

ORIGINAL ARTICLE

Allelopathic interactions of linoleic acid and nitric oxide increase the competitive ability of *Microcystis aeruginosa*

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The frequency and intensity of cyanobacterial blooms are increasing worldwide with major societal and economic costs. Interactions between toxic cyanobacteria and eukaryotic algal competitors can affect toxic bloom formation, but the exact mechanisms of interspecies interactions remain unknown. Using metabolomic and proteomic profiling of co-cultures of the toxic cyanobacterium *Microcystis aeruginosa* with a green alga as well as of microorganisms collected in a *Microcystis* spp. bloom in Lake Taihu (China), we disentangle novel interspecies allelopathic interactions. We describe an interspecies molecular network in which *M. aeruginosa* inhibits growth of *Chlorella vulgaris*, a model green algal competitor, via the release of linoleic acid. In addition, we demonstrate how *M. aeruginosa* takes advantage of the cell signaling compound nitric oxide produced by *C. vulgaris*, which stimulates a positive feedback mechanism of linoleic acid release by *M. aeruginosa* and its toxicity. Our high-throughput system-biology approach highlights the importance of previously unrecognized allelopathic interactions between a broadly distributed toxic cyanobacterial bloom former and one of its algal competitors.

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Introduction

The frequency and intensity of toxin-producing cyanobacterial blooms (CBs) are increasing worldwide because of nutrient over-enrichment from human population growth, urbanization, agricultural and industrial expansion and the synergistic effects of global climate change (Paerl, 2009; Paerl and Paul, 2012; Rigosi *et al.*, 2014, 2015). This threatens the sustainability of freshwater ecosystems by negatively affecting water and habitat quality, drinking water supplies and food webs (Carmichael, 2001; Paerl, 2008). In the United States of America, eutrophication and resultant CBs have been estimated to cost from ten to hundreds of billions of dollars per year (Dodds *et al.*, 2008; Hamilton *et al.*, 2014). In China, numerous large, shallow lakes such as Lake Taihu and Lake Chaohu have suffered serious CBs from late

spring to autumn since the 1990s (Duan *et al.*, 2009; Jiang *et al.*, 2014). Clearly, CBs are a growing environmental, ecological and economic concern worldwide.

Freshwater CBs are promoted by increasing loads of nitrogen (N) and phosphorus (P), light, rising temperatures and altered precipitation patterns accompanying climate change (Paerl and Paul, 2012; Wang *et al.*, 2012). However, the biotic factors triggering toxic CBs are still poorly understood. Allelopathy, the production and release of biomolecules to inhibit or kill competitor species, can alter plankton community composition (Uronen *et al.*, 2007). The study of the roles and mechanisms of allelopathic interactions between algal competitors and cyanobacteria in bloom and toxicity dynamics are of increasing interest (Paerl *et al.*, 2001; Leão *et al.*, 2009; Beversdorf *et al.*, 2013). Even though pre-conditioned media of cyanobacteria cultures have been shown to inhibit the growth of algal competitors (Borowitzka, 2016), the underlying mechanisms remain largely unknown and the importance of allelopathic interactions in the establishment of toxic CBs is yet to be demonstrated.

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A range of secondary metabolites (for example, anatoxin, microcystin and microcarbonin A) have been isolated that can be toxic to biota (from zooplankton to humans) and negatively affect the growth of algal competitors; however, the complex network of interactions at the molecular levels has remained elusive (Borowitzka, 2016). One widely distributed toxic cyanobacterial genus that forms increasingly problematic blooms is *Microcystis*. *Microcystis* is known to inhibit photosynthesis in the dinoflagellate *Peridinium gatunense* via production of microcarbonin A (Sukenic *et al.*, 2002), and can also inhibit the growth of several green algal species with a as yet unknown mechanism (Ma *et al.*, 2015; Bittencourt-Oliveira *et al.*, 2015).

Here, we disentangled the complex network of interactions mediating the negative allelopathic effect(s) of *Microcystis aeruginosa* on one of its competitors, the green alga, *Chlorella vulgaris*—two dominant species often co-occurring in blooms (Cai *et al.*, 2014). We cultivated both species separated by a dialysis membrane in the laboratory and analyzed the effects on each other at the proteome and metabolome levels as well as on the biochemistry of the signaling molecules involved. We further explored the presence of cyanobacterial allelopathic molecules in Lake Taihu (China) and monitored changes in the microbial community structure during a *Microcystis* bloom.

Materials and methods

Algal culturing techniques

Axenic cultures of *C. vulgaris* (strain FACHB-24) and *M. aeruginosa* (strain FACHB-905) were obtained from the Institute of Hydrobiology (Chinese Academy of Sciences, Wuhan, China). These algae were grown in sterilized BG-11 liquid medium and incubated in an environmental chamber at 25 ± 0.5 °C under cool white fluorescent lights ($46 \mu\text{mol m}^{-2} \text{s}^{-1}$, 12 h light/12 h dark). The cell density of cultures was monitored by spectrophotometry daily, and 6 and 12 h time points were added on the first day. For the cell density measurements, we used a regression equation relating cell density ($y \times 10^5 \text{ ml}^{-1}$), as determined by a hemacytometer, and the optical density at 685 nm or OD685 (x), that is, $y = 162.1x + 1.4$ ($R^2 = 99.34\%$) for *C. vulgaris* (Qian *et al.*, 2013), and $y = 34.1x + 0.7$ ($R^2 = 99.17\%$) for *M. aeruginosa* (Qian *et al.*, 2010). All manipulations followed sterile techniques under a laminar flow hood.

Co-culture experimental design

A co-culture device was used to investigate allelopathic interactions between *M. aeruginosa* and *C. vulgaris* (Paul *et al.*, 2009; Poulson-Ellestad *et al.*, 2014). Permeable dialysis cellulose membrane tubing (pore size 12 kDa, 76 mm \times 49 mm; Sigma-Aldrich, St Louis, MO, USA) was used to separate the cultures of both species in co-culturing

experiments (Poulson-Ellestad *et al.*, 2014). Dialysis membrane tubing was cut into sections of ~ 35 cm, soaked overnight in deionized water and then rinsed eight times with deionized water according to the manufacturer's recommendations. One end of the dialysis membrane tubing was closed by tying a knot, autoclaved at 121 °C for 15 min, and the other end of the dialysis bag was suspended with a sterilized wire, which was tied to a glass rod. This device allowed for transport of allelopathic compounds from *M. aeruginosa* through the dialysis membrane over the course of the experiment, but cell contact between species was prevented (Paul *et al.*, 2009).

The sterilized dialysis system was kept under laminar flow until use. Preparation of algal cultures first involved transferring 200 ml of *C. vulgaris* cultures into a sterilized 1000 ml glass beaker. Then, a dialysis bag filled with 200 ml of fresh medium was inoculated with *M. aeruginosa* cultures via the Teflon tubing, and submerged into glass beakers containing the *C. vulgaris* cultures. The co-culturing device was agitated at 90 rotations per min on a laboratory shaker. Reverse co-cultures with *C. vulgaris* inside the dialysis bag and *M. aeruginosa* in the glass flask were also performed, and no differences in growth rates were measured in either of the co-culturing systems used. Control cultures were also prepared similarly, except that only one species was cultivated in both the dialysis bag and the beaker. Different initial cell densities of *M. aeruginosa* were tested (from 7.5×10^4 to 3×10^5 cells per ml), but the initial cell density of the *C. vulgaris* cultures remain constant to 1.8×10^6 cells per ml, resulting in ratios of initial cell density of *C. vulgaris* to *M. aeruginosa* of 6–24. Cell densities of *M. aeruginosa* were representative of cell density measured using a hemocytometer in Lake Taihu water samples for that species (from 1×10^4 cells per ml in oligotrophic situations and reaching 3×10^5 cells per ml at the bloom peak). The initial cell density chosen for *C. vulgaris* was within twofold of cell densities measured in Lake Taihu.

After 1–4 days of co-culture, the cell yield was measured and the relative growth inhibition of *C. vulgaris* was calculated with the following equation: $100 \times \text{OD control} - \text{OD treatment} / \text{OD control}$ (OD, optical density). OD control and OD treatment refer to the optical density at 685 nm of the control monocultures and of the treatment (co-cultures). Total nitrogen and total phosphorus were also measured at the start and at the end of the 4-day experiments. All subsequent measurements in algal cultures described below were done using co-cultures with a ratio of initial cell density of *C. vulgaris* to *M. aeruginosa* of 12:1 as well as with monocultures.

Cellular and subcellular structure analysis

The observation of the *C. vulgaris* cell surface microstructure was performed using a ZEISS SUPRA 55 high-performance field emission scanning

electron microscope (Carl Zeiss Meditec AG, Jena, Germany). The *C. vulgaris* cells cultured alone or co-cultured with *M. aeruginosa* for 4 days (*C. vulgaris* to *M. aeruginosa* initial cell density ratio of 12:1) were harvested by centrifugation at 7000 r.p.m. and fixed with 2.5% glutaraldehyde, 0.4% paraformaldehyde and 0.1 M sodium phosphate buffer (pH 7.2) for 24 h. The fixed algae were dehydrated in absolute alcohol and dried with hexamethyldisilazane, and the dry samples were coated with a 0.5–1 cm² area of conductive adhesive. Images of *C. vulgaris* cell surface were processed with the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Subcellular structures were visualized using transmission electron microscopy. Algal samples from the control and co-culture were fixed with 1.0% OsO₄ and embedded in epoxy resin. The samples were then cut into ultra-thin sections (70–90 nm) using a Reichert Ultracut S ultramicrotome and stained sequentially with uranyl acetate and lead citrate. A JEM-1230 transmission electron microscope (JEOL Ltd., Tokyo, Japan) was used to observe the ultrastructure of *C. vulgaris*.

Measurement of chlorophyll fluorescence parameters
MINI-PAM-II (Walz, Effectich, Germany) was used to measure F_v/F_m ratio (maximum photosynthetic efficiency of photosystem II), relative electron transport rates and photochemical quenching (qP) of *C. vulgaris* cultured alone or co-cultured with *M. aeruginosa* for 4 days (ratio of *C. vulgaris* to *M. aeruginosa* initial cell density of 12:1). The algae were placed in a dark environment for 15 min before the measurements of chlorophyll fluorescence parameters.

Metabolomic analyses

Approximately 0.3 g of *C. vulgaris* or *M. aeruginosa* was collected by centrifugation after 4 days of co-culture or of monocultures (control) for the analysis of the metabolome of each species. Algal cells were dissolved with 8 ml solvent (including methanol: water = 4:1 v/v; 400 μ l chloroform; 20 μ l internal standard) and sonicated for 6 min (power = 950 W, frequency = 20 kHz) on ice using a Ultrasonic cell crusher SCIENTZ-II D. Algal homogenates were then centrifuged at 12 000 r.p.m. for 15 min and supernatants were collected for further purification. In parallel, *C. vulgaris* cultures (grown in co-cultures with *M. aeruginosa* or in monocultures) were filtered on 0.7- μ m Whatman GF/F fiberglass filters at 0.1 MPa and the filtrates were collected for metabolomic analysis of the culture medium. All supernatants were then freeze-dried, dissolved in 500 μ l of 80% methanol and derivatized with 80 μ l N, O-Bis(trimethylsilyl) trifluoroacetamide (15 mg ml⁻¹) including 1% chlorotrimethylsilane. Each 2 μ l aliquot of the derivatized solution (algae homogenate or culture medium) was injected in a splitless mode

into the 7890B-5977A GC/MSD GC-MS system (Agilent Technologies, Palo Alto, CA, USA). Metabolite separation was carried out on a non-polar DB-5 capillary column (30 m \times 250 μ m I.D., J&W Scientific, Folsom, CA, USA), with high-purity helium as the carrier gas at a constant flow rate of 1.0 ml min⁻¹. The GC oven temperature program was as follows: beginning at 90 °C, followed by a 10 °C min⁻¹ ramp up to 180 °C, a 5 °C min⁻¹ ramp up to 240 °C, a 25 °C min⁻¹ ramp up to 290 °C and a final 11 min maintenance at 290 °C. The electron impact ion source was held at 260 °C with a filament bias of -70 V. Full-scan mode (m/z 50–450) was used, with an acquisition rate of 37.132 spectrum per s in the MS setting. All the MS data were finally analyzed using the ChromaTOF software (v 4.34, LECO, St Joseph, MI, USA).

Proteomic analyses

After 4 days of co-culture or in monocultures (control), ~0.3 g of algal fresh weight per sample of *C. vulgaris* or *M. aeruginosa* was collected by centrifugation, extracted and digested with trypsin for analysis of the proteome of each species by 8-plex isobaric tags for relative and absolute quantitation (iTRAQ). Equal amounts of iTRAQ-labeled (AB Science, Framingham, MA, USA) peptides from each group were mixed and resolved into 15 fractions by high-performance liquid chromatography followed by Q Exactive plus hybrid quadrupole-Orbitrap mass spectrometry (Thermo Fisher Scientific, Waltham, MA, USA). The resulting mass spectrometry/mass spectrometry data were processed using the Mascot search engine (v.2.3.0) and searched against a Uniprot *Chlamydomonas* database for *C. vulgaris* or a *M. aeruginosa* protein database for *M. aeruginosa* using MaxQuant (v1.0.13.13) with a false discovery rate of 1% and peptide ion score set to ≥ 20 . Mass error was set to 10 p.p.m. for precursor ions and 0.02 Da for fragment ions.

Functions were attributed to the quantified proteins using gene ontology annotation, Kyoto Encyclopedia of Genes and Genomes pathway analysis, functional enrichment and secondary structure analysis. Two replicates with tag swapping were conducted for each of the two biological replicates.

Detection of intracellular reactive oxygen species, nitric oxide content, superoxide dismutase activity, pyruvate, acetyl-CoA and linoleic acid cell contents

The amount of reactive oxygen species (ROS) per cell produced by *C. vulgaris* when co-cultured or not (control) with *M. aeruginosa* for 6 h to 4 days were measured using the cell-permeant probe 2',7'-dichlorodihydrofluorescein diacetate following the manufacturer's instructions (Beyotime Institute of Biotechnology, Haimen, China). Quantification of nitric oxide (NO) in *C. vulgaris* cells was detected with the fluorescent dye 4-amino-5-methylamino-

2',7'-difluorofluorescein diacetate (Beyotime Institute of Biotechnology, Nantong, China). Fifty milliliters of algal cultures were collected by centrifugation at 7000 r.p.m., and were used to measure superoxide dismutase (SOD) activity as well as pyruvate and acetyl-CoA contents (Qian *et al.*, 2008). SOD activity was measured using a SOD kit (Dojindo Laboratories, Kumamoto, Japan). The pyruvate and acetyl-CoA cell content was determined using commercially available pyruvate (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) and acetyl-CoA kits (Jiangsu, Comin, Suzhou, China) following the instructions provided by the manufacturer. Total linoleic acid (LA) concentrations at each station of Lake Taihu as well as in co-cultures of *M. aeruginosa* and *C. vulgaris* (4 days, treatment 12:1) and in control monocultures were analyzed by gas chromatography (Work *et al.*, 2010). Further information on ROS, NO and LA analyses is provided in Supplementary Materials and Methods.

Effects of exogenous NO on the growth and LA synthesis in monocultures and co-cultures

We evaluated the effects of different NO concentrations on cell growth and LA synthesis in co-cultures of both species or in monocultures of *M. aeruginosa* over 96 h. The NO concentrations were adjusted by adding different concentrations (0.05, 0.1, 0.25 and 0.5 μM) of sodium nitroprussate (SNP, Sigma Company, St Louis, MO, USA), a well-known NO donor or of 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (c-PTIO, Sigma Company; 0.05, 0.1, 0.25 and 0.5 μM), a NO scavenger, in the culture medium. We also tested the influence of c-PTIO (0.05, 0.1, 0.25, 0.5, 2 and 4 μM) addition on the growth of *C. vulgaris* in monocultures and on NO concentrations in these cultures. The effect of co-cultures of both species (initial cell density ratio of *C. vulgaris* to *M. aeruginosa* of 12:1) for 4 days on NO concentrations was also studied. The pH of each culture was adjusted to the same initial value before the experiments.

Effects of different putative allelochemical molecules on C. vulgaris growth and NO production

We tested the effects of different potential allelopathic molecules (LA, methyl palmitoleate, microcystin, mono (2-ethylhexyl-phtalate); 1 $\mu\text{g l}^{-1}$), which were previously identified as candidates mediating the allelopathic interactions of *M. aeruginosa* on *C. vulgaris*, on the growth of *C. vulgaris* in monocultures. The effects of LA addition (20–60 $\mu\text{g l}^{-1}$ LA; the most potent allelochemical) on the growth of *C. vulgaris* in monocultures and NO concentrations were also determined.

Field sampling

Lake Taihu is located in the Yangtze Basin, bordering Shanghai, Jiangsu, and Zhejiang provinces in the

southeastern part of China (30°55'40"–31°32'58"N; 119°52'32"–120°36'10" E). To compare the difference between aquatic microbial communities in the region of algal blooms and non-algal blooms, eight sampling transects encompassing the northern half of Lake Taihu were conducted during 10–11 August 2015, when a CB was present. Further information on eight sampling transects and microbial diversity analyses are provided in Supplementary Materials and Methods.

Statistical analyses

Statistical significance among data for the biochemical and physiological measurements was tested by analysis of variance followed by the Dunnett's *post hoc* test using the StatView 5.0 program (Statistical Analysis Systems Institute, Cary, NC, USA). When the probability (*P*) was less than 0.05, the means were considered significantly different. The metabolomic data sets were imported into the SIMCA-P+ 14.0 software package (Umetrics, Umeå, Sweden). All analyses were performed in triplicate unless otherwise stated. All data are presented in the tables and figures as mean \pm s.e.m.

Results and Discussion

M. aeruginosa inhibits C. vulgaris growth

C. vulgaris growth was significantly inhibited by *M. aeruginosa*. After 1–4 days of co-culture, the cell yield of *C. vulgaris* decreased by 9–24%, using initial cell number ratio of *C. vulgaris* to *M. aeruginosa* varying from 24:1 to 6:11 (Figure 1a). As the inhibitory effect of *M. aeruginosa* on the growth of *C. vulgaris* did not increase significantly when the initial cell number ratio decreased from 12:1 to 6:1, we chose the treatment with an initial cell number ratio of *C. vulgaris* to *M. aeruginosa* of 12:1 for all subsequent analyses involving co-cultures. In contrast, the growth of *M. aeruginosa* did not change significantly when co-cultured with *C. vulgaris* (data not shown). Over the 4-day experiment, total dissolved P and N concentrations remained constant over time and did not change significantly among treatments (co-cultures with a 12:1 *C. vulgaris* to *M. aeruginosa* initial cell density ratio and control monocultures of *C. vulgaris*; Supplementary Figure S1). Both species grew exponentially over the exposure time in the co-cultures or monocultures. This suggested that the inhibitory effect of *M. aeruginosa* on the growth of *C. vulgaris* was mediated by allelopathy rather than exploitation competition. Moreover, the mean pH was similar in the co-cultures and in monocultures of *C. vulgaris* (pH increasing from 7.1 to 7.6 from day 1 to day 4), indicating that *M. aeruginosa* allelopathy was not related to changes in the pH of the medium. This is in accordance with the reported allelopathic effects of *M. aeruginosa* on several other species of green algae (Bittencourt-Oliveira *et al.*, 2015; Ma *et al.*,

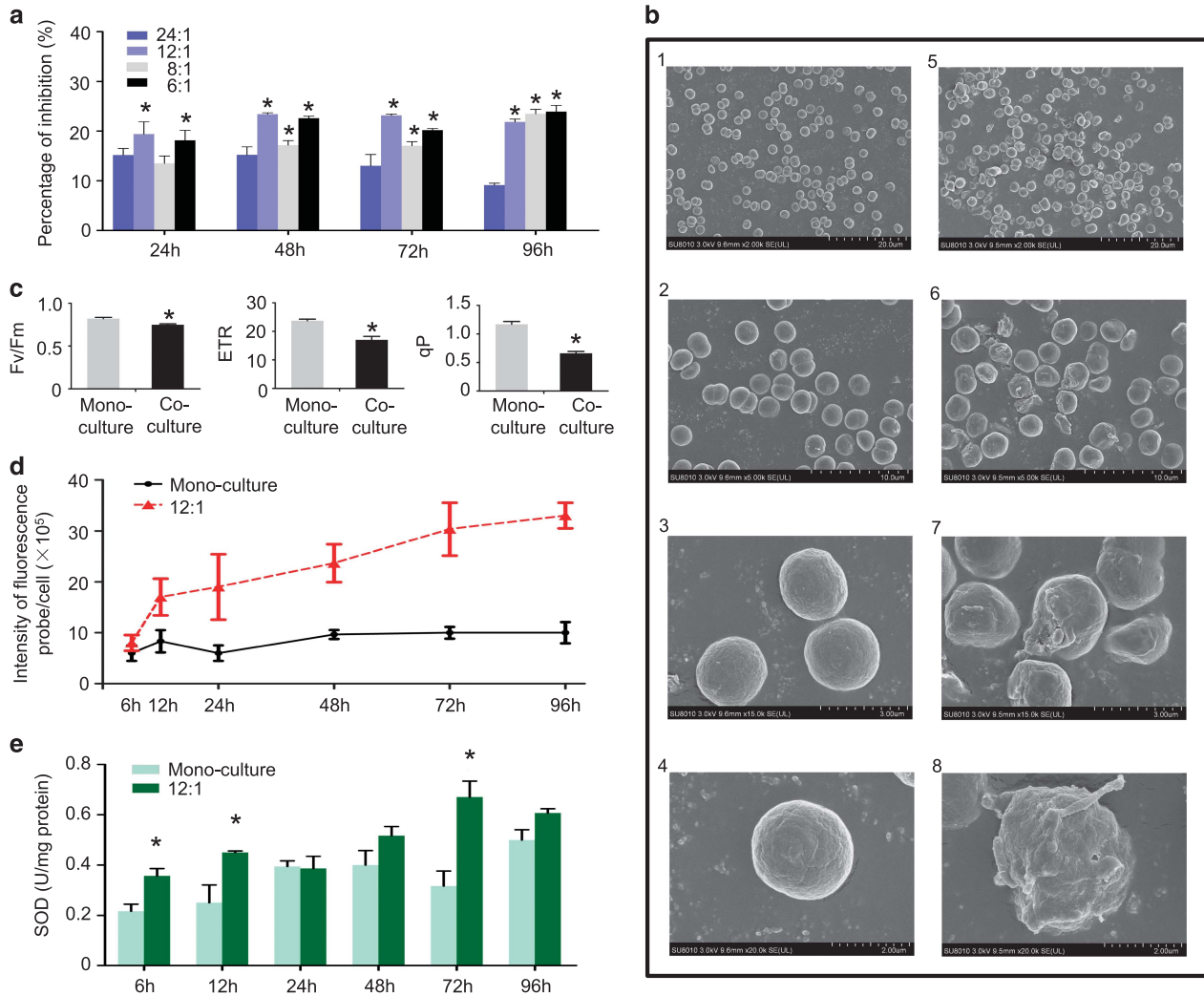


Figure 1 Effect of *Microcystis aeruginosa* on *Chlorella vulgaris* physiology. (a) Effects of *M. aeruginosa* allelopathy on the cell yield of *C. vulgaris* over 4 days with different initial cell density ratios of *C. vulgaris* to *M. aeruginosa*. Inhibition of cell yield (%) of *C. vulgaris* is calculated in co-cultures of *M. aeruginosa* and *C. vulgaris* compared to control monocultures of *C. vulgaris* in the dialysis system without the presence of *M. aeruginosa*. (b) The effect of *M. aeruginosa* allelopathy on the cell surface of *C. vulgaris* as visualized by scanning electron microscope (SEM). *C. vulgaris* monoculture (left images) and co-culture (right images). (c) Effects of *M. aeruginosa* allelopathy on photosynthetic efficiency (F_v/F_m), photochemical quenching (qP) and relative electron transport rate (ETR) of *C. vulgaris*. (d) Effects of *M. aeruginosa* allelopathy on ROS content of *C. vulgaris*. ROS content is expressed in relative units of fluorescence intensity per cell. (e) Effects of *M. aeruginosa* allelopathy on SOD activity of *C. vulgaris*. In b–f, an initial cell density ratio of *C. vulgaris* to *M. aeruginosa* of 12:1 was used in the co-culture and algae were cultivated for 4 days. Monoculture represents treatments with *C. vulgaris* grown in the dialysis system without the presence of *M. aeruginosa*. Asterisks represent statistically significant differences relative to control (* $P < 0.05$, analysis of variance (ANOVA)). Values are mean \pm s.e.m. ($n = 4$).

2015). Scanning electron microscope analyses also indicated that the abundance of dividing cells of *C. vulgaris* in the treatment (co-culture) group decreased by twofold relative to the control group (*C. vulgaris* in monoculture; Figure 1b). Approximately 20% of total cells were dividing in the control group, while ~10% of cells in the treatment (co-culture) group were dividing (Figure 1b), indicating that *M. aeruginosa* inhibited cellular division processes in *C. vulgaris*.

Proteomic and metabolomic response of algae

Co-cultures affected the synthesis of several proteins and metabolites in both species (Tables 1,2 and

Supplementary Table S1). We detected and quantified 41 differentially expressed proteins (18 under-expressed and 23 over-expressed) in *C. vulgaris* in response to the presence of *M. aeruginosa* (Table 1), whereas there were 25 differentially expressed proteins in *M. aeruginosa* (9 under-expressed and 16 over-expressed) when co-cultured with *C. vulgaris* (Supplementary Table S1). On the basis of our proteomics and metabolomics analyses, *M. aeruginosa* allelopathy has an impact on multiple metabolic pathways involved in energy generation and metabolism, including glycolysis, carbon fixation and fatty-acid biosynthesis. *M. aeruginosa* allelopathy significantly inhibited the photosynthetic capability of *C. vulgaris*, as evidenced by the decreased

Table 1 The effect of *M. aeruginosa* allelopathy on the proteome of *C. vulgaris*

Up or down expression	Protein names and categories	Ratio	P-value
<i>Photosynthesis</i>			
△	Cytochrome b6-f complex subunit 4	0.7	3.44E-03
▼	Photosystem I iron-sulfur center	0.4	6.82E-09
▼	Light-independent protochlorophyllidoreductase subunit N	0.7	2.04E-02
▼	Photosystem II protein D1	0.5	6.51 E-04
▼	Photosystem II reaction center protein H	0.7	1.04E-04
▼	Photosystem I reaction center subunit IX	0.6	2.4 E-02
▼	Photosystem I P700 chlorophyll a apoprotein A	0.2	2.28E-04
▼	Ribulose biphosphate carboxylase large chain	0.3	1.50E-02
▼	Ribulose biphosphate carboxylase small chain	0.4	2.27E-03
▼	Ferredoxin—NADP reductase	0.7	7.88E-05
△	Cytochrome <i>f</i>	1.3	2.97E-05
<i>Membrane transporters</i>			
▼	1-acyl-sn-glycerol-3-phosphate acyltransferase	0.5	4.23E-02
△	'Cytochrome b-c1 complex subunit Rieske, mitochondrial'	1.4	3.80E-02
<i>Protein homeostasis</i>			
▼	'30S ribosomal protein S8, chloroplastic'	0.3	3.88E-02
△	40S ribosomal protein S4	1.4	2.24E-07
△	40S ribosomal protein S6	1.4	5.95E-03
△	Ribosomal protein S7	1.4	6.56E-03
△	40S ribosomal protein S27	1.5	7.57E-03
△	40S ribosomal protein S8	1.4	3.03E-03
△	60S ribosomal protein L13	1.4	1.97E-05
△	40S ribosomal protein S18	1.6	4.41E-05
△	Peptidyl-prolylcis-trans isomerase (fragment)	1.5	1.06 E-02
<i>Energy</i>			
▼	Kinesin-like protein	0.7	3.47E-03
△	Glucose-6-phosphate 1-dehydrogenase	1.5	2.18E-02
△	ATP synthase F0 subunit beta	1.6	1.25E-04
△	Glyceraldehyde-3-phosphate dehydrogenase (fragment)	1.3	2.14E-02
△	Fructose-biphosphate aldolase	1.7	4.92E-04
△	Glycerol-3-phosphate dehydrogenase [NAD(+)]	1.4	1.21E-03
<i>The uptake and transportation of nitrogen</i>			
▼	Nitrate transporter NTR	0.4	2.90E-02
△	Nitrate reductase	1.6	4.77E-02
△	Ferredoxin-nitrite reductase	1.4	2.85E-03
<i>Stress and defense</i>			
△	Glutaredoxin	1.4	8.78 E-06
△	Cysteine synthase	1.6	2.24 E-03
<i>Signal transduction</i>			
△	Putative nitric oxide synthase	1.5	4.47E-02
<i>Stability and repair of DNA structure</i>			
▼	Histone H2B	0.4	3.29E-02
▼	Histone H4	0.5	8.67E-05
▼	Heat shock protein 90	0.6	6.41E-03
▼	GrpE protein homolog (fragment)	0.5	1.04E-02
<i>Nucleoside metabolic process</i>			
△	Adenylosuccinatelyase	1.5	9.78 E-07
<i>Fatty-acid metabolism</i>			
△	Acyl-coenzyme A synthase	1.4	2.65 E-07
△	3-Oxoacyl-[acyl-carrier-protein] synthase	1.3	3.31 E-03

Differentially expressed proteins that were significantly over (△) and under (▼) expressed by > 1.2 and < 0.83, respectively, in *C. vulgaris* exposed to *M. aeruginosa* allelopathy relative to protein expression in *C. vulgaris* monocultures are shown and categorized in metabolic pathways and cellular functions.

expression of 10 proteins involved in electron transfer in the chloroplast (for example, cytochrome b6-f complex subunit 4: 0.7-fold, photosystem I iron-sulfur center: 0.4-fold of the control, see Table 1) as

well as a threefold decrease in the expression of ribulose biphosphate carboxylase (Rubisco) large-chain protein. The negative allelopathic effect of *M. aeruginosa* on *C. vulgaris* photosynthesis also

Table 2 Cellular *M. aeruginosa* metabolites whose concentrations increased (Δ) or decreased (\blacktriangledown) when grown in co-cultures with *C. vulgaris*

Up or down expression	Candidate metabolite	Biological subcategory	Fold change	P-value
Δ	Methyl palmitoleate	Fatty-acid biosynthesis	7.7	1.67E-08
Δ	Linoleic acid	Fatty-acid biosynthesis	5.1	2.07E-03
Δ	Mono(2-ethylhexyl)phthalate	Fatty-acid biosynthesis	5.2	3.07E-03
\blacktriangledown	Oxalic acid	Tricarboxylic acid cycle/osmoregulation	0.5	1.21E-08
\blacktriangledown	Xanthine	Purine metabolism	0.6	2.11 E-03
\blacktriangledown	Phenylacetic acid	Phenylalanine metabolism	0.5	2.05E-08
\blacktriangledown	3-Hexenedioic acid	Organic acid metabolism	0.7	1.99E-05
\blacktriangledown	Pyrrole-2-carboxylic acid	Organic acid metabolism	0.8	5.12E-04
\blacktriangledown	Cytidine-monophosphated egrprod	Pyrimidine metabolism	0.7	3.34E-07
\blacktriangledown	3-hydroxy-L-proline	Arginine and proline	0.5	4.47E-05
\blacktriangledown	Erythrose	Metabolism glycolysis	0.8	2.17E-05
\blacktriangledown	Digitoxose	Glycolysis	0.6	2.35E-06

Metabolites that were significantly over (Δ) and under (\blacktriangledown) expressed in *M. aeruginosa* grown in co-cultures with *C. vulgaris* relative to control monocultures of *M. aeruginosa* are shown and categorized in metabolic pathways and cellular functions.

translated into a significant decrease of F_v/F_m , electron transport rate and qP by 8.7%, 28.3% and 43.6% relative to the control after 4 days of co-culture, respectively (Figure 1c), which are indicative of a decrease of photosynthetic efficiency of photosystem II (Qian *et al.*, 2010, 2011; Kiran Kumar Reddy *et al.*, 2014). A significant 3.3-fold decrease in glucose cell content, a 1.3- to 1.7-fold increase in the cell content of the two key glycolysis enzymes (glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase, ATP synthase F0 subunit beta; Table 1), as well as an increase in ATP synthase F0 subunit beta cell content were observed.

In concert, these results suggest that the enzymes involved in glucose oxidation and energy production (via the glycolytic pathway and the tricarboxylic acid cycle) were enhanced in *C. vulgaris*, potentially boosting NADPH synthesis. Moreover, we measured a 1.4-fold increase in ribulose-5-phosphate (an intermediate in the pentose phosphate pathway) concentration in *C. vulgaris* exposed to *M. aeruginosa* allelopathy as well as a 1.5-fold increase in the abundance of one key enzyme of the pentose phosphate pathway (that is, glucose-6-phosphate 1-dehydrogenase). This likely compensated for the decrease in photosynthetically produced NADPH (Kletzien *et al.*, 1994), which is the main reducing potential utilized for the generation of Asc and reduced glutathione (Noctor *et al.*, 1998) under stress conditions (Gill and Tuteja, 2010; Zhao *et al.*, 2015). The expression of enzymes involved in β -oxidation (acyl-coenzyme A synthase) and synthesis (3-oxoacyl-[acyl-carrier-protein] synthase) of fatty acids also significantly increased in *C. vulgaris* by 1.4- and 1.3-fold relative to control, respectively, in response to *M. aeruginosa* allelopathy. Although pyruvate cell content did not change significantly, acetyl-CoA (precursor of fatty acids) amount per cell increased significantly by 1.3-fold (Supplementary Figure S2).

Our results suggest that the synthesis of the biochemical machinery associated with both fatty-acid synthesis and oxidation was stimulated in *C. vulgaris*

exposed to *M. aeruginosa*. The perturbations that impacted *C. vulgaris* lead to a 1.9-fold increase of lignoceric acid production as well as to a decrease in LA and beta-mannosylglycerate by 0.7- and 0.5-fold, respectively (Supplementary Table S2). The induction of fatty-acid synthesis combined with the differential expression of a range of osmoprotective compounds (sarcosine, proline, DL anabasine, allo-inositol and 2-hydroxyvaleric acid; Supplementary Table S2) is suggestive of membrane-restructuring processes taking place in response to possible changes in membrane permeability.

Underexpression of the photosynthetic machinery combined with overexpression of enzymes involved in fatty-acid β -oxidation suggest that *C. vulgaris* became carbon-limited as recently demonstrated in the diatom *Thalassiosira pseudonana* exposed to the allelopathic molecules released by the toxic dinoflagellate *Karenia brevis* (Poulson-Ellestad *et al.*, 2014).

The significant decrease in expression of the proteins histone H4 and histone H2B (involved in nucleosome assembly and chromosomal segregation in the G2 phase) by 2- and 2.5-fold in *C. vulgaris* exposed to *M. aeruginosa* allelopathy relative to control monocultures is consistent with the SEM results showing cell division inhibition in *C. vulgaris* (Figure 1b). *M. aeruginosa* also induced atrophy of the *C. vulgaris* nucleus based on transmission electron microscopy analysis (Supplementary Figure S3).

C. vulgaris signaling compounds

ROS production in *C. vulgaris* increased by 3.3-fold in co-cultures with *M. aeruginosa* (Figure 1d), even though the expression of two antioxidant enzymes increased by 1.4- and 1.6-fold and SOD activity significantly increased between 6 h and 3 days of exposure (Figure 1e). In parallel, we measured a significant 2.5-fold decrease in nitrate transporter expression (Table 1). Hence, it is possible that the recently discovered ROS-induced decrease in N

assimilation in the diatom *Phaeodactylum tricornutum* (Rosenwasser et al., 2014) could also occur in *C. vulgaris* exposed to *M. aeruginosa*.

In contrast, expression of proteins involved in NO production in *C. vulgaris* increased (for example, nitrate reductase: 1.6-fold; putative NO synthase (putative NOS): 1.5-fold; see Table 1). These proteins are involved in reshuffling and recycling of internal nitrogen stores and the synthesis of NO, which is increasingly recognized as a key cell-signaling molecule in algae (Yordanova et al., 2010). Furthermore, our metabolomics data indicate that the cell content of sarcosine (a metabolite in the putative pathway leading to NO production via NOS) markedly increased by 10.7-fold (Supplementary Table S2). Such a high NO production, which is further measured and discussed later in the manuscript, could play several signaling roles. These include programmed cell death as demonstrated in *Chlamydomonas reinhardtii* (Yordanova et al., 2010), influence on cell development and division as demonstrated in higher plants (Ferrer and Ros Barcel, 1999) and could also influence *M. aeruginosa* allelopathy.

C. vulgaris affects *M. aeruginosa* metabolism

In the presence of *C. vulgaris*, *M. aeruginosa* increased the expression of proteins involved in the synthesis of fatty acids by 1.5- to 2.3-fold (long-chain fatty-acid CoA ligase, acyl-carrier protein, acyl-carrier protein), energy generation by 1.3- to 1.5-fold (for example, citrate synthase, ATP synthase subunits), photosynthesis by 1.4- to 1.6-fold (Psb28, Ycf3) and amino acid by 1.7-fold (threonine synthase; Supplementary Table S1). Since NO produced by *C. vulgaris* is the main signaling molecule affecting *M. aeruginosa* allelopathy and LA production (see below), it is also likely that NO affects energy generation via tricarboxylic acid cycle and photosynthesis. NO is indeed known to have a positive effect on plant development and differentiation (Tan et al., 2008; Fröhlich and Durner, 2011).

Interspecies network of interactions

At the metabolomics level, cell content of several fatty acids and metabolites particularly increased in *M. aeruginosa* co-cultured with *C. vulgaris*: methyl palmitoleate (7.7-fold), LA (5.1-fold), mono (2-

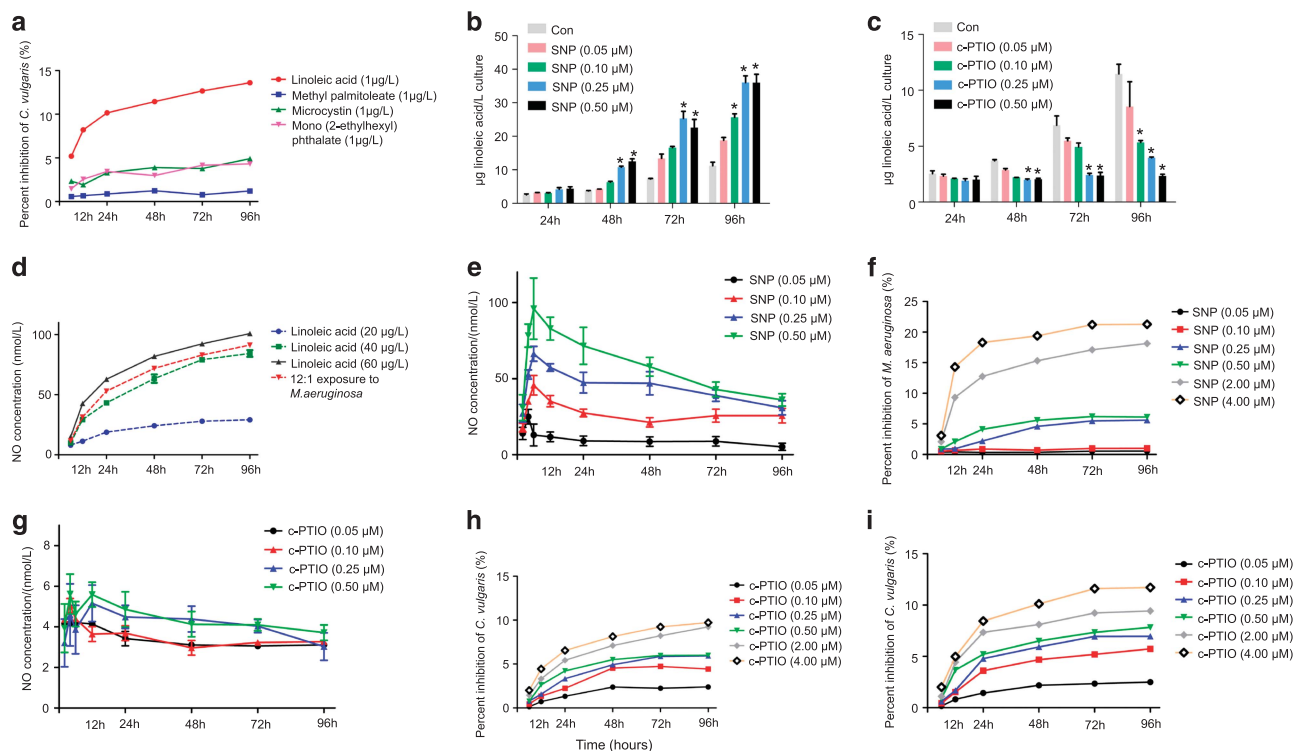


Figure 2 The effect of multiple exogenous metabolite additions on *C. vulgaris*. (a) Cell yield inhibition (%) of *C. vulgaris* in monoculture exposed to $1 \mu\text{g l}^{-1}$ linoleic acid (LA), methyl palmitoleate, microcystin and mono (2-ethylhexyl) phthalate for different periods of time. (b) The effect of SNP (NO donor) on LA production ($\mu\text{g l}^{-1}$) in monocultures of *M. aeruginosa*. (c) The effect of c-PTIO (NO scavenger) on LA ($\mu\text{g l}^{-1}$) production in *M. aeruginosa* cultivated in co-cultures. (d) NO concentrations (nmol l^{-1}) in *C. vulgaris* with monoculture and co-culture exposed to 20–60 $\mu\text{g l}^{-1}$ LA for different periods of time. (e) The effect of SNP on NO concentrations (nmol l^{-1}) in monocultures of *M. aeruginosa*. (f) The effect of SNP on cell yield of *M. aeruginosa* in monocultures. (g) The effect of c-PTIO on the cell yield of *C. vulgaris* cultivated in co-cultures. (h) Cell yield inhibition (%) of *C. vulgaris* alone exposed to 0.05–4 μM c-PTIO for different periods of time. (i) The effect of c-PTIO on the cell yield of *C. vulgaris* cultivated in co-cultures. Controls in a, h are *C. vulgaris* monocultures grown in the BG-11 medium without addition of metabolites or c-PTIO. Asterisks represent statistically significant differences relative to control ($*P < 0.05$, ANOVA). Values are mean \pm s.e.m. ($n = 3$).

ethylhexyl) phthalate (5.2-fold; Table 2) and microcystin (3.2-fold; independently analyzed by high-performance liquid chromatography). Furthermore, the concentration of methyl palmitoleate and LA in co-culture medium also increased by 8.7- and 9.4-fold relative to the control, respectively (Supplementary Table S3). To explore the allelopathic potential of these molecules, we added them individually to *C. vulgaris* monocultures. The cell yield of *C. vulgaris* after 4 days of exposure to $1 \mu\text{g l}^{-1}$ of LA, methyl palmitoleate, microcystin and mono (2-ethylhexyl) phthalate was inhibited by 13.6%, 1.2%, 4.9% and 4.3%, respectively, relative to untreated controls (Figure 2a), suggesting that LA could be an important allelopathic molecule produced by *M. aeruginosa*. The LA (Figures 2b and c, see control bars) and microcystin concentrations we used were representative of those measured in co-cultures. The fatty-acid LA has been previously shown to inhibit growth, as chlorophyll production of phytoplankton including green algae, although at high concentrations ($> \text{mg l}^{-1}$; Ikawa *et al.*, 1996; Chiang *et al.*, 2004; Wu *et al.*, 2006). However, our results suggest that trace LA concentrations can be an effective allelopathic molecule in short-term (4 days) growth experiments of *C. vulgaris*. The toxicity targets and action mechanisms of fatty acids remain poorly understood in phytoplankton and

other microorganisms (Desbois and Smith, 2010). LA may adsorb on or penetrate into the cell and then induce metabolic perturbations in *C. vulgaris*.

Analyses of LA production by *M. aeruginosa* in the presence or absence of NO (Figures 2b and c) also indicate that *M. aeruginosa*-induced NO production in *C. vulgaris* stimulates LA production by *M. aeruginosa* and enhances the negative allelopathic interaction with *M. aeruginosa*. Indeed, we measured LA concentration in cultures with *M. aeruginosa* alone (Figure 2b) and found that *M. aeruginosa* produced little LA (see control bars). However, in the presence of NO, LA concentration was induced (Figure 2b). Moreover, the LA concentration was higher in *M. aeruginosa* grown in co-cultures (Figure 2c, see control bars) than in *M. aeruginosa* monocultures alone (Figure 2b, see control bars). Quantitative analysis of NO production in laboratory algal cultures with/without addition of LA (Figures 2c and d) indicates that LA, which is produced by *M. aeruginosa* according to our metabolomic study, is the key molecule stimulating NO production by *C. vulgaris*. Indeed, under our experimental conditions, we observed that the addition of 20 to $60 \mu\text{g l}^{-1}$ LA (close to those measured in *M. aeruginosa* grown in co-cultures, see Figure 2c control bars after 4 days) in cultures of *C. vulgaris* alone increased NO production to 84.2 nm after

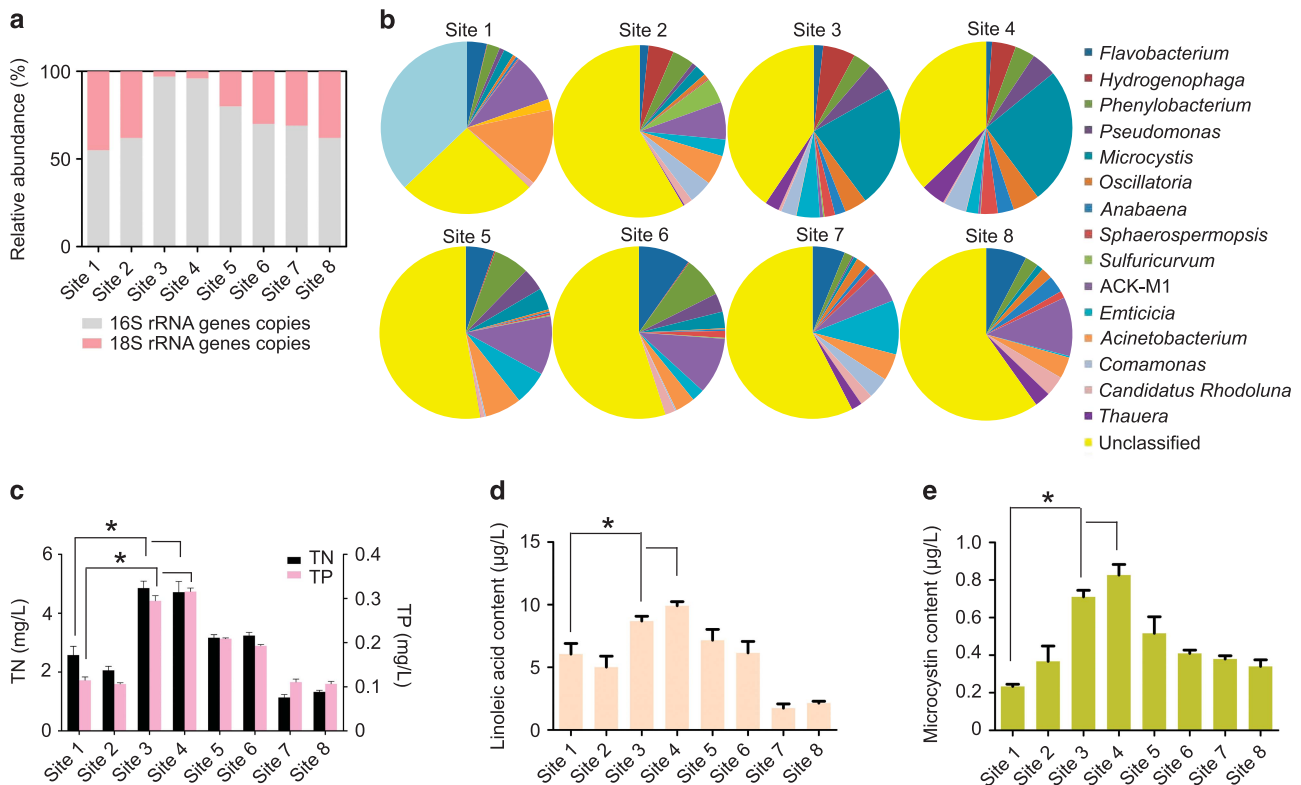


Figure 3 Aquatic microbial community profile analyses. (a) The mean relative abundance of 16S rRNA genes and 18S rRNA genes copies in surface samples collected in Lake Taihu. (b) Genera-level taxonomic distribution of the major aquatic microorganism community profile. Total nitrogen (TN) and total phosphorus (TP);(c), LA (d) and particulate microcystin concentration (e) in surface water of Lake Taihu over the sampling period (mean of the eight stations). Asterisks represent statistically significant differences between treatments ($*P < 0.05$, ANOVA). Data are means \pm s.e.m. ($n = 4$ per site).

4 days of exposure, which was close to the NO concentration produced by *C. vulgaris* after 4 days in co-cultures (91.2 nM NO; Figure 2d), indicating that LA is a key allelopathic molecule inducing NO release from *C. vulgaris*.

To further demonstrate that NO produced by *C. vulgaris* plays an essential role in stimulating *M. aeruginosa* allelopathy, we added SNP (NO donor) and c-PTIO (NO scavenger) to adjust the NO concentration in the medium of cultures of *M. aeruginosa* alone or in co-cultures. We found that NO concentration in *M. aeruginosa* increased in a concentration-dependent manner with SNP (Figure 2e). NO concentration in *M. aeruginosa* increased significantly after 6 h of exposure, and reached about 66.3 nM in the presence of 0.25 μM SNP (Figure 2e), which was similar to the NO concentration (~52.9 nM) measured in *C. vulgaris*, which was cultivated in co-cultures for 24 h (initial cell number ratio of *C. vulgaris* to *M. aeruginosa* = 12:1; Figure 2d). Experiments carried out in the presence of 0.25 μM SNP in monocultures of *M. aeruginosa* revealed that this NO concentration did not affect *M. aeruginosa* growth significantly (Figure 2f). At the same time, LA levels reached 36.1 μg l⁻¹ in the *M. aeruginosa* monoculture (Figure 2b), which represents a 3.2-fold increase relative to that of the control (without added SNP) after 4 days of exposure (Figure 2b). This increase was close to that found in our metabolomics analysis, where LA concentration in *M. aeruginosa* increased by 5.1-fold relative to the control after 4 days of co-culture (Table 2). This indicated that NO produced by *C. vulgaris* induced LA excretion from *M. aeruginosa*. The addition of c-PTIO into co-culture medium scavenged *C. vulgaris*-produced NO completely (Figure 2g). At the same time, the

total LA concentration in the *C. vulgaris* compartment of the co-culture system decreased by fivefold after 4 days of exposure to 0.5 μM c-PTIO relative to the c-PTIO-free control without addition of c-PTIO (Figure 2c).

Taken together, our results clearly showed that NO could be an important signaling molecule regulating the production of secondary metabolites (such as LA) in *M. aeruginosa*, indicating that *M. aeruginosa* allelopathy to *C. vulgaris* can be enhanced in the presence of *C. vulgaris*. Furthermore, NO scavenging by c-PTIO treatment (≥0.05 μM) could maintain optimal *C. vulgaris* growth alone (Figure 2h) or in co-culture experiments (Figure 2i), highlighting the fact that additional NO-scavenging processes could inhibit or suppress the negative allelopathic interactions between *M. aeruginosa* and *C. vulgaris* and that the strength of *M. aeruginosa* allelopathy should be maximized when both species are close to each other such as in aggregates or algal colonies.

Cyanobacteria allelopathy in nature

The absolute abundance of cyanobacterial 16S rRNA was much higher at stations 3 and 4 than in other stations of Lake Taihu (Supplementary Table S4). The absolute abundance of eukaryotic organisms (18S rRNA), however, increased much less than that of prokaryotic organisms at stations 3–4 (Supplementary Table S5). We also found that the relative abundance of eukaryotic algae decreased significantly in parallel with the CBs (Figure 3a). The operational taxonomic unit data revealed that ~70% of aquatic microorganisms belonged to cyanobacteria at stations 3 and 4, while cyanobacteria at other sampling sites occupied only 40% of the aquatic microbial community. At stations 3 and 4 the

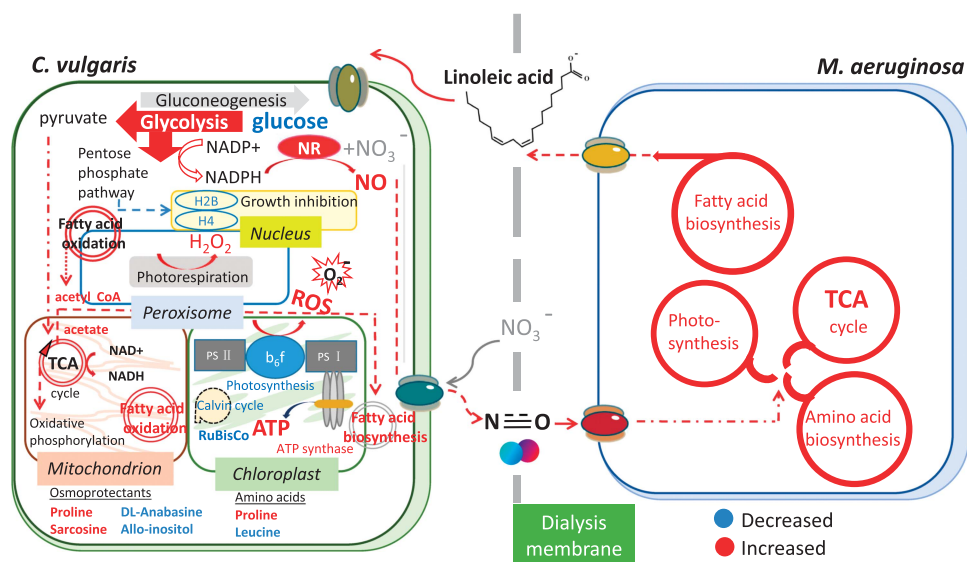


Figure 4 Conceptual model of the allelopathic interactions between *M. aeruginosa* and *C. vulgaris* at the cellular level. The network of cellular pathways, enzymes and metabolites in *C. vulgaris* due to *M. aeruginosa* allelopathy is derived from biochemical, metabolomics and proteomics analyses. The biochemical pathways (arrows) and metabolites in blue and red are increased and decreased, respectively.

percentage of *Microcystis* increased by approximately eightfold relative to other sites (Figure 3b). Although these differences in microbial abundance among lake stations could be related, at least partly, by an increase in nutrient (N and P) concentrations (Figure 3c), we found that *Microcystis* allelopathy could also shape the structure of the microbial community. Indeed, the concentration of the main allelopathic compound of *M. aeruginosa*, LA was approximately fivefold higher in *Microcystis* spp.-rich stations of Lake Taihu (8.5–10.4 µg LA/L) than at other stations, in which no *Microcystis* blooms occurred (~2 µg l⁻¹; Figure 3d). Microcystin concentrations were only between 0.2 and 0.8 µg l⁻¹ among the eight stations, indicating that it was not the main allelopathic compound in Lake Taihu (Figure 3e), which is in line with the findings of Babica et al. (2007).

Conclusions

Our high-throughput system biochemical and metabolic approach unraveled for the first time the intricate allelopathic interactions between a bloom-forming toxic cyanobacterial species and an algal competitor. We disentangled the network of interactions explaining the negative allelopathic effect of *M. aeruginosa* on *C. vulgaris*. *M. aeruginosa* inhibits the growth of *C. vulgaris* mainly via the release of LA and also takes advantage of NO, a cell-signaling compound produced by *C. vulgaris* (as schematized in Figure 4), which stimulated a positive feedback mechanism of LA release by *M. aeruginosa* as well as its inhibitory effect. Our results highlight the important and previously unrecognized interspecies allelopathic interactions involved in the development and persistence of toxic *M. aeruginosa* blooms in freshwater ecosystems.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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