالأصباغ. وقد أوضح تحليل المكون الرئيسى والتحليل العنقودي الهرمي أن العينات التي تمت معاملتها باستخدام MG منفصلة بوضوح عن عينة السيطره في كلا النوعين من الطحالب. بناءً على بيانات خريطة الحرارة يتأثر مستوى تركيز الكربوهيدرات والأحماض الأمينية بشدة بالمعالجة الحيوية لصبغ MG مقارنة بصبغة CV.

الكلمات المفتاحية: أصباغ الآزو، الطحالب الدقيقة، الأيضات، التحليل متعدد المتغيرات، مطيافية الرنين المغناطيسي النووي