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Interactions between *Microcystis aeruginosa* and coexisting bisphenol A at different nitrogen levels

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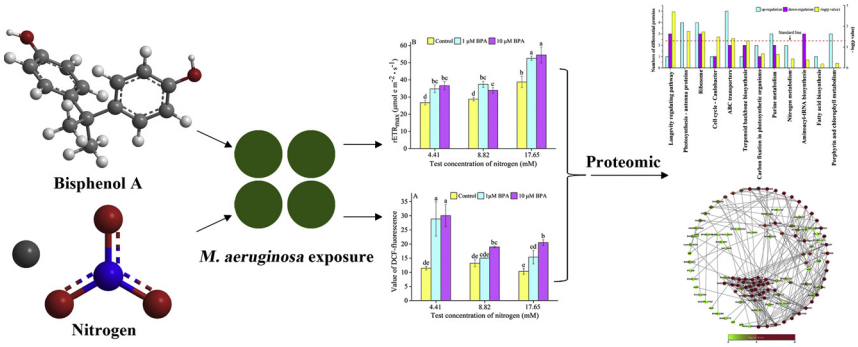
GRAPHICAL ABSTRACT



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ABSTRACT

Microcystis aeruginosa is known as a main contributor of cyanobacterial bloom. However, factors that drive its formation and dispersion remain poorly understood. The cellular-level responses to nutrient drivers of eutrophication were investigated. The results showed that growth rate of *M. aeruginosa* was significantly enhanced with the increasing bisphenol A (BPA) and nitrogen (N) level. Stress of BPA significantly inhibited cellular density, chlorophyll-a content across all the nutrient conditions, while Fv/Fm and rETRmax value were promoted by BPA. Responses of reactive oxygen species (ROS) value, superoxide dismutase (SOD) activity and malodialdehyde (MDA) content indicated that nitrogen deficiency and BPA caused oxidative stress to *M. aeruginosa*. Besides, nitrogen and BPA regulated the production and release of microcystins (MCs). *M. aeruginosa* exposed to BPA caused 95 up-regulated proteins, which was primarily associated with photosynthesis, nitrogen metabolism, glycolysis/glyconeogenesis and carbon fixation in photosynthetic organisms. The 91 down-regulated proteins were related to quorum sensing, longevity regulating and cell cycle-caulobacter, confirming that the driving force of regulating the change of cellular density and genes expression weakened. These findings provide important clues to elucidate the combined regulatory mechanisms of cyanobacterial blooms triggered by endocrine-disrupting compounds and environmental factors and help to effectively prevent and reduce cyanobacterial blooms.

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1. Introduction

In recent decades, toxin-producing cyanobacterial harmful algal blooms driven by anthropogenic eutrophication has become a major threat to fresh waters worldwide, which poses a significant risk to human health and many drinking water supplies [1,2]. *M.* aeruginosa is known as the main contributor to harmful cyanobacterial blooms [3]. Several studies have demonstrated that anthropogenic pollutants and environmental factors can together affect the cyanobacterial cells growth and cyanotoxins production, which might control the cyanobacterial blooms to some extent. For example, coexisting spiramycin inhibited the cyanobacterial cells' growth at a high nutrient level and stimulated MCs production in *M. aeruginosa* at a low nitrogen level [4]. Under hypersalinity stress, the biomass Se and intracellular microcystins in *M. aeruginosa* were released and reduced by 47% and 74%, respectively, resulting in the increasing levels of Se and microcystins in the water column [5]. An herbicide pentachlorophenol was observed to regulate the growth of *M. aeruginosa*, and the regulating effect changed from promotion to inhibition as the exposure concentration increased [6]. An antibiotic amoxicillin was found to enhance the growth of *M. aeruginosa*, but regulated the production and release of microcystins at the higher phosphorus levels [7]. In turn, *M. aeruginosa* can be used as a model strain for evaluating the toxic effects of anthropogenic pollutants on phytoplankton. In the meantime, the great contribution of nitrogen in cyanobacterial blooms and biological community structure made it a key research subject. Besides, nitrogen limitation was observed to regulate photosynthetic production across terrestrial, freshwater, and marine systems [8] and functioned in regulating the expression of microcystin biosynthesis (mcy) genes [9,10].

Bisphenol A (BPA), one of the most abundant endocrine-disrupting compounds, was found in diverse aquatic environment due to its manufacturing, usage, aging, and disposal of BPA related consumer products [11–13]. The occurrence of BPA has been reported in various environmental matrices, especially in natural waters. Huang et al. [14] reviewed the concentration of BPA in surface waters from different regions of china, and reported that BPA level was generally $< 1.0\ \mu g\ L^{-1}$. Jin et al. [15] detected that the BPA concentration ranged from 4.2 to 14, 5.9 to 141, and 4.4 to 107 ng L^{-1} in Taihu Lake, Liaohe River and Hunhe River, China, respectively. Zhang et al. [16] detected that BPA concentration ranged from 410 to 2990 ng L^{-1} with the average value of 1535 ng L^{-1} in Jialu River in Henan Province, China. Arnold et al. [17] found that BPA concentrations in drinking (tap) water were 0.099, 0.014, and 0.317 μg L^{-1} from North America, Europe and Asia, respectively. Yang et al. [18] detected that BPA concentration in surface water of the Pearl River Delta, South China was 4377 ng L^{-1}. It has been well documented that BPA is related to adverse health effects in wildlife and humans [19]. These findings have made BPA a worldwide concern.

However, how is the cyanobacteria bloom regulated by the combined effects of environmental factors and anthropogenic pollutants on cyanobacteria? Study on the combined regulatory mechanism of the effects of BPA and nitrogen on the growth responses, microcystin responses, photosynthetic responses, and antioxidant responses in *M. aeruginosa* at cellular and proteomic level may provide some important clues. A novel isobaric tag for relative and absolute quantification (iTRAQ) labeling quantitative proteomic technology was found to be a useful tool to elucidate the interaction mechanism of photosynthesis-related proteins, MC-synthesis-related proteins and antioxidant stress-related proteins under the condition of interaction between BPA and *M. aeruginosa* at different nitrogen levels. Its large potential in revealing the biological synergic and antagonistic effects of anthropogenic contaminants and environmental factors at the cellular level will be informative with respect to the evaluation of toxicity effect on nutrient elements or anthropogenic contaminants or their combination. These observed results provide important clues to elucidate the combined regulatory mechanism of cyanobacterial blooms regulated by endocrine-disrupting compounds and environmental factors and help effectively prevent and reduce cyanobacterial blooms.

2. Materials and methods

2.1. Strains and reagents

The MCs-producing strain *Microcystis aeruginosa* FACHB905 was purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB-Collection, Wuhan, China). It was cultivated on the sterilized BG11 standard medium. Sodium nitrate was purchased from Shanghai Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Bisphenol A (BPA) (CAS number: 80-05-7) was purchased from Sigma Aldrich (St. Louis, MO, USA). Besides, MC-LR standards were purchased from Sigma Aldrich (St. Louis, MO, USA) (purity ≥ 95% by HPLC).

2.2. Microbial culture and bisphenol A treatment

BG11 medium was used as base medium with the modification of nitrogen levels to produce three nutrient treatments. Nitrate was used as a N source to generate final N concentration of 4.41, 8.82, and 17.65 mM. Nitrate nitrogen at 17.65 mM is the standard N level in BG11 medium. Cultures of *M. aeruginosa* were grown for 7 d to reach the exponential growth phase in sterilized BG11 medium at 25 °C with a light-dark interval of 12: 12 h (4200 lx). Cells were separated from the BG11 medium by centrifugation at 6000 × g for 10 min at 4 °C and then washed three times with sterilized distilled water to prepare the cyanobacterial suspensions at an initial density of 0.84×10^6 cells mL^{-1} in a 1000 mL Erlenmeyer flasks containing 500 mL of modified BG11 medium. In each group, modified BG11 medium with BPA at the environmentally relevant nominal concentration of 1 μM or 10 μM was used as the treatment group at 25 °C with a light-dark interval of 12: 12 h (4200 lx). Modified BG11 medium without BPA was used for the control group. Measurements were conducted at 12:00 on each day to minimize differences in protein expression and cell physiology over the course of a day. Each treatment has three biological replicates.

2.3. Analysis of cell concentration and determination of specific growth rates

Cultures of *M. aeruginosa* were grown for 7 d and aseptically sampled daily to measure the cell density using a hemocytometer under a Zeiss Axioskop microscope (Zeiss, USA) at magnification of 400× [6]. The specific growth rate constant (μ) was calculated using exponential regression according to the cell density. The chlorophyll-a content (pg per cell) was measured based on a published method [20]. 10 mL cell culture was centrifuged at 6000 × g for 10 min at 4 °C, and the supernatant was removed. The extract was thoroughly dissolved in 90% acetone and left at 4 °C overnight in darkness, then centrifuged at 10,000 × g for 10 min at 4 °C. Absorbances at 630, 645, 663 and 750 nm were measured using a UV–vis spectrophotometer (L6S, lengguang, China), with a 90% acetone solution used as the blank. The chlolophyll-a concentrations were calculated according to Eq. (1).

$$\text{Chl-a}\ (\mu g\ mL^{-1}) = [11.64 \times (OD_{663}\text{-}OD_{750})\text{-}2.16 \times (OD_{645}\text{-}\ OD_{750}) + 0.10 \times (OD_{630}\text{-}\ OD_{750})] \times V_1 \times V^{-1} \times \delta^{-1}, \quad (1)$$

Where: V is the volume of the sample; V_1 is the constant volume of the extracted cultures each; δ is the range of the cuvette.

2.4. Analysis of cellular responses

Cultures of *M. aeruginosa* for 7 d were harvested and then were used for analyzing the chlorophyll fluorescence. Specifically, the cyanobacterial cells were incubated in darkness for 15 min, and then a

PhytoPAM fluorescence monitoring system (Walz, Germany) was used to measure the minimum fluorescence (F_0) and the maximum fluorescence (Fm) under a low and saturating actinic light photons, respectively, according to a previous study [21]. The maximum photochemical quantum yield of photosystem II (PSII) was calculated using the equation of Fv/Fm = (Fm- F_0) /Fm. The maximum relative electron transport rate (rETRmax) was directly obtained from the relative value of the plateau phase in the rapid light curve.

MC-LR was the main focus of all the detected MC variants, and it has been verified to account for more than 55% of the total MCs produced in *M. aeruginosa* [22]. During the last day of the culture experiment, 40 mL of algal culture medium was removed from the flasks, and then passed through a 13 mm diameter nylon syringe filter (0.22 μm pore size) to measure the extracellular and intracellular contents of MC-LR, respectively. The culture medium was extracted with Oasis HLB cartridges, and the algal cells was subjected to ultrasonic-assisted extraction followed by Oasis HLB cartridge cleanup process [22]. After extraction, an Agilent 1200 series high performance liquid chromatograph was used for the analysis of MC-LR. Release of MCs was defined as the percentage of extracellular MCs to total MCs, where C_{ex} and C_{in} were the extracellular and intracellular concentrations of MCs (μg L^{-1}). The MC production amount (fg per cell) was defined as the sum of C_{ex} and C_{in} divided by the cell density of *M. aeruginosa*.

2.5. Analysis of antioxidant responses

M. aeruginosa in each test group cultivated for 7 d were harvested by centrifugation at 6000 × g for 10 min at 4 °C for measuring the ROS value, SOD activity and MDA content. The detailed measurement methods of these indices are supplied in Text S1.

2.6. Protein extraction, quantification and digestion

The cyanobacterial cells before and after 1 μM BPA exposure for 7 d, were harvested for protein extraction. The detailed procedures of extraction, quantification and digestion of cyanobacterial cellular proteins were provided in Text S2.

2.7. iTRAQ labeling, desalination and identification

The detailed descriptions of the protein labeling, desalination and identification were provided in Text S3.

2.8. Statistical analyses

Data are expressed as the mean ± standard deviation in the calculations. The statistical analysis of the correlation among BPA and nitrogen treatment and cellular physiological and biochemical response characteristics was performed by SPSS version 23.0 using Duncan's post-hoc test ($p < 0.05$). Each treatment has three biological replicates.

3. Results

3.1. Cellular responses to nitrogen and bisphenol A

According to Table 1, the cyanobacterial cell growth rate was positively correlated with increasing N concentrations at equal BPA concentrations ($p < 0.05$). The highest growth rate was observed at the highest N level of 17.65 mM without BPA-treated group, which was 0.231 d^{-1} (Table 1). BPA exposure significantly inhibited growth rate in all nutrient conditions (one-way and two-way ANOVA $p < 0.05$) (Tables 1 and 2). However, BPA exposure with high concentration enhanced cyanobacterial cell growth rate at each N level compared to low concentrations of BPA. In the meantime, increasing the N concentration significantly enhanced cyanobacterial cell density at equal BPA concentrations ($p < 0.05$). Nitrogen and BPA significantly affected the synthesis of chlorophyll-a in a similar manner as the growth rate (Table 2). BPA exposure significantly restrained the synthesis of chlorophyll-a across all nutrient conditions ($p < 0.05$) (Table 1). As shown in Table 2 and Fig. 1a, nitrogen and BPA significantly affected the Fv/Fm ($p < 0.05$). Fv/Fm was markedly increased as the N concentration enhanced. Surprisingly, significant stimulation effect of BPA on Fv/Fm was also observed across all nutrient conditions ($p < 0.05$). Nitrogen and BPA affected the value of ETR in a similar manner as the Fv/Fm (two-way ANOVA $p < 0.05$) (Table 2 and Fig. 1b). The value of ETR also markedly increased as the N concentration promoted at each BPA concentration, and was significantly stimulated by BPA at all nutrient conditions ($p < 0.05$).

The nitrogen concentration significantly influenced the cyanobacterial cell MC-production amount and MC release ($p < 0.05$) (Table 1). The MC-production amount presented a positive correlation with the increasing N concentration ($p < 0.05$) (Table 1). In the meantime, BPA exposure also significantly stimulated the production of MC-LR in *M. aeruginosa* at each single N level ($p < 0.05$). Owing to the increasing cell density, the concentration of extracellular MCs generally presented a significantly decreasing trend. Nitrogen concentration was significantly inhibitory to the release of extracellular MCs. Surprisingly, BPA exposure markedly stimulated the release of MCs across all the N concentration.

The intensity of DCF fluorescence in Fig. 2a indicates that ROS level in *M. aeruginosa* was significantly influenced by the N concentration ($p < 0.05$). The ROS level at the low N group of 4.41 mM was higher than that at the moderate and high N groups at each BPA concentration, respectively. BPA exposure triggered a significant enhancement of ROS levels in cyanobacterial cells at each N level ($p < 0.05$). Besides, BPA exposure dosage showed a positive correlation with ROS level at each N concentration. The SOD activities generally markedly elevated as the N concentration increased ($p < 0.05$) (Fig. 2b). Additionally, BPA significantly restrained SOD activities in each N group. The two-way ANOVA analysis of N and BPA revealed a significant correlation with the observed ROS value and SOD activity (Table 2). The MDA content was significantly inhibited with the increasing N concentration ($p < 0.05$) (Fig. 2c). The MDA content was significantly stimulated ($p < 0.05$) by BPA in all nutrient condition and showed a positive correlation with the BPA exposure dosage (Fig. 2c). The highest content of MDA was observed in 10 μM of BPA-treated group at the lowest N level, which was 1.47-fold higher than that in the non-BPA-treated control group.

3.2. Proteomic responses to nitrogen and bisphenol A

To further elucidate the regulatory mechanism of BPA, the alterations in the related protein expression of *M. aeruginosa* under exposure to BPA at standard N level for 7 d were investigated compared with the control. The test concentration (1μM) group, which was more close to the environmental contamination level of BPA in natural waters, was selected as the target group. A total of 2379 proteins were identified (Table S1). Among these proteins, 186 of them were significantly differentially expressed (> 1.2-fold change, $p < 0.05$) between the treated and untreated cyanobacteria (Table S2). Among these differentially expressed proteins, the 95 and 91 of them were significantly up- and down-regulated expressed in *M. aeruginosa* exposed to BPA group compared to non-BPA-treated group (Fig. 3). GO (Gene Ontology) enrichment analysis was performed to classify the cellular components, molecular functions, and biological processes that these differentially expressed proteins were involved in Fig. 4a and b–c. The cellular component analysis revealed that most of the proteins with altered expression were associated with light-harvesting complex, phycobilisome, cytoplasmic part, organelle, macromolecular complex, membrane and ribosome. Among the 186 differentially expressed proteins, 109 of them have notable molecular functions, mainly involving in transmembrane transporter activity, structural constituent of ribosome,

Table 1
Growth rate, cell density on the last day of exposure, synthesis of chlorophyll-a, and production and release of MCs in response to different concentrations of BPA and nitrogen (the mean and standard deviation of three replicates are shown, and different lowercase letters represent different significant differences).

NO_3^--N (mM)	BPA concentration μM	Growth rate (d^{-1})	7d-cell density (10^6 mL^{-1})	Chlorophyll-a (pg cell^{-1})	7d-MC-content per cell (fg cell^{-1})	Extracellular MCs (μg L^{-1})	Intracellular MCs (μg L^{-1})	Release of MCs (%)
4.41	0	0.199 ± 0.001b	2.23 ± 0.004b	0.137 ± 0.010bc	19.87 ± 0.39 g	33.73 ± 1.23gh	14.66 ± 0.40 g	30.30 ± 0.27ef
	1	0.185 ± 0.003a	2.13 ± 0.012a	0.122 ± 0.007a	21.07 ± 0.06 g	32.82 ± 0.05gh	15.76 ± 0.18 g	32.44 ± 0.25d
	10	0.189 ± 0.004a	2.16 ± 0.017a	0.132 ± 0.004b	27.68 ± 0.06f	34.67 ± 0.01 g	17.77 ± 0.17f	33.88 ± 0.22c
8.82	0	0.226 ± 0.003 cd	2.42 ± 0.022 cd	0.175 ± 0.000e	39.00 ± 0.35e	41.62 ± 1.04f	21.57 ± 0.61e	34.14 ± 1.12c
	1	0.198 ± 0.001b	2.23 ± 0.010b	0.132 ± 0.001b	42.38 ± 2.13d	44.67 ± 0.40e	27.34 ± 1.45c	37.95 ± 1.15b
	10	0.204 ± 0.002b	2.27 ± 0.016b	0.140 ± 0.002c	42.86 ± 1.90d	48.17 ± 0.84d	35.07 ± 0.69b	42.13 ± 0.07a
17.65	0	0.231 ± 0.005d	2.46 ± 0.038d	0.180 ± 0.004e	59.77 ± 1.18c	55.06 ± 0.99c	23.31 ± 1.36d	29.73 ± 0.85f
	1	0.204 ± 0.004b	2.27 ± 0.059b	0.137 ± 0.003bc	64.45 ± 0.40b	58.49 ± 0.57b	26.98 ± 1.94c	31.54 ± 1.41de
	10	0.218 ± 0.003c	2.37 ± 0.144c	0.155 ± 0.003d	75.63 ± 0.19a	62.42 ± 0.06a	37.45 ± 0.57a	37.50 ± 0.36b

Table 2
Summary of two-way ANOVA analysis between the effects of nitrogen and BPA on the growth rate and various cellular contents of *M. aeruginosa*.

Dependent variables	Factors	DF	F	*P*
Growth rate	Nitrogen	2	76.32	P < 0.001
	BPA	2	56.866	P < 0.001
	Nitrogen × BPA	4	3.026	P < 0.05
7d-cell density	Nitrogen	2	76.32	P < 0.001
	BPA	2	56.866	P < 0.001
	Nitrogen × BPA	4	3.026	P < 0.05
Chlorophyll-a content	Nitrogen	2	148.048	P < 0.001
	BPA	2	218.803	P < 0.001
	Nitrogen × BPA	4	22.678	P < 0.001
MC-production ability	Nitrogen	2	601.103	P < 0.001
	BPA	2	27.201	P < 0.001
	Nitrogen × BPA	4	314.152	P < 0.01
Extracellular percentage of MCs	Nitrogen	2	56.002	P < 0.001
	BPA	2	57.600	P < 0.001
	Nitrogen × BPA	4	56.801	P < 0.001
Fv/Fm	Nitrogen	2	7.673	P < 0.01
	BPA	2	15.693	P < 0.001
	Nitrogen × BPA	4	11.683	P < 0.001
$rETR_{max}$	Nitrogen	2	80.708	P < 0.001
	BPA	2	34.645	P < 0.001
	Nitrogen × BPA	4	57.677	P < 0.001
ROS	Nitrogen	2	11.663	P < 0.001
	BPA	2	19.640	P < 0.001
	Nitrogen × BPA	4	15.651	P < 0.001
MDA	Nitrogen	2	11.619	P < 0.001
	BPA	2	94.245	P < 0.001
	Nitrogen × BPA	4	52.932	P < 0.001
SOD	Nitrogen	2	11.325	P < 0.001
	BPA	2	8.356	P < 0.01
	Nitrogen × BPA	4	9.840	P < 0.001

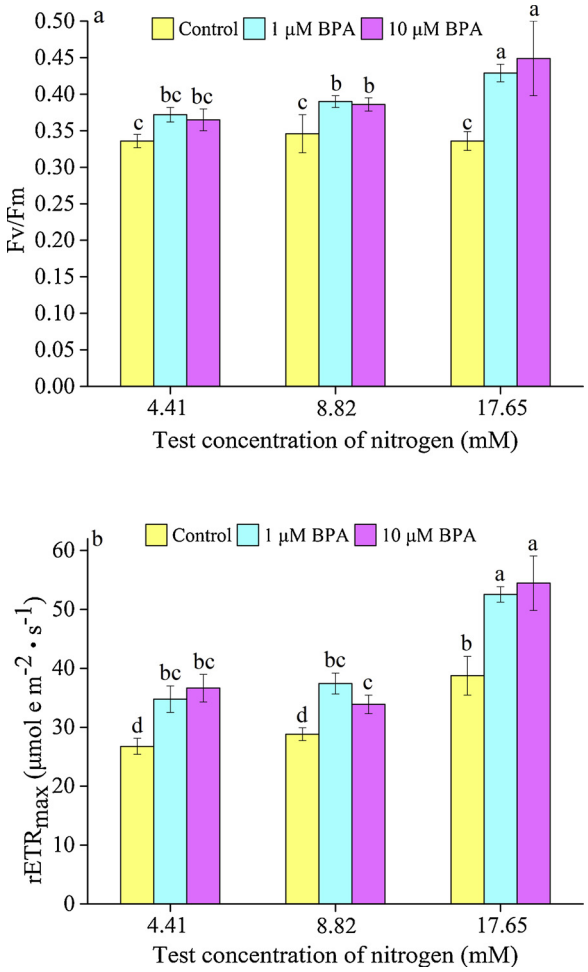

Fig. 1. Effects of bisphenol A (BPA) on (a) the Fv/Fm value and (b) the rETRmax value in *M. aeruginosa* at different nitrogen levels under BPA exposure for 7 d. Significant differences in the means among different test concentrations of BPA and nitrogen are indicated by 'a, b', at $p < 0.05$ according to a parametric one-way ANOVA coupled with a post-hoc comparison. Error bars represent standard deviations (n = 3).

transporter activity, phosphoenolpyruvate carboxykinase activity. The biological process analysis indicated that 85 of the 186 proteins with altered expression were related to cellular nitrogen compound biosynthetic and metabolic process, oxidation-reduction process, translation and ion homeostasis.

DAVID (Database for Annotation, Visualization and Integrated Discovery) analysis was performed to elucidate the metabolic pathways of these altered proteins involved in. The results (Table S3) indicated that the 186 differentially expressed proteins participated in different metabolic pathways in *M. aeruginosa* ($p < 0.05$) with significant enriched level are mainly involved in photosynthesis, ribosome, longevity regulating pathway, cell cycle-caulobacter and terpenoid backbone biosynthesis. Furthermore, to better explain which pathways associated with cellular homeostasis are governed by up- or down-regulated proteins, the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis was utilized. The results are shown in Fig. 5 that among all the related pathways, only photosynthesis, porphyrin and chlorophyll metabolism, nitrogen metabolism, fatty acid biosynthesis, glycolysis/gluconeogenesis, RNA degradation and citrate cycle were significant pathways governed by the up-regulated proteins, especially the photosynthesis (with enriched score of 1.76). There were also 15 metabolic pathways triggered by down-regulated proteins shown in Table S3.

4. Discussion

The growth of microalgae is limited by nutrient supply including

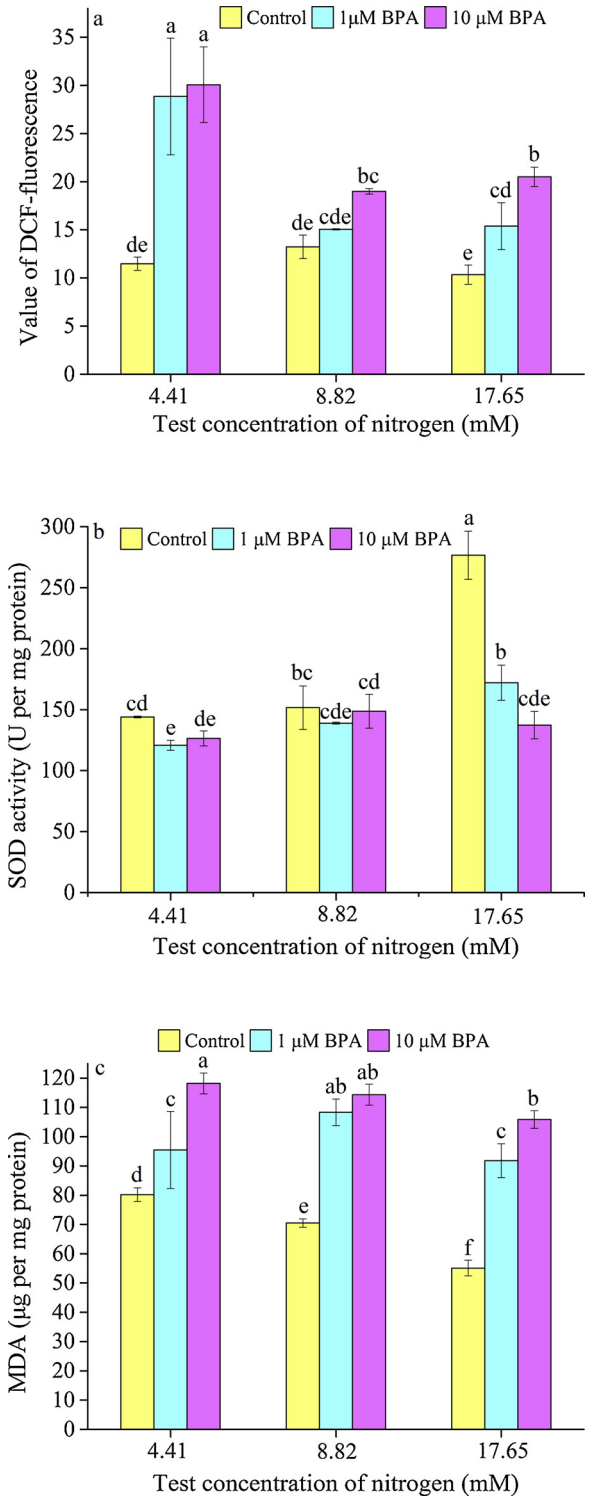

Fig. 2. Effect of BPA on the (a) ROS level, (b) SOD activity and (c) MDA content of *M. aeruginosa* at different nitrogen levels under BPA exposure for 7 d. Mean and standard deviation of three replicates are shown; different alphabet letters indicate a significant difference in the mean among groups at each nitrogen level at $p < 0.05$ according to a parametric one-way ANOVA testing coupled with a post-hoc comparison. Error bars represent standard deviations.

nitrogen and phosphorus [23,24]. In this study, nitrogen acted as the limiting factor and positively regulated the growth of *M. aeruginosa*. According to Table 1, significant stimulation effect of nitrate nitrogen on the growth of *M. aeruginosa* was observed. The up-regulated nitrite transport system substrate-binding protein (NrtA) might be the main

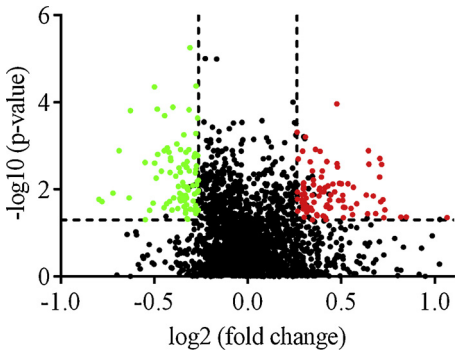

Fig. 3. Distribution of the differentially expressed proteins under BPA stress. The differentially expressed proteins with a fold change of ≥1.2-fold or ≤ 0.83-fold and $p \leq 0.05$ are shown in the Volcano plot. The Y-axis represents the p value in the form of log 10; the X-axis represents the fold change in protein expression in the form of log 2 ratio. The green dots indicate down-regulated proteins, and the red dots indicate up-regulated proteins (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

regulator of this effect. The protein NrtA, an important enzyme associated with nitrogen metabolism and ABC transporters, participated in encoding the active nitrate transport system of the cyanobacterium and played an key roles in cyanobacterial cell's growth and was involved in the active transport of nitrite [25]. Thus, the up-regulated expression of protein NrtA facilitated more cellular nitrate uptake for enhancing cyanobacterial cell growth. Previous studies have observed that many environmental contaminants, including antibiotics and pesticides, could restrain the growth of *M. aeruginosa* [26,27]. Toxicity of BPA in *M. aeruginosa* also followed this pattern. An explanation of this BPA-dependent inhibition was related to the regulation of the following proteins. Branched-chain amino acid transport system ATP-binding protein (LivF) and neutral amino acid transport system ATP-binding protein (NatE) are ABC transporters in the periplasm, which is an important class of transmembrane transporters that are involved in the import and export of amino acids. The LivF gene encoding hydrophilic proteins of 255 amino acids, played an important role in growth defect [28]. NatE gene encoding ATPases, functioned in cellular optimal growth [29]. The down-regulation of LivF and NatE suggested the growth of cyanobacterial cells did not reach the optimal state and was restrained to a certain extent. This finding was confirmed in Table 1. Besides, the protein LivF also participated in quorum sensing together with fused signal recognition particle receptor (FtsY). Quorum sensing was involving in controlling the expression of genes in general and optimizing the growth conditions of cyanobacteria with changes in external conditions. Cyanobacterial cells communicated by quorum sensing to coordinate the production of cyanobacterial growth, photosynthesis, MCs production factors. The down-regulated expression of FtsY and LivF led to the inhibition of quorum sensing pathway, implying that the driving force of regulating the alteration of cellular density and genes expression weakened. This can be the reason for the inhibition of growth of cyanobacterial cells.

BPA exposure led to decreased synthesis of chlorophyll-a, especially at lower nitrogen levels. Xiang et al. [30] obtained similar result that the synthesis of chlorophyll-*a* was inhibited in *Cylindrospermopsis raciborskii* as a stress response to BPA. The main reason was that chlorophyll biosynthesis proteins ChlL and ChlP were down-regulated. The chlorophyll fluorescence parameters associated with Fv/Fm and ETR were efficient ways to monitor photosynthetic response of plant under various stress conditions [31]. Fv/Fm and the value of ETR were markedly increased as the N concentration promoted at each BPA concentration, and were significantly stimulated by BPA at all nutrient conditions (two-way ANOVA $p < 0.05$) (Table 2 and Fig. 1). This was the result of significantly improved photosynthesis. Photosynthesis

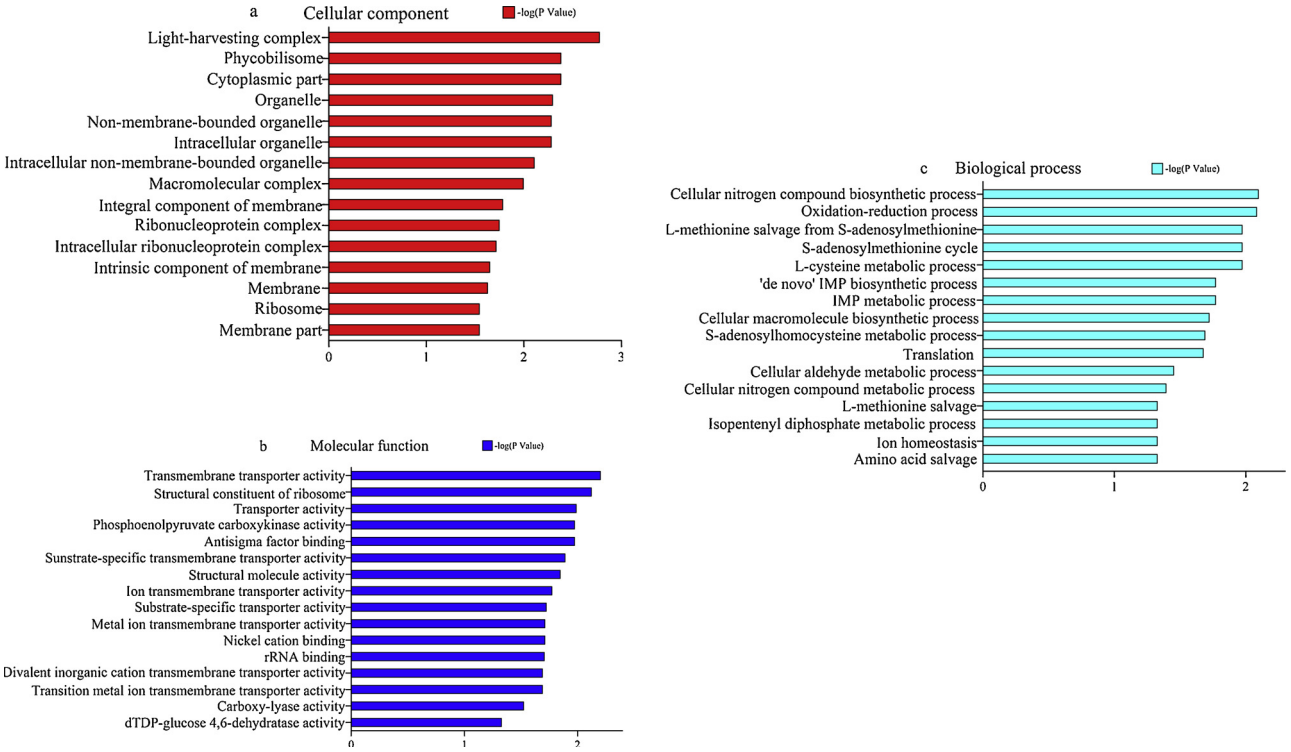

Fig. 4. GO classification of the differentially expressed proteins in *M. aeruginosa* at different nitrogen levels under BPA exposure for 7 d. (a) The 15 components for cellular components, (b) molecular function, and (c) biological process of the differentially expressed proteins are presented along with their respective enrichment score represented as a *P* value.

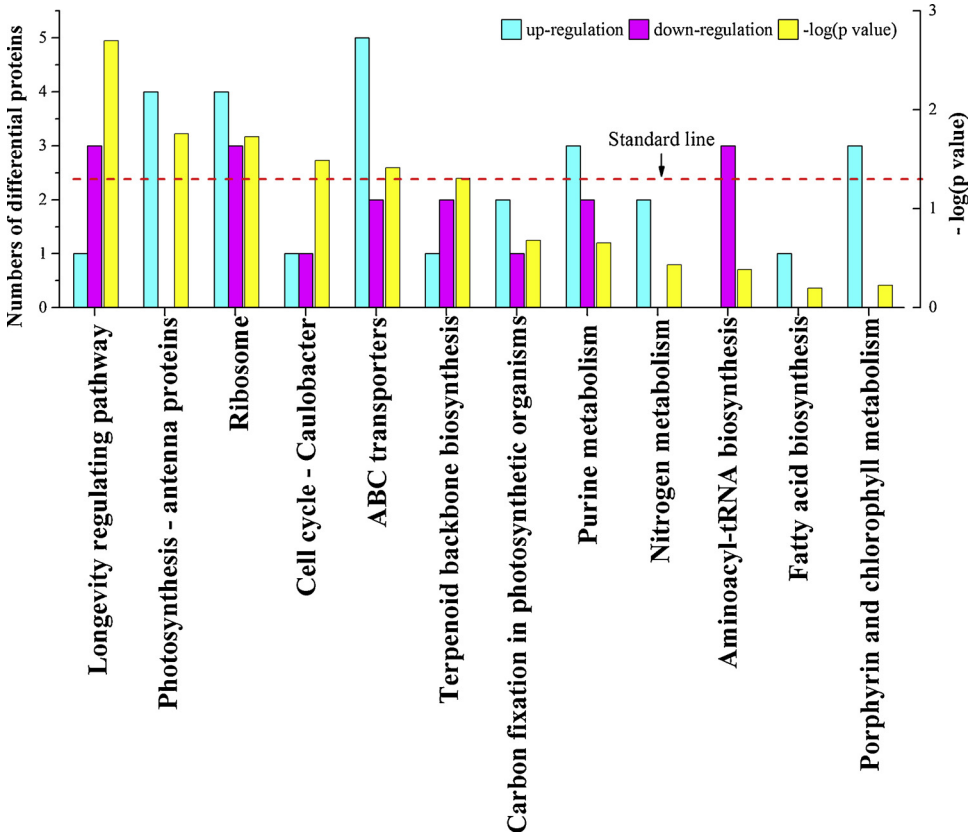

Fig. 5. Pathways associated with up- and down-regulated proteins between the post-exposure BG11 with BPA group and pre-exposure BG11 group were determined using a DAVID analysis. An enhanced score [-log(*p* value)] of ≥1.3 threshold (red dotted line) was considered significant (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

were mainly involved in proteins phycocyanin alpha chain (CpcA), phycocyanin beta chain (CpcB), photosystem I subunit III (PsaF) and photosystem II cytochrome b559 subunit alpha (PsbE). Protein CpcA and CpcB were vital and essential genes that encoded phycocyanin, and phycocyanin was an essential component of phycobiliproteins. Phycobiliproteins together with linker polypeptides, has large potential in absorbing light energy and effectively delivering light energy to the reaction center containing chlorophyll [32]. The up-regulated expression of protein cpcA and cpcB suggested that the synthesis of phycocyanin was stimulated in *M. aeruginosa* exposed to 1 μM BPA, implying that BPA could promote more light energy to be delivered to the reaction center. Protein PsbE participated in a secondary electron transport pathway that helps protect PSII from photo-damage. The up-regulated of protein PsbE implied that the reaction center was protected and photosynthesis efficiency was elevated. Further support for this inference was provided by the results for the chlorophyll flulrescenece parameters, including Fv/Fm and rETRmax values in Fig. 1. Protein PsaF has been shown to facilitate electron transfer from plastocyanin to the reaction center - P700, situated in subunits I_a and I_b [33]. Hence, the up-regulated psaF speeded up the transfer of electron to P700 to improve the photosynthesis efficiency. The current study observed significantly elevated Fv/Fm and rETRmax values treated by BPA ($p < 0.05$) (Table 1 and Fig. 1) were the best evidence of these results.

The MC-production ability per cell was stimulated by nitrogen sufficiency and BPA exposure, while the release of MCs were promoted by nitrogen deficiency and BPA exposure (Table 1). Production of MCs was regulated by microcystin synthases, including protein mcyB, mcyD, mcyA, and mcyC [34]. Protein mcyB was significantly up-regulated, while proteins including mcyD, mcyA and mcyC were stably expressed in the present study. Furthermore, the expression of protein mcyB in response to nitrogen and BPA varied in a similar tendency as MC-production ability, indicating that MC-production was regulated by nitrogen and BPA at the proteomic level. Zhang et al. [35] observed similar result that coexisting PAH contaminant promoted MC-production. Ye et al. [27] observed stimulated release of MCs under exposure to a chiral pesticides. Synthesis and release MCs could improve the competitiveness of *M. aeruginosa* against other microalgae to resist the external environment and prevent themselves from being preyed [36]. It has been also reported that the production of MCs is directly related to the function of cell division [37]. MCs may protect the cell against oxidative stress by binding to cysteine residues on redox-sensitive proteins, effectively blocking attack by ROS, which was another explanation for BPA-stimulated release of MCs. The LivF gene encoding hydrophilic proteins of 255 amino acids, played an important role in growth defect [28]. NatE gene encoding ATPases, functioned in cellular optimal growth [29]. The down-regulation of LivF and NatE and up-regulation of McyB was considered the mechanism to synthesize and release MCs in order to improve their competitiveness against other microalgae to resist the external environment [36]. The production and release of MCs were stimulated by BPA even at the lowest N level, indicating that MCs was more sensitive than growth rate in response to the stress cause by BPA.

To confirm the hypothesis that the oxidant damage might have occurred in the low N concentration treatment group, which was based on the results of cellular growth response, the ROS value, MDA contend and SOD activity were measured to confirm the predicted metabolic responses to different N and BPA treatments (Fig. 2a and b–c). A variety of stress conditions are known to cause enhanced production of ROS, for example, high light intensity and carbon depletion [38]. Previous study has also founded that metabolic activities, mainly respiration and photosynthesis, frequently result in the production of ROS [39]. To avoid the potential damage of ROS, cells have evolved protection mechanisms that include antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and peroxidases [40].

The low growth rate of cyanobacteria cells associated with low nitrogen concentration in this study, which demonstrated that the growth of cyanobacteria cells was stressed under low nitrogen condition, resulting in the more generation of ROS. In addition, MDA was used as an indicator of the destruction of cell membrane lipids by peroxidation caused by external environmental stresses [41]. The significantly higher MDA content found in cells grown at low nitrogen concentrations in the present study therefore further indicates the generation of ROS in *M. aeruginosa* cells incubated with low nitrogen concentrations. The antioxidant SOD mainly plays a role in scavenging ROS to protect cells against external stress [42]. SOD activity in this study was dramatically lower at high phosphorus concentrations, suggesting that a low-nitrogen environment might cause severe membrane damage in *M. aeruginosa*.

These functional proteins, including 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (IspD), phosphoenolpyruvate carboxykinase (ATP) (PckA) and phosphoglycerate kinase (Pgk), superoxide dismutase (SodB), ATP-dependent Clp protease (ClpP) and 5-(carboxyamino) imidazole ribonucleotide synthase (PurK), might be the biomarkers to regulate the antioxidant responses. The IspD gene participates in terpenoid backbone biosynthesis which is an essential metabolic process that is sensitive to chemical inhibition [43]. The up-regulation of protein IspD suggested that more IspD enzyme inhibitors were synthesized to resist BPA stress in *M. aeruginosa.* This finding was confirmed by the results of DCF-fluorescence intensity and MDA content (Fig. 2a and c). Phosphoenolpyruvate carboxykinase (ATP) (PckA) and phosphoglycerate kinase (Pgk) were key enzymes involving in photosynthetic carbon fixation and glycolysis/gluconeogenesis. Phosphoenolpyruvate carboxykinase (ATP) (PckA) catalyzed phosphoenolpyruvate and fixed CO_2 with ADP to transform into oxaloacetate and generating ATP, and then the oxaloacetate and ATP entered into TCA cycle to produce more energy for a variety of cyanobacterial metabolic activities. Phosphoglycerate kinase (Pgk) is a transferase that catalyzes the reversible transfer of a phosphate group from 1,3-bisphosphoglycerate (1,3-BPG) to ADP producing 3-phosphoglycerate (3-PG) and ATP. The up-regulation of PckA and Pgk suggested the ATP synthesis in *M. aeruginosa* was improved during BPA exposure process, which implied that more energy was provided to stimulate cyanobacterial growth, MCs-production and photosynthesis. This was consistent with the results showed in Table 1and Fig.1.

Superoxide dismutase (SodB), ATP-dependent Clp protease (ClpP) and 5-(carboxyamino) imidazole ribonucleotide synthase (PurK) were involving in longevity regulating pathway with the enriched score of 2.70 (Fig. 5). As a member of the iron/manganese superoxide dismutase family, SodB transforms toxic superoxide into hydrogen peroxide and diatomic oxygen and clears mitochondrial reactive oxygen to protect cells against cell death [44]. The down-regulated biosynthesis of SodB implied the inhibited SOD activities and being insufficient for scavenging intracellular ROS, which was consistent with the significantly decreasing SOD activities and increasing ROS and MDA content detected in the current study (Fig. 2a and b–c). ATP-dependent Clp protease proteolytic subunit (ClpP), an essential component to form the protein complex of Clp protease, also participated in cell cycle-caulobacter pathway (Table S3). It has been reported about the cell cycle-caulobacter pathway, the ClpP subunit associates with ClpX, referring to as the ClpP-ClpX protease system, which is an autolyzed system that governs normal cell division and proliferation [45]. The ClpX chaperone responsible for substrate recognition has ATPase activity to provide energy for the unfolded substrate. The unfolded substrate will be fed into the serine-type peptidase proteolytic core to form the ClpP subunit, which has the function of preventing protein aggregation and disassembling preformed complexes or aggregates [46]. Direct evidence shows that the Clp-protease is involved in a program of circadian-regulated proteolysis, in which proteins are targeted for degradation by the ClpX–P1–P2 complex at specific circadian times, as well as in response to a dark pulse in cyanobacteria [47]. Down-regulation of ClpP leads to a decreased ability to depredate proteins and triggers damage occurrence from external factors. 5-(carboxyamino) imidazole

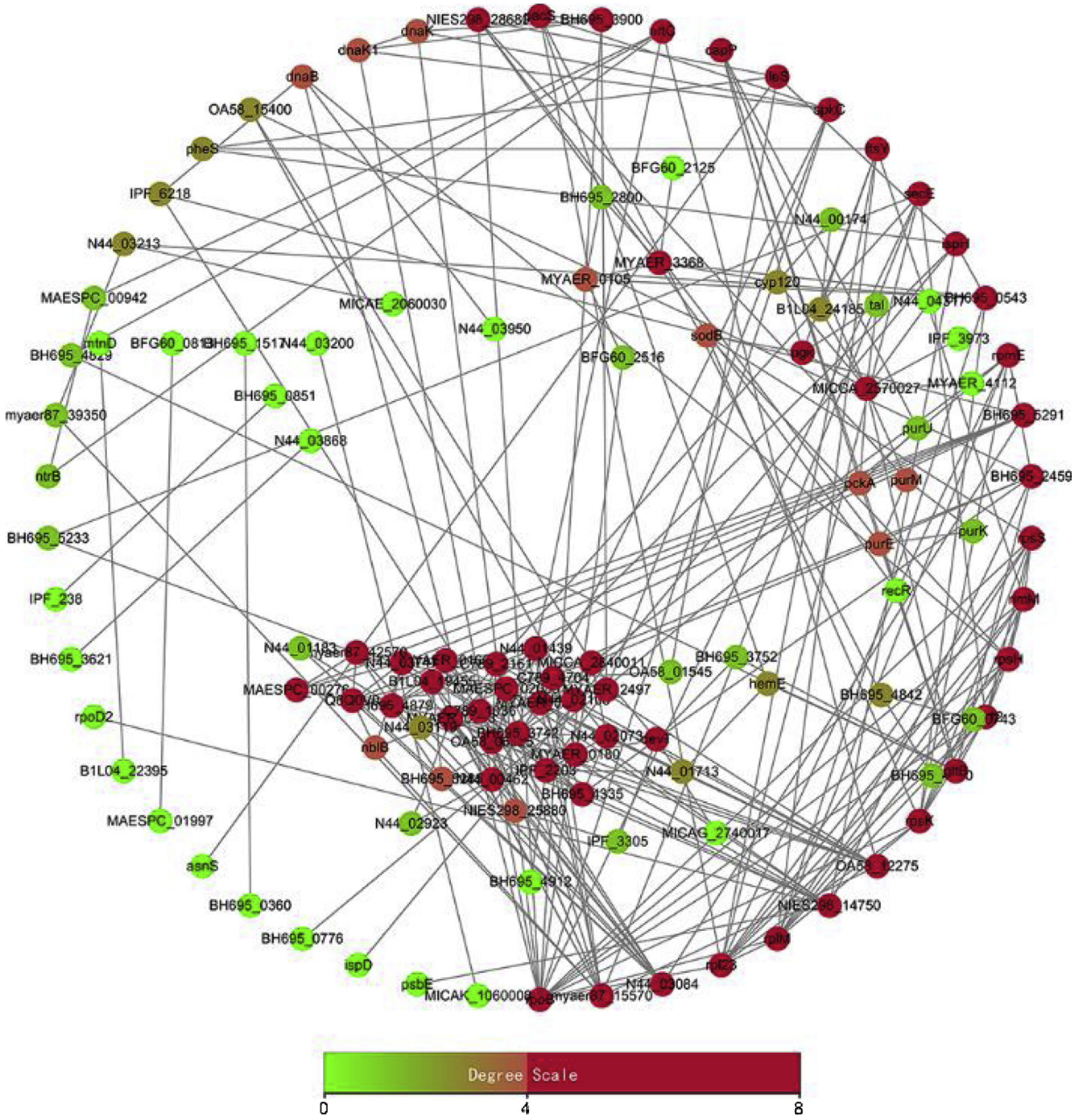

Fig. 6. The protein and protein interaction networks of all differentially expressed proteins between the post-exposure BG11 with BPA group and pre-exposure BG11 group. The protein-protein interaction networks were built using STRING 10.5 with a high confidence level (0.7) and all available prediction methods. The nodes are represented as circles and edges as lines. The number of edges at each node (degree) is indicated by the color.

ribonucleotide synthase (PurK) belongs to the family of ligases, specifically those forming generic carbon-nitrogen bonds. PurK is involved in the purine biosynthetic pathway associated with the conversion from aminoimidazole ribonucleotide to carboxyaminoimidazole ribonucleotide. It had been reported PurK implied unexpected physiological importance under low-oxygen environments in cyanobacteria [48]. The down-regulation of PurK may suppress the synthesis of proteins regulating the cyanobacterial cells normal growth.

After BPA exposure of 7 d, a metabolic network among up- and down-regulated differentially expressing proteins were found in Fig. 6, in which RpoB, RpiM and GltB were three key nodes. The co-occurrence proteins, PurK, PurE and PurK in this network were mainly related to purine metabolism. The co-expression proteins, RplW, RplM, RpsK, RpsL, RpsS, Rps8 and MAE_11870 in this network were mainly involved in ribosome. PckA, GltB, HemE, PacS and PetE were mainly associated with nitrogen metabolism, carbon fixation in photsynthetic organisms and porphyrin and chorophyll metabolism. AsnS, PheS and IleS were related to aminoacyl-tRNA biosynthesis. MAE_13620, RpoB, DnaB, RplW, RplM, RpsK, RpsL, RpsS, Rps8, RpiM, RpmE, FtsY, Pgk and Tal were associated with RNA processing, protein synthesis, cell cycle, longevity regulation, quorum sensing and energy production. The combined interaction of these differentially expressed proteins enabled cyanobacterial cells to adjust their metabolic pathway to adapt to and resist the stress triggered by the external environment to maintain dynamic cellular homeostasis in *M. aeruginosa*.

5. Conclusion

This study investigated the combined regulatory mechanism of the effects of BPA and nitrogen on the growth responses, microcystin

responses, photosynthetic responses, and antioxidant responses in *M. aeruginosa*. This is also the first study on the whole proteome response of cyanobacteria to endocrine-disrupting compounds. Longevity regulating, photosynthesis, cell cycle-caulobacter, glycolysis/gluconeogenesis and ABC transporters were the most dominant metabolic pathways in response to nitrogen and BPA. Proteins, including NarE, NrtA, FtsY, LivF and mcyB were the candidate biomarkers for controlling the cyanobacterial cells growth and MCs production. Proteins, including CpcA, CpcB, PsbE and PsaF were the regulator of photosynthesis. Proteins, including IspD, Pck and Pgk, SodB, ClpP and PurK, might be the biomarkers to regulate the antioxidant responses in *M. aeruginosa* under exposure of BPA.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.02.030.

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