Allelopathic Effects of Two Organic Acids on *Microcystis Aeruginosa*

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**Abstract:** We selected two organic acids, heptanoic acid and benzoic acid, with the same carbon length but with different molecular structures to study their mechanisms of algal inhibition and to examine their combined algal inhibition ability. The results showed that the algal inhibition ability of benzoic acid was greater than that of heptanoic acid. The median lethal concentrations of benzoic acid and heptanoic acid were 55.02 mg/L and 83.90 mg/L, respectively. Heptanoic acid and benzoic acid could decrease the Total Superoxide Dismutase (T-SOD) and Catalase (CAT) activities of *Microcystis aeruginosa* and cause increased Malondialdehyde (MDA) production. The T-SOD and CAT activities initially increased and then decreased. The MDA content quickly decreased after reaching a maximum value. Benzoic acid had a greater effect on the antioxidant enzyme system of *Microcystis aeruginosa* than heptanoic acid. A hybrid evaluation was performed on the combined algal inhibition effect of heptanoic acid and benzoic acid. The results indicated that benzoic acid and heptanoic acid showed a synergistic effect and that their compound algal inhibition effect was superior to the separate effects of the two organic acids acting independently.

1. Introduction
Due to the rapid development of the economy and the swift growth of the population, water eutrophication is gradually increasing \[1\] \[2\]. It is becoming more difficult to manage increasingly frequent algal blooms using traditional physical and chemical algae removal methods \[3\] \[4\]. Therefore, some novel, high-efficiency and safe algae removal technologies are in rapid development. Based on plant allelopathy, the direct application of allelochemicals to inhibit algal propagation has become a new and important method of efficiently treating eutrophic waters \[5\]. As an important type of secondary metabolite, with the most species secreted by aquatic plants in natural waters, organic acid allelochemicals play an important role in the study of algal inhibition.

According to general research, the inhibition mechanism of broad-spectrum allelochemicals, such as organic acids, on all types of algae mainly consists of disrupting the operation and photosynthesis capability of the cell membrane structures and antioxidant enzyme systems of algal cells \[6\] \[7\]. Additionally, organic acids may affect the synthesis of microcystic toxins. Ma Ruixia \[8\] found 3 organic acid allelochemicals (ferulic acid, benzoic acid and p-hydroxybenzoic acid) produced from
the decay process of wheat straw that had an inhibitory effect on the activity of nitrate reductase at a specific concentration and pH value and might interfere with the synthesis of microcystic toxins. Liu et al. [9] found that high concentrations of organic acid components of *Canna indica* could reduce the chlorophyll content in *Microcystis aeruginosa* and produce oxidative stress. The algal inhibition mechanism of organic acids might also be closely related to their chemical structures. Wu et al. [10] found that fatty acids changed the permeability of cell membranes, which caused the leakage of cell contents, further damaged the membrane structures and thus affected the synthesis of microcystic toxins. Jiang Wenxin et al. [11] studied the inhibitory effect of palmitic acid and stearic acid on the growth of *Microcystis aeruginosa* [12] and compared the structure-activity relationship of algal inhibition of long-chain saturated fatty acids. Their results showed that the algal inhibition ability of a long-chain fatty acid was related to the carbon chain length and the degree of unsaturation of the fatty acid. Although many types of organic acid allelochemicals exist, the mechanisms of action of many organic acids remain unknown. Heptanoic acid and benzoic acid, which were selected for this research, are two organic acid allelochemicals with the same carbon length but with different molecular structures. Their impact on algal blooms is seldom reported. Our study used the common algae of algal blooms, *Microcystis aeruginosa*, for algal inhibition experiments, utilized various physiological indexes of algal liquid to measure algal inhibition, compared the differences in allelopathy of the two organic acids, examined the algal inhibition ability of the combination of both organic acids, supplemented the existing research on the allelopathic algal inhibition mechanism of organic acids and provided a theoretical reference for the application of organic acids for algal inhibition.

2. Experimental materials

2.1 Selection of experimental materials
Heptanoic acid and benzoic acid are organic acids that have been isolated and identified from plants with allelopathic properties. They are fatty acids and aromatic acids with the same carbon length but with different molecular structures and chemical properties. Heptanoic acid (98%) was purchased from Aladdin Chemistry Co., Ltd. Benzoic acid was purchased from Sinopharm Chemical Reagent Co., Ltd. as a type of analytical reagent. *Microcystis aeruginosa* was obtained from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (serial number FACHB-905).

2.2 Preparation of experimental materials
(1) *Microcystis aeruginosa* in the logarithmic phase of growth
The algae filament of *Microcystis aeruginosa* was inoculated in sterilized BG11 culture medium, and amplification culture was performed in a light incubator. The light intensity of the incubator was set to 3000 lx, and the temperature was approximately 25±1.0°C. *Microcystis aeruginosa* was used for the experiment after reaching the logarithmic phase of growth.

(2) Preparation of the stock solutions of the two organic acids
A sterilized BG11 culture solution was used for the ultrasonic dissolution of heptanoic acid and benzoic acid. A 1.0 g·L⁻¹ stock solution was prepared for storage at a temperature of 4°C after filtration by a 0.22 μm microfiltration membrane.

(3) Preparation of the mixed organic acids
Heptanoic acid and benzoic acid were mixed at a ratio of 1:1. A sterilized BG11 culture solution was used for the ultrasonic dissolution. A 1.0 g·L⁻¹ stock solution was prepared for storage at 4°C.

3. Experimental contents and methods

3.1 Experimental contents
Moderate amounts of *Microcystis aeruginosa* in the logarithmic phase of growth were used to
inoculate sterilized 250 mL triangular flasks. Stock solutions of heptanoic acid and benzoic acid and the mixed organic acids were added. Then, 150 mL of sterilized BG11 culture medium was added to each flask to achieve final total concentrations of the two organic acids and the mixed organic acids of 5 mg·L⁻¹, 10 mg·L⁻¹, 20 mg·L⁻¹, 50 mg·L⁻¹ and 100 mg·L⁻¹. The initial OD₆₈₀ value of the algal culture was 0.06±0.005. The control group did not contain any allelochemicals. Each group contained 3 parallel samples that were placed in a light concussion incubator. The temperature was set at 28±1.0°C, and the light intensity was set at 3500 lx. The OD₆₈₀ of the algal liquid was measured every 24 h. The Total Superoxide Dismutase (T-SOD) activity, Catalase(CAT) activity and malondialdehyde (MDA) content were measured every 48 h.

3.2 Measurement methods

(1) Measurement of the number of algal cells
In the experiment, the algal density was represented by the OD₆₈₀, which was measured by an ultraviolet-visible spectrophotometer.

(2) Measurement of the T-SOD activity
The T-SOD activity in the algal cells was calculated using the following formula:
T-SOD activity (U.cell⁻¹) = (control OD₅₅₀-measurement OD₅₅₀)/control OD₅₅₀ /50% x the total volume of reaction liquid (mL)/sampling amount (mL) /algal density (cell.mL⁻¹)

(3) Measurement of the CAT activity
The CAT activity of the algal cells was calculated by measuring the OD₄₀₅ value.
CAT activity (U.cell⁻¹) = (control OD₄₀₅-measurement OD₄₀₅) x 271 x 1/60 x sampling amount / algal density (cell.mL⁻¹)

(4) Measurement of the MDA content
MDA content (nmol/algae) = measurement OD₅₃₂ - control OD₅₃₂/standard OD₅₃₂ - blank OD₅₃₂ x 10 nmol/mL/algae/mL

(6) Calculation of the half maximal inhibitory concentration
The half maximal inhibitory concentration (EC₅₀) of the allelochemicals was calculated by the Bliss method using R procedural programming [13].

(7) Evaluation of the combined effect of the mixed acids
Combined index methods were adopted to evaluate the combined algal inhibition effect of benzoic acid and heptanoic acid, which mainly included the toxic unit method [14], additive index method [15] and similarity index method [16].

4. Experimental results and analysis

4.1 Effects of the two organic acids on the algal density of Microcystis aeruginosa
As shown in Figure 1 and Figure 2, benzoic acid and heptanoic acid both inhibited the growth of Microcystis aeruginosa, but their inhibitory abilities were different. The half maximal inhibitory concentration of heptanoic acid (83.90 mg/L) was greater than that of benzoic acid (55.02 mg/L). Therefore, heptanoic acid exhibits a weak algal inhibition ability. When the concentration of heptanoic acid was less than 20 mg/L, the growth of Microcystis aeruginosa was slightly accelerated. The algal inhibition rate of 100 mg/L of heptanoic acid was 48.63% on the 7th day. The algal inhibition effect of the heptanoic acid was consistent with the “hormesis effect” of some allelochemicals described in existing research. The algal inhibition rate of 100 mg/L of benzoic acid reached 99.92% on the 7th day after the experiment. However, the algal inhibition rate of low concentrations of benzoic acid was very low. When the concentration of benzoic acid was less than 20 mg/L, the algal inhibition rate of was less than 14% on the 7th day.
4.2 Effects of the two organic acids on the antioxidant enzyme activity of Microcystis aeruginosa

As important constitutive enzymes in an enzymatic defense system, SOD and CAT supplement each other and maintain the dynamic steady state of the Reactive oxygen species (ROS) level in algal cells. When *Microcystis aeruginosa* is under severe stress, the ROS level can increase and damage the algal cells. When ROS cannot be cleared in a timely manner, the activities of SOD and CAT are inhibited.

(1) Effects of the two organic acids on the T-SOD activity of *Microcystis aeruginosa*

As displayed in Figure 3, the T-SOD activities of the control group and the treatment groups with 5 mg/L and 10 mg/L of the two organic acids were stable during the experiment, and heptanoic acid had little effect on the T-SOD activity of *Microcystis aeruginosa*, which might be responsible for its weak allelopathic inhibition of *Microcystis aeruginosa*. The T-SOD activities of the treatment groups with medium and high concentrations of benzoic acid increased to varying degrees on the 1st day of the experiment. Except for the group treated with 100 mg/L of benzoic acid, the T-SOD activities of the other groups treated with benzoic acid continued to increase on the 3rd day of the experiment. Meanwhile, the T-SOD activity of the group treated with 100 mg/L of benzoic acid began to sharply decrease on the 3rd day, which probably occurred because the high concentration of benzoic acid caused the ROS level in *Microcystis aeruginosa* to exceed a certain threshold. The SOD activity reached a specific limit, and the ROS balance could not be maintained, which damaged the algal cell membranes and biological macromolecules and obviously reduced the SOD activity.
Figure 3. Effects of the two organic acids on the T-SOD activity of *Microcystis aeruginosa*.

(2) Effects of the two organic acids on the CAT activity of *Microcystis aeruginosa*

As shown in Figure 4, the CAT activity of *Microcystis aeruginosa* showed different responses to various concentrations of the two organic acids. As displayed in Figure 4, heptanoic acid had a relatively small effect on the CAT activity of *Microcystis aeruginosa*. The CAT activity of the group treated with 100 mg/L of heptanoic acid was obviously decreased on the 7th day of the experiment; however, the CAT activities of the other treatment groups exhibited small fluctuations during the experiment. The CAT activities of the groups treated with benzoic acid increased on the 1st day and started to decrease on the 3rd day of the experiment. In addition, the rate of decrease was directly proportional to the amount of organic acids added. The results showed that the CAT activity of *Microcystis aeruginosa* might be stimulated by benzoic acid. However, the CAT system of *Microcystis aeruginosa* was severely damaged and exhibited reduced activity with continued stress, which increased the difficulty of maintaining the hydrogen peroxide balance in algal cells. The accumulation of hydrogen peroxide continued to damage structures such as the cell membranes and various enzyme systems, which thus formed a vicious cycle and affected the metabolism of the algal cells. CAT and SOD are important components of the antioxidant enzyme system in algal cells. According to the experimental results, the two enzymes had similar responses to benzoic acid, possibly because the two enzymes are isozymes. The ROS level was maintained in the body of the algal cells, possibly because the two enzymes exhibit similar stress responses to adverse factors in the environment [17].

Figure 4. Effects of the two organic acids on the CAT activity of *Microcystis aeruginosa*.

4.3 Effects of the two organic acids on the MDA content of *Microcystis aeruginosa*

MDA is produced from the peroxidative reaction of *Microcystis aeruginosa* in response to environmental stress. Under normal circumstances, changes in the MDA content of the algal cells reflect the degree of damage of algal cells or membrane structures [18]. As shown in Figure 5, the two organic acids affected the MDA content of *Microcystis aeruginosa* to varying degrees. The MDA content of the groups treated with a high concentration of the two acids quickly increased, reaching a
maximum value on the 1st day and sharply decreasing on the 3rd day of the experiment, which indicated that the algal cells were seriously damaged. At this time, the algal density of Microcystis aeruginosa exhibited a state of slow growth. The cell membranes were damaged. However, many of the Microcystis aeruginosa cells did not immediately die; rather, the widespread death of algal cells occurred slowly during prolonged stress, which also indicated that damage to the cell membrane structures and the accumulation of MDA resulted in the death of Microcystis aeruginosa.

Figure 5. Effects of two organic acids on the MDA content of Microcystis aeruginosa.

4.4 Evaluation of the algal inhibition effect of the combined organic acids

Figure 6 shows the combined effect of heptanoic acid and benzoic acid, mixed in a ratio of 1:1, on the growth of Microcystis aeruginosa. Compared to the control group, the various treatment groups showed slow growth or death of algal cells. The algal density of the various treatment groups was lower than that of the control group on the 7th day of the experiment. Various treatment groups showed an attenuated increase in algal density compared to that in the control group starting on the 2nd day. Over time, the gap between the algal densities of the various treatment groups and the control group became increasingly wider, which indicates that the combination of the two organic acids had a strong inhibitory effect on Microcystis aeruginosa. The algal inhibition rate gradually increased with increasing concentrations of the organic acids. When the combined algal inhibition abilities of the two organic acids were compared to the separate effects of each acid, treatment with both heptanoic acid and benzoic acid showed a stronger algal inhibition ability than treatment with heptanoic acid and benzoic acid separately, which indicates that the combination of the two acids might have a synergistic effect on algal inhibition. As shown in Table 3-1, the half maximal inhibitory concentrations were calculated on the 7th day of the experiment: the concentration of benzoic acid was 55.02 mg/L; the concentration of heptanoic acid was 83.90 mg/L; the concentration of the benzoic acid and heptanoic acid mixture in equal proportions was 48.11 mg/L. The data further showed that the compound algal inhibition effect of benzoic acid and heptanoic acid was superior to the separate effects of the two organic acids.
Figure 6. Effect of the two organic acids, mixed in a ratio of 1:1, on the algal density of *Microcystis aeruginosa*.

The toxic unit method, additive index method and mixture toxic index method were adopted to evaluate the combined effect of the mixture of the two organic acids. The results are shown in Table 3-2. Benzoic acid and heptanoic acid showed a synergistic effect. The combined effect of the two organic acids indicates that the mixture of different organic acids might produce algal inhibition characteristics different from those in the case of the single organic acids, which could provide a basis for the development of a mixed algistat in the future.

Table 3-1 Half maximal inhibitory concentrations of the separate and combined organic acids

| Allelochemicals               | Half maximal inhibitory concentration/mg·L⁻¹ |
|------------------------------|---------------------------------------------|
| Benzoic acid                 | 55.02                                       |
| Heptanoic acid               | 83.90                                       |
| Benzoic acid + heptanoic acid| 48.11                                       |

Table 3-2 Evaluation parameters of the combined effect of the two organic acids

| Allelochemical | Method | Parameter | Evaluation |
|----------------|--------|-----------|------------|
| Benzoic acid   | TU     | 0.44 0.29 | 0.73 1.66  | - - | Synergy |
|                | AI     | 0.44 0.29 | 0.73 1.66  | 0.37 - | Synergy |
| Heptanoic acid | Mₜ₁    | 0.44 0.29 | 0.73 1.66  | - 1.62 | Synergy |

5. Conclusions
(1) Heptanoic acid and benzoic acid had an inhibitory effect on *Microcystis aeruginosa*. Benzoic acid exhibited a stronger algal inhibition ability than heptanoic acid. The median lethal concentration of benzoic acid was 55.02 mg/L, while the median lethal concentration of heptanoic acid was 83.90 mg/L.

(2) The two organic acids decreased the SOD and CAT activities of *Microcystis aeruginosa* and increased the MDA content. Benzoic acid had a greater effect on the antioxidant enzyme system of *Microcystis aeruginosa* than heptanoic acid.

(3) The two organic acids were mixed at a 1:1 ratio to evaluate their combined algal inhibition effect. Benzoic acid and heptanoic acid had a synergistic effect, and their compound algal inhibition effect was superior to the separate effects of the two organic acids.

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