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Toxicon

DOI:

[10.1016/j.toxicon.2019.10.002](https://doi.org/10.1016/j.toxicon.2019.10.002)

Published: 05/12/2019

Peer reviewed version

[Cyswllt i'r cyhoeddiad / Link to publication](#)*Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA):*

Rzymkic, P., Evans, D., Murphy, P., & Kokocinski, M. (2019). A study of polymethoxy-1-alkenes in *Raphidiopsis (Cylindrospermopsis) raciborskii* and *Aphanizomenon gracile* isolated in Poland. *Toxicon*, 171, 51-53. <https://doi.org/10.1016/j.toxicon.2019.10.002>

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1 **A study of polymethoxy-1-alkenes in *Raphidiopsis (Cylindrospermopsis) raciborskii* and**
2 ***Aphanizomenon gracile* isolated in Poland**

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11

12 **Abstract**

13 Previous studies indicated that teratogenic polymethoxy-1-alkenes (PMAs) are produced by
14 phylogenetically diverse cyanobacteria taxa, **however corresponding studies on the occurrence**
15 **of PMAs in European cyanobacteria is lacking.** Herein, the **presence of PMAs** in strains of
16 *Raphidiopsis raciborskii* and *Aphanizomenon gracile* isolated **from surface waters** in Poland
17 **was studied** using **nuclear magnetic resonance** and mass spectrometry. No PMAs were detected
18 **in any of the strains investigated**, indicating that production of these compounds **may be**
19 geographically diversified. Further studies are necessary to elucidate mechanisms of
20 cyanobacterial PMAs synthesis.

21

22 **Keywords:** Polymethoxy-1-alkenes; Cyanobacteria; *Cylindrospermopsis raciborskii*;
23 *Aphanizomenon gracile*

24

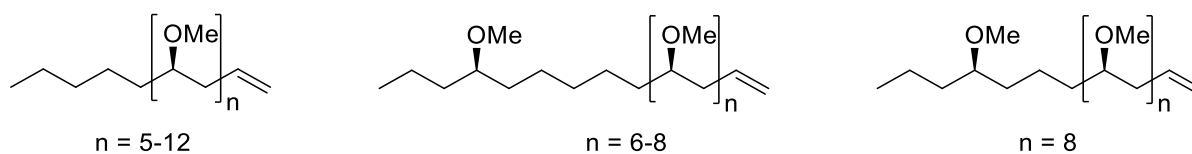
25 The toxicity of cyanobacteria attracts ongoing attention, and over the **past few** decades,
26 **the** structures of numerous toxic metabolites have been identified, their biosynthesis pathways
27 elucidated and **their potential** ecological roles proposed. **The most extensively studied**
28 **cyanobacterial compounds include the hepatotoxic cyclic peptides, microcystins and**
29 **nodularins, cytotoxic alkaloid cylindrospermopsin, the neurotoxic alkaloids saxitoxins and**
30 **anatoxin-a, and more recently a neurotoxic amino acid β -methylamino-l-alanine (Poniedziłek**
31 **et al. 2012; Rzymiski et al. 2014; Testai et al. 2016; Buratti et al. 2017; Chernoff et al. 2017). In**
32 **general,** production of cyanotoxins has been found to be a species and strain-specific feature,
33 and **seems to** be geographically diversified. Despite the extensive research, **a significant** number
34 of cyanobacteria have **shown *in vivo* and *in vitro* toxicity** (including human cells), **however** the
35 metabolites **responsible for this activity** were not identified. This **is of** particular concern **for**
36 European strains belonging to the Nostocales order such as *Raphidiopsis (Cylindrospermopsis)*
37 *raciborskii* and some members of the traditional “*Aphanizomenon*” genus (Bernard et al. 2003;
38 Fastner et al. 2003; Antal et al. 2011; Acs et al. 2013; Poniedziłek et al. 2015; Vehovszky et
39 al. 2015; Rzymiski et al. 2017; Falfushynska et al. 2019), and highlights the need for further
40 explorations in this area.

41 Polymethoxy-1-alkenes (PMAs) represent a group of lipophilic compounds and varying
42 in chain length (Fig. 1). Initially, PMAs were identified in *Tolypothrix conglutinate* (Mynderse
43 and Moore 1979), *Scytonema ocellatum* (Mori et al. 1991a, b) and later, in **an** Israeli strain of
44 *Aphanizomenon ovalisporum* (Banker et al. 2000), **an** Australian strain of *R. raciborskii* and
45 other cyanobacterial species (Jaja-Chimedza et al. 2015). There is, however no information on
46 **the** production of PMAs in any cyanobacteria occurring in European surface waters.
47 Considering that PMAs may exert significant teratogenic action by triggering developmental
48 dysfunctions (as presented using the zebrafish (*Danio rerio*) embryo model; Jaja-Chimedza et

49 al. 2012, 2015)), it is imperative to evaluate whether cyanobacteria strains previously **shown to**
50 **be toxic are producing these metabolites.**

51 In this study the presence of PMAs in strains of *Aphanizomenon gracile* and *R.*
52 *raciborskii* isolated from Western Poland was tested using nuclear magnetic resonance (NMR)
53 and mass spectrometry (MS). These strains do not produce cylindrospermopsin (**CYN**)
54 (although a **CYN** producing strain of *A. gracile* has been reported in Poland; see Kokociński et
55 al. 2013), microcystins, saxitoxins, anatoxin-a, β -methylamino-L-alanine or diaminobutyric
56 acid (with exception of *A. gracile* LWI-Ag **in which it was found at levels** below the level of
57 quantification). **However, selected extracts of these strains were found to exhibit relevant**
58 **toxicities in human and fish cells *in vitro*** (Poniedziałek et al. 2015; Rzymiski et al. 2017a;
59 Falfushynska et al. 2019). **This, in turn, highlighted** the necessity to investigate whether any of
60 these strains is capable of **producing PMAs** (Rzymiski et al. 2017b).

61



63

Figure 1. Chemical structure of known PMA variants.

64 Three strains of *R. raciborskii* and four strains of *A. gracile* (**Fig. S1**) were isolated **from**
65 **water samples from freshwater lakes in Western Poland (Table 1). Single trichomes were**
66 **isolated under light and inverted microscopes using elongated glass Pasteur pipette and**
67 **transferred to sterile WC medium prepared according to Guillard and Lorenzen (1972). This**
68 **procedure was repeated until cyanobacterial monocultures were obtained.** All cultures were
69 maintained to stationary phase reaching 10^6 trichomes L^{-1} and then processed for analysis. **All**
70 **strains of *R. raciborskii* had characteristic morphological features for this species *viz* solitary**
71 **trichomes, with straight cylindrical cells which are constricted at the cross-walls, together with**

72 terminal heterocytes at one or both ends of the trichomes with a characteristic elongated drop-
73 lake shape. Apical cells were clearly narrowed if heterocytes were not present. *A. gracile*
74 similarly had characteristic morphological features viz solitary trychomes, with straight
75 cylindrical cells constricted at the cross-walls together with often elongated and narrowed
76 terminal cells with intercalary and solitary barrel-like shape heterocytes. Cylindrical, elongated
77 akinetes with a characteristic cup-shaped sheath formation were rarely observed under culture
78 conditions.

79 The PMAs were analyzed using two methods and initially, NMR analysis was
80 employed. The analysis was targeted towards the identification of known PMAs and also the
81 presence of potential homologous compound which high contain extra [-CH=CH-]_n, [-
82 CH₂CH(OMe)-]_n or [-CH₂CH₂-]_n fragments. The biomass of each strain was collected by
83 centrifugation and ultrasonicated on ice for complete lysis as previously described (Rzymiski et
84 al. 2017a). The obtained samples were evaporated to dryness using a rotary evaporator (Buchi
85 R124 Rotavapor) and the residue dried under high vacuum. ¹H NMR spectra of the CDCl₃
86 soluble and D₂O soluble material from the residue were recorded using a Bruker Ultrashield
87 Plus 400 MHz instrument. Inspection of the regions 5.00-6.00 ppm (vinylic protons) and 3.00-
88 3.70 ppm (*MeOCH*, methyl and methine protons) did not clearly indicate the presence of PMAs
89 on comparison with literature NMR data since no analytical standards were available and no
90 database for these compounds exist (Mori et al. 1991a, Mori et al. 1991b, Mynderse and Moore
91 1979). In order to determine if any of the known PMAs were present in the residues obtained,
92 but below the detection limits of NMR analysis, the samples were further investigated by mass
93 spectrometry.

94 **Table 1.** Polish strains of *R. raciborskii* and *A. gracile* screened for PMAs production in this
95 study.

Species/Strain	Place of isolation	Coordinates
<i>Raphidiopsis raciborskii</i>		
LBO-Cr	Lake Boczowskie	52°19'10"N, 14°56'47"E
LBY-Cr	Lake Bytyńskie	52°29'55"N, 16°30'30"E
LBU-Cr	Lake Buszewskie	52°32'34"N, 16°22'38"E
<i>Aphanizomenon gracile</i>		
LWI-Ag	Lake Witobelskie	52°15'55"N, 16°43'32"E
LPN-Ag	Lake Pniewskie	52°30'41"N, 16°14'27"E
LBN-Ag	Lake Bnińskie	52°12'02"N, 17°06'59"E
LJE-Ag	Lake Jelonek	52°32'04"N, 17°35'17"E

96

97 **To this end, a separate set of sample of** cyanobacterial biomass was collected by
98 centrifugation, freeze-dried, then suspended in CHCl₃, stirred vigorously for 16 h then filtered
99 **through** a sintered filter apparatus. This procedure was repeated a total of three times and the
100 combined filtrates were dried over anhydrous magnesium sulphate and the solvent removed
101 under reduced pressure. The residue obtained was then dried under high vacuum until constant
102 weight. The material obtained was fractioned using silica gel chromatography eluting with a
103 stepwise gradient of ethyl acetate in petrol (10%, 20%, 40% and 100%). Each fraction was
104 collected and evaporated to dryness. MS analysis was performed either on a Xevo G2-S QTof
105 or a LTQ Orbitrap XL or on a ThermoQuest Finnigan LCQ Deca mass spectrometer. **Conditions**
106 **of analysis, ionization method and m/z for all species are given in the Supplementary Material.**
107 Data from these analyses also did not indicate molecular ions **reported previously by Jaja-**
108 **Chimedza et al. (2012; 2015)** to represent known PMAs, thus suggesting that the investigated
109 strains of *R. raciborskii* and *A. gracile* are not producers of these metabolites. This is the first
110 reported investigation for **the presence of PMAs in** European cyanobacteria.

111 **In conclusion,** the findings of the present study, **despite being** negative, are important in
112 view of **the** limited information **available** on **the presence** PMAs in cyanobacteria, particularly
113 from European freshwaters, and in terms of ecological and health risk assessment if one

114 considers the disruptive, teratogenic action of these metabolites (Jaja-Chimedza et al. 2012).
115 Our results also indicate that production of PMAs may potentially be geographically diversified
116 with Australian strains of *R. raciborskii* capable of their synthesis (Jaja-Chimedza et al. 2015),
117 on contract to those occurring in Central Europe. One should however note that this is a
118 preliminary study and further investigations screening more diverse strains are required before
119 final conclusions can be drawn in this regard. The exclusion of PMAs production by tested
120 strains does not unambiguously exclude any teratogenic effect, as other cyanobacteria species
121 have been found to produce teratogenic compounds such as retinoic acids and carotenoid
122 glycosides (Wu et al. 2012; Jaja-Chimedza et al. 2017), which were not considered here. Further
123 research is required to elucidate the as yet unknown biosynthesis of PMAs, identify potential
124 factors regulating their synthesis and to evaluate the frequency of occurrence in European
125 cyanobacteria. Moreover, continued study is needed in order to elucidate the toxicity previously
126 observed for selected European strains of *R. raciborskii* as well as teratogenicity of various
127 cyanobacteria species.

128

129 **Acknowledgements**

130 Thanks are given to Rolf Kraehenbuehl, Shôn Jones and Bela Paizs for support with mass
131 spectrometry analysis.

132

133 **Funding**

134 This study has been partly funded by Beacon+. Beacon+ has been part-funded by the European
135 Regional Development Fund through the Welsh Government. EU Funds: Investing in Wales.

136

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