Heavy-metal pollution alters dissolved organic matter released by bloom-forming *Microcystis aeruginosa*

Haiming Wu, a Li Lin, b Guangzhu Shen a and Ming Li a,b,c*

The risk of heavy metals to aquatic ecosystems has been paid much attention worldwide in recent years, however, the knowledge on effects of heavy metals on dissolved organic matter (DOM) released by *Microcystis* was quite poor, especially in eutrophic lakes. The aim of this study is to investigate effects of heavy metals on DOC release of *Microcystis* using EEM-PAFAFAC analysis. *Microcystis aeruginosa* was batch cultured for 18 days in BG-11 medium treated with a range of concentrations of Cu\(^{2+}\) (0.02, 0.05, 0.10 and 0.25 mg L\(^{-1}\)) and Zn\(^{2+}\) (0.10, 0.20, 0.50 and 1.00 mg L\(^{-1}\)), to investigate the effects of heavy metals on DOM release of *Microcystis*. The cultures without addition of Cu\(^{2+}\) or Zn\(^{2+}\) were set as controls. Cell density in the treatment of 0.25 mg L\(^{-1}\) Cu\(^{2+}\) was 25% less than that in the control, but addition of 0.25 mg L\(^{-1}\) Zn\(^{2+}\) had little effect on *Microcystis* growth. Moderate levels of Cu\(^{2+}\) (0.05 and 0.10 mg L\(^{-1}\)) and high level of Zn\(^{2+}\) (1.00 mg L\(^{-1}\)) stimulated DOC production of *M. aeruginosa* on day 10; moderate levels of Zn\(^{2+}\) (0.10–0.50 mg L\(^{-1}\)) stimulated DOC production on day 18 compared to the control. Four components of DOM: two humic-like components (C1 and C3) and two protein-like components (C2 and C4), were identified by fluorescence excitation–emission matrix spectroscopy combined with parallel factors (EEM-PAFAFAC) analysis. The fluorescence intensity of the four components increased when *Microcystis* was treated with moderate level of Cu\(^{2+}\) (0.05 and 0.10 mg L\(^{-1}\)) on day 10. In addition, the composition of DOM produced by *Microcystis* was not affected by heavy metals. Our results suggested that *Microcystis* increased DOM production in logarithmic phase by altering cellular secretion, but large biomass was the effective measure for *M. aeruginosa* to reduce toxicity of heavy metals in the stationary phase.

1. Introduction

Heavy metal pollution is a serious issue resulting from anthropogenic activities that poses a severe risk to the environment, ecosystems, and human health. a The risks to humans of heavy-metal pollution in freshwater ecosystems are especially high because of the enrichment of heavy metals in aquatic food chains. a Despite the application of many environmental technologies to keep heavy-metal concentrations within safe levels, the distribution of heavy metals in aquatic ecosystems, and their toxicity to fish, phytoplankton, and other aquatic animals, still requires investigation to gain a better understanding of the risks of heavy metal pollution in aquatic ecosystems over the last several decades. a,b

Cyanobacterial bloom in lakes and reservoirs is another environmental issue resulting from anthropogenic activities. a This phenomenon causes serious environmental problems, such as oxygen depletion, unpleasant odors, and toxin production. a However, bloom-forming cyanobacteria, including the most widely distributed example, *Microcystis*, have been reported as carbon sources for zooplankton in eutrophic lakes. b *Microcystis* also releases dissolved organic matter (DOM) into water, including polysaccharides and amino acids. b DOM is an important energy source for heterotrophic bacteria, which vary dramatically in response to varying environments. c,d Therefore, the variation in DOM released by *Microcystis* under different environmental conditions should be well understood.

The concentration of dissolved organic carbon (DOC) has been shown to increase with decreasing nitrate in a eutrophic lake (Lake Taihu, China) with *Microcystis* blooms. e Furthermore, Yang and Kong f reported that the concentrations of extracellular polysaccharides (EPS) and the main component of DOM released by *Microcystis* were higher when treated with a lower nutrient concentration. These results demonstrated that DOM concentration is negatively correlated with nutrient level. The effects of temperature and light intensity on the EPS of...
Microcystis have also been well studied.\textsuperscript{12} Bi et al.\textsuperscript{13} indicated that increasing Pb concentration promoted EPS production in Microcystis. Herzi et al.\textsuperscript{14} showed that the DOC concentration of *Alexandrium catenella* varied greatly when exposed to different concentrations of Pb and Zn. Therefore, it could be deduced that heavy metals can affect DOM production by *Microcystis*. Accordingly, heavy metal pollution can affect DOM dynamics in aquatic ecosystems by influencing DOM release from *Microcystis*, especially in eutrophic lakes where *Microcystis* blooms occur frequently. However, the effects of heavy metals on DOM release by *Microcystis* are poorly understood, and should be investigated with regard to quantity and quality.

Fluorescence excitation–emission matrix spectroscopy combined with parallel factor analysis (EEM-PARAFAC) is a rapid and efficient method for identifying the composition of complex organic matter, and has been widely applied to characterize DOC in lakes and algal EPS.\textsuperscript{15–27} Xu et al.\textsuperscript{18} analyzed extracellular polymeric substances from *Microcystis* by EEM-PARAFAC and successfully determined four components belonging to protein-like and humic-like substances. Additionally, Herzi et al.\textsuperscript{14} analyzed the DOM released by *Alexandrium catenella* exposed to heavy metals using the same method.

The aim of this study is to investigate the effects of heavy metals on DOC release in *Microcystis* using EEM-PARAFAC analysis. *Microcystis aeruginosa*, a common bloom-forming *Microcystis* species distributed worldwide, was chosen as the test organism. Cu and Zn were chosen as the test heavy metals because copper sulfate is often added into lakes as an agent to remove *Microcystis*\textsuperscript{29} and the Zn concentration is always higher than those of other heavy metals in lakes and reservoirs.\textsuperscript{30} Furthermore, Cu and Zn are necessary trace elements for photosynthesis.\textsuperscript{31} The results of this work increase understanding of the effects of heavy metals on energy flows and carbon cycles in aquatic ecosystems.

## 2. Materials and methods

### 2.1 Organisms

*M. aeruginosa* (FACHB 469) was provided by the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences. This strain was cultivated axenically in BG-11 medium for more than three months and existed as single cells before experiments.

### 2.2 Culture conditions

The alga was batch cultured in BG-11 medium (120 mL) in a 250 mL conical flask with varying Cu\textsuperscript{2+} and Zn\textsuperscript{2+} concentrations. The concentrations were adjusted to 0, 0.02, 0.05, 0.10, and 0.25 mg L\textsuperscript{−1} Cu\textsuperscript{2+} and 0, 0.10, 0.20, 0.50, and 1.00 mg L\textsuperscript{−1} Zn\textsuperscript{2+} using CuCl\textsubscript{2} (5 mg L\textsuperscript{−1}) and ZnCl\textsubscript{2} (10 mg L\textsuperscript{−1}) solutions, respectively. Different Cu\textsuperscript{2+} and Zn\textsuperscript{2+} concentration ranges were selected because Cu\textsuperscript{2+} was more toxic than Zn\textsuperscript{2+} to *Microcystis*.\textsuperscript{32} Concentrations of 0 mg L\textsuperscript{−1} were used as the control. Six replicates were carried out for each treatment. All samples were cultured at 25 °C in a light–dark cycle (12 hours each) using 50 μmol per photons per m\textsuperscript{2} per s. The culture experiment was performed for 18 days. The flasks were shaken two or three times daily by hand to prevent cells from adhering to the inner flask walls.

### 2.3 Culture enumeration

The cell density of *M. aeruginosa* was measured every two days by counting cells in a hemocytometer three times under an optical microscope (Olympus CX31, Olympus Corporation) at 400× magnification. If the three counts differed by less than 10%, the average value was calculated as the final cell density. Otherwise, additional counting was carried out.

### 2.4 Analysis of DOM and DOC

Samples were collected twice, on days 10 and 18, as three replicates. The first collection represented the logarithmic phase, while the second represented the stationary phase. The algal sample was centrifuged at 16 000 × g for 10 min and the supernatants were then filtered through a membrane with 0.45 μm pore size. The filtrate was used for the analysis of DOC concentration and fluorescence EEM spectra. DOC concentration was analyzed using a total organic carbon analyzer (TOC-L CPN, Shimadzu, Japan). Fluorescence EEM spectra were measured using a fluorescence spectrometer (F97 Pro, Leng-guang Tech., China) in scan mode with a xenon lamp (700 V) at room temperature. All cuvettes were rinsed with 5% HNO\textsubscript{3} solution before analysis. The excitation (Ex) wavelengths were 200–450 μm with a step length of 5 nm, while the emission (Em) wavelengths were 250–500 μm with step length of 2 nm. The scan speed was set at 6000 nm min\textsuperscript{−1}.

### 2.5 Data analysis

All data were presented as mean ± standard deviation. PARAFAC modeling was executed in MATLAB 7.0 software (Mathworks, Natick, MA) using the DOMFluor toolbox.\textsuperscript{33} There was no outlier sample in the leverage comparison. Residual and split-half analyses were used to determine the correct numbers of components. Analysis of variance (ANOVA) was conducted to analyze the differences among different treatments using Tukey’s post hoc test with SPSS 10.0 software.

## 3. Results and discussion

### 3.1 Growth of *M. aeruginosa*

The cell density of *M. aeruginosa* increased rapidly from day 2 to day 14, after which the growth slowed gradually (Fig. 1). The cell density in the 0.25 mg L\textsuperscript{−1} Cu\textsuperscript{2+} treatment was 25% lower than that in the control throughout incubation. *M. aeruginosa* growth in the 0.02 and 0.05 mg L\textsuperscript{−1} Cu\textsuperscript{2+} treatments was similar to that of the control. Cell density in the 0.10 mg L\textsuperscript{−1} Cu\textsuperscript{2+} treatment (3.8 × 10\textsuperscript{7} cells per mL) was slightly lower than that of the control (3.9 × 10\textsuperscript{7} cells per mL). The maximum cell densities were 3.8, 4.1, 4.1, 3.9, and 3.7 × 10\textsuperscript{7} cells per mL, respectively, in the control and 0.10, 0.20, 0.50, and 1.00 mg L\textsuperscript{−1} Zn\textsuperscript{2+} treatments, respectively.

Tsai\textsuperscript{24} reported that the minimum amount of Cu\textsuperscript{2+} required to inhibit *M. aeruginosa* growth was 0.16 mg L\textsuperscript{−1}. Wu et al.\textsuperscript{25} indicated that 0.25 mg L\textsuperscript{−1} Cu\textsuperscript{2+} significantly inhibited
Microcystis growth. Deng et al.\textsuperscript{26} showed that 0.10 mg L\textsuperscript{-1} Cu\textsuperscript{2+} significantly decreased the Chl-a content in \textit{M. aeruginosa}. Polyak et al.\textsuperscript{27} demonstrated that 0.25 mg L\textsuperscript{-1} Zn\textsuperscript{2+} inhibited \textit{M. aeruginosa} growth. Our results matched with the toxicities of Cu\textsuperscript{2+} and Zn\textsuperscript{2+} reported elsewhere.

### 3.2 DOC concentrations

DOC concentrations in the control were 74 and 120 mg L\textsuperscript{-1} on days 10 and 18, respectively (Fig. 2). On day 10, the DOC concentrations in the 0.05 and 0.10 mg L\textsuperscript{-1} Cu\textsuperscript{2+} and 1 mg L\textsuperscript{-1} Zn\textsuperscript{2+} treatments were greater than that of the control. The maximum value of approximately 98 mg L\textsuperscript{-1} appeared in the 1 mg L\textsuperscript{-1} Zn\textsuperscript{2+} treatment. On day 18, the DOC concentrations in all Zn\textsuperscript{2+} treatments were significantly higher than that of the control, except for that of the highest Zn\textsuperscript{2+} treatment. Unexpectedly, the DOC concentration in the sample treated with the highest level of Cu\textsuperscript{2+} decreased to 100 mg L\textsuperscript{-1}, while samples treated with moderate levels of Cu\textsuperscript{2+} showed no obvious differences compared with the control. Reports on the effects of heavy metals on DOC concentration in \textit{Microcystis} are rare. Bi et al.\textsuperscript{13} reported that Pb stimulated the production of extracellular polysaccharide (EPS) in \textit{Microcystis}. Herzi et al.\textsuperscript{14} indicated that the DOM released by \textit{A. catenella} was promoted by 0.78 mg L\textsuperscript{-1} Zn\textsuperscript{2+}, with the results similar to ours obtained from \textit{M. aeruginosa} in this study. These results indicated that heavy metals may promote DOC production in \textit{Microcystis}.

The cellular DOC content is shown in Fig. 3. In general, the cellular DOC content on day 10 was larger than that on day 18. The cellular DOC content increased with increasing Cu\textsuperscript{2+} concentration on day 10, but differences among different Cu\textsuperscript{2+} treatments were not significant on day 18. Zn\textsuperscript{2+} addition had little effect on the cellular DOC content on day 10, except in the case of 1 mg L\textsuperscript{-1} Zn\textsuperscript{2+} treatment. However, the production of cellular DOC content was promoted when treated with moderate levels of Zn\textsuperscript{2+}.

The DOC concentrations in culture medium exposed to different levels of Cu\textsuperscript{2+} on day 18 were found to be significantly lower than those in the medium amended with different levels of Zn\textsuperscript{2+}. Although both Cu and Zn are necessary trace elements for photosynthesis, the Zn content (15–50 mg kg\textsuperscript{-1} dry weight) in plants is always higher than that of Cu (1–20 mg kg\textsuperscript{-1} dry weight).\textsuperscript{21} In the current study, the growth and cellular DOC content of \textit{Microcystis} on day 18 were significantly promoted when treated with moderate levels of Zn\textsuperscript{2+}, but inhibited when treated with Cu\textsuperscript{2+}. This result implied that \textit{Microcystis} photosynthesis was promoted when treated with moderate level of Zn\textsuperscript{2+}, causing the DOC concentration to increase.

### 3.3 Composition of EEM spectra

All fluorescence EEM spectra of DOM were successfully decomposed into a four-component model based on residual and split-half analyses. The contours and Ex/Em loadings of the four components are shown in Fig. 4. The first component (C1) gave a peak at $\lambda_{\text{Ex/Em}} = 355/458$ nm. This component was

\[ \text{Fig. 1 Growth curves of} \ M. \text{aeruginosa at varying Cu}\textsuperscript{2+} \text{and Zn}\textsuperscript{2+} \text{concentrations.} \]

\[ \text{Fig. 2 DOC concentrations in culture medium at varying Cu}\textsuperscript{2+} \text{and Zn}\textsuperscript{2+} \text{concentrations on days 10 and 18. *Indicates values of each component in the treatment that were significantly different from that in the control (p < 0.05).} \]
a humic acid-like compound also described in Region V by Chen et al.29 The second component (C2) gave maximum intensities at an excitation wavelength of 235 nm and centered between emission wavelengths of 250 and 400 nm. This component was identified as an aromatic protein-like compound.29–31 The third component (C3) gave two peaks ($\lambda_{\text{Ex/Em}} = 280/474$ nm and $\lambda_{\text{Ex/Em}} = 415/474$ nm) and was defined as a UVC humic acid-like compound with a high molecular weight.30,32,33 The last component (C4) gave a peak at $\lambda_{\text{Ex/Em}} = 285/358$ nm and was defined as a protein-like compound containing tryptophan.18,29,34

Therefore, the four components identified from the fluorescence spectra were two humic-like components (C1 and C3) and two protein-like components (C2 and C4). Xu et al.18 identified three protein-like compounds and a humic-like compound in the EPS of Microcystis. Aoki et al.35 found a fulvic acid-like compound, a protein-like compound, and an unknown compound in the DOM of M. aeruginosa. Yang et al.36 distinguished a tryptophan-like component and two humic acid-like compounds. The wavelength coverage of excitation and emission in the current study was much wider than previous studies, resulting in more humic acid-like components being identified.

### 3.4 Variations in fluorescence contributions

The fluorescence intensity of all four components in the DOM had increased by day 10 when treated with moderate levels of Cu$^{2+}$ (0.05 and 0.10 mg L$^{-1}$) (Fig. 5). However, Zn$^{2+}$ had only a small effect on fluorescence intensity. Nevertheless, the fluorescence intensity of C1 increased when treated with 0.10 mg L$^{-1}$ Cu$^{2+}$ and 0.50 mg L$^{-1}$ Zn$^{2+}$. The results in the stationary phase were completely different than in the logarithmic phase. For Cu$^{2+}$ treatments, no significant difference was observed between the fluorescence intensities of different treatments and the control, except that the values of C3 and C4 treated with 0.02 mg L$^{-1}$ Cu$^{2+}$ were significantly higher than those of the control. Additionally, the fluorescence intensities of C1, C3, and C4 in the DOM improved when treated with moderate levels of Zn$^{2+}$ (0.10–0.50 mg L$^{-1}$).

The relationships among the fluorescence intensities of different components, cell densities, and DOC concentrations...
in the logarithmic phase (day 10) and stationary phase (day 18) are shown in Tables 1 and 2, respectively. On day 10, the DOC concentration was neither significantly related to the fluorescence intensities of all components, nor significantly related to cell density (Table 1). Furthermore, we found that there was no significant difference in the percentage of the four components calculated from their fluorescence intensities among the different treatments on day 10 (data not shown). These results showed that the increase in fluorescence intensity of the four components on day 10 was due to increased DOC production, but with no change in DOM composition. Moreover, the DOC concentration was not correlated with cell density on day 10, indicating that the increase in DOC concentration induced by the addition of Cu²⁺ and Zn²⁺ (Fig. 2) was not caused by the influence of heavy metals on the biomass of _M. aeruginosa_, but by the variation in cellular secretion of DOM during the logarithmic phase (Fig. 3).

Although DOC concentrations of the cell and the culture medium treated with moderate levels of Zn²⁺ were greater than that in the control sample on day 18 (Fig. 2 and 3), the cellular DOC content showed no obvious variations on day 18 under treatment with varying concentrations of Cu²⁺. Moreover, the DOC concentration was significantly correlated with cell density on day 18 (Table 2). Therefore, it was deduced that the increase

![Fluorescence intensities of the four components in the DOM of _M. aeruginosa_ on days 10 and 18. *Indicates values of each component in the treatment that were significantly different from that in the control (p < 0.05).](image)

**Table 1** Pearson correlation among fluorescence intensities of different components, cell densities, and DOC concentrations in the logarithmic phase (day 10). Data were obtained from both Cu²⁺ and Zn²⁺ treatments

<table>
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<th></th>
<th>Cell density</th>
<th>DOC</th>
<th>Total</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
</tr>
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<td>1.000</td>
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<tr>
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<td>1.000</td>
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<tr>
<td>C1</td>
<td>0.479</td>
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<td>0.961**</td>
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<tr>
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<td>0.465</td>
<td>0.978**</td>
<td>0.889**</td>
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<td>C3</td>
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<td>0.931**</td>
<td>0.989**</td>
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<td>0.918**</td>
<td>0.987**</td>
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* Total indicates total fluorescence intensity. *Correlation is significant at the 0.05 level (two-tailed); **correlation is significant at the 0.01 level (two-tailed).
in DOC concentration induced by Cu\(^{2+}\) and Zn\(^{2+}\) treatment in the stationary phase (Fig. 2) was caused by the influence of heavy metals on biomass of *M. aeruginosa*, but not by variations in cellular secretion. The component percentage amounts calculated from their fluorescence intensities on day 18 were similar among different treatments. On day 18, the fluorescence intensities of all four components were significantly related to the total fluorescence intensity. Therefore, similar to day 10, the DOM composition was not affected by heavy metal addition on day 18.

Extracellular polymeric substances (EPS) are the main components of DOM released by *Microcystis*.\(^{18}\) EPS has been reported to play a role in protecting algae against heavy metal toxicity\(^{17,18}\) and other chemical stresses.\(^{29,40}\) Humic acid-like compounds have been used to chelate heavy metals.\(^{39}\) Herzi *et al.*\(^{44}\) also suggested that both tryptophan-like compounds and proteins could chelate heavy metals to reduce their toxicity. Our results showed that the DOM composition produced by *Microcystis* was not affected by heavy metal addition, but that the concentration of each component increased when treated with heavy metal. It was deduced that all four compounds identified in the current study could be used to chelate heavy metals to reduce their toxicity toward *M. aeruginosa*. However, *Microcystis* increased DOM production in the logarithmic phase by altering cellular secretion, but high biomass was the effective measure for *M. aeruginosa* to reduce the toxicity of heavy metals in the stationary phase. Xu *et al.*\(^{48}\) indicated that two protein-like components and a humic acid-like component were significantly correlated with the cell density of *M. aeruginosa* culture in low nutrient medium and standard medium. Our results differed from theirs because heavy metal addition in the current study altered the DOM production of *M. aeruginosa*.

Our results demonstrated that 0.05 and 0.1 mg L\(^{-1}\) Cu\(^{2+}\) improved DOM production in *M. aeruginosa* during the logarithmic phase, but 0.25 mg L\(^{-1}\) Cu\(^{2+}\) reduced DOM production. In addition, 0.10–1.00 mg L\(^{-1}\) Zn\(^{2+}\) stimulated DOM release from *M. aeruginosa* in different growth phases. All above concentrations of Cu\(^{2+}\) and Zn\(^{2+}\) have been detected in natural waters bodies, including Lake Taihu\(^{48}\) and Lake Qarun.\(^{41}\) Therefore, the DOC concentrations in these waters would increase due to the effects of heavy metals on DOM release by *Microcystis* and other phytoplankton. The increasing DOC would affect carbon and nitrogen cycles,\(^{42}\) the abundance and community of aquatic microorganisms,\(^{43}\) and the fate and transport of hazardous materials\(^{44,45}\) in natural waters. Therefore, the effects of heavy metals in relatively safe concentrations on aquatic ecosystems should be considered because they can alter DOM production by phytoplankton especially for *Microcystis*.

### 4. Conclusions

(1) During the logarithmic phase, moderate levels of Cu\(^{2+}\) (0.05 and 0.10 mg L\(^{-1}\)) and high levels of Zn\(^{2+}\) (1.00 mg L\(^{-1}\)) stimulated DOC production of *M. aeruginosa*. In the stationary phase, moderate levels of Zn\(^{2+}\) (0.10–0.50 mg L\(^{-1}\)) stimulated DOC production, but high levels of Cu\(^{2+}\) (0.10 mg L\(^{-1}\)) decreased DOC concentration compared with the control.

(2) Four components were identified from fluorescence spectra by EEM-PARAFAC, consisting of two humic-like components (C1 and C3) and two protein-like components (C2 and C4). The fluorescence intensities of all four components in the DOM were improved on day 10 when treated with moderate levels of Cu\(^{2+}\) (0.05 and 0.10 mg L\(^{-1}\)), but the fluorescence intensities of C1, C3, and C4 in the DOM on day 18 were improved when treated with moderate levels of Zn\(^{2+}\) (0.10–0.50 mg L\(^{-1}\)).

(3) The DOM composition produced by *Microcystis* was not affected by heavy metal addition. *Microcystis* increased DOM production in the logarithmic phase by altering cellular secretion, but high biomass was the effective measure for *M. aeruginosa* to reduce the toxicity of Cu and Zn in the stationary phase.

### Acknowledgements

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### References


| Table 2 Pearson correlation among fluorescence intensities of different components, cell densities, and DOC concentrations in the stationary phase (day 18). Data were obtained from both Cu\(^{2+}\) and Zn\(^{2+}\) treatments* |
| --- | --- | --- | --- | --- | --- |
| Cell density | DOC | Total | C1 | C2 | C3 |
| Cell density | 1.000 | | | | |
| DOC | 0.739* | 1.000 | | | |
| Total | 0.783** | 0.812** | 1.000 | | |
| C1 | 0.842** | 0.827** | 0.912** | 1.000 | |
| C2 | 0.592 | 0.590 | 0.829** | 0.547 | 1.000 |
| C3 | 0.713* | 0.729* | 0.960** | 0.769** | 0.942** | 1.000 |
| C4 | 0.409 | 0.588 | 0.865** | 0.617 | 0.871** | 0.920** | 1.000 |

* Total indicates total fluorescence intensity. *Correlation is significant at the 0.05 level (two-tailed); **correlation is significant at the 0.01 level (two-tailed).
2 A. Altnda and S. Yi, Chemosphere, 2005, 60, 552–556.