# Characterization of *Microcystis* Strains by Alkyl Sulfides and β-Cyclocitral

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Cyanobacteria of the genus *Microcystis* have been found to liberate large amounts of β-cyclocitral. While all seven strains of *Microcystis* studied possessed this property, it has not been found in any other cyanobacterial species. This chemical characteristic of the genus *Microcystis* enables the unequivocal systematic arrangement of controversial species. Another chemical property allows two chemotypes to be distinguished within the genus *Microcystis*: one exhibits an intensive release of isopropylmethyl disulfide, diisopropyl disulfide and diisopropyl trisulfide, substances which have not been previously found in any microorganism. The second chemotype excretes dimethyl disulfide and in some cases also dimethyl trisulfide and dimethyl tetrasulfide. Species producing isopropyl sulfides emanate an intensive sulphurous malodour.

## Introduction

Some cyanobacteria and unicellular freshwater algae are known to produce volatile sulphur compounds. Occurrence of dimethyl sulfide is a characteristic feature in cultures of the freshwater diatoms *Asterionella*, *Nitzschia* and *Diatoma* and is most likely derived from dimethyl β-propiothetin [1]. *Ochromonas danica* [2] and *Poterioochromonas malhamensis* [3] which both belong to the Chrysophyceae and which have been intensively studied excrete a number of different sulphur compounds. Besides dimethyl disulfide, dimethyl trisulfide and dimethyl tetrasulfide several methyl thioesters are liberated in high amounts under microaerobic conditions by these organisms. Studies on freshwater green algae have not yet led to the detection of any volatile organic sulphur compound [4]. However, the excretion of $H_2S$ has been observed in Chlorophyceae [5]. The major sulphur compounds of Euglenophyceae (*Astasia*, *Euglena*) have been reported to be mercaptans [5]. The identity of those, however, has not been determined. Dimethyl disulfide, dimethyl sulfide, methyl thiol and $H_2S$ have been reported to occur in unicellular and filamentous cyanobacteria [4]. While studying naturally occurring blooms of *Anabaena* and laboratory cultures of *Microcystis aeruginosa*, Jenkins and coworkers [6] identified isopropyl thiol and dimethyl disulfide in the latter species. Since *Microcystis* contributes to most blooms observed in european impondments, a more complete study of several *Microcystis* strains was undertaken to get more description of the volatile sulphur components excreted by this ecologically most important genus.

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## Materials and Methods

### *Origin of Microcystis strains*

Axenic *Microcystis* PCC 7806 was supplied by R. Rippka, Institut Pasteur. After every transfer this strain was controlled for sterility by microscopy and plating on nutrient agar. The other strains used were non-axenic. *M. aeruginosa* B 46.80, originally isolated by M. Rieper, was obtained from the Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität Göttingen, and *M. aeruginosa* strain Hindák from L. Kováčik, Československá Akademie věd Botanický Ústav, Hydrobotanické Oddělení, Třeboň, Czechoslovakia. *M. aeruginosa* isolated by W. E. Scott from the Hartbeespoort Dam, South Africa, and *Microcystis* PCC 7005 were kind gifts of G. H. J. Krüger, Botany Department, Bloemfontein, South Africa. *M. aeruginosa*, originally isolated by W. E. Scott from the Rietvlei Dam, South Africa, was kindly supplied by M. J. Amann, Institut für Chemische Pflanzenphysiologie der Universität Tübingen. A sample from a cyanobacterial bloom occurring in the lake Federsee/SW-Germany in 1983 contained *M. wesenbergii* and *M. viridis*.

*Small and mass culture of Microcystis*

The different *Microcystis* strains were grown at 25 °C in 300 ml Erlenmeyer flasks in a medium suitable for planktonic cyanobacteria [7]. Illumination of approximately 1000 lx ($5 \times 10^{-4}$ W/cm$^2$) was supplied with fluorescent tube-lights. A scale-up of the culture was performed with *Microcystis* PCC 7806 using a 30 liter tower type plant [8]. The tower was gassed with a mixture of 0.2% (v/v) $CO_2$ in air (1 l/min) and illuminated from three directions with three fluorescent tube-lights each of 1000 lx.

*Analysis of sulphur compounds and β-cyclocitral*

50–100 ml portions of cultures of different *Microcystis* strains were stripped of volatile compounds after addition of 20% NaCl (w/v) as described previously [9]. The stripped compounds were adsorbed on 150 mg Tenax TA filled odour traps, subsequently thermally desorbed and then transferred with a hydrogen gas stream onto a cooled (0 °C) 50 m glass capillary column coated with UCON 50 HB 5100 of a gas chromatograph (model Carlo Erba 4200). The separation of the components was performed using hydrogen as the carrier gas and a temperature program (0–180 °C, 5 °C/min). To enable simultaneous detection of the separated components with both a flame ionization detector (FID) and a sulphur selective detector (SSD), the exit of the capillary column was split with a home-made glass splitter. The quartz capillary branches of the splitter supplied the FID and SSD with equal amounts of the gas stream. The signals of each detector were recorded on two separate recorders: each attenuated differently. The same column and procedure of application as stated above were used to obtain mass spectra on a gas chromatograph – mass spectrometer combination.

*Origin and synthesis of reference substances*

Diisopropyl disulfide was purchased from Aldrich, the other chemicals from Fluka (Neu-Ulm, W-Germany). Isopropylmethyl disulfide was prepared by co-photolysis of dimethyl disulfide and diisopropyl disulfide for 80 h in quartz cuvettes according to Gupta *et al.* [10]. A procedure of Banerji and Kalena [11] was applied for the synthesis of isopropyl trisulfide. Diimidazolyl sulfide was obtained by the reaction of N-trimethylsilyl imidazole with sulphur dichloride. This substance was then stirred in hexane solution with isopropyl thiol to give isopropyl trisulfide.

## Results

*Microcystis* PCC 7806 liberates volatile sulphur compounds in such quantities that a few ml of culture suspension of this cyanobacterium are sufficient to produce a strong malodour which can easily be recognized*. This strain grew readily in mass culture using growth conditions suitable for *M. aeruginosa* [8]. Mass culture of this strain in a thirty liter tower type plant with a suspension width of 3.5 cm provided a continuous supply of the sulphur compounds for analysis. The volatile substances stripped from the culture vessel with the aeration gas stream were adsorbed on a Tenax TA filled odour trap which was mounted at the gas vent of the plant. Sampling times of 15–30 min were sufficient to obtain intense peaks from the sulphur selective detector after gas chromatographic separation on a glass capillary column. Similar peak patterns were obtained after stripping 100 ml of axenic standing cultures. A chromatogram of the volatile substances from such a culture simultaneously detected with a FID and a SSD is presented in Fig. 1. Three prominent sulphur peaks were obtained. The mass spectra of these components (Figs. 2 and 3) exhibited intense molecular ions, which are typical for most sulphur compounds, and characteristic fragment ions, such as of $m/z$ 45 ($HCS^+$), 66 ($H_2S_2^+$) and 80, 108, 140 (M-42) indicating a hydrogen transfer accompanying elimination of an alkene fragment. The structural formulas isopropylmethyl disulfide (4-methyl-2,3-dithiapentane), diisopropyl disulfide (2,5-dimethyl-3,4-dithiahexane) and diisopropyl trisulfide (2,6-dimethyl-3,4,5-trithiaheptane) fitted the fragmentation patterns and retention times of these three unknown sulphur substances. Confirmation of identity was achieved by synthesis of reference substances with these structures. The other *Microcystis* strains were analyzed in a similar way using 100 ml of cell suspension of standing cultures. The sulphur compounds identified are listed up in Table I, together with the occurrence of

* I thank R. Rippka, Institut Pasteur, Paris, whose observation of the malodour of this culture gave me the impulse for making this study.

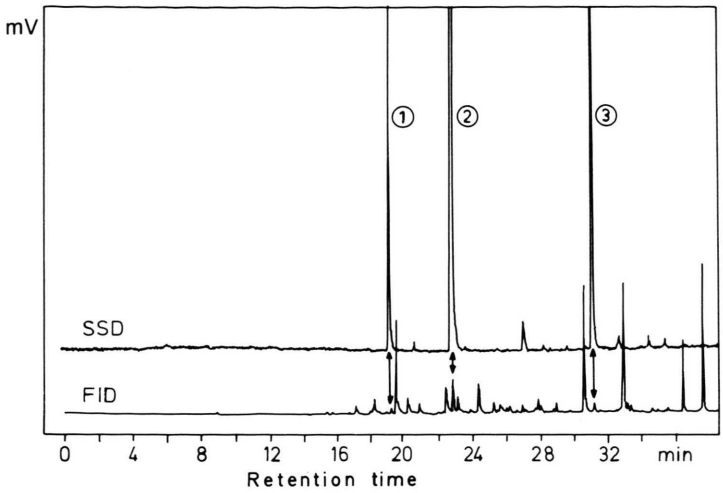


Fig. 1. Gas liquid chromatogram (WCOT glass capillary column UCON 50 HB 5100, 0–180 C, 5 °C/min, $H_2$ carrier gas) of the volatile sulphur compounds of an axenic culture of *Microcystis* PCC 7806. The components were simultaneously detected with a FID (lower trace) and a SSD (upper trace). 1. Isopropylmethyl disulfide, 2. diisopropyl disulfide, 3. diisopropyl trisulfide.

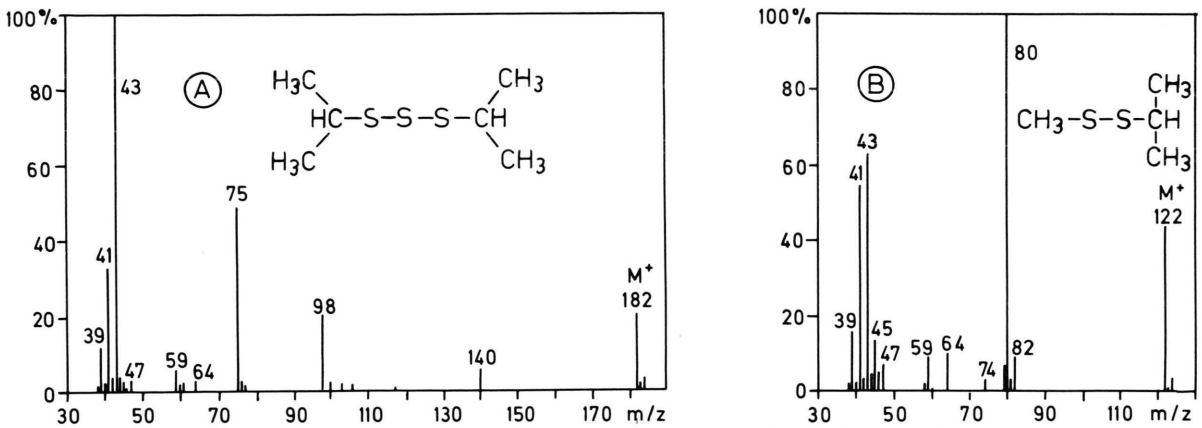


Fig. 2. Electron impact mass spectra (80 eV) of diisopropyl trisulfide (A) and methylisopropyl disulfide (B). Both compounds were isolated from an axenic culture of *Microcystis* PCC 7806.

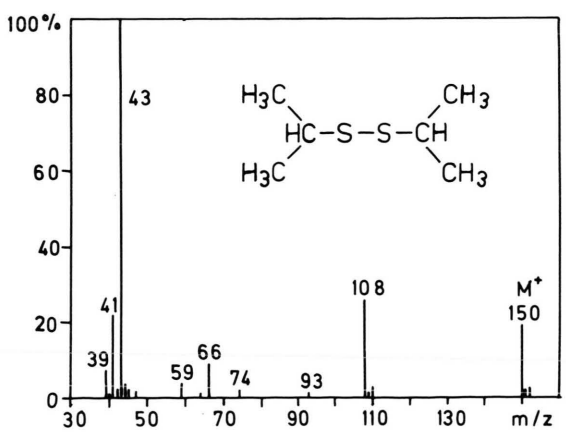


Fig. 3. Electron impact mass spectrum (80 eV) of diisopropyl disulfide, the major volatile sulphur component of *Microcystis* PCC 7806.

Table I. Occurrence of dimethyl disulfide ($MS_2M$), dimethyl trisulfide ($MS_3M$), dimethyl tetrasulfide ($MS_4M$), isopropylmethyl disulfide ($IS_2M$), diisopropyl disulfide ($IS_2I$), diisopropyl trisulfide ($IS_3I$) and β-cyclocitral (β-Cyc) in different strains of *Microcystis* (+++ major compound, ++ minor compound, + trace amounts, − below the detection limit). The sulphur components were detected with the SSD, β-cyclocitral with the FID signal.

| Strain | $MS_2M$ | $MS_3M$ | $MS_4M$ | $IS_2M$ | $IS_2I$ | $IS_3I$ | β-Cyc |
|---|---|---|---|---|---|---|---|
| *Microcystis* PCC 7806 | − | − | − | ++ | +++ | ++ | +++ |
| *M. aeruginosa* (Rietvlei) | ++ | − | − | +++ | ++ | − | +++ |
| *M. aeruginosa* SAUG 46.80 | + | − | − | − | − | − | +++ |
| *Microcystis* PCC 7005 | +++ | + | + | − | − | − | +++ |
| *M. aeruginosa* (Hartbeespoort) | +++ | − | − | − | − | − | ++ |
| *M. aeruginosa* (Hindák) | +++ | + | − | − | − | − | +++ |
| *M. wesenbergii*/*M. viridis* (Phytoplankton sample) | ++ | − | − | − | − | − | +++ |

β-cyclocitral which was determined after addition of 20% NaCl to the culture suspension. While marked differences in the occurrence of alkyl sulfides were observed in the strains studied, β-cyclocitral was a constant feature of all strains tested.

## Discussion

*Microcystis* is a unicellular colony forming cyanobacterium. The arrangement of the cells in the colony, the shape and delimitation of the mucilage together with cell number and size, are the morphological features used to differentiate species within the genus *Microcystis* [12]. Under laboratory conditions promoting intense growth, isolated colonies soon disintegrate, and single cell cultures are obtained. In addition, the ability to produce gas vacuoles, a property used to differentiate between the genus *Synechocystis* and *Microcystis* [13], is often lost during plating [14]. A consequence is that such gas vacuole free laboratory cultures can no longer be distinguished from the genus *Synechocystis* by morphological methods. Since the determination of even the genus of cultures is a problem, it should be clear that any attempt to extend the level of determination to that of the species will be still more difficult.

Study of the volatile compounds clearly showed that all *Microcystis* strains investigated were able to liberate large amounts of β-cyclocitral. This compound was first isolated from a bloom of *M. wesenbergii* [15]. No such ability has been observed in strains of *Synechocystis*. Though only a limited number of *Microcystis* strains isolated from several locations in Europe and South Africa has been investigated, it seems very likely that the release of substantial amounts of β-cyclocitral is restricted to the genus *Microcystis*. Thus, the identification of β-cyclocitral provides an easy means of deciding whether to place systematically controversal organisms in this genus. This chemical characterization is especially suitable for laboratory cultures which have lost their characteristic features during prolonged cultivation.

β-Cyclocitral contributes to the odour emanating from *Microcystis* mass developments only during the collapse of a bloom. Sulphur compounds are, however, excreted steadily by growing organisms. The amounts are large enough to severely affect the environment. The components responsible for the distinctive sulphurous odour are isopropylmethyl disulfide, diisopropyl disulfide and diisopropyl trisulfide. These substances have not been found before in any microorganism. The corresponding *n*-propyl sulfides have been described before for *Bacillus*, *Micrococcus*, *Mycobacterium*, *Pseudomonas* and *Klebsiella* species grown on nutrient supplemented with propylcysteine [16] or on onions [17]. Unawareness of the existence of these organic sulphur substances in *Microcystis* has often led to the assumption that hydrogen sulfide is the cause of the sulphurous odour liberated during blooms. While studying *Microcystis aeruginosa*, Jenkins and coworkers [6] identified isopropyl thiol and held this substance responsible for the typical sulphurous odour. In the investigation presented here, no indication was found for the existence of this compound. However, different growth conditions may

have been responsible for the occurrence of the reduced form of this compound.

When different strains were compared, isopropyl sulfides proved to be restricted to two strains. The other strains produced dimethyl disulfide as the most prominent compound accompanied in some cases by dimethyl trisulfide and dimethyl tetrasulfide. In the latter strains not even small amounts of isopropyl sulfides could be detected. Thus, two groups of *Microcystis aeruginosa* which are morphologically identical can be distinguished. Members of the one group excrete isopropyl sulfides (easily be recognized by sniffing) and members of the other liberate much smaller amounts solely of methyl sulfides and do not have a typical "sulphurous" odour. The occurrence of dimethyl disulfide, together with isopropyl sulfides, in *M. aeruginosa* strain Rietvlei can be explained in two ways: either this culture is not monoclonal and represents a mixture of two different strains or it is an additional chemotype with the features of both groups. *M. wesenbergii* which is distinguishable morphologically from the other strains by its greater cell diameter did not show a pattern of the sulphur compound production which would be suitable to separate this species from the others by chemical criteria.

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