



Article

# Inhibition Effect of Ionic Liquid [Hmim]Cl on *Microcystis* Growth and Toxin Production

Yang Liu <sup>1,\*</sup> , Yijie Zhang <sup>1</sup>, Yousef Sultan <sup>1,2</sup> , Peng Xiao <sup>3</sup> , Li Yang <sup>4</sup>, Hanyang Lu <sup>1</sup> and Bangjun Zhang <sup>1,\*</sup>

<sup>1</sup> College of Life Sciences, Henan Normal University, Xinxiang 453007, China; zhangyj2013@126.com (Y.Z.); sultanay18@hotmail.com (Y.S.); lhyang8168@163.com (H.L.)

<sup>2</sup> Department of Food Toxicology and Contaminants, National Research Centre, Dokki, Cairo 12622, Egypt

<sup>3</sup> College of Life and Environmental Science, Wenzhou University, Wenzhou 325035, China; pxiao@wzu.edu.cn

<sup>4</sup> Wuhan Imagination Science and Technology Development Co., Ltd., Wuhan 443000, China; bioyangli85@163.com

\* Correspondence: ly@htu.cn (Y.L.); zhangbangjun@htu.edu.cn (B.Z.); Tel.: +86-0373-3326-282 (Y.L.)

**Abstract:** Ionic liquids (ILs) are known as “green solvents” and widely used in industrial applications. However, little research has been conducted on cyanobacteria. This study was conducted to investigate the toxicity of ionic liquids ([Hmim]Cl) on *Microcystis aeruginosa* PCC7806. The EC<sub>50</sub> (72 h) of [Hmim]Cl on the growth of *Microcystis aeruginosa* PCC 7806 was 10.624 ± 0.221 mg L<sup>-1</sup>. The possible mechanism of toxicity of [Hmim]Cl against *M. aeruginosa* PCC 7806 was evaluated by measuring cell growth, photosynthetic pigment contents, chlorophyll fluorescence transients, cell ultrastructure, and transcription of the microcystin-producing gene (*mcyB*). The concentrations of chlorophyll *a* and carotenoids were significantly reduced in treated *M. aeruginosa* cultures. The results of chlorophyll fluorescence transients showed that [Hmim]Cl could destruct the electron-accepting side of the photosystem II of *M. aeruginosa* PCC 7806. Transmission electron microscopy demonstrated cell damage including changes in the structure of the cell wall and cell membrane, thylakoid destruction, and nucleoid disassembly. The transcription of the *mcyB* gene was also inhibited under [Hmim]Cl stress. In summary, this study provides new insights into the toxicity of [Hmim]Cl on cyanobacteria.

**Keywords:** [Hmim]Cl; toxicology; aquatic ecosystem; microorganism; photosynthesis



**Citation:** Liu, Y.; Zhang, Y.; Sultan, Y.; Xiao, P.; Yang, L.; Lu, H.; Zhang, B. Inhibition Effect of Ionic Liquid [Hmim]Cl on *Microcystis* Growth and Toxin Production. *Int. J. Environ. Res. Public Health* **2022**, *19*, 8719. <https://doi.org/10.3390/ijerph19148719>

Academic Editors: Paul B. Tchounwou and Charles Madenjian

Received: 15 May 2022

Accepted: 17 July 2022

Published: 18 July 2022

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

## 1. Introduction

Demand for green technologies has increased rapidly in recent years. Ionic liquids (ILs) have attracted great attention as promising organic solvents. ILs are low-temperature molten salts, which possess many unique physiochemical characteristics and excellent solvent properties (negligible vapor pressure, high thermal, chemical, and electrochemical stability) [1]. Therefore, ILs have been considered to be “green” substitutes for conventional volatile organic compounds (VOCs) used in synthetic and analytical processes [2]. Although ILs can reduce the potential risk of air pollution due to their negligible vapor pressures, they may pose ecological risks to aquatic ecosystems, due to their high solubility in water. In addition, many studies have found that ILs show poor biodegradability [3].

Algae are crucial primary producers for the food chain in aquatic ecosystems and play an important role in nutrient cycling. Algae have been used for hazard assessment of aquatic contaminants [4,5], because of their rapid response to environmental changes. Several studies have been done on the toxicity of ILs on aquatic organisms, such as green algae [6], diatoms [7], cladocerans [8], mussels [9], and fish [10]. However, little research has been conducted on cyanobacteria [11].

Global warming and eutrophication have enhanced the proliferation of cyanobacteria in freshwater bodies [12,13]. *Microcystis aeruginosa* (Kützing) Kützing 1846 is one of the most widespread and dominant cyanobacterial species [14,15]. *M. aeruginosa* also has the capability of producing secondary metabolites such as microcystin, the synthesis of

which is sensitive to the surrounding environment [16,17]. Thus, the response of *M. aeruginosa* to IL stress can be used for assessing the impact of ILs on aquatic ecosystems. Oxygenic photosynthesis of cyanobacteria is easily affected by hazardous substances such as ILs [18]. The chlorophyll fluorescence measurement is an important index to evaluate the performance of photosynthesis processes and is usually measured via the redox states of photosystem II (PSII) and the electron-donating and -accepting sides of the active reaction centers. Krause et al. [19] and Strasser and Strasser [20] established the JIP-test, which conducts a kinetic analysis of the light-induced chlorophyll fluorescence rise curve based on the theory of biofilm energy flow. The JIP-test reflects the physiological state of cyanobacterial PSII by detecting the effects of different factors on its structure and function [18,21,22].

The compound 1-hexyl-3-methylimidazolium chloride, which is abbreviated as [Hmim]Cl, is one of the widely used ILs [23]. Until now, there has been no available study concerning the effects of [Hmim]Cl on cyanobacteria. The current study aims to illustrate the possible toxicological mechanism of [Hmim]Cl on cyanobacteria from the view of cell growth, photosynthesis, and gene expression.

## 2. Materials and Methods

The *M. aeruginosa* PCC 7806 was provided by the Institute of Hydrobiology, Chinese Academy of Sciences, and was cultured in a BG11 liquid medium [24]. The culture was maintained at a light–dark cycle of 12 h:12 h with a light intensity of 3000 lx under a controlled temperature (28 °C). The [Hmim]Cl (99% purity) was purchased from the Chengjie Chemical Company (Shanghai, China).

### 2.1. Measurement of Cell Growth and Photosynthetic Pigments Content

The [Hmim]Cl was prepared and added into the *M. aeruginosa* PCC 7806 culture with a cell density of  $10^4$  cells mL<sup>-1</sup>. The final concentrations of [Hmim]Cl were set at 5, 10, 20, and 30 mg L<sup>-1</sup>. All experiments were performed in triplicate. The cell density and pigment contents in the control and treated cultures were measured after 24 and 72 h of incubation. To measure the pigment contents, the cultures were centrifuged for 10 min at 8000 rpm, and the supernatants were discarded. The chlorophyll *a* (Chl *a*) and carotenoids were extracted from cell pellets using 80% acetone for 24 h in darkness at 4 °C. Absorbance of the extracts was measured using a spectrophotometer at 663, 645, and 450 nm. The following equations set by Richards et al. (1952) [25] were used for the calculation of the pigment contents.

### 2.2. Measurement of Chlorophyll Fluorescence Transients

The polyphasic rise of chlorophyll fluorescence transients was detected by PEA (AquaPen-C AP-C 100, Photon Systems Instruments, The Czech Republic). The JIP transients were measured after exposure to [Hmim]Cl stress for 24 h and 72 h, respectively. Cells from the control and treated cultures were placed on special plastic clips. The parameters of absorption (ABS/RC), trapping (TR<sub>0</sub>/RC), electron transport (ET<sub>0</sub>/RC), dissipation (DI<sub>0</sub>/RC), the probability that a trapped exciton moves an electron into the electron transport chain beyond QA ( $\psi_0$ ), the maximum quantum yield of primary photochemistry ( $\varphi P_0$ ), the quantum yield of electron transport ( $\varphi E_0$ ), the probability that an absorbed photon is dissipated ( $\varphi D_0$ ), relative fluorescence values  $[(1 - V_K/V_J)_r]$ , and the performance indexes of photosystem II were calculated from the chlorophyll fluorescence transients [26,27]. The intensity of actinic light was set at 30  $\mu$ mol photons/(m<sup>2</sup>·s). The equations used for calculating the above parameters were as follows [27]:

$$\text{ABS/RC} = (M_0/V_J)(1 - F_0/F_M) \quad (1)$$

$$\text{TR}_0/\text{RC} = M_0/V_J \quad (2)$$

$$\text{ET}_0/\text{RC} = (\text{TR}_0/\text{RC})\psi_0 \quad (3)$$

$$DI_0/RC = (ABS/RC) - (TR_0/RC) \quad (4)$$

$$\varphi P_0 = 1 - (F_0/F_M) \quad (5)$$

$$\psi_0 = 1 - V_J \quad (6)$$

$$\varphi E_0 = (1 - F_0/F_M)(1 - V_J) \quad (7)$$

$$\varphi D_0 = F_0/F_M \quad (8)$$

$$PI_{ABS} = [RC/ABS][\varphi P_0/(1 - \varphi P_0)][\psi_0/(1 - \psi_0)] \quad (9)$$

$$(1 - V_K/V_J)_r = [(1 - V_K/V_J)_{\text{treatment}}]/[(1 - V_K/V_J)_{\text{Control}}] \quad (10)$$

### 2.3. Observation of Cell Ultrastructure

Changes in the ultrastructure of the treated *M. aeruginosa* cells at 10 and 30 mg L<sup>-1</sup> of [Hmim]Cl were detected using transmission electron microscopy. After 48 h of exposure, culture cells were centrifuged for 15 min at 4000 rpm under 4 °C, and the supernatant was discarded. The cell pellets were fixed with 2.5% glutaraldehyde solution. The samples were sent to the Institute of Hydrobiology, Chinese Academy of Sciences, for electron microscopic analysis. Changes in the ultrastructure of the treated cells were identified by comparison with the control group.

### 2.4. Transcription of *mcyB* Gene

#### 2.4.1. RNA Extraction and Reverse Transcription

The cells of *M. aeruginosa* PCC 7806 were harvested after 24 h and 72 h of exposure to each concentration of [Hmim]Cl. The cultures were centrifuged at 6000 rpm for 10 min, and the supernatant was discarded. Cell pellets were re-suspended in a Trizol reagent (Invitrogen, Waltham, MA, USA) and then frozen and thawed for three times. Total RNAs were extracted following the manufacturer's manual of Trizol reagent and dissolved with diethyl pyrocarbonate-treated water. Reverse transcription was conducted using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany).

#### 2.4.2. Determination of *mcyB* Gene Transcription

The primers 30F and 108R were used for the detection of the *mcyB* gene [16], whereas 5'-GGACGGGTGAGTAACGCGTA-3' and 5'-CCCATTGCGGAAAATCCCC-3' were used to amplify 16S rRNA gene as the internal control [28]. The transcription of *mcyB* in *M. aeruginosa* was determined by qPCR after 24 h and 72 h of exposure to each concentration of [Hmim]Cl. The qPCR reaction was prepared with final volumes of 10 µL containing 5 µL of Master Mix (SYBR Green, CWBIO, China), 0.2 µL of each primer (10 µmol L<sup>-1</sup>), and 0.4 µL cDNA and 4 µL of sterile water. The qPCR conditions were set as follows: 95 °C for 10 min; 40 cycles at 95 °C for 15 s, 59 °C for 30 s, and 72 °C for 30 s. The relative transcription level of the *mcyB* gene was calculated using 2<sup>-ΔΔCt</sup> where ΔΔCt = (Ct<sub>target gene</sub> - Ct<sub>16S rrm</sub>)<sub>stress</sub> - (Ct<sub>target gene</sub> - Ct<sub>16S rrm</sub>)<sub>control</sub> [29].

### 2.5. Statistical Analysis

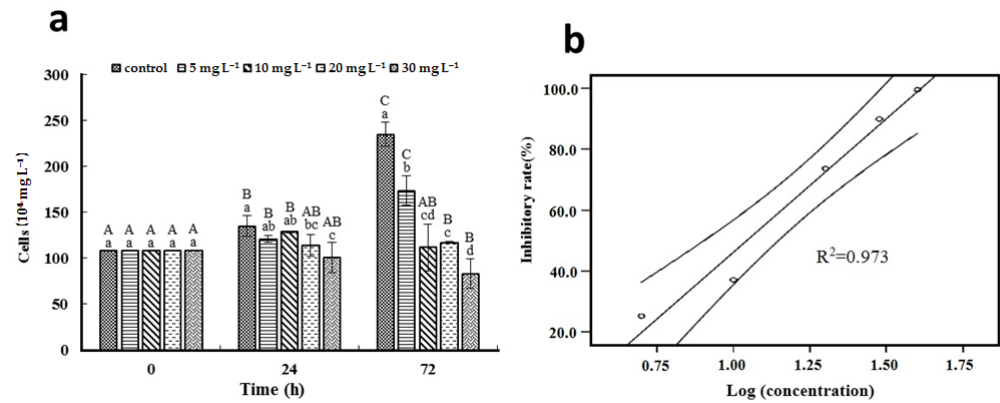
The experimental data were analyzed by SPSS 20.0 (ANOVA, LSD). Differences were considered to be significant at *p* < 0.05.

## 3. Results

### 3.1. Inhibition of [Hmim]Cl on *M. aeruginosa* PCC 7806 Growth

As displayed in Figure 1a, cell growth was not affected by [Hmim]Cl at the concentrations of 5 and 10 mg L<sup>-1</sup> at 24 h. However, cell growth was significantly inhibited by higher concentrations of [Hmim]Cl with 25% decrease at 30 mg L<sup>-1</sup>. After 72 h, the cell density of the control culture and the culture exposed to 5 mg L<sup>-1</sup> of [Hmim]Cl increased by 118% and 61%, respectively. For cultures exposed to higher concentrations of [Hmim]Cl, cell density decreased after 72 h of exposure. No growth was observed at 10 mg L<sup>-1</sup> of

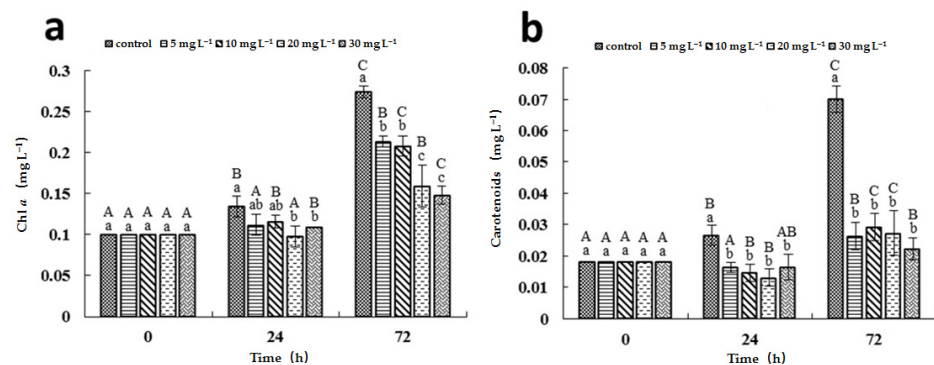
[Hmim]Cl, and cell density decreased by 23% in culture exposed to 30 mg L<sup>-1</sup> of [Hmim]Cl at 72 h compared with 0 time. [Hmim]Cl at the concentrations of 10, 20, and 30 mg L<sup>-1</sup> inhibited cell growth by >50% when compared with the control culture after 72 h of incubation. Regression curves of the logarithmic values of [Hmim]Cl concentrations against the inhibitory rate after 72 h of exposure is shown in Figure 1b. The calculated EC<sub>50</sub> of [Hmim]Cl on the growth of *M. aeruginosa* was 10.624 ± 0.221 mg L<sup>-1</sup>.



**Figure 1.** Effect of [Hmim]Cl on *M. aeruginosa* PCC 7806 growth. (a) Cell density after 24 h and 72 h of incubation. (b) Regression analysis between [Hmim]Cl concentration and inhibitory rate after 72 h. Average values ± standard deviation (*n* = 3). Different uppercase letters represent significant differences (*p* < 0.05) between exposure times within the same treatment. Different lowercase letters represent significant differences (*p* < 0.05) between treatments within the same exposure time.

### 3.2. Effects of [Hmim]Cl on the Pigment Contents

The effects of [Hmim]Cl on the pigment contents were similar to those on the cell growth (Figure 2). The Chl *a* concentration in the control culture increased by 34.5% and 174.2% after 24 h and 72 h of incubation, respectively. However, little changes were observed in the treated cultures at 24 h when compared with 0 time. Although the Chl *a* concentrations in the treated cultures increased after 72 h of exposure, they remained significantly lower than in the control culture. The inhibition of [Hmim]Cl on carotenoids production was more apparent than that on Chl *a* production. The increase of the carotenoids' concentrations in the treated cultures was lower than 50% in comparison to three times in the control culture after 72 h of incubation.

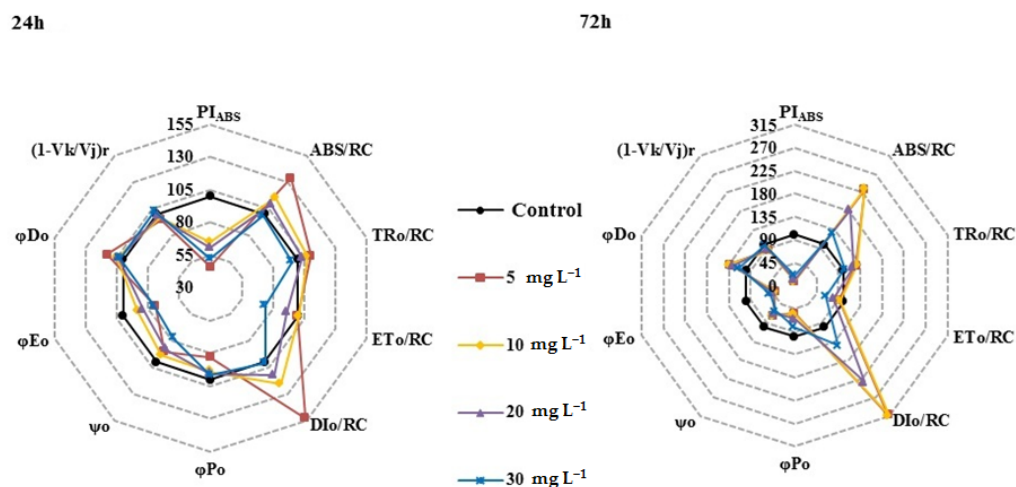


**Figure 2.** Effects of [Hmim]Cl on the photosynthetic pigment content of *M. aeruginosa* PCC 7806. (a) Chl *a* concentration; (b) carotenoids concentration. Different uppercase letters represent significant differences (*p* < 0.05) between exposure times within the same treatment. Different lowercase letters represent significant differences (*p* < 0.05) between treatments within the same exposure time.

### 3.3. Characteristics of Chlorophyll Fluorescence Transients

The parameters of the JIP test are illustrated as a radar plot (Figure 3). At 24 h, TR<sub>0</sub>/RC, φP<sub>0</sub>, φD<sub>0</sub>, and (1 - V<sub>K</sub>/V<sub>J</sub>)<sub>r</sub> were not affected by [Hmim]Cl, whereas PI<sub>ABS</sub> was

significantly inhibited at all concentrations of [Hmim]Cl. The parameters ABS/RC and  $DI_0/RC$  at  $5 \text{ mg L}^{-1}$  were significantly larger than those of the control culture and  $ET_0/RC$  and  $\psi_0$  were significantly lower at  $30 \text{ mg L}^{-1}$ .

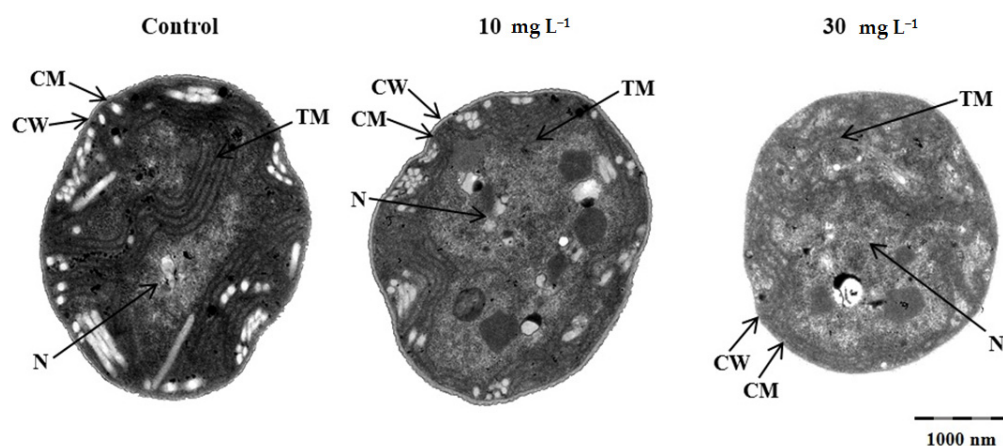


**Figure 3.** Radar plots of the parameters originated from chlorophyll fluorescence transients after 24 h and 72 h of [Hmim]Cl treatment.

The effects of [Hmim]Cl on chlorophyll fluorescence transients were more apparent at 72 h compared with those at 24 h. The  $\phi P_0$  value was significantly lower than that of the control culture after 72 h of exposure.  $DI_0/RC$  was doubled at 5 and 20  $\text{mg L}^{-1}$  and increased 3 times at 10  $\text{mg L}^{-1}$  when compared with the values at 24 h. The ABS/RC was significantly increased at all concentrations at 72 h, whereas  $PI_{ABS}$  was significantly inhibited.

### 3.4. Effect of [Hmim]Cl on the Cell Ultrastructure

Figure 4 shows the cell ultrastructure of *M. aeruginosa* PCC 7806 at 10 and 30  $\text{mg L}^{-1}$  of [Hmim]Cl after 48 h of incubation. Exposure to 10  $\text{mg L}^{-1}$  of [Hmim]Cl resulted in cell damage including thylakoid disorganization and nucleoid diffusion. More severe cell damage was observed at 30  $\text{mg L}^{-1}$  of [Hmim]Cl, including extensive disassembly of intracellular structures and disorganization of the cell wall and cell membrane.

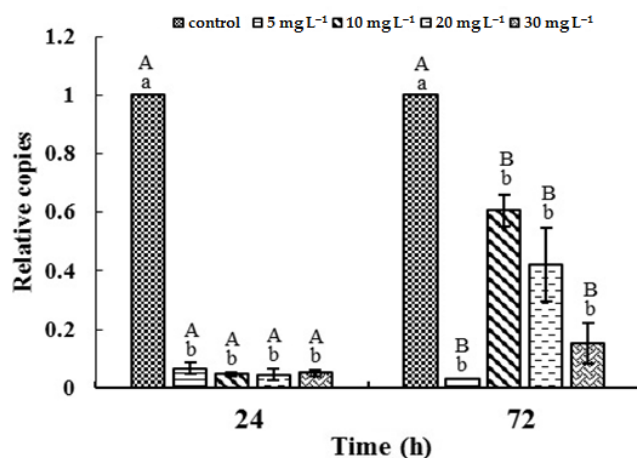


**Figure 4.** Effects of [Hmim]Cl on the ultrastructure of *M. aeruginosa* PCC 7806 after 48 h of exposure at 10 and 30  $\text{mg L}^{-1}$ , CW: cell wall; CM: cell membrane; TM: thylakoid membrane; N: nucleoid.

### 3.5. Transcription of *mycB* Gene

The relative expression of *mycB* gene at different concentrations of [Hmim]Cl was significantly lower than that of the control culture (Figure 5). With the exception of

5 mg L<sup>-1</sup>, the gene expressions in treated cultures were increased significantly after 72 h of incubation.



**Figure 5.** Effects of increasing [Hmim]Cl concentrations on the transcription of *mcvB* gene. Different uppercase letters represent significant differences ( $p < 0.05$ ) between exposure times within the same treatment. Different lowercase letters represent significant differences ( $p < 0.05$ ) between treatments within the same exposure time.

#### 4. Discussion

Recently, several studies have been published regarding the structural characteristics, toxicity, environmental fate, and application of ILs [30,31]. Although ILs have been validated as green solvents instead of the hazardous organic solvents, they can negatively affect the aquatic environment [3].

A toxicological assessment of ILs in aquatic organisms involves bacteria (*Vibrio fischeri*), green algae (*Selenastrum capricornutum*, *Oocystis submarina*), diatoms (*Cyclotella meneghiniana*), mollusks (*Physa acuta*), vertebrates (*Danio rerio*, *Rana nigromaculata*, *Cyprinus carpio*), and plants (*Lemna minor*) [7]. A few kinds of aquatic organisms are inferior and simple, and their cell membrane is the first target that can be affected by ILs [32,33]. ILs could cause the destruction of a membrane lipid structure and the change of composition due to their properties as ionic surfactants. The long alkyl chain of [Hmim]Cl in the cationic part increases the lipophilicity and consequently interacts with the phospholipid bilayer and hydrophobic part of membrane proteins. This leads to the leakage of cellular contents and then cell death [34]. Such interpretation can explain the decrease of the *M. aeruginosa* cell density in the current study. The experimental results revealed that the EC<sub>50</sub> (72 h) of [Hmim]Cl for the growth of *M. aeruginosa* PCC 7806 was  $10.624 \pm 0.221$  mg L<sup>-1</sup>, referring to the low biodegradability as mentioned in the previous studies on imidazole or pyridine ILs [35–37].

Previous study revealed that ILs can inhibit algal photosynthesis by causing a decrease in the content of chlorophyll, thereby affecting the growth of algae [38,39]. Similarly, the contents of both Chlorophyll *a* and carotenoids were significantly decreased after 72 h of exposure to [Hmim]Cl. These results may be attributed to the long alkyl chain of [Hmim]Cl, which can lead to a leakage of cellular contents [30,37,40].

Chlorophyll fluorescence transients could be used to indicate the structure and function of PSII [27] and have become an important method to examine the response of plants and cyanobacteria to environmental stress [41]. According to the energy-flow model diagram of Strasser and Strasser (1995) [20],  $\phi P_0$  can represent the electron-transfer performance of the electron donor side of the PSII reaction center. After 72 h of exposure,  $\phi P_0$  was significantly inhibited, indicating that the electron donor side of the PSII reaction center of *M. aeruginosa* is the target site of [Hmim]Cl.  $\psi_0$  and  $\phi E_0$  can reflect the performance of the electron acceptor side of the PSII reaction center. Inhibition of  $\psi_0$  and  $\phi E_0$  by [Hmim]Cl indicated that the electron acceptor side of the PSII reaction center is also the target site

of [Hmim]Cl.  $ABS/RC$ ,  $TR_0/RC$ ,  $DI_0/RC$ , and  $\phi D_0$  values increased after extending the exposure time. These results indicate that although exposure to [Hmim]Cl leads to partial reaction center inactivation or cleavage, the efficiency of the active reaction center is improved. The values of  $(1 - V_K/V_J)_r$  can represent the state of the oxygen-evolving complex of PS II [26]. However, the relative value of  $(1 - V_K/V_J)_r$  showed no significant changes when compared with the control culture (Figure 3). Therefore, the oxygen-evolving complex was not the target of [Hmim]Cl. On the other hand,  $PI_{ABS}$ , which is the performance index of photosynthesis, was markedly decreased in all treatments. The results from this study show that [Hmim]Cl would cause serious damage to the photosynthetic system of *M. aeruginosa*. It also proves that  $PI_{ABS}$  is more sensitive to reflect the changes of the photosynthetic system.

Previous investigation reported that ionic liquids can impair cells via lipid peroxidation [42] and DNA damage [43]. The results of ultrastructural observation by TEM indicate that both the thylakoid membrane and the cellular membrane were all damaged by [Hmim]Cl at  $30 \text{ mg L}^{-1}$ . These results suggest that the mechanism of the toxicity of [Hmim]Cl against *M. aeruginosa* PCC 7806 may also be involved in the damage of membrane. The experimental data show that [Hmim]Cl had a short-time strong inhibition on the transcription of *mcyB*, which recovered slowly after 72 h. Given that there is a positive relationship between the growth rate and microcystin production [44], the rapid inhibition of *mcyB* transcription provided further evidence for the toxic effects of [Hmim]Cl on *M. aeruginosa* at transcription level. It also reflects that the potential threat indicated by [Hmim]Cl caused the cells to allocate energy toward cell division instead of toxin production.

The results of this study found that an adverse effect was showed on *M. aeruginosa* under different concentrations of [Hmim]Cl, but this is just the indoor research. If many ILs flowed into the aquatic ecosystem, threats may be superimposed on the algae with other substances, such as heavy metals and pesticides. The combined toxicity of ILs requires further study.

## 5. Conclusions

The main conclusions may be summarized as follows.

- i. The  $EC_{50}$  of [Hmim]Cl on *M. aeruginosa* PCC 7806 was  $10.624 \pm 0.221 \text{ mg L}^{-1}$  after 72 h of exposure.
- ii. [Hmim]Cl could destruct the electron-accepting side of the photosystem II of *M. aeruginosa* PCC 7806.
- iii. Cellular ultrastructure examination indicated that the distortion of the thylakoid membrane and the loss of the integrity of the cell membrane were associated with [Hmim]Cl treatment and concentration.
- iv. The transcriptional profiles of *mcyB* were depressed.

**Author Contributions:** Conceptualization, Y.L. and B.Z.; methodology, Y.L.; software, Y.Z. and Y.S.; validation, Y.Z., Y.S. and L.Y.; formal analysis, Y.Z., P.X. and Y.S.; investigation, L.Y. and H.L.; data curation, P.X. and H.L.; writing—original draft preparation, Y.L.; writing—review and editing, Y.L. and Y.S.; supervision, Y.L. and B.Z.; project administration, Y.L. and B.Z.; funding acquisition, Y.L. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was supported by the Key Scientific Research Projects of Henan Province college youth backbone teacher (2021118), the National Key Research and Development Program of China (2021YFE0112000).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The authors declare that all data and materials are available to be shared on a formal request.

**Acknowledgments:** We thank Renhui Li for providing the strains of *Microcystis aeruginosa* and Gaofei Song for finishing the electron microscopy photos.

**Conflicts of Interest:** The authors declare no conflict of interest. The company (Wuhan Imagination Science and Technology Development Co. Ltd.) had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

## References

- Mei, X.; Yue, Z.; Ma, Q.; Dunya, H.; Mandal, B.K. Synthesis and electrochemical properties of new dicationic ionic liquids. *J. Mol. Liq.* **2018**, *272*, 1001–1018. [[CrossRef](#)]
- Gathergood, N.; Garcia, M.T.; Scammells, P.J. Biodegradable ionic liquids: Part I. Concept, preliminary targets and evaluation. *Green Chem.* **2004**, *6*, 166–175. [[CrossRef](#)]
- Cvjetko Bubalo, M.; Radošević, K.; Radojčić Redovniković, I.; Halambek, J.; Gaurina Srček, V. A brief overview of the potential environmental hazards of ionic liquids. *Ecotoxicol. Environ. Saf.* **2014**, *99*, 1–12. [[CrossRef](#)]
- Blaise, C.R. *Practical Laboratory Applications with Microalgae for Hazard Assessment of Aquatic Contaminants*; Richardson, M.L., Ed.; Ecotoxicology Monitoring VCH: Weinheim, Germany, 1993; pp. 83–108.
- Lewis, M.A. Algae and Vascular Plant Tests. In *Fundamentals of Aquatic Toxicology: Effects, Environment Fate, and Risk Assessment*; Rand, G.M., Ed.; Taylor and Francis: Washington, DC, USA, 1995; pp. 135–170.
- Liu, H.; Zhang, X.; Chen, C.; Du, S.; Dong, Y. Effects of imidazolium chloride ionic liquids and their toxicity to *Scenedesmus obliquus*. *Ecotoxicol. Environ. Saf.* **2015**, *122*, 83–90. [[CrossRef](#)]
- Flieger, J.; Flieger, M. Ionic Liquids Toxicity—Benefits and Threats. *Int. J. Mol. Sci.* **2020**, *21*, 6267. [[CrossRef](#)]
- Pretti, C.; Chiappe, C.; Baldetti, I.; Brunini, S.; Monni, G.; Intorre, L. Acute toxicity of ionic liquids for three freshwater organisms: *Pseudokirchneriella subcapitata*, *Daphnia magna* and *Danio rerio*. *Ecotoxicol. Environ. Saf.* **2009**, *72*, 1170–1176. [[CrossRef](#)]
- Tsarpali, V.; Belavgeni, A.; Dailianis, S. Investigation of toxic effects of imidazolium ionic liquids, [bmim][BF<sub>4</sub>] and [omim][BF<sub>4</sub>], on marine mussel *Mytilus galloprovincialis* with or without the presence of conventional solvents, such as acetone. *Aquat. Toxicol.* **2015**, *164*, 72–80. [[CrossRef](#)]
- Costa, S.P.F.; Azevedo, A.M.O.; Pinto, P.C.A.G.; Saraiva, M.L.M.F.S. Environmental Impact of Ionic Liquids: Recent Advances in (Eco)toxicology and (Bio)degradability. *ChemSusChem* **2017**, *10*, 2321–2347. [[CrossRef](#)]
- Pham, T.P.T.; Cho, C.-W.; Yun, Y.-S. Environmental fate and toxicity of ionic liquids: A review. *Water Res.* **2010**, *44*, 352–372. [[CrossRef](#)]
- Salmaso, N.; Boscaini, A.; Capelli, C.; Cerasino, L. Ongoing ecological shifts in a large lake are driven by climate change and eutrophication: Evidences from a three-decade study in Lake Garda. *Hydrobiologia* **2017**, *824*, 177–195. [[CrossRef](#)]
- Li, R.H.; Cheng, Y.; Geng, R.Z.; Cheng, X.L.; Xiao, P.; Zhang, H.; Liu, Y.; Ma, Z.L. Taxonomic separation and combination of cyanobacterial genera *Cylindrospermopsis* and *Raphidiopsis*. *J. Henan Norm. Univ.* **2022**, *50*, 39–46.
- Abdel-Rahman, G.N.; Sultan, Y.Y.; Hassoub, M.A.; Marrez, D. Cytotoxicity and antibacterial activity of the blue green alga *Microcystis aeruginosa* extracts against human cancer cell lines and foodborne bacteria. *Egypt. J. Chem.* **2020**, *63*, 4095–4105. [[CrossRef](#)]
- Liu, Y.; Chen, Y.; Fang, H.; Lu, H.; Wu, X.; Yu, G.; Nakano, S.-I.; Li, R. Relationship between morphospecies and microcystin-producing genotypes of *Microcystis* species in Chinese freshwaters. *J. Oceanol. Limnol.* **2021**, *39*, 1926–1937. [[CrossRef](#)]
- Kurmayer, R.; Kutzenberger, T. Application of Real-Time PCR for Quantification of Microcystin Genotypes in a Population of the Toxic Cyanobacterium *Microcystis* sp. *Appl. Environ. Microbiol.* **2003**, *69*, 6723–6730. [[CrossRef](#)]
- Ding, W.; Shangguan, Y.; Zhu, Y.; Sultan, Y.; Feng, Y.; Zhang, B.; Liu, Y.; Ma, J.; Li, X. Negative impacts of microcystin-LR and glyphosate on zebrafish intestine: Linked with gut microbiota and microRNAs? *Environ. Pollut.* **2021**, *286*, 117685. [[CrossRef](#)]
- Zhao, B.; Wang, J.; Gong, H.; Wen, X.; Ren, H.; Lu, C. Effects of heat stress on PSII photochemistry in a cyanobacterium *Spirulina platensis*. *Plant Sci.* **2008**, *175*, 556–564. [[CrossRef](#)]
- Krause, G.H.; Weis, E. Chlorophyll Fluorescence and Photosynthesis: The Basics. *Annu. Rev. Plant Biol.* **1991**, *42*, 313–349. [[CrossRef](#)]
- Strasser, B.J.; Strasser, R.J. Measuring Fast Fluorescence Transients to Address Environmental Questions: The JIP-Test. In *Photosynthesis: From Light to Biosphere*; Mathis, P., Ed.; KAP Press: Dordrecht, The Netherlands, 1995; pp. 977–980.
- Lu, C.; Vonshak, A. Characterization of PSII photochemistry in salt-adapted cells of cyanobacterium *Spirulina platensis*. *New Phytol.* **1999**, *141*, 231–239. [[CrossRef](#)]
- Bueno, M.; Fillat, M.F.; Strasser, R.J.; Maldonado-Rodríguez, R.; Marina, N.; Smienk, H.; Gómez-Moreno, C.; Barja, F. Effects of lindane on the photosynthetic apparatus of the cyanobacterium *Anabaena*. *Environ. Sci. Pollut. Res.* **2004**, *11*, 98–106. [[CrossRef](#)]
- Bilgili, S.; Atac, A.; Bardak, F. Theoretical and experimental investigation of the spectroscopic features of and interionic interactions in 1-hexyl-3-methylimidazolium chloride, 1-hexyl-3-methylimidazolium tetrafluoroborate and 1-hexyl-3-methylimidazolium hexafluorophosphate ionic liquids. *J. Mol. Liq.* **2020**, *301*, 112468. [[CrossRef](#)]
- Rippka, R.; Deruelles, J.; Waterbury, J.B.; Herdman, M.; Stanier, R.Y. Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria. *Microbiology* **1979**, *111*, 1–61. [[CrossRef](#)]



25. Richards, F.A.; Thompson, T.G. The estimation and characterization of plankton populations by pigment analyses. II. A spectrophotometric method for the estimation of plankton pigments. *J. Mar. Res.* **1952**, *11*, 156–172.
26. Appenroth, K.-J.; Stöckel, J.; Srivastava, A.; Strasser, R. Multiple effects of chromate on the photosynthetic apparatus of *Spirodelia polyrhiza* as probed by OJIP chlorophyll a fluorescence measurements. *Environ. Pollut.* **2001**, *115*, 49–64. [[CrossRef](#)]
27. Christen, D.; Schönmann, S.; Jermini, M.; Strasser, R.J.; Défago, G. Characterization and early detection of grapevine (*Vitis vinifera*) stress responses to esca disease by in situ chlorophyll fluorescence and comparison with drought stress. *Environ. Exp. Bot.* **2007**, *60*, 504–514. [[CrossRef](#)]
28. Shao, J.; Wu, Z.; Yu, G.; Peng, X.; Li, R. Allelopathic mechanism of pyrogallol to *Microcystis aeruginosa* PCC7806 (Cyanobacteria): From views of gene expression and antioxidant system. *Chemosphere* **2009**, *75*, 924–928. [[CrossRef](#)]
29. Shao, J.; He, Y.; Li, F.; Zhang, H.; Chen, A.; Luo, S.; Gu, J.-D. Growth inhibition and possible mechanism of oleamide against the toxin-producing cyanobacterium *Microcystis aeruginosa* NIES-843. *Ecotoxicology* **2015**, *25*, 225–233. [[CrossRef](#)]
30. Pham, T.P.T.; Cho, C.-W.; Min, J.; Yun, Y.-S. Alkyl-chain length effects of imidazolium and pyridinium ionic liquids on photosynthetic response of *Pseudokirchneriella subcapitata*. *J. Biosci. Bioeng.* **2008**, *105*, 425–428. [[CrossRef](#)]
31. Amde, M.; Liu, J.-F.; Pang, L. Environmental Application, Fate, Effects, and Concerns of Ionic Liquids: A Review. *Environ. Sci. Technol.* **2015**, *49*, 12611–12627. [[CrossRef](#)]
32. Kumari, P.; Pillai, V.V.; Benedetto, A. Mechanisms of action of ionic liquids on living cells: The state of the art. *Biophys. Rev.* **2020**, *12*, 1187–1215. [[CrossRef](#)]
33. Vilar, M.; Rodrigues, T.; Silva, L.; Pacheco, A.; Ferrão-Filho, A.; Azevedo, S. Ecophysiological Aspects and *sxt* Genes Expression Underlying Induced Chemical Defense in STX-Producing *Raphidiopsis raciborskii* (Cyanobacteria) against the Zooplankter *Daphnia gessneri*. *Toxins* **2021**, *13*, 406. [[CrossRef](#)]
34. Kulacki, K.J.; Lamberti, G.A. Toxicity of imidazolium ionic liquids to freshwater algae. *Green Chem.* **2007**, *10*, 104–110. [[CrossRef](#)]
35. Docherty, K.M.; Joyce, M.V.; Kulacki, K.J.; Kulpa, C.F. Microbial biodegradation and metabolite toxicity of three pyridinium-based cation ionic liquids. *Green Chem.* **2010**, *12*, 701–712. [[CrossRef](#)]
36. Zhang, C.; Wang, H.; Malhotra, S.V.; Dodge, C.J.; Francis, A.J. Biodegradation of pyridinium-based ionic liquids by an axenic culture of soil Corynebacteria. *Green Chem.* **2010**, *12*, 851–858. [[CrossRef](#)]
37. Shao, Y.; Du, Z.; Zhang, C.; Zhu, L.; Wang, J.; Wang, J. Acute Toxicity of Imidazole Nitrate Ionic Liquids with Varying Chain Lengths to Earthworms (*Eisenia foetida*). *Bull. Environ. Contam. Toxicol.* **2017**, *99*, 213–217. [[CrossRef](#)]
38. Hui, M.; Peng, X.J.; Ning, D.; Chen, J. Toxicity of [C<sub>8</sub>mim]PF<sub>6</sub> to aquatic organisms. *China Environ. Sci.* **2009**, *29*, 1196–1201. (In Chinese, English Summary)
39. Sena, D.W.; Kulacki, K.J.; Chaloner, D.T.; Lamberti, G.A. The role of the cell wall in the toxicity of ionic liquids to the alga *Chlamydomonas reinhardtii*. *Green Chem.* **2010**, *12*, 1066–1071. [[CrossRef](#)]
40. Cho, C.-W.; Pham, T.P.T.; Jeon, Y.-C.; Vijayaraghavan, K.; Choe, W.-S.; Yun, Y.-S. Toxicity of imidazolium salt with anion bromide to a phytoplankton *Selenastrum capricornutum*: Effect of alkyl-chain length. *Chemosphere* **2007**, *69*, 1003–1007. [[CrossRef](#)]
41. Nakai, S.; Yamada, S.; Hosomi, M. Anti-cyanobacterial fatty acids released from *Myriophyllum spicatum*. *Hydrobiologia* **2005**, *543*, 71–78. [[CrossRef](#)]
42. Zhang, B.; Li, X.; Chen, D.; Wang, J. Effects of 1-octyl-3-methylimidazolium bromide on the antioxidant system of *Lemna minor*. *Protoplasma* **2012**, *250*, 103–110. [[CrossRef](#)]
43. Li, X.-Y.; Jing, C.-Q.; Zang, X.-Y.; Yang, S.; Wang, J.-J. Toxic cytological alteration and mitochondrial dysfunction in PC12 cells induced by 1-octyl-3-methylimidazolium chloride. *Toxicol. Vitro* **2012**, *26*, 1087–1092. [[CrossRef](#)]
44. Oh, H.-M.; Lee, S.J.; Jang, M.-H.; Yoon, B.-D. Microcystin Production by *Microcystis aeruginosa* in a Phosphorus-Limited Chemostat. *Appl. Environ. Microbiol.* **2000**, *66*, 176–179. [[CrossRef](#)] [[PubMed](#)]