Quantitative Proteomic Analysis of Nile Tilapia Brain Following Sub-Chronically Exposed to Toxic Cyanobacteria Microcystis aeruginosa

XU, Hai
Institute of Environmental Health and Ecological Security, School of Environment and Safety Engineering, Jiangsu University

DING, Yuling
Institute of Environmental Health and Ecological Security, School of Environment and Safety Engineering, Jiangsu University

QIU, Xuchun
Institute of Environmental Health and Ecological Security, School of Environment and Safety Engineering, Jiangsu University

SHIMASAKI, Yohei

他

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Quantitative Proteomic Analysis of Nile Tilapia Brain Following Sub–Chronically Exposed to Toxic Cyanobacteria *Microcystis aeruginosa*

Hai XU[1]*, Yuling DING[1], Xuchun QIU[1], Yohei SHIMASAKI and Yuji OSHIMA

Laboratory of Marine Environmental Science, Faculty of Agriculture,
Kyushu University, Fukuoka 812–8581, Japan
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In the present study, we investigated the effects of *Microcytis* on Nile tilapia brain. Male Nile tilapia were exposed to lyophilized *Microcytis aeruginosa* for 28 days. An isobaric Tags for Relative and Absolute Quantitation (iTRAQ) approach coupled with nano high–performance liquid chromatography–tandem mass spectrometry (nano HPLC–MS/MS) analysis was employed to detect and identify the differential expressed proteins. A total of 49 proteins were successfully identified and categorized into functional classes that mostly included metabolism, ubiquitin–proteasome, signal transduction and transporting, indicating that *Microcytis* toxicity in the fish brain is complex and diverse. Our proteomic analyses added new perspective to *Microcytis* neurotoxicity in aquatic organisms.

Key words: *Microcystis*; Proteomics; Brain; Nile tilapia

INTRODUCTION

Toxic cyanobacterial blooms in freshwaters represent a serious human health and ecological problem worldwide, and they are mostly dominated by *Microcystis aeruginosa* (Chen *et al*., 2016; Malbrouck and Kestemont, 2006). *M. aeruginosa* and other cyanobacteria can produce a range of toxins including microcystins. Up to now, more than 100 variants of microcystins have been isolated from *Microcytis* and cultures, and the toxicity of microcystins is particular well documented in aquatic organisms (Puddick *et al*., 2014). However, in the real aquatic environment, aquatic organisms, such as fish, are not only exposed to isolated microcystins but rather to *Microcystis* cells and lysates that contain other bioactive substances in *Microcystis* blooms. Some previous studies have showed that crude extracts from *Microcystis* cells could have greater effects than those of purified microcystins (Palíková *et al*., 2007; Rogers *et al*., 2011). Therefore, the toxicity of *Microcystis* is needed to further study.

The brain is an organ that serves as the center of the nervous system in all vertebrate animals. Many previous studies have demonstrated that microcystins could accumulate in brain and affect swimming behavior of fish, suggesting the probable neurotoxicity of microcystins (Hu *et al*., 2016). Furthermore, a previous study demonstrated that microcystins can cause neurotocity in fish at protein levels (Wang *et al*., 2010). However, the effects of *Microcystis* on the fish brain are limited.

Nile tilapia (*Oreochromis niloticus*) is an important economic species and also is considered as bio–indicator for aquatic environmental contaminants, due to its significant tolerance to environmental stress. In previous studies, brain cholinesterases (ChE) in Nile tilapia were evaluated considering its potential use in bio–monitoring neurotoxic contaminations in tropical environments (Pathiratne *et al*., 2008). In the present study, to evaluate the toxic effects of *Microcystis* on fish brain, we used isobaric tags for relative and absolute quantitation (iTRAQ), a powerful proteomics technique to detect proteome profile of *Microcystis*–exposed Nile tilapia brain.

MATERIALS AND METHODS

Preparation of Exposure Solutions

The lyophilized cells of *Microcystis aeruginosa* was obtained from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (Wuhan, China). For exposure of Nile tilapia, lyophilized *Microcystis* was reconstituted back to its original nominal concentration of 100 mg of lyophilized cells/L.

Experimental Design

This experiment was conducted based on our previous study (Chen *et al*., 2017). Briefly, male Nile tilapia weighing between 40 and 50 g at the beginning of the experiment were obtained from the fish farm of Freshwater Fisheries Research Center, Chinese Academy of Fishery Science (Wuxi, China). Adults were acclimatized in dechlorinated tap water tanks for 14 days prior to the experiment at a water temperature of 28 ± 0.5°C and a 14 h light : 10 h dark cycle. Then, using static–renewal procedure, six Nile tilapia were randomly distributed into an aquarium as a group and exposed to lyophilized *Microcystis* and control (fish water) for 28 days in triplicate at each treatment group. In the *Microcystis* group, measured MC–LR concentration was 8.3 ± 0.5 μg of MC–LR equiv L^{-1}. After 28 days exposure, all fish were anesthetized on ice for sampling. Whole brain (brain and pituitary) tissues were removed from fish and wrapped in aluminum foil and immediately

[1] Institute of Environmental Health and Ecological Security, School of Environment and Safety Engineering, Jiangsu University, Zhenjiang 212013, China
* Corresponding author (E–mail: xuhai@ujs.edu.cn)

snap frozen in liquid nitrogen until analysis.

Quantitative proteomic analysis

Quantitative proteomic analysis was conducted according to previous method (Wu *et al*., 2017). The protein concentration was determined using the BCA Protein Assay kit (Pierce). For iTRAQ labeling, a total of 100 μg of protein from each sample was denatured, reduced and alkylated according to the protocol of TMT reagent kit (Pierce Biotechnology, Rockford, USA). Samples were then digested by trypsin (Promega) overnight at 37°C. iTRAQ 4–plex was used in the present study, which can technically only measure four samples in one run, therefore, there were two biological replicates for control group and *Microcystis* group in this study and each biological replicate included six brains. Proteome Discoverer software (Thermo Fisher Scientific, version 3.0.5126) was used for relative quantification of proteins, and the expression level was considered statistically significant if $p < 0.05$ and exhibited a fold change >1.2 for up–regulation and < 0.83 for down–regulation as described (Unwin *et al*., 2006).

GO and pathway enrichment analysis

Based on previous methods (Wu *et al*., 2007), proteins with significant response to *Microcystis* exposure in tilapia brains were classified into the biological function and signaling pathway using the online functional annotation tools, such as DAVID functional annotation cluster tool (http://david.abcc.ncifcrf.gov) and KEGG pathway (http://www.genome.jp/pathway. html).

RESULTS AND DISCUSSION

In this study, the protein profiles of brain of tilapia exposed to lyophilized *Microcystis* for 28 days were investigated by iTRAQ–based proteomic analysis. Quantitative proteomic profiling was performed to identify differential protein expression of exposed tilapia compared with the control. Different iTRAQ reagents were used to distinguish peptides obtained from samples in control and *Microcystis*–exposed conditions. Protein quantification based on the relative amounts of the different iTRAQ labels was obtained for all identified. Compared with the control, 49 proteins were further quantified as showing differential expression by iTRAQ analysis, including 17 up–regulated proteins and 32 down–regulated proteins upon *Microcystis* exposure (Table 1).

Of the identified proteins, numerous differentially expressed proteins are known to be associated with the glycolytic pathway (glycerol–3–phosphate dehydrogenase, glucosamine–6–phosphate isomerase, pyruvate carboxylase) and ATP production (NADH dehydrogenase [ubiquinone] iron–sulfur protein 7, NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6). In addition, there were some other proteins relevant to metabolism, functions included protein synthesis (60S ribosomal protein L4, 60S ribosomal protein L5), nucleotides metabolism (exosome complex exonuclease RRP44), and other metabolism. Differential regulation of these proteins may suggest that brain tissue in exposed organisms is compensating for the increased energy requirement resulting from *Microcystis* toxicity.

Ubiquitin–proteasome system is a cellular pathway responsible for the degradation of short–lived, misfolded and damaged proteins (Dantuma and Bott, 2014). We detected two differentially expressed proteins proteasome subunit alpha type–1 and ubiquitin carboxyl–terminal hydrolase 8 belong to this system. The proteasome is an essential regulator that control cell–cycle progression and apoptosis, and the hydrolase plays an important regulatory role at the level of protein turnover by preventing degradation. These proteins may collaborate to affect the turnover of proteins when organisms were stressed by *Microcystis*. Therefore, our study presented a new clue to understanding the toxicological mechanisms underlying *Microcystis*–induced cellular responses.

Microcystis treatment caused a significant effect on signal transduction in tilapia brain. Signal transducer and activator of transcription 3 (STAT3) belongs to the STAT family of transcription factors that serve important functions by regulating the expression of effector genes and by regulating cell differentiation, survival and apoptosis. The specific deletion of STAT3 could cause inflammation in animals (Welte *et al*., 2003). An interesting finding of this study is that *Microcystis* inhibited the expression of serine/threonine protein phosphatase 2A (PP2A) 55 kDa regulatory subunit B alpha isoform (PPP2R2A). It is well–known that microcystin–LR (MC–LR), a byproduct of *Microcystis*, can inhibit protein serine/threonine phosphatases 1 and 2A (PP1 and PP2A) which is one of MC–LR's toxicological mechanisms (Chen *et al*., 2016). In the present study, PPP2RA2 was inhibited, indicating *Microcystis* produced MC–LR and exerted toxic effects on tilapia brain. Unlikely, guanine nucleotide–binding protein subunit gamma (Gγ) was slightly increased by *Microcystis* exposure. The heterotrimeric guanine nucleotide binding proteins (G proteins) consist of three distinct subunits, Gα, Gβ and Gγ, and each of them has many isoforms. The role of Gγ in signaling by G proteins still remains to be clarified. Usually, γ subunits would encode any specificity associated with $\beta\gamma$ dimers (Smrcka, 2008). Some recent studies indicated Gγ7 might be associated with behavioral changes (Schwindinger *et al*., 2003), and Gγ3 might be associated with metabolism (Schwindinger, *et al*., 2004). However, more detailed experiments will be required to sort out their individual specific functions. Overall, these proteins were affected suggesting an indirect evidence of neurotoxicity in tilapia brain with *Microcystis* exposure.

In addition, several proteins involved in transporting were also affected by *Microcystis*. Protein transport protein Sec23A plays a critical role in generating transport vesicles from the endoplasmic reticulum. In zebrafish, knockout of Sec23A can lead to abnormal skeleton and cuticle morphogenesis (Lang *et al*., 2006). In the present study, the overexpression of Sec23A might

Table 1. List of differentially expressed proteins in Nile tilapia brain

Protein name	Accession	Unused protein score	Peptide	Fold change (mean ± SD, *p* value)
abhydrolase domain containing 11	gil542227478	35.4	1	0.69 ± 0.1, 0.009
alpha–1,3/1,6–mannosyltransferase ALG2 isoform X2	gil542196192	45.97	2	1.52 ± 0.09, 0.002
ankyrin repeat domain–containing protein 13D	gil348514207	347.84	5	0.52 ± 0.25, 0.031
carboxy–terminal domain RNA polymerase II polypeptide A small phosphatase 1–like	gil348518153	235.89	11	0.7 ± 0.1, 0.009
CBP80/20–dependent translation initiation factor isoform X3	gil542236989	235.67	4	0.67 ± 0.2, 0.044
exosome complex exonuclease RRP44	gil542200210	49.37	1	0.82 ± 0.03, 0.002
cytoplasmic dynein 1 light intermediate chain 2	gil542169933	82.06	2	1.22 ± 0.1, 0.023
glycerol–3–phosphate dehydrogenase, mitochondrial isoform X2	gil908534948	175.67	3	0.81 ± 0.12, 0.043
huntingtin–interacting protein K	gil908545048	30.23	1	0.71 ± 0.15, 0.027
nuclear pore complex protein Nup133	gil348533307	0	1	0.83 ± 0.04, 0.003
oxysterol–binding protein	gil542223794	0	1	1.34 ± 0.09, 0.005
pyruvate carboxylase	gil542176617	0	1	0.81 ± 0.11, 0.039
protein disulfide–isomerase	gil348535980	0	1	0.77 ± 0.12, 0.034
piggyBac transposable element–derived protein 5	gil542200201	50.43	1	1.34 ± 0.04, 0
succinyl–CoA ligase [GDP–forming] subunit beta	gil348510544	32.88	1	0.74 ± 0.03, 0
T–complex protein 1 subunit alpha	gil348524895	28.87	1	0.77 ± 0.05, 0.003
mitochondrial import inner membrane translocase subunit Tim8 A	gil348515419	139.35	6	0.74 ± 0.05, 0.001
tetratricopeptide repeat protein 9A	gil348515875	0	2	0.81 ± 0.12, 0.047
ubiquitin carboxyl–terminal hydrolase 8	gil908424722	0	1	1.27 ± 0.11, 0.016
golgi membrane protein 1	gil348517276	176.31	8	0.53 ± 0.21, 0.021
zinc finger protein 462	gil542245462	0	1	1.37 ± 0.15, 0.017
small ubiquitin–related modifier	gil348510887	133.18	4	0.72 ± 0.13, 0.022
prominin–1–A–like isoform X1	gil908430253	108.85	7	0.81 ± 0.07, 0.01
ras–related protein Rab–8B	gil348500156	108.2	2	0.8 ± 0.09, 0.022
NADH dehydrogenase [ubiquinone] iron–sulfur protein 7	gil348523115	95.97	2	1.9 ± 0.05, 0
proteasome subunit alpha type–1	gil348535954	88.81	7	0.76 ± 0.07, 0.007
60S ribosomal protein L4	gil348505894	85.64	5	0.82 ± 0.09, 0.028
protein transport protein Sec23A	gil908523787	78.38	2	1.23 ± 0.1, 0.02
signal transducer and activator of transcription 3 isoform X2	gil908443166	71.44	1	0.71 ± 0.05, 0.002
glucosamine–6–phosphate isomerase	gil542175243	59	1	1.51 ± 0.12, 0.003
mitochondrial 2–oxoglutarate/malate carrier protein	gil348542252	56.18	2	0.78 ± 0.07, 0.007
poly(rC)–binding protein 2–like	gil348502908	44.34	1	1.21 ± 0.11, 0.033
deubiquitinating protein VCIP135	gil348530660	41.25	1	1.41 ± 0.23, 0.037
peptidyl–prolyl cis–trans isomerase FKBP1A–like	gil348539069	36.25	2	0.82 ± 0.1, 0.04
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6	gil348529568	28.87	1	1.2 ± 0.01, 0
guanine nucleotide–binding protein subunit gamma	gil348517895	28.08	2	1.22 ± 0.12, 0.034
XK–related protein 4	gil348519554	0	1	1.69 ± 0.22, 0.008
caspase–1–A–like	gil542245237	27.72	1	0.83 ± 0.08, 0.022
isocitrate dehydrogenase [NADP] cytoplasmic	gil348524104	132.82	7	0.46 ± 0.03, 0
KH domain–containing, RNA–binding, signal transduction–associated protein 1	gil542229266	21.53	1	0.56 ± 0.01, 0
bifunctional ATP–dependent dihydroxyacetone kinase/FAD–AMP lyase (cyclizing)	gil348539830	107.15	6	0.67 ± 0.02, 0
stathmin–like	gil542222293	25.16	3	0.74 ± 0.13, 0.028
3–mercaptopyruvate sulfurtransferase–like	gil348511484	35.85	1	0.75 ± 0.16, 0.049
serine/threonine–protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform	gil348505464	35.93	1	0.79 ± 0.04, 0.002
zinc finger protein ZPR1	gil542185769	0	2	0.8 ± 0.08, 0.013
fasciculation and elongation protein zeta–1	gil348524665	52.84	1	0.81 ± 0.03, 0.001
glycerophosphodiester phosphodiesterase 1–like	gil348525150	63.75	3	1.21 ± 0.11, 0.032
uncharacterized protein	gil542203758	118.5	3	1.23 ± 0.13, 0.041
60S ribosomal protein L5	gil542223459	0	1	1.33 ± 0.19, 0.041

[a] Unused protein score indicates a measure of the protein confidence for a detected protein.
[b] Peptide indicates peptide sequence number matching a protein
[c] The values are calculated as the ratio of *Microcystis*–exposed to Non–exposed label.

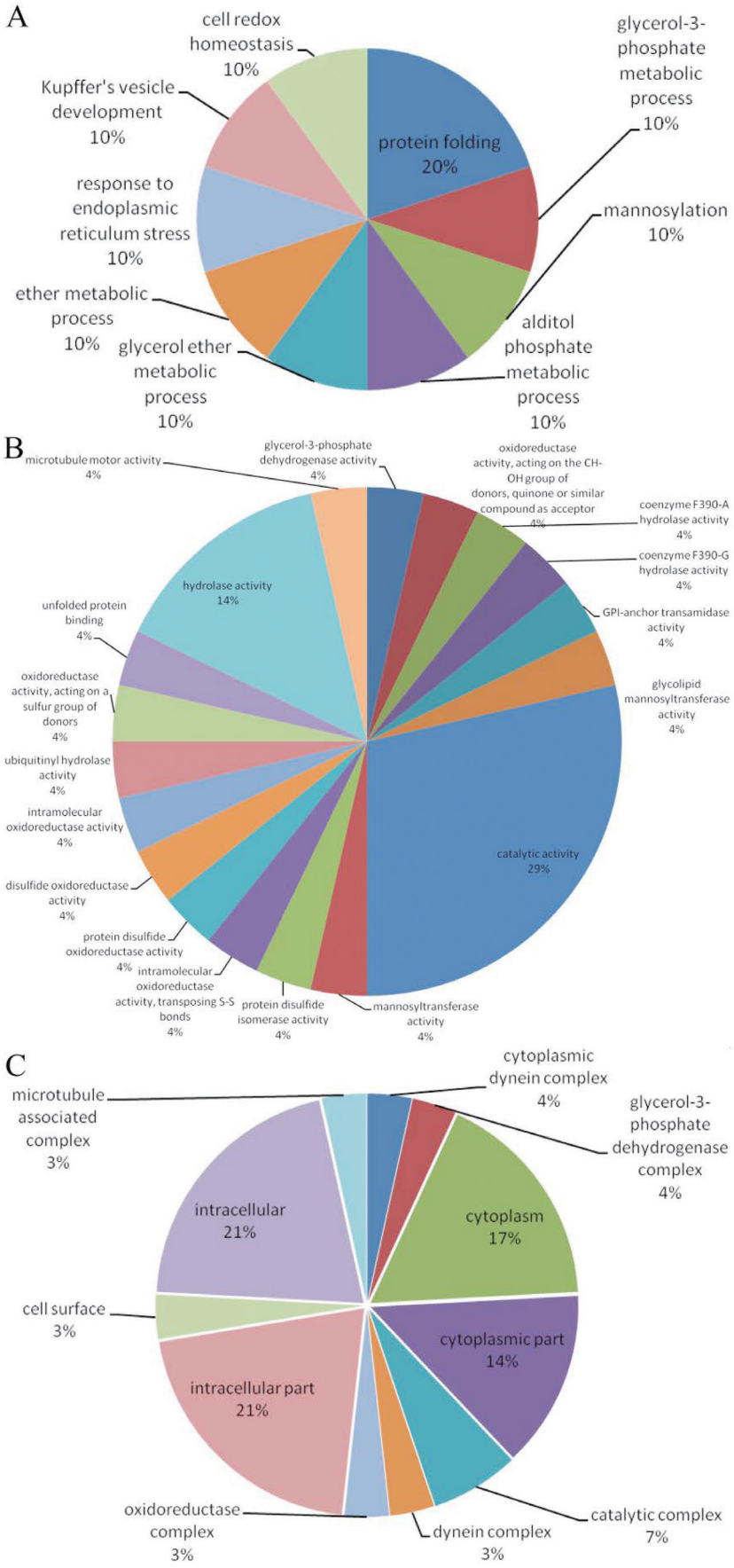

Fig. 1. GO categories enrichment analysis of 49 differentially expressed proteins in accordance with (A) biological process; (B) molecular function; (C) cellular component.

be a compensatory effect of the cells in fighting against *Microcystis* attack, which needed to further study. Mitochondrial 2–oxoglutarate/malate carrier protein (SLC25a11) is a member of SLC25 family. SLC25a11 mediates the export of oxoglutarate in exchange for a dicarboxylate and contributes to mitochondrial uptake of glutathione (GSH). Inhibition of SLC25a11 decreases mitochondrial GSH levels (Zhong *et al*., 2008). SLC25a11 may also modulate apoptosis. A previous study indicated that knockdown of SLC25a11 induced apoptosis in mouse insulimoma cells (Gallo *et al*., 2011). Therefore, the inhibition of SLC25a11 in the present study indicated the *Microcystis* caused cellular damage in the brain.

There were also many other proteins in tilapia brain affected by *Microcystis* exposure; however, functions of some of them are still not clear. Therefore, based on analysis above, we performed functional analysis of all the differentially expressed proteins using the online annotation tool, DAVID, one of the most widely used online resources for protein function classification (Huang *et al*., 2009), and a particular category of biological process, molecular function and cellular component involved in tilapia brain triggered by *Microcystis* could be identified. Among these enriched categories, the biological processes most represented by proteins included protein folding (Fig. 1, A). Molecular functions most represented by proteins detected in the brain included catalytic activity and hydrolase activity (Fig. 1, B). The cellular components that had the greatest number of annotated proteins included intracellular and cytoplasm (Fig. 1, C). The KEGG pathway analysis for the identified proteins showed that these pathways were mostly related to TCA cycle.

In conclusion, the present study performed a sub–chronically exposure of male Nile tilapia to *Microcystis* with the aim to understand the neurotoxicity of *Microcystis* at proteomic levels. We found that *Microcystis* affected energy associated proteins, proteins involved in ubiquitin–proteasome system and signal transduction, transporting proteins, suggesting that *Microcystis* toxicity to the tilapia brain was complex and diverse. These results also provided meaningful evidence for the safety of *Microcystis*, including the neurotoxicity and dysfunction of the central nervous system following sub–chronically exposure. However, further works are required to elucidate the potential mechanisms of neurotoxicity of *Microcystis* in fish.

AUTHOR CONTRIBUTIONS

H. Xu designed the study, performed the proteomic analysis and bioinformatic analysis, analyzed the data and wrote the paper. Y. Ding analyzed the data. X. Qiu performed the proteomic analysis. Y. Shimasaki and Y. Oshima supervised the work. All authors assisted in editing of the manuscript and approved the final version.

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