Polyphenols and fatty acids responsible for anti-cyanobacterial allelopathic effects of submerged macrophyte Myriophyllum spicatum
S. Nakai, G. Zou, T. Okuda, W. Nishijima, M. Hosomi and M. Okada

ABSTRACT
Myriophyllum spicatum is known to inhibit the growth of cyanobacteria such as Microcystis aeruginosa by releasing anti-cyanobacterial allelochemicals. The allelochemicals possibly responsible for the inhibition include five polyphenols and three fatty acids, but the extent to which these are indeed responsible for the anti-cyanobacterial effects is unclear. The goal of this research was to determine the contribution of these compounds to the allelopathic effect of M. spicatum on M. aeruginosa. We first collected information on the release rates of these compounds and then added the compounds to a cyanobacterial medium on the basis of their release rates so as to simulate their excretion by M. spicatum. Addition of the polyphenols and fatty acids inhibited the growth of M. aeruginosa, and the interaction of the polyphenols and fatty acids was additive. The EC50 of a polyphenol and fatty acid mixture was compared with that of M. spicatum itself as previously determined in a mixed culture system in which M. spicatum and M. aeruginosa were incubated. The former was about 1.9 times higher than that of the latter, the implication being that the inhibitory effect of the polyphenols and fatty acids contributed about 53% of the allelopathic effect of M. spicatum. This paper is the first to describe allelochemicals that account for a half of the anti-cyanobacterial allelopathic effect of a macrophyte.

Key words | allelopathy, cyanobacterial growth inhibition, fatty acid, Myriophyllum spicatum, polyphenol

INTRODUCTION
Severe outbreaks of cyanobacteria in eutrophic ponds and lakes result in serious problems with regard to the suitability of these bodies of water as, inter alia, water-supply reservoirs, habitats for the production of commercially important fish, or both. Certain macrophytes produce and release metabolites as allelochemicals to inhibit cyanobacterial growth (e.g. Gross 1999; Hong et al. 2011). The perception is therefore that the allelopathic effect of macrophytes contributes to the maintenance of clear-state conditions in shallow aquatic systems (Scheffer 1998; Hilt & Gross 2008). The results of many cyanobacterial assays with water samples collected from macrophyte vegetation areas (Hilt et al. 2006; Mulderij et al. 2006; Takeda et al. 2011) and macrophyte culture solutions (Jasser 1995; Gross et al. 1996; 2003; Nakai et al. 1999, 2008; Mulderij et al. 2003, 2005) are consistent with the existence of anti-cyanobacterial macrophytic metabolites. These results suggest the potential appeal of such macrophytic vegetation for control of cyanobacterial blooms.

Identification of the causal compounds should precede use of macrophytes to prevent cyanobacterial bloom. Previous studies have implicated a submerged macrophyte, Myriophyllum spicatum, as the cause of the allelopathic inhibition of the growth of cyanobacteria (Gross et al. 1996; Nakai et al. 1999). It is well known today that the genus Myriophyllum produces anti-cyanobacterial compounds. Saito et al. (1989) confirmed the existence of polyphenols such as eugeniin (EUG) and its derivatives gallic and ellagic acids (GA and EA) in an extract of M. brasiliense. M. verticillatum produces hexahydroxydiphenoyl- and galloyl-glucose esters, quercetin-glycoside (Bauer et al. 2009), and three phenylpropanoid glucosides that

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each includes a galloyl group (Aliotta et al. 1992). In addition to EUG and its derivatives (Gross 2003), extracts of *M. spicatum* contain other polyphenols such as pyrogallic and protocatechuic acids (Planas et al. 1981). Further studies have confirmed the release of five polyphenols, (+) catechin (CAT, Nakai et al. 2000), EUG (Gross et al. 1996), EA and GA acids (Gross et al. 1996; Nakai et al. 2000), and pyrogallic acid (PA, Nakai et al. 2000) from *M. spicatum*. Other studies (see Nakai & Hosomi 2005) have revealed that *M. spicatum* also releases two or three of the anti-cyanobacterial fatty acids: nonanoic (NA), cis-6-octadecenoic (6OA) and cis-9-octadecenoic acid (9OA), although because of analytical limitations it is unclear whether it releases both 6OA and 9OA.

The identification of anti-cyanobacterial metabolites is the first step to reveal the compounds that cause the anti-cyanobacterial allelopathy of macrophytes. Further research should address whether the identified compounds account for observed allelopathic effects. Furthermore, from a practical standpoint there is a need to determine whether macrophytes living at natural light intensities release allelochemicals in sufficient amounts to inhibit cyanobacterial growth. With respect to the anti-cyanobacterial metabolites released from *M. spicatum*, EUG is likely to play a major role in the allelopathic effect of *M. spicatum* (Gross 1999). However, a recent study has shown that the five polyphenols (CAT, EUG, EA, GA, and PA) contribute less than 70% of the allelopathic effect of *M. spicatum* on the cyanobacterium *Microcystis aeruginosa* (Matsushima et al. 2008). This discovery led us to hypothesize that the aforementioned fatty acids also contribute to the allelopathy of *M. spicatum*.

If these polyphenols and fatty acids are the allelochemicals responsible for the allelopathy of *M. spicatum*, the hypothesis that allelopathic inhibition of cyanobacterial growth occurs in the aquatic environment can be tested by determining whether these compounds inhibit cyanobacterial growth at concentrations resembling natural exudation from stands of *M. spicatum*. If the hypothesis is true, the use of these compounds could facilitate the investigation of impacts from the release of *M. spicatum* allelochemicals on aquatic ecosystems, because use of *M. spicatum* plants for this purpose would likely cause other effects that result, for example, from competition for nutrients (Fitzgerald 1969). To facilitate the determination of the extent to which the identified polyphenols and fatty acids are responsible for the allelopathy of *M. spicatum*, our initial goal was to reproduce in experimental systems the pattern of release of the allelochemicals by *M. spicatum*. As the first step in this study we therefore generated a dataset of release rates of the polyphenols and fatty acids. We then investigated the inhibition of cyanobacterial growth caused by the concurrent action of the polyphenols and fatty acids released at rates that simulated their excretion by *M. spicatum*. Finally, we determined the contribution of these compounds to the allelopathic effect of *M. spicatum* on *M. aeruginosa*.

**MATERIALS AND METHODS**

*M. spicatum* and *M. aeruginosa*

*M. spicatum* plants, including apical shoots with a length of about 15 cm, were collected from the Toyoda-yousui irrigation channel, Tokyo, Japan, and incubated in five-times diluted Gorham’s medium (Zehnder & Gorham 1960) at 25 °C under a continuous light intensity of 60 μmol m⁻² s⁻¹ (Matsushima et al. 2008). *M. aeruginosa* (NIES-87) was obtained from the microbial collection of the National Institute for Environmental Studies (NIES), Japan, and it was cultured in 40 mL of five-times diluted Gorham medium in a 200 mL Erlenmeyer flask on a shaker table at a rotation rate of 70 rpm under the same conditions used for *M. spicatum* incubation.

**Release rates of allelochemicals**

A previous study has already reported measurements of the release rates of the five polyphenols (CAT, EA, EUG, GA, and PA) (Matsushima et al. 2008) in cultures of *M. spicatum* grown in five-times diluted Gorham’s medium. The manner followed coexistence assays in which *M. spicatum* and *M. aeruginosa* were cultivated in this medium to confirm the inhibitory effect of the plant body on cyanobacterial growth (Nakai et al. 1996). In this study we therefore determined the release rates of fatty acids in the same manner as in the previous studies (Nakai et al. 1996; Matsushima et al. 2008). After washing *M. spicatum* with tap and MilliQ water, the plant body was added at 720 g dry wt m⁻³ to 200 mL of five-times diluted Gorham’s medium in 500 mL Erlenmeyer flasks for measurement of fatty acid concentrations in duplicate. The cultivation was repeated three times at least. This macrophyte concentration is equivalent to 10 g wet wt L⁻¹ based on the dry/wet weight ratio (0.072) that we obtained in a preliminary experiment. A concentration of 10 g wet wt L⁻¹ was sufficient for *M. spicatum* to inhibit the growth of *M. aeruginosa* completely in the coexistence system (Nakai et al. 1996). The fatty acids in the culture solution
were extracted using a solid extraction cartridge (OASIS HLB, Waters, USA), derivatized using diazomethane, and finally analyzed with a gas chromatograph equipped with a mass selective detector (GC/MS, Hewlett Packard 6890/5973 series, Germany) as reported by Nakai & Hosomi (2005).

**Contribution of the polyphenols and fatty acids to the allelopathic effect**

In order to determine the contribution of the polyphenols and fatty acids to the allelopathic effect of *M. spicatum* on *M. aeruginosa*, their inhibitory effect on the growth of *M. aeruginosa* was compared with that caused by *M. spicatum*. The inhibitory effect of *M. spicatum* itself on the growth of *M. aeruginosa* has already been investigated by coexistence assays (Nakai et al. 1996). The inhibitory effect of the allelochemicals on the growth of *M. aeruginosa* was investigated by simulating their release from *M. spicatum* in a mixed culture system.

*M. aeruginosa* was inoculated into 40 mL of five-times diluted Gorham’s medium in a 200 mL Erlenmeyer flask to which we added every 24 h a methanol solution containing an amount of these allelochemicals (CAT>95.0%, NA>98%, 6OA>95%, 9OA>99%, Tokyo Kasei, Japan; EA min. 99.0%, GA min. 98%, PA min. 99.0% Wako Pure Chemical Industries, Japan; EUG, >95%, Nagara Science, Japan) daily released from 144, 288, and 720 g dry wt mg⁻³ of *M. spicatum* to mimic the excretory behavior of *M. spicatum* (Table 1). Note that a big variance was observed for the values of EA and NA. As the causes were not clarified and in question, we decided to use the average of the dataset in the control experiments, the same amount of methanol was added. The assays were carried out in triplicate, and the assay for mixtures of the polyphenols and fatty acids was repeated to check reproducibility of the result. During the cultivation period, we monitored the growth of *M. aeruginosa* by microscopic observation using a Thoma hemocytometer (Hishigaki, Japan).

Note that the maximum values for 6OA and 9OA in Table 1 were determined on the assumption that *M. spicatum* released either 6OA or 9OA, because the GC/MS analysis could not separate 6OA and 9OA. The different values for 6OA and 9OA were due to their different response factors in the GC/MS analysis. Unless otherwise stated, we assumed the release rates of 6OA and 9OA to be 3 nmol g dry wt⁻¹ h⁻¹, because 6 nmol g dry wt⁻¹ h⁻¹, is approximately the average of the maximum values for the 6OA and 9OA release rates in Table 1. In the cyanobacterial assays to investigate whether the relative amounts of 6OA and 9OA in the mixture affected the inhibition of *M. aeruginosa* growth, the maximum release rates of these two fatty acids (7.1 nmol g dry wt⁻¹ h⁻¹ for 6OA and 5.3 nmol g dry wt⁻¹ h⁻¹ for 9OA) were used to prepare the mixture on the assumption that either 6OA or 9OA was released from *M. spicatum*.

**Table 1**

<table>
<thead>
<tr>
<th>Allelochemicals (Molecular weight)</th>
<th>Release rate (nmol g dry wt⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polyphenols</strong></td>
<td></td>
</tr>
<tr>
<td>CAT (290.3)</td>
<td>0.55 (0.28)</td>
</tr>
<tr>
<td>EA (302.2)</td>
<td>11 (16)</td>
</tr>
<tr>
<td>EUG (938.7)</td>
<td>22 (7.7)</td>
</tr>
<tr>
<td>GA (170.1)</td>
<td>2.5 (0.72)</td>
</tr>
<tr>
<td>PA (126.1)</td>
<td>2.4 (1.5)</td>
</tr>
<tr>
<td><strong>Fatty acids</strong></td>
<td></td>
</tr>
<tr>
<td>NA (158.2)</td>
<td>5.3 (6.6)</td>
</tr>
<tr>
<td>6OA (282.5)</td>
<td>&lt;7.1 (1.8)</td>
</tr>
<tr>
<td>9OA (282.5)</td>
<td>&lt;5.3 (1.7)</td>
</tr>
</tbody>
</table>

*Calculated from the reported value (Matsushima et al. 2008) using a g dry wt/g wet wt ratio of 0.072.*

*Based on the assumption that either 6OA or 9OA is released.*

**Statistical analysis**

A one-way ANOVA followed by a Tukey test was carried out for the normalized maximum growth to determine the statistical significance of the inhibitory effect of the mixtures of polyphenols and fatty acids on the assumptions of normal probability distribution and homogeneity of variance. In order to analyze the concurrent actions of the polyphenols and fatty acid groups, the concentrations at which the polyphenols and/or fatty acid groups reduced the growth of *M. aeruginosa* to 50% of the control growth (EC₅₀) and their 95% confidence intervals were calculated by a simple linear regression analysis, and the values were used for the isobologram analysis (Holstein & Hohl 2001; Tallarida 2006).

**RESULTS AND DISCUSSION**

**Release rates of the fatty acids**

The concentrations of the fatty acids increased linearly with time in the *M. spicatum* culture medium (Figure 1). As the observed concentrations of the fatty acids were linear functions of time, their release rates (nmol g dry wt⁻¹ h⁻¹)
were calculated by dividing the slopes of the relationships (nmol m\(^{-3}\) h\(^{-1}\)) by the \textit{M. spicatum} concentration (g dry wt m\(^{-3}\)).

A summary of the release rates (Table 1) reveals that the EUG release rate was the highest among the eight compounds, and the release rate of CAT was the lowest. Because of the high content of EUG in the plant body of \textit{M. spicatum}, Gross (1993) suggested that EUG plays a major role in the allelopathic effect of \textit{M. spicatum}. A series of measurements in this study and a previous one (Matsushima et al. 2008) also showed that EUG is the major compound excreted by this plant among the eight investigated.

**Growth inhibition by the allelochemicals**

When the polyphenols and fatty acids equivalent to 720 g dry wt m\(^{-3}\) of \textit{M. spicatum} were added to the cyanobacterial medium on the basis of their release rates (Table 1), the maximum growth of \textit{M. aeruginosa} was only 8% of the maximum control growth (Figure 2). In contrast, a coexistence assay in a previous study showed that this concentration of live \textit{M. spicatum} could reduce the growth of \textit{M. aeruginosa} to virtually zero (Nakai et al. 1996), although other anti-cyanobacterial mechanisms such as nutrient competition might occur under the coexistence of macrophytes (Fitzgerald 1969). This comparison indicates that the polyphenols and fatty acids are the allelochemicals that contribute much of the allelopathic effect of \textit{M. spicatum} on \textit{M. aeruginosa}.

In order to quantify their contribution, further experiments were carried out to elucidate the quantitative relationships between cyanobacterial growth inhibition effects and doses of these allelochemicals.

Examination of the dose–response relationships between the allelochemicals and growth inhibition effects as represented by normalized maximum growth curves (Figure 3) revealed that the equivalent concentration of \textit{M. spicatum} at which the mixture of polyphenols and fatty acids reduced the growth of \textit{M. aeruginosa} to 50% of the control value (EC\(_{50}\)) was 134 g dry wt m\(^{-3}\). When we added the polyphenols and fatty acid groups separately, the EC\(_{50}\)s were 206 and 416 g dry wt m\(^{-3}\), respectively. These results indicate that the polyphenols and fatty acid groups contribute together to the allelopathic effect of \textit{M. spicatum}. Further examination of the concurrent action of these two groups on the inhibition of \textit{M. aeruginosa} growth revealed that the EC\(_{50}\) of the mixture lay within the area of additive interactions defined by the 95% confidence ranges of the EC\(_{50}\) values of each group (Figure 4). This result indicates that the interaction of the polyphenols and fatty acid groups on the inhibition of \textit{M. aeruginosa} growth was additive.
In this series of cyanobacterial assays, the composition of mixtures of five polyphenols and three fatty acids added to the cyanobacterial medium was determined on the assumption that both 6OA and 9OA were released from *M. spicatum* at 3 nmol g dry wt^{-1} h^{-1}. However, the composition of 6OA and 9OA in the mixture would not change the inhibitory effect of the mixture on the growth of *M. aeruginosa* (vide infra).

Effect of 6OA/9OA composition in the mixture on the inhibition of cyanobacterial growth

In order to determine whether the composition of 6OA and 9OA affected the inhibition of *M. aeruginosa* growth, we prepared polyphenol and fatty acid mixtures equivalent to 144 g dry wt m^{-3} of *M. spicatum* to carry out assays on *M. aeruginosa* on the assumption that either 6OA or 9OA was released from *M. spicatum*. We compared the inhibition of *M. aeruginosa* growth by mixtures of polyphenols and fatty acids that contained (i) 6OA but no 9OA, (ii) 9OA but no 6OA, and (iii) 6OA and 9OA (Figure 5).

In a previous study, 6OA demonstrated a stronger inhibitory effect on *M. aeruginosa* growth than 9OA, and the EC_{50} of 9OA to *M. aeruginosa* was 2.1 times higher than that of 6OA (Nakai & Hosomi 2005). A different degree of growth inhibition was therefore expected when the mixtures with different 6OA/9OA composition were used. Although the order of normalized maximum growth (Figure 5) was consistent with the reported result (Nakai & Hosomi 2005), the values of normalized maximum growth were within a range from 39 to 47%. In addition, the observed difference was statistically insignificant between 6OA and 6OA/9OA (p-value=0.052), between 6OA/9OA and 9OA (p-value=0.22), or between 6OA and 9OA (p-value=0.53). Although we did not test another 6OA:9OA ratio, the results obtained under these conditions (Figure 5) indicate that the compositions of 6OA and 9OA in the mixture might not affect the inhibition of *M. aeruginosa* growth by the polyphenol and fatty acid mixture.

Contribution of the polyphenol and fatty acid groups to the allelopathy

It is well known that the genus *Myriophyllum* produces anti-cyanobacterial polyphenols (Saito et al. 1989; Aliotta et al. 1992; Gross et al. 1996; Gross 1999; Nakai et al. 2000; Bauer et al. 2009), and much research has focused on the anti-cyanobacterial effects of polyphenols (Gross 2005; Hilt et al. 2006; Matsushima et al. 2008; Bauer et al. 2009; Zhu et al. 2010). However, the results of this study have demonstrated that fatty acids also contribute to the allelopathic effect of *M. spicatum*.

The dose-response relationship between the polyphenol and fatty acid mixture and the inhibition of *M. aeruginosa* growth (Figure 5) indicates that the EC_{50} of the mixture was 134 g dry wt m^{-3}. In contrast, we determined the EC_{50} of *M. spicatum* itself to be 72 g dry wt m^{-3} (equivalent to 1 g wet wt L^{-1}) from a coexistence assay with a mixed culture system in which we cultivated *M. spicatum* and *M. aeruginosa* together (Nakai et al. 1996). As the former is about 1.9 times the latter, the comparison implies that the inhibitory effect of these allelochemicals on *M. aeruginosa* accounts for about 53% of the allelopathic inhibitory effect of *M. spicatum* itself. This paper is the first to report
allelochemicals that account for a half of the anti-cyanobacterial allelopathic effect of a macrophyte.

CONCLUSIONS

In this study, a dataset of release rates of the five polyphenols and three fatty acids excreted by M. spicatum was created. The release rate of was 5.3 nmol g dry wt\(^{-1}\) h\(^{-1}\). We estimated the release rates of 6OA and 9OA acids to be less than 7.1 and 5.3 nmol g dry wt\(^{-1}\) h\(^{-1}\), respectively. Based on the estimated release rates, the five polyphenols and three fatty acids were added to a cyanobacterial medium to simulate the release behavior of M. spicatum in a mixed culture system containing M. spicatum and M. aeruginosa. The fact that the addition of these polyphenols and fatty acids inhibited the growth of M. aeruginosa confirmed that they are among the allelochemicals that contribute to the allelopathic effect of M. spicatum on M. aeruginosa. The interactive effect on growth inhibition of the polyphenol and fatty acid groups was additive. The EC\(_{50}\) of the polyphenol and fatty acid mixture was about 1.9 times that of M. spicatum itself. This result implies that the inhibitory effect of the polyphenols and fatty acids on M. aeruginosa is about 53% that of M. spicatum itself. These allelochemicals can therefore account for 53% of the allelopathic effect of M. spicatum.

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