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Article

Effect of Selected Plant Extracts and D- and L-Lysine on the Cyanobacterium *Microcystis aeruginosa*

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Abstract: We tested extracts from *Fructus mume*, *Salvia miltiorrhiza* and *Moringa oleifera* as well as L-lysine and D-Lysine as curative measures to rapidly suppress the cyanobacterium *Microcystis aeruginosa* NIVA-CYA 43. We tested these compounds under similar conditions to facilitate comparisons. We hypothesized that for each compound, relatively low concentrations—*i.e.*, 5–50 mg L^{-1}, would reduce *M. aeruginosa* biomass. At these low concentrations, only L-lysine caused a decline in *M. aeruginosa* biomass at ≥4.3 mg L^{-1}. *F. mume* extract was effective to do so at high concentrations, *i.e.*, at ≥240 mg L^{-1}, but the others were virtually non-effective. Low pH caused by organic acids is a probable explanation for the effect of *F. mume* extract. No complete wipe-outs of the experimental population were achieved as Photosystem II efficiency showed a recovery after six days. L-lysine may be effective at low concentrations—meaning low material costs. However, the effect of L-lysine seems relatively short-lived. Overall, the results of our study did not support the use of the tested plant extracts and amino-acid as promising candidates for curative application in *M. aeruginosa* bloom control.

Keywords: eutrophication control; mitigation; natural algaecide; nuisance cyanobacteria

1. Introduction

Excessive nutrient loading (eutrophication) of lakes and ponds is the major cause of water blooms [1,2] and surface scums of cyanobacteria (blue-green algae). Scums often accumulate at the leeside shores [3] and because cyanobacteria can produce potent toxins, such scums pose a threat to human and animal health [4,5]. Consequently, blooms and scums of cyanobacteria may hamper the use of surface waters through swimming bans, or may impact irrigation and lead to drinking water shortages [3,6,7].

Microcystis aeruginosa is one of the world wide and most frequently encountered bloom-forming cyanobacteria in freshwater bodies, several of its strains produce powerful toxins—microcystins [8,9]. In the Netherlands, accumulations of cyanobacteria are reported to contain microcystin concentrations well over 10,000 μg L^{-1} [10,11]. Such high toxin concentrations conflict with societal demands for safe water and need to be dealt with according to the European Water Framework Directive (WFD) [12] and the EU Bathing Water Directive (BWD) [13].

Nutrient reduction—with a focus on phosphorus control [14], is considered the most effective way to mitigate cyanobacterial nuisance [15]. However, methods to this effect often operate longer-term, which leave water authorities with a great need to instantaneously curate cyanobacterial nuisances at the moment they occur. For example, at official bathing areas the BWD demands that "when cyanobacterial proliferation occurs and a health risk has been identified or presumed, adequate management measures shall be taken immediately to prevent exposure". Accordingly, curative methods are wanted to immediately suppress the proliferation of cyanobacteria or (safely) end a massive bloom with the purpose to provide—at least in the summer season, access to the water for drinking, irrigation, aquaculture, industry and recreation [16].

Many curative products to control cyanobacteria are proposed as Columbus's Egg to the Dutch water authorities. These water authorities have a preference for biological curative measures above chemical approaches, because the former are considered environmental friendly. The biological anti-cyanobacterial products are viewed, and reported, as promising in controlling cyanobacteria [17,18]. The majority of these products are compounds extracted from plant tissues and tested on *M. aeruginosa* [17–19]. In such testing, extracts from *Fructus mume* [19], *Salvia miltiorrhiza* (Chinese Danshen) [20] and extracts from seeds of the pan tropical tree *Moringa oleifera* [21] are observed to express anti-cyanobacterial activity against *M. aeruginosa*. In addition, the amino acid L-lysine is reported as active against cyanobacteria [22–24]. However, with respect to D-Lysine reports are less conclusive; while Kaya and Sano [22] reported that D-Lysine was equally suppressive to *Microcystis* as L-Lysine, others found no effect of D-Lysine on *Microcystis* [25].

The literature data on effective concentrations against cyanobacteria of the above mentioned plant extracts and amino acids are not comparable, as different studies used quite dissimilar test conditions [18] (Appendix Table A1). Importantly, several of these studies most likely did not use agitation of the cultures (Table A1), whereas mixing enhances biomass [26] and was recently found to affect growth inhibition by a filtrate from a *Tychonema* culture towards *M. aeruginosa* [27]. In our current study, we tested the effect of filtrates from *Fructus mume*, *Salvia miltiorrhiza*, and *Moringa oleifera* as well as L-lysine and D-Lysine on growth of *M. aeruginosa*, under similar experimental conditions with continuous shaking. For each compound we hypothesized that a sufficiently high concentration will reduce the growth of *M. aeruginosa* and diminish *M. aeruginosa* biomass. We relate our results to

similar studies reported in literature and discuss our results regarding the feasibility of the use of these compounds in actual mitigation of blooms of cyanobacteria. As previously reported in the literature, we also observed a strong effect of *F. mume* on the solution pH.

2. Materials and Methods

2.1. Test Organism

The cyanobacterium *Microcystis aeruginosa* NIVA-CYA 43 was obtained from the Norwegian Institute for Water Research (NIVA, Oslo, Norway). A stock culture of this strain was grown in a 1000 mL Erlenmeyer flask containing 400 mL modified WC medium [28] and that was closed with a cellulose plug. To promote a dense green culture, the culture flask was placed in a Gallenkamp ORBI-SAFE Netwise Orbital Incubator at 24 °C under continuous light of 80 µmol quanta m^{-2} s^{-1} and continuous orbital shaking at 60 rpm. This strain of *M. aeruginosa* is completely uni- and bicellular under the given growth conditions and does not contain any measurable amounts of microcystin [29]. Inoculums for the experiments were taken from the late-log phase dense culture and diluted to ~10 µg L^{-1} in fresh modified WC medium in 2 L Erlenmeyer's.

2.2. Test Compounds

Dried fruits (Fructus) of *Prunus mume* and the roots of *Salvia miltiorrhiza* were purchased from NatuurApotheek (Pijnacker, The Netherlands). Seeds of *Moringa oleifera* were obtained from the Miracle Trees Foundation (Rotterdam, The Netherlands). The amino acids D-lysine (CAS Number 923-27-3) and L-lysine (CAS Number 56-87-1) were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands).

2.3. Preparation Extracts

The fruit tissue of *Fructus mume* was separated from the kernel by cutting with a knife. The kernels were discarded. Whereas the purchased *F. mume* were obtained as dried material, the roots of *S. miltiorrhiza* and seeds of *M. oleifera* were not dry when obtained. Drying was done in a stove at 50 °C for 96 h. *M. oleifera* seed coats were removed after drying. Each dried tissue was separately grinded to a powder using a mortar and pestle. Of these ground tissues 24 g of the *F. mume* tissue and 37 g root tissue of *S. miltiorrhiza* were boiled separately for 30 min in Millipore water under continuous stirring using a magnetic stirrer. Evaporation losses were compensated with Millipore water such that for each a final concentration of 100 g L^{-1} was obtained. These suspensions were centrifuged for seven min at 3000 rpm, where after the supernatants were filtered through a glass-fiber filter (GF/A, Whatman International Ltd., Maidstone, UK) and transferred to 250 mL flasks. The filtrates were autoclaved and stored in the dark at room temperature until further use.

For *M. oleifera* 10 g powder was added to 1 L Millipore water, stirred for 30 min, filtered through a glass-fiber filter (GF/C Whatman) and transferred to a 1 L flask that was closed and stored in a refrigerator until further use.

Stock solutions for D-lysine and L-lysine were prepared by adding aliquots of 4.0 g to separate flasks and dissolving it in 250 mL Millipore water yielding final concentration of the stocks of 16.0 g L^{-1}.

The pH of the extracts were: *F. mume*, pH = 3.1; *S. miltiorrhiza*, pH = 5.2; *M. oleifera* seed extract, pH = 4.5. The corresponding electric conductivity (EC) of the extracts were 9500, 4300 and 362 µS cm^{-1}. The pH of the L-lysine stock solution was 5.2, for the D-lysine pH was 5.7, while the EC was 6800 and 6700 µS cm^{-1}, respectively.

2.4. Dose-Range Finding Experiment

A dose-range finding experiment was done to determine how much of the stock solutions of the *F. mume*, *S. miltiorrhiza* extracts and which concentration of the lysine stereo-isomers should be used in the bioassay. The active dose range for the *M. oleifera* extracts was based on [21]. A dilution series of each stock solution was made in WC medium such that the added volume was always 1 mL. The range finding experiment was done in triplicate in 100 mL Erlenmeyer flasks containing 49 mL of *M. aeruginosa* suspensions in WC medium to which 1 mL of *F. mume, S. miltiorrhiza* or L-lysine was added yielding concentrations of 0, 3 and 300 mg L^{-1} for *F. mume* and *S. miltiorrhiza*, and 0, 1, 5, 10, 40 mg L^{-1} for L-lysine. The initial *M. aeruginosa* concentration was 14.4 (±0.7) µg L^{-1} chlorophyll-a (CHLa) for *F. mume* and *S. miltiorrhiza* and 27.7 (±1.1) µg L^{-1} for the L-lysine trial as determined with a PhytoPAM phytoplankton analyzer (Heinz Walz GmbH, Effeltrich, Germany). The flasks were incubated for two days (*F. mume*, *S. miltiorrhiza*) or three days (L-lysine) in a Gallenkamp ORBI-SAFE Netwise Orbital Incubator at 24 °C under continuous light of 80 µmol quanta m^{-2} s^{-1} and continuous orbital shaking at 60 rpm, where after CHLa was measured again.

2.5. Bioassay

Based on the range finding experiment, different amounts of extracts and D- and L-lysine were tested (Table 1). For each extract and D- and L-lysine serial dilutions were made in WC medium such that for every desired concentration 1 mL was added to 49 mL *M. aeruginosa* suspensions in 100 mL Erlenmeyer flasks. The flasks were closed with a cellulose plug. The bioassay experiment consisted of a 4 day pretreatment growth period to allow *M. aeruginosa* to adapt to the prevailing conditions and a six day post treatment period. The initial *M. aeruginosa* concentration was ~10 µg L^{-1}. CHLa concentrations (µg L^{-1}) and Photosystem II efficiencies (Φ_{PSII}) were determined using a PhytoPAM phytoplankton analyzer (Heinz Walz GmbH, Effeltrich, Germany). The biovolume concentration (μm^3 mL^{-1}) was measured using a CASY cell counter (Schärfe System Gmbh., Reutlingen, Germany). The measurements were taken four days (4 d), one day (1 d) and just before (0 d) the application of the treatments as well as three and six days after the application (*i.e.*, 3 d and 6 d). Just after the application of the treatments and at the end of the experiment pH and electric conductivity were measured using a WTW pH/cond 340i meter. The experiment was done in fivefold. The experimental flasks were divided systematically over two incubators (Gallenkmap, ORBI-SAFE Netwise) set at identical conditions; 24 °C under continuous light of 80 µmol quanta m^{-2} s^{-1} and continuous orbital shaking at 60 rpm, *i.e.*, three replicates were placed in one and the other two in the other incubator. Light intensity was checked with a Li-COR Li-185B quantum/radiometer/photometer connected with a LI-190SB quantum sensor.

Table 1. Amounts of dried *Fructus mume*, *Salvia miltiorrhiza* and *Moringa oleifera* that were extracted in water and tested in different concentrations against *Microcystis aeruginosa* NIVA-CYA 43, including the concentration ranges for the amino-acids D- and L-lysine tested.

F. mume **(mg L^{-1})**	***S. miltiorrhiza*** **(mg L^{-1})**	***M. oleifera*** **(mg L^{-1})**	***D*- and *L*-lysine (mg L^{-1})**
0	0	0	0
30	40	4	0.5
60	80	8	1.4
120	160	16	4.3
240	320	32	13
480	640	64	38.9

2.6. Data Analysis

In controls and treatments where exponential growth was observed over the entire experimental period, the slope of the regression line between natural log-transformed CHLa concentrations and time was used to estimate the population growth rate. In treatments where growth was affected, growth rates (μ) were determined from the difference in CHLa concentrations at the start of the application and after 6 days according: $\mu = \frac{\{ln(CHL_{6d}) - ln\,(CHL_{0d}\}}{6}$. In addition, growth rates were also calculated similarly based on changes in biovolume concentrations. For each compound the estimated growth rates (μ) at different concentrations were compared by a one-way ANOVA or when prerequisites for parametric testing were not met by Kruskal-Wallis One Way Analysis of Variance on Ranks. As some products caused a decline in population growth yielding negative growth rates, finite rates of increase of the population (λ) were calculated ($\lambda = e^{\mu}$) based on which EC50 values were determined by four parameter logistic regression in the tool pack SigmaPlot version 12.5. The effect of each extract/compound on Φ_{PSII} was tested by running repeated measure ANOVAs in the statistical program IBM SPSS version 19 (IBM Corporation, Armonk, NY, USA).

3. Results

3.1. Range Finding Assay

The range finding assay revealed that *F. mume* and *S. miltiorrhiza* extracts and L-lysine can inhibit growth of *M. aeruginosa* (Table 2). Based on these results the concentration range was further extended.

3.2. Bioassay

The CHLa concentration of *M. aeruginosa* populations was not affected by *F. mume* extract up to 60 mg L^{-1}, reflected in strong exponential increase over time (Figure 1A). However, at 120 mg L^{-1} the increase in CHLa concentration was reduced, while it even declined after adding the extract in 240 and 480 mg L^{-1} (Figure 1A). Consequently, growth rates were significantly different; they were on average 0.47 d^{-1} in controls and *F. mume* extract up to 60 mg L^{-1}, but negative at the two highest doses tested (Table 3). Extract of *S. miltiorrhiza* had no effect on *M. aeruginosa* CHLa concentrations up to 80 mg L^{-1}. However, *S. miltiorrhiza* extracts at 160,320 and 640 mg L^{-1} did lower CHLa concentrations (Figure 1B).

The growth rates were on average 0.48 d^{-1} in controls and *S. miltiorrhiza* extracts up to 80 mg L^{-1} Although growth rates were significantly reduced at higher doses, at the highest *S. miltiorrhiza* dose tested (640 mg L^{-1}) growth was still 75% of the growth measured in controls, indicating considerable increase in *M. aeruginosa* population even in this highest dose (Table 3). D-lysine in the concentration range tested had no effect on the course of the CHLa concentrations in *M. aeruginosa* populations (Figure 1C) and growth rates were similar (Table 3). L-lysine caused a decline in CHLa concentrations in concentration at 4.3 mg L^{-1} and higher (Figure 1D), which was also expressed in significantly reduced growth rates (Table 3). Extract of *M. oleifera* seeds had no effect on CHLa concentrations up to dose 8 mg L^{-1} (Table 3). At 16 mg L^{-1} and higher *M. oleifera* extracts lowered the CHLa concentrations (Table 3). Three groups of significantly different growth rates were observed: 0–8 mg L^{-1} (group a), 16 and 32 mg L^{-1} (group ab) and 64 mg L^{-1} (group b) (Table 3; Figure 1E).

The biovolume-based growth rates in controls and *F. mume* extract up to 60 mg L^{-1} were on average 0.30 d^{-1}, they were slightly lower in 120 mg L^{-1} (Table 4). No growth was observed after addition of *F. mume* extract at 240 and 480 mg L^{-1} (Table 4). Only the highest dose of *S. miltiorrhiza* extract (640 mg L^{-1}) reduced the biovolume-based growth rate to 73% of that in the control, but this difference was not statistically significant (Table 4). D-lysine had no effect on biovolume-based growth rates, whereas L-lysine completely blocked growth in concentration of 4.3 mg L^{-1} and more (Table 4). The biovolume-based growth rate of *M. aeruginosa* in extract of *M. oleifera* seeds at 64 mg L^{-1} was 75% of that in control, but the difference was not statistically significant due to rather large within group variability (Table 4). The biovolume-based growth rates in controls (Table 4) were about 65% of the CHLa-based growth rates (Table 3).

The EC_{50} of *F. mume* extract to *M. aeruginosa* was 148 mg L^{-1} for CHLa-based growth and 128 mg L^{-1} for biovolume-based growth, while EC_{50} of L-lysine was 2.2 mg L^{-1} for CHLa-based growth and 1.7 mg L^{-1} for biovolume-based growth (Table 3 and 4). The EC_{50} for *S. miltiorrhiza*, *M. oleifera* and D-lysine were outside the concentration range tested, *i.e.*, higher than 640, 64 and 38.9 mg L^{-1}, respectively (Tables 3 and 4).

Table 2. Growth rates (d^{-1}) of *Microcystis aeruginosa* NIVA-CYA 43 grown for three days in different concentrations of L-lysine, including *F*- and *P*-value of a one-way ANOVA, as well as growth rates of *M. aeruginosa* exposed for two days to different concentrations of extracts from *Fructus mume* or *Salvia miltiorrhiza*, including *F*- and *P*-values of a two-way ANOVA. Values between brackets indicate one standard deviation (n = 3). Similar letters (^{a,b,c} and $^{\alpha,\beta,\gamma}$) indicate homogenous groups that are not different at the p = 0.05 level (Holm-Sidak method).

Growth Rate (d^{-1})				
Concentration	**L-lysine**	**Concentration**	***F. mume***	***S. miltiorrhiza***
0	0.67 (0.03) ^{a}	0	0.46 (0.05) $^{\alpha}$	0.46 (0.05) $^{\alpha}$
1	0.52 (0.02) ^{b}	3	0.45 (0.02) $^{\alpha}$	0.40 (0.05) $^{\alpha}$
5	0.21 (0.02) ^{c}	300	−0.35 (0.04) $^{\beta}$	0.05 (0.04) $^{\gamma}$
10	0.17 (0.01) ^{c}	Extract	$F_{1,17}$ = 37.3	p < 0.001
40	0.16 (0.04) ^{c}	Concentration	$F_{2,17}$ = 428.9	p < 0.001
	$F_{4,14}$ = 282.3 p < 0.001	Interaction	$F_{2,17}$ = 56.7	p < 0.001

Figure 1. Course of chlorophyll-a concentrations (CHL-*a*, µg L^{-1}) in *Microcystis aeruginosa* NIVA-CYA 43 populations grown for four days (−4 to 0) in absence and six days (0 to 6) in presence of filtered extracts from *Fructus mume* (**A**); *Salvia miltiorrhiza* (**B**); or *Moringa oleifera* (**C**); and in different concentrations D-lysine (**D**) or L-lysine (**E**). Error bars indicate one standard error (n = 5).

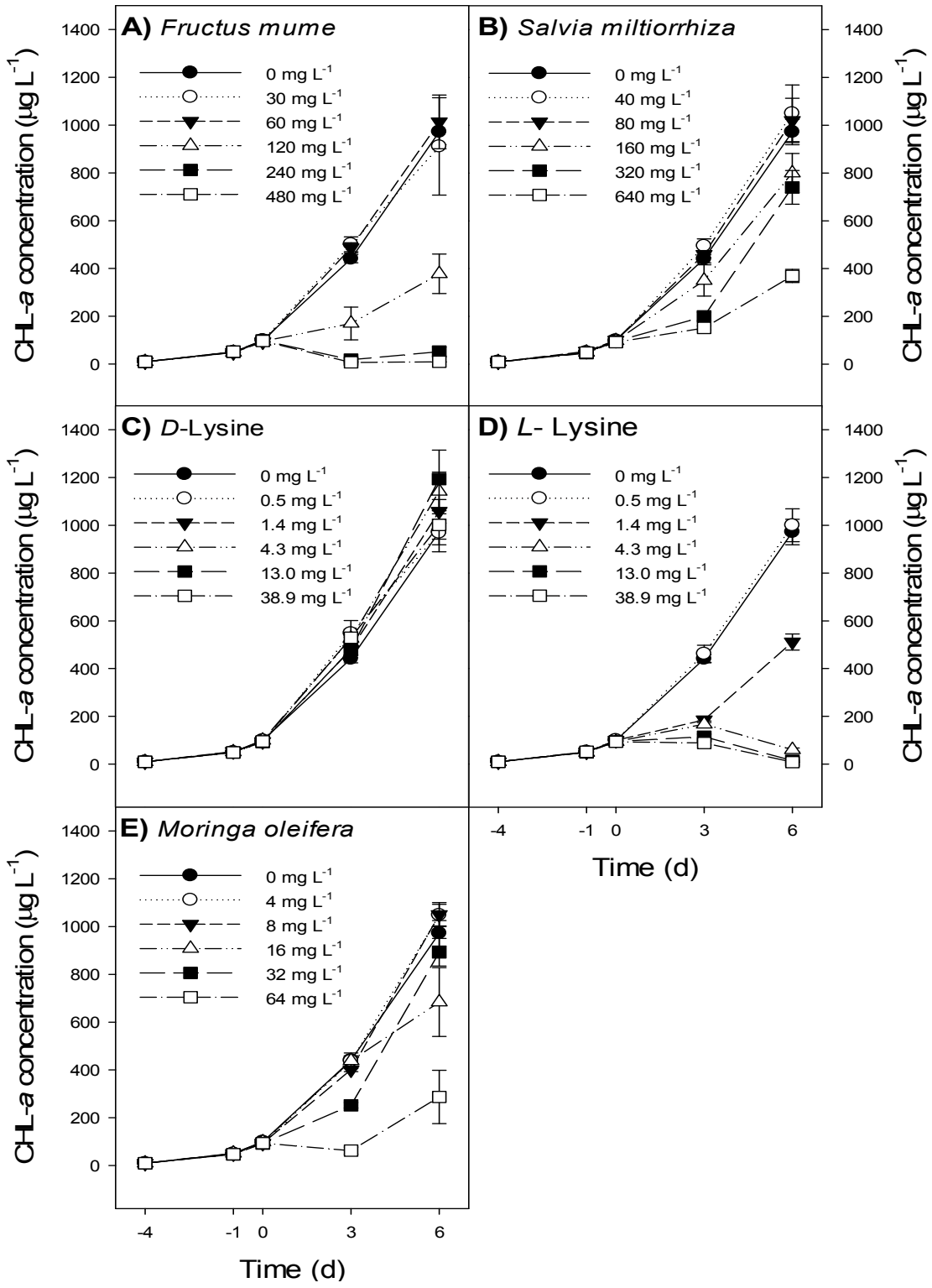


Table 3. Chlorophyll-*a* based growth rates (d^{-1}) of *Microcystis aeruginosa* NIVA-CYA 43 exposed to different concentrations (C, mg L^{-1}) of extracts from *Fructus mume* , *Salvia miltiorrhiza*, *Moringa oleifera* and D-and L-lysine, including *F*- and *P*-values of one-way ANOVAs, or *H*-values of Kruskal-Wallis One Way Analysis of Variance on Ranks. Values between brackets indicate one standard deviation (n = 5). Similar letters per column (^{a,b,c}) indicate homogenous groups that are not different at the p = 0.05 level. Also given are EC_{50} values (mg L^{-1}) and adjusted r^2 of non-linear regression (four parameter logistic curve).

F. mume		***S. miltiorrhiza***		**D-Lysine**		**L-Lysine**		***M. oleifera***	
[C]	**growth (d^{-1})**	**[C]**	**growth (d^{-1})**	**[C]**	**growth (d^{-1})**	**[C]**	**growth (d^{-1})**	**[C]**	**growth (d^{-1})**
0	0.47 (0.01) ^{a}	0	0.47 (0.01) ^{a}	0	0.47 (0.01) ^{a}	0	0.47 (0.01) ^{a}	0	0.47 (0.01) ^{a}
30	0.45 (0.08) ^{a}	40	0.48 (0.02) ^{a}	0.5	0.48 (0.01) ^{a}	0.5	0.48 (0.01) ^{a}	4	0.48 (0.01) ^{a}
60	0.48 (0.03) ^{a}	80	0.48 (0.02) ^{a}	1.4	0.48 (0.01) ^{a}	1.4	0.38 (0.01) ^{ab}	8	0.48 (0.01) ^{a}
120	0.33 (0.05) ^{ab}	160	0.44 (0.02) ^{b}	4.3	0.49 (0.01) ^{a}	4.3	−0.02 (0.01) ^{b}	16	0.43 (0.07) ^{ab}
240	−0.04 (0.03) ^{ab}	320	0.42 (0.02) ^{b}	13	0.49 (0.02) ^{a}	13	−0.09 (0.02) ^{b}	32	0.45 (0.02) ^{ab}
480	−0.12 (0.01) ^{b}	640	0.35 (0.02) ^{c}	38.9	0.48 (0.02) ^{a}	38.9	−0.13 (0.02) ^{b}	64	0.26 (0.11) ^{b}
	H_5 = 26.6		$F_{5,34}$ = 40.6		$F_{5,34}$ = 1.94		H_5 = 31.0		H_5 = 20.7
	p < 0.001		p < 0.001		p = 0.118		p < 0.001		p < 0.001
EC_{50} (mg L^{-1})									
	148		>640		>38.9		2.2		>64
r^2_{adj}									
	0.994		---		---		0.998		---

Table 4. Biovolume-based growth rates (d^{-1}) of *Microcystis aeruginosa* NIVA-CYA 43 exposed to different concentrations (C, mg L^{-1}) of extracts from *Fructus mume*, *Salvia miltiorrhiza*, *Moringa oleifera* and D-and L-lysine, including *F*- and *P*-values of one-way ANOVAs, or *H*-values of Kruskal-Wallis One Way Analysis of Variance on Ranks. Values between brackets indicate one standard deviation (n = 5). Similar letters per column (^{a,b,c}) indicate homogenous groups that are not different at the p = 0.05 level. Also given are EC_{50} values (mg L^{-1}) and adjusted r^2 of non-linear regression (four parameter logistic curve).

F. mume		***S. miltiorrhiza***		**D-Lysine**		**L-Lysine**		***M. oleifera***	
[C]	**growth (d^{-1})**	**[C]**	**growth (d^{-1})**	**[C]**	**growth (d^{-1})**	**[C]**	**growth (d^{-1})**	**[C]**	**growth (d^{-1})**
0	0.31 (0.05) ^{a}	0	0.31 (0.05) ^{a}	0	0.31 (0.05) ^{a}	0	0.31 (0.05) ^{a}	0	0.31 (0.05) ^{a}
30	0.28 (0.04) ^{a}	40	0.30 (0.00) ^{a}	0.5	0.30 (0.00) ^{a}	0.5	0.30 (0.00) ^{a}	4	0.31 (0.02) ^{a}
60	0.31 (0.07) ^{a}	80	0.30 (0.04) ^{a}	1.4	0.30 (0.04) ^{a}	1.4	0.30 (0.04) ^{a}	8	0.31 (0.05) ^{a}
120	0.25 (0.06) ^{a}	160	0.31 (0.04) ^{a}	4.3	0.31 (0.04) ^{a}	4.3	0.31 (0.04) ^{a}	16	0.30 (0.04) ^{a}
240	0.00 (0.01) ^{b}	320	0.31 (0.07) ^{a}	13	0.31 (0.07) ^{a}	13	0.31 (0.07) ^{a}	32	0.29 (0.07) ^{a}
480	0.00 (0.01) ^{b}	640	0.23 (0.10) ^{a}	38.9	0.23 (0.10) ^{a}	38.9	0.23 (0.10) ^{a}	64	0.23 (0.05) ^{a}
	$F_{5,34}$ = 51.6		$F_{5,34}$ = 1.57		$F_{5,34}$ = 1.57		$F_{5,34}$ = 1.57		$F_{5,34}$ = 2.00
	p < 0.001		p = 0.201		p = 0.201		p = 0.201		p = 0.109
EC_{50} (mg L^{-1})									
	128		>640		>38.9		1.7		>64
r^2_{adj}									
	0.985		---		---		0.991		---

In all incubations, the Φ_{PSII} changed significantly over time (Figure 2; Table 5). Φ_{PSII} declined with higher concentrations of *F. mume* extract over the first three days of exposure, but all increased again

between day 3 and 6 (Figure 2A). In *S. miltiorrhiza*, the rmANOVA also indicated a significant extract concentration effect (Table 5), but here the highest doses caused a significant increase in Φ_{PSII} (Figure 2B). D-lysine had no effect on Φ_{PSII} of *M. aeruginosa* (Figure 2C; Table 5), while L-lysine caused a significant drop in Φ_{PSII} over the first three days where after Φ_{PSII} increased again in all treatments (Figure 2D). *M. oleifera* seed extract had no significant effect on Φ_{PSII} of *M. aeruginosa* (Figure 2E; Table 5).

Figure 2. Course of the Photosystem II efficiencies in *Microcystis aeruginosa* populations grown for four days (−4 d to 0 d) in the absence and six days (0 d to 6 d) in the presence of filtered extracts from *Fructus mume* (**A**); *Salvia miltiorrhiza* (**B**); in presence of different concentrations D-lysine (**C**); L-lysine (**D**); and in *Moringa oleifera* seed extract (**E**). Error bars indicate one standard error (n = 5).

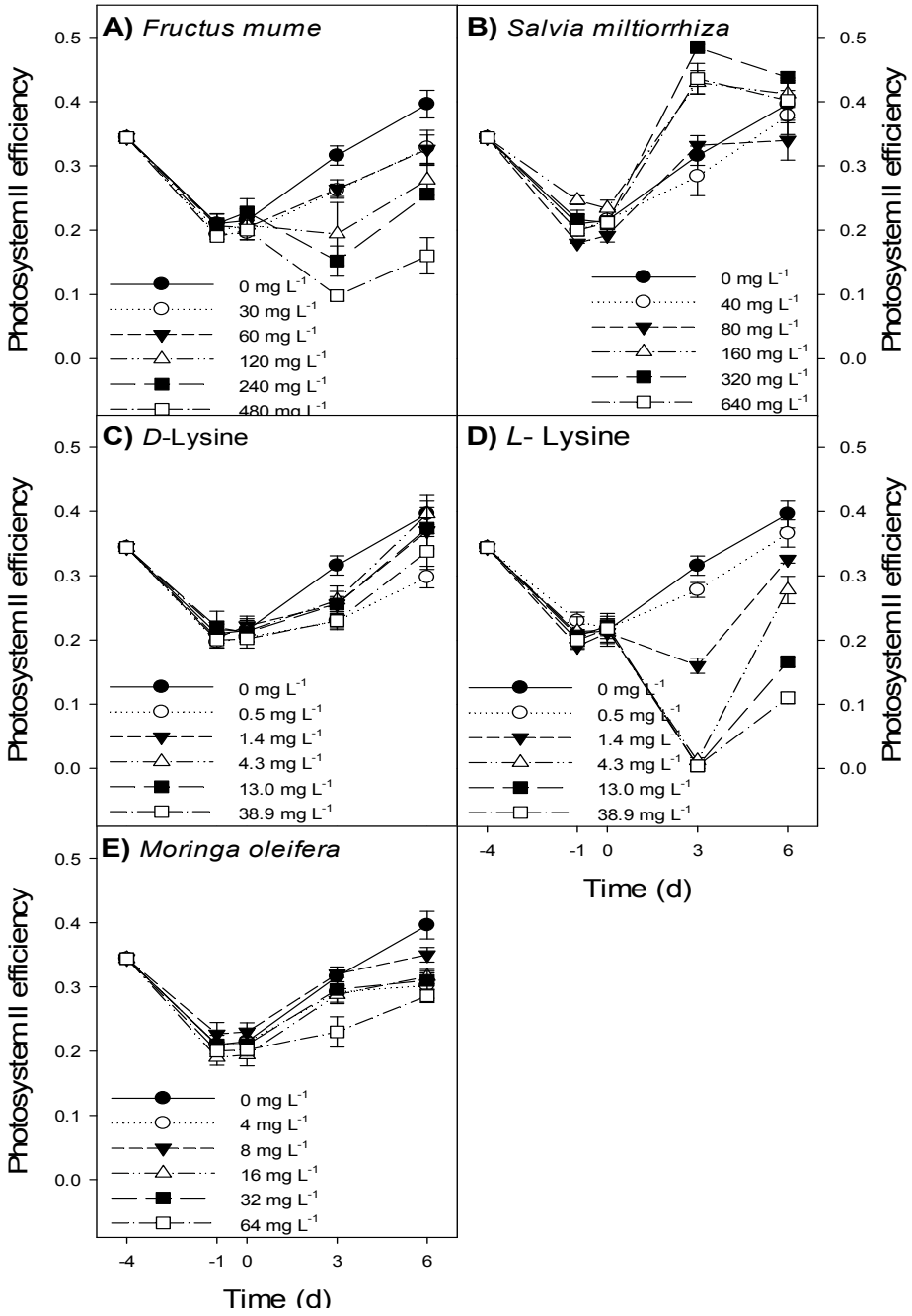
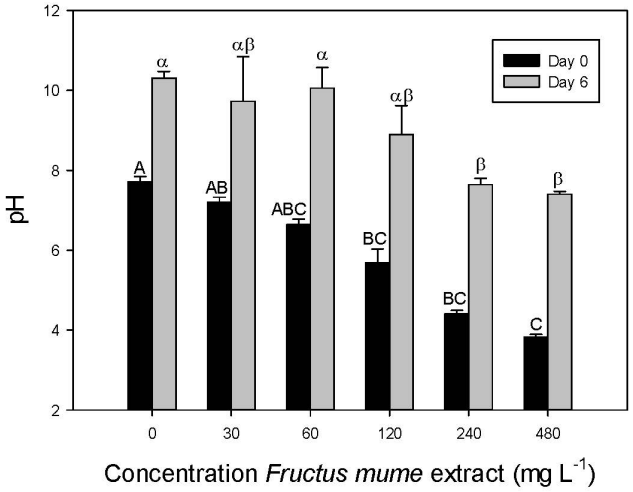


Table 5. Summary of repeated measures ANOVAs of Photosystem II efficiencies in *Microcystis aeruginosa* NIVA-CYA 43 populations exposed to different concentrations of extracts from *Fructus mume, Salvia miltiorrhiza, Moringa oleifera* and D-and L-lysine. Significant differences ($p < 0.05$) are indicated in bold. If needed (Mauchly's test), degrees of freedom (df) were corrected using Greenhouse-Geisser estimates of sphericity if epsilon <0.75 or the Huynh-Feldt correction if epsilon >0.75.

		F. mume			***S. miltiorrhiza***	
		Tests of Within-Subjects Effects				
Source	df	F	*P*	df	*F*	*P*
Time	3.79	84.4	**<0.001**	4	134.4	**<0.001**
Time × concentration	18.9	8.13	**<0.001**	20	4.52	**<0.001**
Error	53.1			60		
		Tests of between-Subjects Effects				
Source	df	*F*	*P*	df	*F*	*P*
Concentration	5	9.91	**<0.001**	5	6.24	**0.003**
Error	15			15		
		D -lysine			**L -lysine**	
		Tests of Within-Subjects Effects				
Source	df	*F*	*P*	df	*F*	*P*
Time	3.82	193.6	**<0.001**	4	183.1	**<0.001**
Time × concentration	19.1	2.29	**0.008**	20	26.2	**<0.001**
Error	57.3			60		
		Tests of between-Subjects Effects				
Source	df	*F*	*P*	df	*F*	*P*
Concentration	5	1.06	0.422	5	21.8	**<0.001**
Error	15			15		
		M. oleifera				
		Tests of Within-Subjects Effects				
Source	df	*F*	*P*			
Time	4	123.7	**<0.001**			
Time × concentration	20	3.48	**<0.001**			
Error	60					
		Tests of between-Subjects Effects				
Source	df	*F*	*P*			
Concentration	5	2.66	0.065			
Error	15					

The addition of D-lysine, L-lysine and *M. oleifera* seed extract had no effect on pH; pH values were between 7.4 and 7.7. The pH of the *M. aeruginosa* populations was slightly decreased with increasing concentrations of *S. miltiorrhiza* extract; pH dropped from 7.7 in controls to 7.2 in the highest dose of *S. miltiorrhiza* extract (640 mg L^{-1}). Extract of *F. mume* caused a significant ($H_5 = 32.7$; $p < 0.001$) decrease in pH with increasing extract concentrations of *F. mume* reaching a pH as low as 3.8 in the highest *F. mume* dose (Figure 3). The pH at the end of the six day incubation period was still significantly different ($H_5 = 26.3$; $p < 0.001$) among *F. mume* treatments, but where controls reached as high as 10.3, pH in the highest dose had recovered to a pH of 7.4 (Figure 3).

Figure 3. Effect of different concentrations filtered extracts from *Fructus mume* on pH of *Microcystis aeruginosa* NIVA-CYA 43 populations just after addition (day 0) and after six days of growth (day 6). Error bars indicate 1SD (n = 5) and similar symbols above the bars indicate homogenous groups that are not different at the p = 0.05 level.



Adding D-or L-lysine to the *M. aeruginosa* populations slightly increased the EC from 278 (±6) µS cm^{-1} in controls to 294 (±3) µS cm^{-1} in 38.9 mg L^{-1} D-lysine and 296 (±3) µS cm^{-1} in 38.9 mg L^{-1} L-lysine. Extract of *M. oleifera* seeds had no effect on EC that remained 279 (±6) µS cm^{-1} in all treatments. In contrast, S. miltiorrhiza extract significantly increased EC (H5 = 23.9; $p < 0.001$) from 278 (±6) µS cm^{-1} in controls to 315 (±3) µS cm^{-1} in 640 mg L^{-1}, while *F. mume* extract significantly elevated (H_5 = 18.4; p = 0.002) EC to 362 (±2) µS cm^{-1} in 480 mg L^{-1}.

4. Discussion

We hypothesized that a sufficiently high concentration of each compound would reduce the growth of *M. aeruginosa* and diminish *M. aeruginosa* biomass. However, only *F. mume* extract at ≥240 mg L^{-1} and L-lysine at ≥4.3 mg L^{-1} caused a decline in *M. aeruginosa* biomass. Despite the low pH values measured (pH 4.4 and 3.8), *M. aeruginosa* recovered in the ≥240 mg L^{-1} *F. mume* treatments. The low pH is most probably caused by organic acids, such as citric acid and malic acid in *F. mume* extract [30]. The inhibiting effect of *F. mume* can be attributed these compounds [19] leading to the low pH as a major driving force for the lytic effects. Another member of the Microcystaceae, *Anacystis nidulans*, died at pH < 4.5 [31]. Likewise, a suspension of "Effective Microorganisms" (EM-A) containing a high concentration of organic acids only affected *M. aeruginosa* growth and reduced biomass when dosed at such high concentrations that the pH had dropped to a pH ~3.5 [32]. This organic acid rich EM formulation caused inhibition in several bacteria, but the growth inhibition disappeared after the pH was adjusted from 3.7 to 6.5 [33]. Hence, low pH as a result of the organic acids seems the most likely explanation for observed decline and growth inhibition in *M. aeruginosa*. The concentration of *F. mume* extract that completely inhibited growth in our study (240 mg L^{-1}) was lower than the 780 mg L^{-1} reported previously [19], which might be caused by slightly different extract procedure (½ h *vs.* 1 h boiling). Also the organic acid content of the *F. mume* fruits depends on their ripeness [34]. A low pH (pH 4.4 and 3.8) as measured in the ≥240 mg L^{-1} *F. mume* treatments, however, is not compliant with

the WFD standards for pH in different lake types (pH ≥ 5.5) [35]. The addition of relatively high amounts of organic matter through a field application of the compounds under study here, as well as their possible consequential lysis of the *M. aeruginosa* cells could imply an increase in nutrient concentrations. Inasmuch as the effects of *F. mume* or L-lysine are only temporary and no complete wipe-out of *M. aeruginosa* occurred, as was evident from the observed recovery in Φ_{PSII}, the cyanobacterium is expected to quickly recover. Moreover, the increased nutrient concentrations may enhance bacterial growth, hence reduce oxygen concentrations, which may lead to an increased release of phosphates from the lake's sediment, therefore aggravate the cyanobacterial nuisance rather than mitigate. Both low pH and relative high organic matter addition present major drawbacks that will not favor decisions by water authorities for applications.

In case of L-lysine, pH seems not to play a role as in the highest dose the pH of 7.4 was very close to those in the controls. Our study confirms results of several other studies that L-lysine can inhibit *M. aeruginosa*. We determined an EC_{50} of around 2 mg L^{-1} and a complete inhibition at 4.3 mg L^{-1}, which is close to values reported in the literature. However, there is considerable among strain and species variability in sensitivity reported in the literature (Table 6). Hence, the generalization "L-lysine is one of most interesting natural antialgal compounds (...) it shows an inhibitory effect against cyanobacteria at 0.6 mg L^{-1}" [18] finds no support in the literature. L-lysine seems to be effective against several nuisance species, but apparently not all, implying more experiments are needed, especially ones including field populations as growth forms of *M. aeruginosa* for example might differ substantially between the laboratory—mostly unicells—and the field—mainly as colonies, often embedded in mucous [36].

Table 6. Overview of effects or no effects of L-lysine on various *Microcystis* strains reported in the literature.

Species/Strain	Effect	Concentration *L*-lysine	Reference
M. aeruginosa N–87, N–98	complete lysis	1.2 mg L^{-1}	[38,39]
M. viridis N–102	"	"	"
M. aeruginosa N–88	lysis	2.4 mg L^{-1}	"
M. wesenbergii N–604	"	"	"
M. aeruginosa N-90	"	5 mg L^{-1}	"
M. viridis	killed	1 mg L^{-1}	[22]
M. aeruginosa N-44, N-298	inhibited, no complete lysis	10 mg L^{-1}	[39]
M. aeruginosa	no effect/inhibition	0.5/5 mg L^{-1}	[25]
M. aeruginosa 3 strains	decline/stabilization	5 mg L^{-1}	[40]
M. viridis NIES 102	inhibited	3.65 mg L^{-1}	[23]
M. novacekii	"	7.3 mg L^{-1}	"
M. aeruginosa TAC 71-1, *M. ichthioblabe*	significant growth	7.3 mg L^{-1}	"
M. wesenbergii N-104, N–111	hardly affected/no effect	10 mg L^{-1}	[38]

D-Lysine had no effect on *M. aeruginosa* in our study, which is in agreement to the findings of others [25]. However, considering the strong among strain/species variability observed in response to L-lysine, it is possible that D-lysine might inhibit growth of other species, such as *M. viridis* [22].

The effect of *S. miltiorrhiza* root extract in our study was less than reported in the literature, where extract of 800 mg L^{-1} caused an inhibition of 95% [20]. This difference could be due to different

extracting procedures: methanolic extracts that were reconstituted in DSMO [20], whereas in our study a water extract was used. The EC_{50} of the water extract in our study was above 640 mg L^{-1}, while others found EC_{50} values of 99 mg L^{-1} for ethyl-acetate, 111 mg L^{-1} for chloroform and 370 mg L^{-1} for methanol extracts [20]. An active compound in the ethyl-acetate extract has been identified as neo-przewaquinone A with a corresponding EC_{50} value of 4.7 mg L^{-1} [37]. It has been proposed that active ingredients with anti-algal activity should be extracted for formulation of a natural algicide [20]. Although the example of *S. miltiorrhiza* root extract shows purification can lower the EC_{50} value and thus application dose, this procedure results in a much higher price [16] and limited availability [18].

The highest dose of *M. oleifera* seed extract (64 mg L^{-1}) reduced CHLa-based growth only by 45% compared to controls and biovolume-based growth only by 26%. However, in another study 20 mg L^{-1} of seed extract caused strong population decline (−0.25 d^{-1}) [21]. A possible explanation might be the different initial cyanobacteria densities between the current study (94.4 µg L^{-1} CHLa) and [21] (13.4 µg L^{-1}), as EC_{50} values increase, and thus sensitivity decreases, with higher initial algal densities [41]. This seems further supported by the experiments in [21] where a blooming density of *M. aeruginosa* (~270 µg L^{-1} CHLa) was less affected by *M. oleifera* seed extract than a lower *M. aeruginosa* density (13.4 µg L^{-1} CHLa). Alternatively, the storage time between the two experiments could have influenced the effectiveness as has been found for the coagulating effect of *M. oleifera* seed extract [42]. The coagulating effect of *M. oleifera* seed extract is another important feature that was not included in the current study as flasks were continuously shaken. Up to 97% of the algae present in raw Nile water could be removed by coagulation with *M. oleifera* seed extracts [43]. Effective coagulation dose (50–500 mg L^{-1}) was reported comparable or higher than that of alum [44], but in general effective doses are rather high; the best removal efficiency of M. aeruginosa was achieved with concentration >200 mg L^{-1} [45], whereas for *M. protocystis* this was 50 or 175 mg L^{-1} depending on the turbidity [46]. Overall, it seems that under blooming conditions of cyanobacteria higher dose of seed extract is needed to reduce the bloom where coagulating properties could act in synergy with the cyanobactericidal effect. Such high dose might, however, come with some serious drawbacks of organic carbon and nutrient inputs that can cause odor, taste, and color problems and promote microorganisms growth [47,48].

Of the compounds examined in this study, L-lysine seemed the most promising fast acting curative intervention. It could decimate *M. aeruginosa* standing biomass at effective concentration of a few mg L^{-1} assuming field populations are equally sensitive as laboratory strains. Applying one ton in a dose of 5 mg L^{-1} could treat 2×10^5 m^3 *M. aeruginosa* infested water at a product cost of less than one cent per m^3 lake water (bulk price L-lysine around € 1500 per ton [49]). Nonetheless, the effect might be short-lived as Φ_{PSII} of *M. aeruginosa* recovered after six days in our study, while a pond treated with 7.3 mg L^{-1} L-lysine showed a strong effect on *Microcystis* during the first 4 days, slight recovery after 5 to 8 days, followed by growth reaching almost pretreatment *Microcystis* densities after 11 days [23]. Similarly, an enclosure of 100 m^2 treated with L-lysine (6.7–7.7 mg L^{-1}) showed a sharp decline in *Microcystis*, but recovery started after one week [50]. Therefore, if L-lysine would have to be applied every two—three weeks to control cyanobacteria, costs will rise substantially over the season. In addition, potential effects of organic carbon and nitrogen enrichment (adding L-lysine at 5 mg L^{-1} would imply a nitrogen enrichment of 0.96 mg N L^{-1}) on ecosystem functioning should be studied first. This is further corroborated by [23], who observed in a repetition of their pond experiment an

almost complete absence of *Microcystis* for almost two weeks, but after a short *Euglena* peak, another cyanobacterium, *Phormidium tenue*, had become dominant. Likewise, in enclosures treated with 20 mg L^{-1} L-lysine, *Microcystis* was strongly inhibited, but replaced by *Euglena* and *Anabaena*, while oxygen concentrations dropped to below 1 mg L^{-1} and ammonium went up to above 3 mg L^{-1} [39]. Treatment of a larger enclosure with L-lysine (6.7–7.7 mg L^{-1}) led oxygen drop to about 70% of that in the control [50]. Also lysis of the *M. aeruginosa* cells could enhance decomposition and reduce oxygen concentrations. Moreover, cell lysis is considered the major mechanism of release of microcystins into the surrounding water [51]. Hence, it remains to be seen if treatment with L-lysine delivers immediate improve of the water quality. Repeated additions of L-lysine are expected to influence water quality, while the replacement with other cyanobacteria [23,39] illustrates that the earlier mentioned among strain and species variability in sensitivity to L-lysine might present a major drawback in its applicability in cyanobacterial control.

Resuming, the results of our study gave no support to any of the tested plant extracts and amino-acid as promising candidates for curative application in *M. aeruginosa* bloom control. It therefore seems that water authorities should focus more on alternative treatments of (often positively buoyant) *Microcystis* blooms, and cyanobacteria blooms in general. Combined coagulant/flocculants and ballast [52] that entrap cyanobacteria cells/colonies as intact cells and sink them to the sediment [53] are one of such promising alternatives.

5. Conclusions

From the results of our controlled experiments to test extracts from *Fructus mume*, *Salvia miltiorrhiza* and *Moringa oleifera* as well as L-lysine and D-Lysine as curative measures to rapidly suppress the cyanobacterium *Microcystis aeruginosa* NIVA-CYA 43 we conclude that:

- *Fructus mume* extract at ≥240 mg L^{-1} and L-lysine at ≥4.3 mg L^{-1} caused a decline in *Microcystis aeruginosa* NIVA-CYA 43 biomass.
- The effect of *F. mume* extracts is most probably caused by low pH.
- *Salvia miltiorrhiza, Moringa oleifera* and D-Lysine had no relevant effect on *M. aeruginosa* biomass.
- All treatments had a short-lived effect only and did not cause a complete wipe-out of *M. aeruginosa*.
- Our results do not favor the application of the compounds tested as method to mitigate blooms of cyanobacteria in the field.

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Author Contributions

Miquel Lürling and Frank van Oosterhout designed the experiment, analyzed the data and wrote the manuscript.

Appendix

Table A1. Overview of experimental conditions, such as medium type, volume of experimental units, inoculum densities, light regime (intensity–light dark regime), temperature, shaking and duration, of studies performed to investigate the effect of plant extracts or lysine on growth of cyanobacteria. Mv = *Microcystis viridis*, Ma = *M. aeruginosa*, Mw = *M. wesenbergii*, Mi = *M. ichthioblabe,* Mn = *M. novacekii*, Pa = *Pseudanabaena articulata*, Pp = *Planktothrix perornata*.

Cyanobacteria	Medium	Volume	Initial density	Light (µmol m^{-2} s^{-1})	Temp	Shaking	Time	Reference
Mv	MA	5 mL	10^5 cells mL^{-1}	??	??	no	48 h	Kaya and Sano 1996 [22]
Ma, Pa, Pp	BG11	550 mL	??	53−12:12 L:D	22 °C	yes	96 h	Zimba *et al*., 2001 [25]
Ma, Mv, Mw	CB	10 mL	10^6 cells mL^{-1}	50−12:12 L:D	22 °C	no	48 h	Hehmann *et al*., 2002 [38]
Ma, Mi, Mn, Mv	CT	100 mL	??	2000 lx	30 °C	no	35 d	Takamura *et al*., 2004 [23]
Ma	BG11	0.2 mL	10^6 cells mL^{-1}	3000 lx−16:8 L:D	25 °C	no	7 d	Yang *et al*., 2009 [19]
Ma	WC	50 mL	13.4, 269 µg CHL*a* L^{-1}	~57−18:6 L:D	22 °C	40 rpm	11,14 d	Lürling and Beekman 2010 [21]
Ma	BG11	20 mL	10^6 cells mL^{-1}	90−14:10 L:D (?)	25 °C (?)	3 × d^{-1}	7 d	Yi *et al*., 2012 [20]
Ma	BG11	??	4.6×10^6 cells mL^{-1} (?)	20−12:12	30 °C	40 rpm	??	Liu *et al*., 2013 [24]

Conflicts of Interest

The authors declare no conflict of interest.

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