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# Comparison of evaluation methods for *Microcystis* cell breakage based on dissolved organic carbon release, potassium release and flow cytometry

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## HIGHLIGHTS

- Flow cytometry-, DOC- and $K^+$-based *Microcystis* rupture evaluation methods were compared.
- The three indicators exhibited strong linear relationships with *Microcystis* cell breakage.
- DOC release was remarkably affected by EOM and strong oxidants.
- $K^+$ release was only impacted by cell intake resulting in deviations in the evaluation.
- Flow cytometry has the broadest application scope and the fewest influencing factors.

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## ABSTRACT

As *Microcystis* cell breakage may decrease the water quality, the accurate evaluation of cell breakage is of concern in drinking water production. Well-known cell breakage indicators such as dissolved organic carbon (DOC) release and potassium release were investigated in comparison with flow cytometry coupled with a fluorescence probe. DOC release, potassium release and ratios of SYTOX Green positive cells (ruptured cells) were calibrated using known degrees of cell breakage (mixtures of live and heated cells). Good linear relationships were observed between the indicators and the known ratios of ruptured cells, with $R^2$ values larger than 0.925. Flow cytometry coupled with a fluorescence probe had the best overall performance, followed by potassium release and then DOC release. Moreover, the influence of factors such as extracellular organic matter (EOM), cell intake, sample conservation and strong oxidants was also studied. EOM and strong oxidants caused the overestimation and underestimation, respectively, of the cell breakage by DOC release. Potassium release had a more extensive application scope than DOC release and was only influenced by intake by residual live cells. Flow cytometry was generally not affected by EOM, cell intake or strong oxidants except during long-term storage.



## 1. Introduction

Algae are ubiquitous in rivers and reservoirs that supply source water for drinking water plants [1,2]. As algal populations increase due to the global eutrophication trend, source water quality will be impaired and drinking water treatment plants may be strongly affected [3,4]. *Microcystis aeruginosa*, which commonly dominates seasonal blooms, is one of the most problematic algal species, causing the release of toxins and organic matter [5,6]. *M. aeruginosa* can produce 0.1–0.2 μg toxins per μg chlorophyll a, indicating a high proportion of the toxins in the biomass. The released organics can serve as the precursors for disinfection byproducts (DBPs) such as trihalomethanes and haloacetic acids, during the water disinfection process using chlorine [7]. Moreover, *Microcystis* biomass, which is rich in organic nitrogen, contributes to the formation of nitrogenous DBPs, increasing the byproduct carcinogenicity by 2–3 orders of magnitude [8].

The removal of *Microcystis* cells and the degradation of their hazardous metabolites are of particular concern in drinking water production. Chemicals including copper sulfate, chlorine, ozone, potassium permanganate and hydrogen peroxide have been used to treat the algae-laden water [1,9]. However, these chemicals usually cause cell breakage, releasing intracellular toxins and organics

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[10,11]. The concentrations of the intracellular toxins are nearly one order of magnitude greater than these of extracellular toxins [12].The intracellular organic matter (IOM) can form more DBPs than extracellular organic matter (EOM) can during chlorination [7]. Therefore, the accurate evaluation of *Microcystis* cell breakage, to provide a basis for the control of cell breakage in water treatment, is of great significance.

Several methods to evaluate *Microcystis* cell breakage evaluation methods have been developed; they can be classified as direct or indirect categories. The direct evaluation method depends on observation via various types of microscopy to count viable cells using the heterotrophic plate count (HPC) method [13,14]. However, cell breakage does not necessarily provoke apparent changes in the cell morphology. In another study, no considerable changes in the morphology were observed for the majority of *Microcystis* cells following exposure to chlorine, but intracellular substances were found to have been released [15]. In the indirect methods, indicators such as dissolved organic carbon (DOC) release, potassium release, toxin release, nitrogen fixation rate and surface zeta potential are commonly used [9,16]. As potassium is actively absorbed into the vacuole of the cell and stored as an enzyme activator, potassium release can manifest cell membrane damage [17]. Chen et al. [16] have investigated the effect of potassium permanganate on *Microcystis* cell integrity using DOC release, potassium release and the zeta potential variation of cell surface as the indicators. Zhou et al. [12] have used potassium release to describe the cell breakage resulting from exposure to copper sulfate, hydrogen peroxide, diuron and ethyl 2-methylacetoacetate. Peterson et al. [9] studied the cell membrane damage of cyanobacteria under exposure to water treatment chemicals via indexes such as the nitrogen fixation rate, DOC release and potassium release, and found that DOC release was less sensitive.

Recently, flow cytometry coupled with a fluorescence probe has increasingly been adopted in the evaluation of algal cell damage resulted from chlorination, ozonation and pre-oxidation with other chemicals [18–22]. Flow cytometry can assay parameters including cell volume, chlorophyll a fluorescence and cell viability, and thus is regarded as a useful tool in toxicity tests with microalgae for various pollutants [23]. With the help of nucleic acid stains such as SYTOX Green and PI which easily penetrate compromised cells and stain nucleic acids but cannot cross the membranes of live cells [24–26], a flow cytometer can quickly perform numerous quantitative and sensitive measurements on each individual cell within a large heterogeneous population [27]. Zamyadi et al. [28] established a fluorescein diacetate/propidium iodide staining method to assess the impact of chlorination on cell integrity and defined the cells with red fluorescence as damaged cells. Fan et al. [29] evaluated the effects of copper sulfate, chlorine, potassium permanganate, hydrogen peroxide and ozone on cyanobacterial cell integrity using flow cytometry with SYTOX Green.

In general, there are several algal cell breakage evaluation methods that have been widely used to study cell damage resulting from water treatment chemicals and environmental stimuli. However, these methods have always been directly adopted in the reported experiments without any calibration. A comparison among the various cell breakage evaluation methods has not yet been performed, leading to a lack of knowledge of the merits, defects and application scopes of these methods. Moreover, the influencing factors have not been carefully investigated either, possibly allowing the overestimation or underestimation of the cell breakage. Accordingly, this study compared three *Microcystis* cell breakage evaluation methods: the DOC release and potassium release and flow cytometry coupled with a fluorescence probe. Moreover, the effects of EOM, cell intake, sample conversation and strong oxidants on the evaluation accuracy were also investigated.

## 2. Materials and methods

### 2.1. Algal culture and organic matter extraction

*M. aeruginosa* (Culture Collection of Algae at the Institute of Hydrobiology, Chinese Academy of Sciences, China) was selected for this study. The algal cultivation methods followed Qu et al. [30]. Axenic cultures were grown in batches in 1 L conical flasks with BG11 medium. The conical flasks were cultured in an incubator at a temperature of 25 °C with 14 h of illumination with 5000 l× each day. The algae were harvested in the log or stationary growth phase. The harvested algal solution was diluted to a cell density of $1 \times 10^6$ cells/L with simulated surface water which was Milli-Q water spiked with 0.5 mM $CaCl_2$, 1.0 mM $NaHCO_3$, and 15.0 mM $NaClO_4$ [8]. These chemicals were added to simulate the ionic strength of surface water. The pH of the *Microcystis* solution was adjusted to 7.5 ± 0.1 with NaOH (0.1 M) and HCl (0.1 M) solutions.

EOM was extracted via the centrifugation method [31,32]. The harvested algal solution was centrifuged at 4000*g* and 4 °C for 15 min, using a high speed refrigerated centrifuge (H2050R, Xiangyi, China). EOM was obtained by filtering the supernatant through a 0.45 μm mixed ester membrane filter (Taoyuan, China). To prepare the algal cell suspension without EOM, the cells remaining at the bottom of the centrifuge tube and those on the filter were collected and resuspended in simulated surface water. To determine the concentration of algogenic organic matter (AOM), the algal cells were ruptured by heating the cell suspension in a water bath (90 °C) for 20 min [33], which caused the color of cell suspension to change from blue-green to yellow brown, as shown in Fig. S1. The flow cytometry spectra (Fig. S2) further verified the cell breakage after heating. Subsequently, the heated cell suspension was centrifuged at 10,000*g* for 15 min. The above filtration step was repeated to obtain the AOM solution which included both EOM and IOM. The three-dimensional excitation-emission matrix (EEM) fluorescence spectrum was characterized for the EOM and AOM using a fluorescence spectrophotometer (F7000, Hitachi, Japan). The details of this measurement have been described elsewhere [30].

### 2.2. Cell breakage evaluation methods

#### 2.2.1. Cell breakage evaluation using DOC release

To determine DOC release, the DOC concentrations of the total organics ($C_t$) of the *Microcystis* cells and of the extracellular organics before and after treatment were determined. As mentioned above, EOM and AOM were extracted through high-speed refrigerated centrifugation and heating with centrifugation, respectively. As shown in Fig. S1, the color of the algal solution changed from blue-green to brown. In addition, the flow cytometry spectra demonstrated that the SYTOX negative cells (live cells) disappeared after the heating treatment (Fig. S2). These results provided evidence of the high sensitivity of algal cells to a high temperature. Therefore, the heating method was used to determine the total organics in algal solution. The concentration of AOM was denoted as $C_t$, and $C$ and $C_0$ referred to the EOM concentrations before and after treatments, respectively. Then, DOC release was calculated using Eq. (1). The concentrations of organics were measured using a TOC analyzer (Multi N/C 2100, JENA, Germany)

$$\text{DOC release}\,(\%) = 100 \times (C - C_0)/(C_t - C_0) \quad (1)$$

#### 2.2.2. Cell breakage evaluation using potassium release

To determine the potassium release of *Microcystis* cells, the concentrations of total potassium ($C_t$), initial extracellular potassium