

Allelopathic effects of pyrogallic acid secreted by submerged macrophytes on *Microcystis aeruginosa*: Role of ROS generation

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ABSTRACT

We investigated the intracellular $O_2^{\cdot-}$, H_2O_2 and OH^{\cdot} generation induced by PA in *Microcystis aeruginosa* using two targeted fluorescent probes and also determining the SOD activity. We found that PA significantly induces the dose-dependent generation of $O_2^{\cdot-}$ and consequently H_2O_2 , OH^{\cdot} . Under dark condition, when exposed to 50 mg L^{-1} PA for 8 h, the $O_2^{\cdot-}$ and H_2O_2/OH^{\cdot} levels in *M. aeruginosa* were 2.00 and 1.91 folds of the respective control. The light enhanced the $O_2^{\cdot-}$ formation. The low concentration of PA (5 mg L^{-1}) activated the SOD, but was inhibited gradually with increase in PA concentrations, especially under light condition. The results of this study indicated the significant generation of $O_2^{\cdot-}$ as precursor of H_2O_2 and OH^{\cdot} , which provided original and direct evidence for the oxidative stress of PA to *M. aeruginosa*. The results also suggested that the futile redox cycling of PA was the main source of excessive intracellular $O_2^{\cdot-}$ and consequent H_2O_2 and OH^{\cdot} , which induced the allelopathic inhibition of *M. aeruginosa*.

Key words: Allelopathy, hydrogen peroxide, hydroxyl radical, oxidative stress, redox cycling, superoxide anion, superoxide dismutase.

INTRODUCTION

In recent decades, the eutrophication of water body and consequent cyanobacteria blooms has considerably increased (6). The developments of cyanobacteria blooms cause many adverse effects on aquatic environments. To date, many methods to control cyanobacteria blooms have been reported (filtration, coagulation, flotation, chemical reagents and biomanipulation), but these methods are not practical due to operational complexity, subsequent secondary pollution or high cost (10). In natural aquatic ecosystems, allelopathy of submerged macrophytes has been widely observed in phytoplankton (19,22,24). As far back as 1949, Hasler and Jones (5) reported that submerged macrophytes reduce the phytoplankton densities in ponds. Mjelde and Faafeng (14) also indicated that the low population of phytoplankton species and the clear-water state were only found in the lakes with high biomass density of submerged macrophytes, even the lakes contained high concentrations of phosphorus ($> 30 \text{ mg m}^{-3}$) after

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investigation of 24 lakes in Norway (14). So application of allelopathic effects of submerged macrophytes against cyanobacteria has been considered as a new alternative to control the cyanobacteria blooms (15).

Researches on allelopathic inhibition of phytoplankton by aquatic macrophytes have different stages: (i) Identification of the effect of submerged macrophytes on phytoplankton by field surveys and co-existence experiments in laboratory, (ii) Isolation of allelochemicals from submerged macrophytes and to assay their toxicity on phytoplankton and (iii) Study of the mechanisms of allelopathic inhibition effects in target organisms and in natural aquatic ecosystems (23).

Myriophyllum spicatum is identified to have strong allelopathic potential to cyanobacteria (21). The pyrogalllic acid (PA) isolated from both *M. spicatum* and its surrounding water is potent allelochemical for inhibiting the growth of *Microcystis aeruginosa* with the EC₅₀ of 0.65-2.97 mg L⁻¹ (16,25). However, the allelopathic mechanism of PA to *M. aeruginosa* is still not fully elucidated.

The oxidative stress due to reactive oxygen species (ROS) generation is one of the important mechanisms of allelopathic inhibition of PA to *M. aeruginosa* (16). In most previous studies, the antioxidant enzymes [e.g. superoxide dismutase (SOD), catalase (CAT) and peroxidases (POD)] were used as biomarkers to indicate the oxidative stress and the generation of ROS indirectly. ROS mainly include superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂), and hydroxyl radical (OH[•]). Wang *et al.* (20) reported that H₂O₂ and OH[•] formation could be induced by PA via redox cycling in *M. aeruginosa* (20). However, O₂^{•-} is the primary product in redox cycling process PA participating and the precursor of H₂O₂ and OH[•]. It is essential to provide the evidence for presence of O₂^{•-} in the redox cycling process in the test organisms exposed to PA. There is, to date, no study dealing with the relationship between PA exposure and O₂^{•-} generation in *M. aeruginosa*.

This investigation aims to elucidate the pathway of ROS generation mediated by PA for better understand the mechanism of the allelopathic effects on cyanobacteria. We investigated the intracellular O₂^{•-}, H₂O₂ and OH[•] formation, respectively, using two targeted fluorescent probes in *M. aeruginosa* after exposure to PA,

MATERIALS AND METHODS

I. Culture conditions

The *M. aeruginosa* (Code: FACHB 905) was purchased from the Freshwater Algae Culture Collection Institute of Hydrobiology, Chinese Academy of Sciences. The *M. aeruginosa* was cultivated in BG11 medium under a 12h:12h light/dark cycle using fluorescent tubes with the light intensity of 55 μmol photons m⁻² s⁻¹ at 25 ± 1 °C and shaken thrice daily for 30s by hand at 8 h intervals.

II. Chemicals

PA was purchased from Chem Service Company (West Chester, PA, USA) and was dissolved in redistilled H₂O. Dihydroethidium (DHE) and 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Beyotime Institute of Biotechnology (Haimen, China) and Sigma-Aldrich (St. Louis, MO, USA) respectively. The stock solutions of DHE and DCFH-DA were prepared respectively in dimethyl

sulfoxide (DMSO, >99.5%, Sigma-Aldrich, St. Louis, MO, USA) with the concentration of 1mM and wrapped with aluminum foil and kept in -20 °C. Cell lysis buffer, BCA Protein Assay Kit, and Total SOD Assay Kit were from Beyotime Institute of Biotechnology (Haimen, China).

III. Exposure of allelochemical PA

The experimental treatments consisted of two factors: (i). Exposure Time 2(8,12 h) and (ii). PA concentrations 5(0,50,20,25 mg L⁻¹). The culture without PA was used as the control group and incubated under the same conditions as PA-treated groups. The exponentially growing cultures of *M. aeruginosa* were used for the experiments with the initial density of 1×10⁶ cells mL⁻¹. The cultures were incubated at 25 ± 1 °C under dark and light conditions respectively.

IV. Detection of intracellular O₂⁻, H₂O₂ and OH[•] production

DHE and DCFH-DA were used to detect the intracellular O₂⁻ level and H₂O₂/OH[•] level, respectively. Both DHE and DCFH-DA are cell permeable indicators, which can diffuse freely across the membrane systems of cells. After penetrating into the cells of test organisms, DHE reacts immediately with O₂⁻ to form ethidium, which intercalates in the deoxyribonucleic acid and exhibits DHE fluorescence. And DCFH-DA after diffusing into the cell will be hydrolyzed by cellular esterase to form the nonfluorescent 2',7'-dichlorodihydrofluorescein (DCFH) which is immediately changed to fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of H₂O₂-peroxidase and OH[•].

The method to determine the O₂⁻, H₂O₂ and OH[•] production was used as per Park *et al.* (17) and Hong *et al.* (7). Briefly, after exposure to the designated doses of PA for 8 and 12 h under light and dark conditions respectively, the cells of control and PA-treated groups were washed with the phosphate buffered saline (PBS) solution (50 mM, pH 7.0) and then resuspended in PBS to adjust the cell density of 1 × 10⁶ cells mL⁻¹. The loading concentrations of DHE and DCFH-DA were 10 μM respectively. After adding the probes, all samples of cultures were incubated under dark at 25 °C with shaking for 30 min, then washed with PBS once again and finally resuspended in PBS. DHE and DCF fluorescences were detected by high speed sorting flow cytometer (FACSAriaTMIII, Becton, Dickinson and Company) at room temperature with emission filters of 610 and 530 nm respectively. For each sample, 10,000 events were collected and detected.

DMSO concentrations did not exceed 2% (v/v) in the solvent control and PA-treated groups, and the results indicated that the concentrations of DMSO added had no effect on O₂⁻, H₂O₂ and OH[•] formation in the test organisms. Three replicates for control and each test concentration were performed. Each experiment was repeated four times.

V. Measurement of cellular SOD activity

The level of cellular SOD activity was measured using the Total SOD Assay Kit which contains a highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). The Enhanced Bicinchoninic Acid (BCA) Protein Assay Kit was used for determination of the protein content in supernatant of *M. aeruginosa* cell homogenates. The level of enzymic activity was expressed as units of SOD activity per microgram protein, where 1 U of SOD was defined as the amount of enzyme required for 50% inhibition of reduction of WST-1.

VI. Statistical analysis

One-way analysis of variance (ANOVA) followed by Dunnet's posttest was done to determine the significant differences between the control and PA-treated groups. Statistical significance was defined as $P < 0.05$. All the statistical analyses were performed using SPSS 13.0 (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

$O_2^{\cdot-}$, H_2O_2 and OH^{\cdot} generation in *M. aeruginosa* induced by PA

For illustrating the possible pathway of generation of ROS mediated by PA, the intracellular $O_2^{\cdot-}$ and H_2O_2/OH^{\cdot} levels in *M. aeruginosa* were detected after exposure to different concentrations of PA for 8 and 12 h under both dark and light conditions respectively. The results of the $O_2^{\cdot-}$ and H_2O_2/OH^{\cdot} levels were displayed in Fig. 1 (a) (b) and (c) (d) correspondingly.

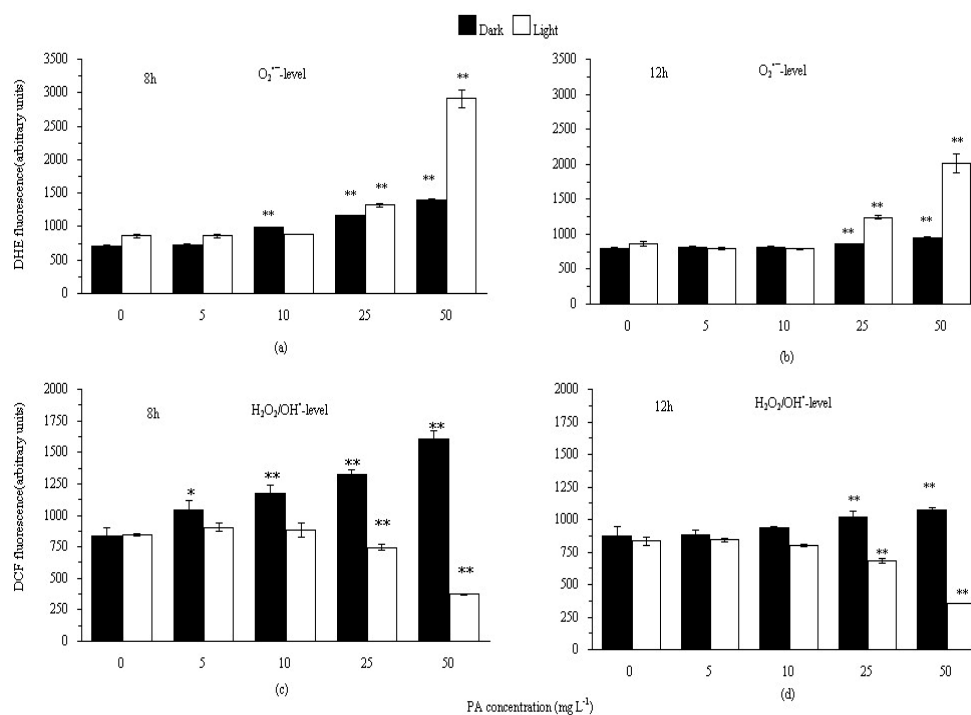


Figure 1. $O_2^{\cdot-}$ and H_2O_2/OH^{\cdot} levels in *M. aeruginosa* exposed to PA for 8 and 12 h under dark and light conditions respectively. * ($P < 0.05$) and ** ($P < 0.01$) indicate a significant difference in $O_2^{\cdot-}$ and H_2O_2/OH^{\cdot} levels compared to the respective control. Each bar represents mean \pm standard deviation.

A dose-dependent increase in $O_2^{\cdot-}$ level was observed under both dark and light conditions, and significant generation of $O_2^{\cdot-}$ was found at exposure concentrations of 25 and

50 mg L⁻¹ ($P < 0.01$) [Fig 1 (a), (b)]. This might suggest that the lower production of O₂^{•-} was rapidly transformed to H₂O₂ by dismutation under low exposure concentrations of PA. Moreover, O₂^{•-} generation was obviously enhanced by the illuminated condition. For instance, O₂^{•-}-level at exposure concentration of 50 mg L⁻¹ under light condition was 2.07 and 2.12 folds of that under dark condition respectively, for exposure time of 8 and 12 h.

From the Fig. 1 (c) (d) it could be found that, opposite trends in H₂O₂/OH[•] level were observed between dark and light conditions. Under dark condition, a dose-response increase of H₂O₂/OH[•] level was seen with high linearity (8h: R²=0.9866 and 12h: R²=0.9761). As shown in Fig. 1 (c) (d), H₂O₂/OH[•] level at exposure concentration of 50 mg L⁻¹ under dark condition was 1.91 and 1.23 folds of the control respectively, for exposure time of 8 and 12 h. However under light condition, significant decrease in H₂O₂/OH[•] level at high exposure concentrations was found ($P < 0.01$). As shown in Fig. 1 (c) (d), H₂O₂/OH[•] level at exposure conc of 50 mg L⁻¹ was only 44% (8h) and 43% (12h) compared to control, respectively.

In cells of organisms, O₂^{•-} can transform to H₂O₂ and consequent OH[•] under a series of redox reactions. As the results of this study, a coincidence of dose-dependent increases of both O₂^{•-} and H₂O₂/OH[•] levels was observed under dark condition. However under light condition, H₂O₂/OH[•] level was significantly lower than control, which suggested that a restriction in transformation from O₂^{•-} to H₂O₂ might exist. For illustrating the phenomenon observed, we detected SOD activity in this study, which has been discussed in next paragraphs.

Effects of PA on cellular SOD activity of *M. aeruginosa*

To illustrate the transformation from O₂^{•-} to H₂O₂, SOD activity in *M. aeruginosa* was detected after exposure to different concentrations of PA for 8 and 12 h under both dark and light conditions respectively (Fig. 2).

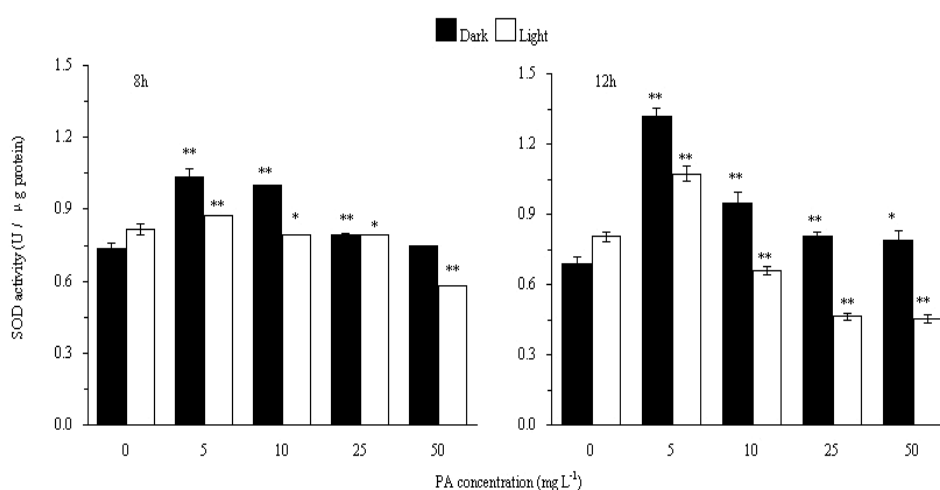


Figure 2. SOD activity of *M. aeruginosa* exposed to PA for 8 and 12 h under dark and light conditions respectively. *($P < 0.05$) and **($P < 0.01$) indicate a significant difference in SOD activity compared to the respective control. Each bar represents mean \pm standard deviation.

Under dark conditions, SOD activities of treatment groups were higher than control group and the activities significantly increased at PA concentrations of 5, 10, 25 mg L⁻¹ ($P < 0.01$) (Fig. 2). The results demonstrated that SOD was activated, which increased the H₂O₂/OH[•] formation [Fig. 1 (c) (d)]. However under light conditions, SOD activity was activated only at low PA concentration (5 mg L⁻¹). When exposed to 10, 25, 50 mg L⁻¹ PA, the SOD activities were found to be lower than the control. At 12 h light exposure, the SOD activities of 10, 25, 50 mg L⁻¹ PA-treated groups were 82.2%, 57.5%, 56.2% compared with that of the control group, respectively. The decrease in SOD activity might result in accumulation of O₂^{•-} and decrease in H₂O₂/OH[•] level, as shown in Fig. 1.

The results showed that SOD activity was obviously increased under low exposure concentration of PA (5 mg L⁻¹). It indicated that the process of transformation from O₂^{•-} to H₂O₂ was activated. But degree of activation declined gradually with increase in PA concentrations, which suggested that high concentrations of PA might inhibit SOD activity especially under light condition.

Possible pathway of ROS generation in *M. aeruginosa* induced by PA

Our results showed that PA induced dose-dependent generation of O₂^{•-} in *M. aeruginosa* significantly under both dark and light conditions. As a reducing agent, it has long been known that PA could autoxidize rapidly. Kondo *et al.* (9) and Long *et al.* (11) reported that PA-containing flavonoids are able to form O₂^{•-} accompanying the process of PA autoxidation. The products of PA autoxidation are semiquinone radical and *o*-quinone (8). According to the electrochemical character, in the presence of intracellular reductants [e.g. NAD(P)H], electrophilic *o*-quinone would undergo a two-electron reduction to form PA again and a one-electron reduction to form corresponding electrophilic semiquinone radical which would also undergo a one-electron reduction to form PA again (2). Thus after uptake by the test organisms, PA leads to futile redox cycling, spending the intracellular NAD(P)H to generate O₂^{•-} continuously (18,20). Namely, PA participated in the redox cycling process in *M. aeruginosa* with the generation of O₂^{•-} as the direct product. The relationship between the allelochemical PA and O₂^{•-} formation in phytoplankton was reported for the first time in this paper.

Moreover in the plant system, the photosynthetic electron transport chain is major source of O₂^{•-} due to high-energy reactions of photosynthesis (1). Mehler (13) originally proposed that the direct reduction of O₂ occurs in the so-called pseudocyclic electron transport, also known as Mehler reaction. So in this study, O₂^{•-} generation via pseudocyclic electron transport might be the cause of enhanced O₂^{•-} in light conditions.

As the products of reduction of molecular oxygen, ROS are formed in a stepwise fashion, since O₂ can only accept one electron at a time due to spin restrictions, wherein O₂ accepts one electron to form O₂^{•-} in the first step (12). O₂^{•-} is unique among the ROS radicals that favours the thermodynamics and enables it to act as both an oxidant and a reductant. Two molecules of O₂^{•-} can transform to H₂O₂ and O₂, the reaction is catalyzed by SOD. In the presence of Fe²⁺ ions, H₂O₂ can transform to OH[•] through Haber-Wiess reaction (H₂O₂ + O₂^{•-} → OH[•] + OH[•] + O₂). In one word, O₂^{•-} is the precursor of H₂O₂ and OH[•] (4). In the research, significant generations of O₂^{•-} and consequent H₂O₂ and OH[•] induced by PA were observed, which provided original and direct evidence for oxidative stress of PA towards *M. aeruginosa*.

ROS radicals, especially OH^\bullet which is the most reactive radical among ROS, are extremely potent oxidants (12). ROS can damage various macromolecules such as cytochromes, proteins, lipids and DNA, which will lead to structural damage and functional or metabolic disorder of cells (3). For example, lipid peroxidation will destroy the integrity of cell membranes (21). So obvious generations of ROS radicals observed in the research could cause serious oxidative damage to *M. aeruginosa*, which might be the mechanism of allelopathic inhibition of PA to *M. aeruginosa*.

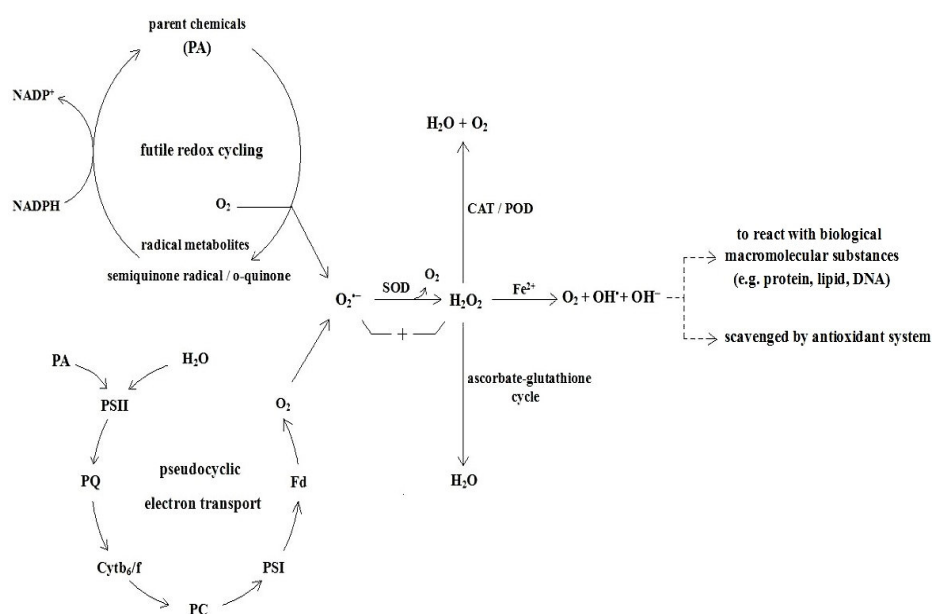


Figure 3. The possible pathway of generation of ROS induced by PA and antioxidant system in *M. aeruginosa*.

To sum up, the futile redox cycling of PA generated $\text{O}_2^{\bullet-}$ continuously in *M. aeruginosa*, which lead to the increase of H_2O_2 and OH^\bullet level in *M. aeruginosa*. Consequently, oxidative stress due to ROS generation inflicted the allelopathic inhibition of *M. aeruginosa*. The pathway of ROS generation induced by PA was shown in Fig. 3.

CONCLUSIONS

The futile redox cycling of PA was the main source of intracellular $\text{O}_2^{\bullet-}$ and consequent H_2O_2 and OH^\bullet , which may be the pathway of ROS generation in *M. aeruginosa*. Significant generations of $\text{O}_2^{\bullet-}$, H_2O_2 and OH^\bullet observed in the research provided original and direct evidence for the oxidative stress of PA towards *M. aeruginosa*, which might be the mechanism of allelopathic inhibition effect of PA to *M. aeruginosa*.

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REFERENCES

1. Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology* **141**:391-396.
2. Cohen, G.M. and d'Arcy Doherty, M. (1987). Free radical mediated cell toxicity by redox cycling chemicals. *British Journal of Cancer* **55**: 46-52.
3. Dziga, D., Suda, M., Bialczyk, J., Urszula, C.P., Lechowski, Z. (2007). The alteration of *Microcystis aeruginosa* biomass and dissolved microcystin-LR concentration following exposure to plant-producing phenols. *Environmental Toxicology* **22**: 341-346.
4. Foyer, C.H. (1997). Oxygen metabolism and electron transport in photosynthesis. In: *Molecular Biology of Free Radical Scavenging Systems* (Ed., J. Scandalios) pp. 587-621. Cold Spring Harbor Laboratory Press, New York.
5. Hasler, A.D. and Jones, E. (1949). Demonstration of the antagonistic action of large aquatic plants on algae and rotifers. *Ecology* **30**: 359-364.
6. Heisler, J.P., Gilbert, J., Burkholder, J., Anderson, D., Cochlan, W., Dennison, W., Dortch, Q., Gobler, C.J., Heil, C., Humphries, E., Lewitus, A., Magnien, R., Marshall, H., Sellner, K., Stockwell, D., Stoecker, D. and Suddleson, M. (2008). Eutrophication and harmful algal blooms: a scientific consensus. *Harmful Algae* **8**: 3-13.
7. Hong, Y., Hu, H.Y. and Li, F.M. (2008b). Physiological and biochemical effects of allelochemical ethyl 2-methyl acetoacetate (EMA) on cyanobacterium *Microcystis aeruginosa*. *Ecotoxicology and Environmental Safety* **71**: 527-534.
8. Janeiro, P. and Brett, A.M.O. (2004). Catechin electrochemical oxidation mechanisms. *Analytica Chimica Acta* **518**: 109-115.
9. Kondo, K., Kurihara, M., Miyata, N., Suzuki, T. and Toyoda, M. (1999). Scavenging mechanisms of (-)-epigallocatechin gallate and (-)-epicatechin gallate on peroxyl radicals and formation of superoxide during the inhibitory action. *Free Radical Biology and Medicine* **27**: 855-863.
10. Lee, T.J., Nakano, K. and Matsumara, M. (2000). Ultrasonic irradiation for blue-green algae bloom control. *Environmental Technology* **22**: 383-390.
11. Long, L.H., Clement, M.V. and Halliwell, B. (2000). Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (-)-epigallocatechin, (-)- epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. *Biochemical and Biophysical Research Communications* **273**: 50-53.
12. Mallick, N. and Mohn, F.H. (2000). Reactive oxygen species: response of algal cells. *Journal of Plant Physiology* **157**: 183-193.
13. Mehler A.H. (1951). Studies on the reactions of illuminated chloroplasts. Mechanism of the reduction of O₂ and other Hill reagents. *Archives of Biochemistry and Biophysics* **33**: 65-77.
14. Mjelde, M. and Faafeng, B. (1997). *Ceratophyllum demersum* (L.) hampers phytoplankton development in some small Norwegian lakes over a wide range of phosphorus level and geographic latitude. *Freshwater Biology* **37**: 355-365.
15. Nakai, S., Inoue, Y., Hosomi, M. and Murakami, A. (1999). Growth inhibition of blue-green algae by allelopathic effects of macrophyte. *Water Science and Technology* **39**: 47-53.
16. Nakai, S., Inoue, Y. and Hosomi, M. (2001). Algal growth inhibition effects and inducement modes by plant-producing phenols. *Water Research* **35**: 1855-1859.

17. Park, W.H., Han, Y.H., Kim, S.H. and Kim, S.Z. (2007). Pyrogallol, ROS generator inhibits As4.1 juxtglomerular cells via cell cycle arrest of G2 phase and apoptosis. *Toxicology* **235**: 130-139.
18. Saeki, K., Hayakawa, S., Isemura, M. and Miyase, T. (2000). Importance of a pyrogallol-type structure in catechin compounds for apoptosis-inducing activity. *Phytochemistry* **53**: 391-394.
19. Wang, H.Q., Wu, Z.B., Zhang, S.H., Cheng, S.P., He, F. and Liang, W. (2008). Relationship between the allelopathic activity and molecular structure of hydroxyl derivatives of benzoic acid and their effects on cyanobacterium *Microcystis aeruginosa*. *Allelopathy Journal* **22**: 205-212.
20. Wang, J., Zhu, J.Y., Liu, S.P., Liu, B.Y., Gao, Y.N. and Wu, Z.B. (2011). Generation of reactive oxygen species in cyanobacteria and green algae induced by allelochemicals of submerged macrophytes. *Chemosphere* **85**: 977-982.
21. Wang, J., Zhu, J.Y., Gao, Y.N., Liu, B.Y., Liu, S.P., He, F. and Wu, Z.B. (2013). Toxicity of allelochemicals released by submerged macrophytes on phytoplankton. *Allelopathy Journal* **31**: 199-210. 413
22. Wu, Z.B., Gao, Y.N., Wang, J., Liu, B.Y., Zhou, Q.H. and Zhang, Y.Y. (2009). Allelopathic effects of phenolic compounds present in submerged macrophytes on *Microcystis aeruginosa*. *Allelopathy Journal* **23**: 403-410.
23. Wu, Z.B., Zhang, S.H., Wu, X.H., Cheng, S.P. and He, F. (2007). Allelopathic interactions between *Potamogeton maackianus* and *Microcystis aeruginosa*. *Allelopathy Journal* **20**: 327-338.
24. Zhang, S.H., Cheng, S.P., Wang, H.Q., He, F. and Wu, Z.B. (2009). Allelopathic interactions between the *Potamogeton* spp and toxic cyanobacteria (*Microcystis aeruginosa*). *Allelopathy Journal* **23**: 379-390.
25. Zhu, J.Y., Liu, B.Y., Wang, J., Gao, Y.N. and Wu, Z.B. (2010). Study on the mechanism of allelopathic influence on cyanobacteria and chlorophytes by submerged macrophyte (*Myriophyllum spicatum*) and its secretion. *Aquatic Toxicology* **98**: 196-203.