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Determination and Occurrence of Retinoids in a Eutrophic Lake (Taihu Lake, China): Cyanobacteria Blooms Produce Teratogenic Retinal

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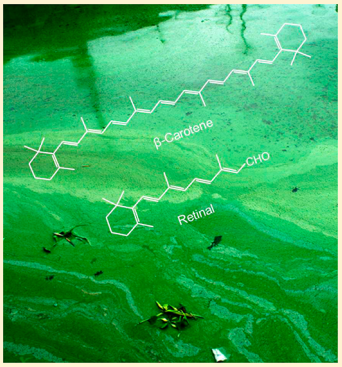
Supporting Information

ABSTRACT: Besides retinoic acids (RAs), some retinoids such as retinal (RAL) and retinol (ROH), which are considered as RA precursors in vertebrates, are also reported to be teratogenic agents. In this study we investigated four RA precursors including RAL, ROH, retinyl palmitate, and β-carotene in the eutrophic Taihu Lake, China, by developing a sensitive analytical method. RAL and β-carotene were widely detected in natural cyanobacteria blooms and lake water. Intracellular concentrations of RAL and β-carotene in blooms were 9.4 to 6.9×10^3 and 3.4 to 1.8×10^5 ng L^{-1}, respectively, and their concentrations in lake water were up to 1.4×10 ng L^{-1} (RAL) and 9.8×10^2 ng L^{-1} (β-carotene). The good correlation between intracellular concentrations of RAL and RAs implied that RAL was involved in the production of RAs by cyanobacteria blooms. Further examination of 39 cyanobacteria and algae species revealed that most species could produce RAL and β-carotene. The greatest amount of RAL was found in *Chlamydomonas* sp. (FACHB-715; 1.9×10^3 ng g^{-1} dry weight). As the main cyanobacteria in Taihu Lake, many *Microcystis* species could produce high amounts of RAL and were thought to greatly contribute to the production of RAL measured in the blooms. Productions of RAL and β-carotene by cyanobacteria were associated with species, origin location, and growth stage. The results in this study present the existence of a potential risk to aquatic animals living in a eutrophic environment from a high concentration of RAL in cyanobacteria blooms and also provide a clue for further investigating the mechanism underlying the biosynthetic pathway of RAs in cyanobacteria and algae.



INTRODUCTION

Retinoids are a class of compounds including vitamin A (retinol, ROH) and its natural analogues such as retinal (RAL) and retinoic acids (RAs), in addition to its synthetic derivatives.[1] During the past decade, environmental retinoids, especially RAs, have become an increasing concern due to their teratogenecities and a potential role in causing deformed amphibians in the field.[2−7] As one of the most potent known animal teratogens, RAs are generally thought of as vertebrate-specific hormones and can be transformed in vivo from RAL, ROH, retinyl esters (REs), and carotenoids,[1] which are collectively called RA precursors. The RA precursors are largely synthesized and widely used in medicines, nutrition supplements, feed additives, the food industry, and cosmetics. It has been extensively proved that an excess of some RA precursors can produce adverse effects in humans and animals.[8] For example, oral administration of retinyl palmitate (RPT) to cynomolgus monkey during early pregnancy can cause abortion and malformation in the embryo.[9] Also, ROH can cause limb malformation in tadpoles with tail amputation,[10] and exposure to RAL and ROH can generate various kinds of deformities in Zebrafish.[11]

Besides endogenous origin in vertebrates as well as artificial synthesis, RAs (including their analogues 4-oxo-RAs) were recently found in some laboratory-cultured cyanobacteria and algae species which are commonly observed in the aquatic environment, as well as in natural cyanobacteria blooms in Taihu Lake, China.[12] Although the biosynthetic pathway of RAs in cyanobacteria and algae remains unclear, considering the widespread presence of carotenoids in cyanobacteria and algae, and the reports of RAL acting as photoreceptors in some green algae and cyanobacteria,[13−16] it can be inferred that these RA precursors should contribute to the production of RAs in cyanobacteria and algae and should also exist in blooms in eutrophic environment. So far, only RAs have been studied for their occurrences and sources in the environment,[5,7,12] leaving the other retinoids, especially RA precursors, uninvestigated. A major hurdle for studying the RA precursors in the environment is the lack of a sensitive analytical method. While liquid chromatography coupled to mass spectrometry (LC-MS or LC-MS/MS) or an ultraviolet detector (LC-UV) is generally used for the determination of retinoids and carotenoids in biological samples,[17−26] an effective sample extraction procedure needs to be developed for the analysis of environmental water samples because of the low levels of retinoids in the environment.

To demonstrate the hypothesis that RA precursors exist in the blooms of a eutrophic environment, four RA precursors including RAL, ROH, RPT, and β-carotene were determined

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in Taihu Lake, the eutrophic lake we investigated previously,[12] by using a unique analytical method developed in this paper. Further examination of 39 representative cyanobacteria and algae species was carried out to determine the specific species that mainly contributed to the production of these RA precursors in cyanobacteria blooms.

MATERIALS AND METHODS

Chemicals and Materials. *all-trans*-ROH, *all-trans*-RAL, RPT, and β-carotene were purchased from Sigma (St. Louis, MO). *all-trans*-RAL-d_5 was obtained from Toronto Research Chemicals (Toronto, Canada). Structures of the analytes are shown in Figure S1, Supporting Information. The organic solvents used in this study were of HPLC grade and purchased from Fisher Chemical (Fair Lawn, NJ). HPLC-grade formic acid was provided by Dima Technology Inc. (Richmond Hill, Ontario, Canada). Ultrapure water was obtained by a Milli-Q synthesis water purification system (Millipore, Bedford, MA).

Field Sample Collection and Preparation. The field samples were taken from Taihu Lake, China, at the same sites during the same sampling campaign as reported in our previous paper[12] (Figure S2, Supporting Information). Briefly, 24 surface (0−0.5 m) samples containing water and cyanobacteria blooms were collected in October 2010, stored in amber bottles, and treated immediately after arrival at the laboratory.

The collected samples were vacuum filtered through GF/C filters (1.2 μm, Whatman), and the phytoplankton (mainly cyanobacteria) remaining on the filters were stored at −20 °C until analysis. A portion of 500 mL of filtrate was mixed with 330 mL of 2-propanol and spiked with RAL-d_5 as surrogate standard, and then the mixture was passed through an Oasis HLB cartridge (6 mL, 500 mg, Waters, Milford, MA) which had been conditioned previously with 6 mL of ethyl acetate, 6 mL of methanol, and 12 mL of 40% aqueous 2-propanol, at a flow rate of 8 mL min^{-1}. The cartridge was dried with nitrogen gas and then eluted with 8 mL of ethyl acetate. The extract was evaporated to dryness under a gentle stream of nitrogen gas at room temperature and reconstituted with 0.2 mL of acetonitrile before injecting into the LC-MS/MS system.

The cyanobacterial cells remaining on the filters were used to measure the intracellular retinoids. The filters were placed in a 50 mL centrifuge tube containing 15−20 mL of acetone and then sonicated in an ultrasonic water bath for 10 min. The cells were soaked into the solvent during the cell disruption to minimize the contact between cells and oxygen. After disruption of cells in an ice bath by use of an ultrasonic cell disrupter system (10−20 s pulse, repeat 10 times, 600−800 W) and centrifugation at 3000*g* for 10 min, the supernatant was transferred to another centrifuge tube, and then 15 mL of fresh acetone was added to the residues. The mixture was mechanically shaken for 5 min and subsequently centrifuged (3000*g*, 10 min). The combined supernatant was concentrated to a final volume of 5 mL by evaporation with nitrogen gas and then diluted with ultrapure water to 50 mL after addition of surrogate standard (RAL-d_5). The mixture was loaded onto the preconditioned Oasis HLB cartridge (6 mL, 200 mg, Waters), which was then dried with nitrogen gas. Analytes were eluted sequentially with 6 mL of ethyl acetate, 3 mL of acetone, and 8 mL of ethyl acetate containing 0.5% formic acid. The combined extracts were dried under nitrogen gas and reconstituted with 0.3 mL of acetonitrile before injection to the LC-MS/MS system.

Preparation of Cultured Cyanobacteria and Algae. Thirty-nine unialgal cultures of freshwater cyanobacteria and algae including 24 cyanophyta, 8 chlorophyta, 6 bacillariophyta, and 1 euglenophyta with the cell numbers more than 10^8 cell L^{-1} were purchased from the Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB), Chinese Academy of Sciences. To compare the species-specific production of RAL and β-carotene, the cultures of cyanobacteria and algae at a similar growth stage, i.e., logarithmic growth stage, were used for analysis. Two axenic cultures of cyanobacteria, *Microcystis aeruginosa* (PCC 7806) and *Synechococcus* sp. (PCC 7002), were from the Pasteur Culture Collection of Cyanobacteria (PCC), France. Laboratory cultivation of *Microcystis flos-aquae* (FACHB-1028) was as described in our previous paper.[12] Briefly, the cyanobacteria strain consisting of three replicates was grown in BG11 medium at 25 °C on a 14 h light/10 h dark cycle. At each sampling time, the cell counting was carried out (n = 6) for each sample and the number of cells (cell L^{-1}) was calculated.

The culture (35 mL) was centrifuged at 6000 rpm for 10 min, and then the cyanobacteria/algae pellets were resuspended in 2−3 mL of phosphate buffer (0.2 M, pH = 6.0) before performing cell disruption as described above. After adding RAL-d_5 as surrogate standard, the sample was extracted three times with 5 mL of hexane/chloroform (3:1, v/v) by mechanically shaking for 5 min, and the combined organic layer was evaporated to dryness under a gentle stream of nitrogen gas. The residue was dissolved in 350 μL of acetonitrile and then injected into the LC-MS/MS system.

Instrumentation. Chromatographic separation of ROH, RAL, and RPT was carried out on an ACQUITY ultra-performance liquid chromatography (UPLC) BEH Phenyl column (2.1 × 100 mm, 1.7 μm, Waters) at a flow rate of 0.3 mL min^{-1} at 40 °C, using a Waters ACQUITY UPLC system consisting of binary solvent manager and sample manager. Mobile phase A was methanol, and mobile phase B was 0.1% formic acid in ultrapure water. The gradient (with respect to mobile phase A) was as follows: 0−1 min, 20−70% A; 1−4 min, 70−100% A; 4−6 min, 100% A; 6−8 min, re-equilibration with 20% A. The injection volume was set to 5 μL.

Chromatographic analysis of β-carotene was carried out on an ACQUITY UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 μm, Waters) at a flow rate of 0.3 mL min^{-1} and 40 °C, using the same Waters UPLC system. Mobile phase A was methanol, and mobile phase B was 0.1% formic acid in ultrapure water. The gradient (with respect to mobile phase A) was as follows: 0−1 min, 20−70% A; 1−6 min, 70−100% A; 6−14 min, 100% A; 14−16 min, re-equilibration with 20% A. The injection volume was set to 5 μL.

The analysis was performed using a Waters Micromass Quattro Premier XE detector equipped with an electrospray ionization (ESI) source. All analytes were determined in positive ion mode (ESI+) by multiple reaction monitoring (MRM), and the optimized parameters are listed in Table S1, Supporting Information. The optimized parameters were as follows: capillary voltage, 3.40 kV; cone voltage, 25.00 V; extractor, 5.00 V; RF lens, 0.5 V; source temperature, 110 °C; desolvation temperature, 350 °C; cone gas flow, 50 L h^{-1}; desolvation gas flow, 600 L h^{-1}; multiplier voltage, 650 V. Argon (99.999%) was used as the collision gas, and argon pressure in the collision cell was kept at 3.5 × 10^{-3} mbar. All data were acquired and processed using MassLynx 4.1 software.

Quality Assurance and Quality Control (QA/AC). Confirmation of the target analytes in the environmental samples was accomplished by comparing the retention time (<2%) and the ratio of the two MRM ion transitions with those of standards

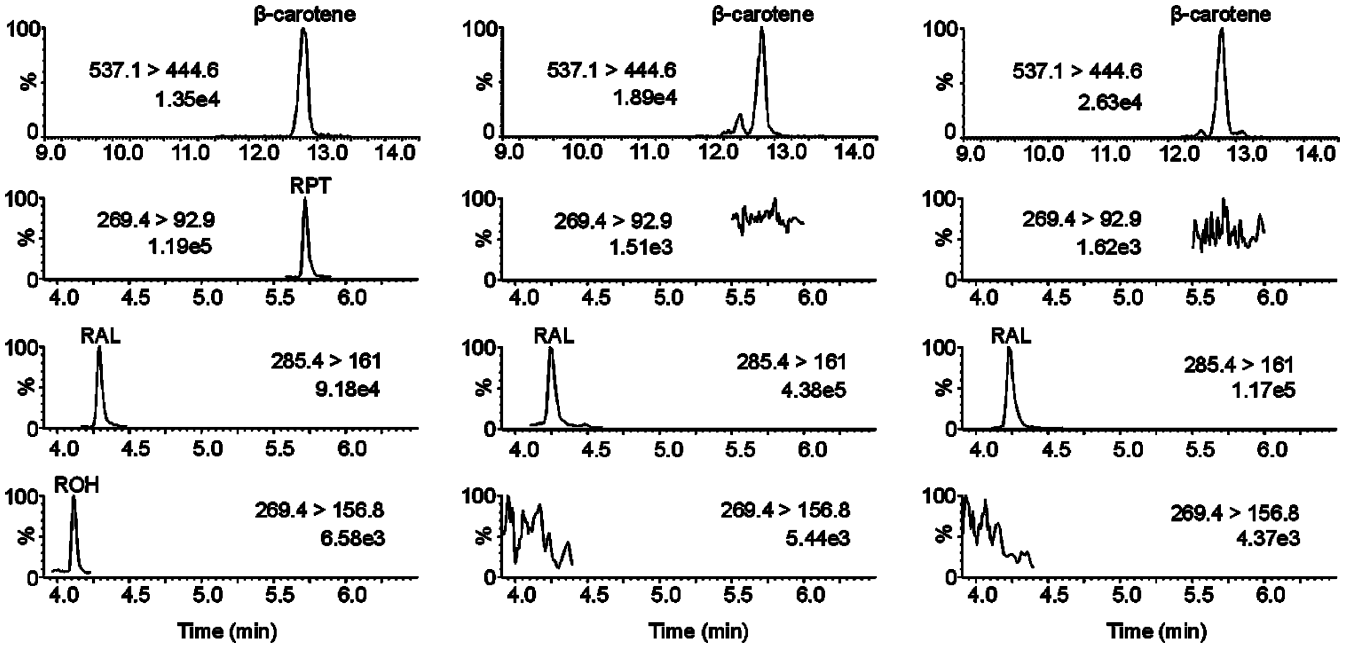

Figure 1. LC-MS/MS chromatograms of retinoids. (Left) Standards (20 μg L^{-1}). (Center) Bloom sample from Taihu Lake, China. (Right) Water sample from Taihu Lake, China. ROH: retinol; RAL: retinal; RPT: retinyl palmitate.

Table 1. Instrument and Method Validation Parameters for Determination of Target Retinoids

compound	LOD (pg) in reference	LOD (pg) in this study	curve (R^2)	recovery (%) in lake water	recovery (%) in cultured algal cells	MDL (ng L^{-1}) in lake water	MDL (ng g^{-1} DW) in cultured algal cells
ROH	7[20]	2	0.9991	91.0 ± 3.6	80.2 ± 1.0	0.4	2.0
RAL	28.4[19]	0.2	0.9976	97.3 ± 4.1	78.1 ± 1.5	0.04	0.3
RPT	377.9[22]	0.4	0.9904	62.6 ± 0.3	95.2 ± 2.1	0.1	0.6
β-carotene	8[26]	0.5	0.9956	61.9 ± 12.0	109.8 ± 7.2	0.08	0.2
RAL-d_5				99.7 ± 8.6	81.2 ± 6.4		

(<20%). Laboratory blanks were analyzed to assess potential sample contamination. Calibration curves were constructed for each retinoid standard solution from 0.02 to 1000 μg L^{-1}. The limit of detection (LOD) is calculated by applying the compound's calibration curve to the noise response of a sample to obtain a value which is then multiplied by a factor of 3. The method detection limit (MDL) was defined as the lowest concentration of the analyte in samples that could be analyzed with the described method generating a signal with a signal-to-noise ratio of 3. Recoveries of target compounds in lake water samples were determined by spiking mixed standards to the water samples and treating as described above with three replicates, while the mixed standards were added to the cultured algal cells for estimating the recoveries in algae samples. The signal suppression was used to evaluate the matrix effect by spiking mixed standards into the extracted lake water or cultured algal cells matrix and then comparing their responses between the spiked matrix and standard solution. The signal suppression was calculated by the following equation:

$$\text{signal suppression (\%)} = 100 \times [1 - (R_{sp} - R_b)/R_s]$$

where R_{sp} is the response of a compound in the sample extract, R_b is the response a spiked compound in unspiked sample extract, and R_s is the response of a spiked compound in the standard solution.

RAL-d_5 was used as the surrogate standard for analyzing RAL and β-carotene to compensate for the matrix-induced signal enhancement/suppression in ionization, the analyte losses during sample preparation, and variations in the instrumental response from injection to injection in LC-ESI-MS/MS analysis. Samples with high β-carotene concentration were diluted before LC-MS/MS analysis to maintain the response of β-carotene within the linear dynamic range of the instrument.

Because retinoids are much more stable under yellow light than under natural light conditions,[27] all handling of samples was done carefully in dark rooms under dim yellow light, and amberized containers were used whenever possible to prevent photoisomerization and photodegradation.[28] Additionally, all cartridges were wrapped in aluminum foil.

RESULTS AND DISCUSSION

Method Performance. Separation of ROH and β-carotene using a BEH Shield RP_{18} column in the HPLC system has been applied to the analysis of biological samples.[26] However, in our preliminary experiment we found that the BEH Shield RP_{18} column showed strong retention for RPT and β-carotene under a flow rate of 0.3 mL min^{-1} which resulted in a wide peak (0.3−0.4 min) and subsequently led to low sensitivity as well as long analysis time. Therefore, BEH Phenyl, BEH C_{18}, BEH C_8, and HSS T3 (Waters) were further evaluated to improve the chromatographic retention and separation of the target compounds. While both BEH Phenyl and BEH C_8 yield reasonable retention (all analytes were eluted within 7 min) and sharp peaks with peak width of 0.1 min for all analytes, the BEH Phenyl column allowed the baseline separation of ROH and RAL (Figure 1, left panel) and selected for the analysis of target

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compounds with the exception of β-carotene. The BEH C_{18} column was used for the quantitation of β-carotene in field samples and laboratory-cultured algae or cyanobacteria samples because the peak of β-carotene overlapped with unknown peaks when using the BEH Phenyl column for separation as shown in Figure S3, Supporting Information.

Although ESI was less used for analyzing retinoids in previous studies than atmospheric-pressure chemical ionization (APCI) because of a lack of linearity of detector response over a wide range of retinoid concentrations,[22,24] in this study we found that the ESI detector provided good sensitivities for the analytes and that the standard curves showed excellent linearity with a correlation coefficient (R^2) better than 0.99 over the concentration range of 0.4−1000 μg L^{-1} for ROH, 0.04−1000 μg L^{-1} for RAL, 0.08−40 μg L^{-1} for RPT, and 0.1−80 μg L^{-1} for β-carotene (Table 1), which was sufficient for environment analysis. The LODs in this study were 0.2−2 pg on the column (Table 1), which were 3.5−945 times lower than those in previous reports where LC-APCI-MS(MS) or HPLC-UV were used for the analysis.[19,20,22,26] The instrumental repeatability was determined by injecting the standard solutions three times during the same day (n = 3) and different days (n = 7), and the instrumental intra- and interday precision was generally below 10%.

So far there is no reported method for analyzing nonpolar RA precursors in environmental water samples. In this study we found that when using solid-phase extraction (SPE) to extract RA precursors from water, addition of organic solvent, such as acetone or 2-propanol, to the water sample could improve the recoveries of target analytes. As shown in Figure 2, the recoveries

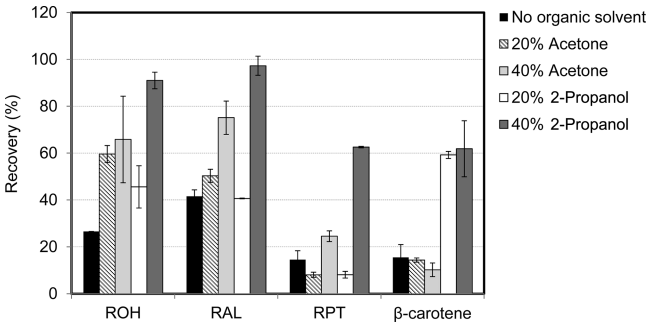

Figure 2. SPE recoveries of target retinoids with different percentages of organic solvent in lake water samples. ROH: retinol; RAL: retinal; RPT: retinyl palmitate.

of target analytes were limited to 14.6−41.6% without addition of any solvent. After addition of acetone or 2-propanol to the water sample, the recoveries of analytes were improved, and 40% 2-propanol in the water sample was found to provide better recoveries for all analytes compared with 40% acetone. Therefore, 2-propanol was selected as the additive solvent to the loading solution in the SPE procedure at a percentage of 40%. A similar phenomenon was observed by Ding et al., who found that the recoveries of 10:1 and 10:2 monosubstituted polyfluoroalkyl phosphates (monoPAP) were largely improved by addition of methanol (25%) to the water sample.[29] This result may be due to the fact that addition of organic solvent helped to improve the retention of some analytes on the HLB cartridge possibly by decreasing the polarity of the sample. Finally, the overall method recoveries for ROH, RAL, RPT, β-carotene, and RAL-d_5 in lake water samples were 91.0 ± 3.6%, 97.3 ± 4.1%, 62.6 ± 0.3%, 61.9 ± 12.0%, and 99.7 ± 8.6%, respectively (Table 1), and the signal suppressions were 5.2 ± 19.9% for ROH, 12.7 ± 3.7% for RAL, 0.05 ± 7.2% for RPT, and −17.7 ± 6.9% for β-carotene. The MDLs of the four target analytes in lake water samples were in the range of 0.04 to 0.4 ng L^{-1} (Table 1), which makes the method applicable for analyzing trace RA precursors in the aqueous environment. The recoveries of ROH, RAL, RPT, β-carotene, and RAL-d_5 in cultured algal cells were 80.2 ± 1.0%, 78.1 ± 1.5%, 95.2 ± 2.1%, 109.8 ± 7.2%, and 81.2 ± 6.4%, respectively, and the signal suppressions were 5.2 ± 19.9% for ROH, 17.3 ± 3.7% for RAL, −2.0 ± 20.6% for RPT, and −2.7 ± 16.5% for β-carotene. The MDLs of the four target analytes were between 0.2 to 2.0 ng g^{-1} dry weight (DW) (Table 1).

RAL and β-Carotene in Lake Water and Cyanobacteria Blooms. As the third largest freshwater lake in China, Taihu Lake is hypereutrophic and the issue of annual cyanobacteria blooms in Taihu Lake has drawn worldwide attention.[30,31] Recently, we found that teratogenic RAs including their analogues, 4-oxo-RAs, widely existed in Taihu Lake and were largely produced by cyanobacteria blooms.[12] In this study, four RA precursors (ROH, RAL, RPT, and β-carotene) were investigated in Taihu Lake, and only RAL and β-carotene were detected. Figure 1 (center and right panels) shows typical UPLC-MS/MS chromatograms of target retinoids in samples from Taihu Lake. The concentrations of RAL and β-carotene in lake water were up to 1.4 × 10 ng L^{-1} and 9.8 × 10^2 ng L^{-1}, respectively (Table 2). RAL and β-carotene were also detected in samples of cyanobacteria blooms, and their concentrations were

Table 2. Concentrations of RAL and β-Carotene in Water Samples and Bloom Materials Collected from Taihu Lake, China, and Cell Density of *Microcystis* at Each Sampling Site

	retinoids in water (ng L^{-1})		retinoids in blooms (ng L^{-1})		
sample	RAL	β-carotene	RAL	β-carotene	cell density of *Microcystis* (cell L^{-1})
1	1.4 × 10	9.0	2.4 × 10	8.6 × 10^2	7.8 × 10^6
2	8.8	8.5	1.4 × 10	1.2 × 10	1.1 × 10^6
3	8.7	9.0	1.1 × 10	2.0 × 10^2	1.3 × 10^7
4	8.4	1.2 × 10	9.4	9.2 × 10	1.3 × 10^6
5	8.1	9.9	9.6	3.4	1.2 × 10^6
6	8.4	6.4	6.2 × 10	2.0 × 10^3	3.1 × 10^8
7	6.5	9.5	3.4 × 10	2.3 × 10^3	2.3 × 10^8
8	1.0 × 10	3.0	3.6 × 10	8.3 × 10^2	2.1 × 10^8
9	8.9	1.5 × 10	2.5 × 10^2	7.0 × 10^3	3.9 × 10^8
10	4.7	6.8	3.2 × 10	9.1 × 10^2	1.1 × 10^8
11	9.9	9.2 × 10	3.0 × 10^3	9.7 × 10^4	4.7 × 10^9
12	8.0	6.8	4.4 × 10	8.6 × 10^2	3.4 × 10^8
13	3.5	1.4 × 10^2	2.3 × 10^2	5.2 × 10^3	4.5 × 10^9
14	6.1	6.4	5.2 × 10	1.6 × 10^3	3.1 × 10^8
15	8.7	7.1	2.7 × 10	2.6 × 10	9.4 × 10^7
16	5.9	3.3 × 10	9.6 × 10	4.7 × 10	6.3 × 10^8
17	5.4	1.2 × 10	4.2 × 10	1.0 × 10^3	1.4 × 10^9
18	2.6	9.1	1.1 × 10	2.9 × 10^2	3.3 × 10^6
19	8.8	6.1	1.5 × 10	3.8 × 10^2	3.9 × 10^7
20	0.5	2.8 × 10	2.0 × 10^3	4.2 × 10^4	1.1 × 10^{10}
21	0.6	3.2 × 10	1.6 × 10^3	2.2 × 10^4	9.1 × 10^{10}
22	ND[a]	6.3 × 10	6.0 × 10^3	6.2 × 10^4	4.5 × 10^{10}
23	0.6	7.1 × 10	9.3 × 10^2	1.3 × 10^4	2.4 × 10^{10}
24	2.4	9.8 × 10^2	6.9 × 10^3	1.8 × 10^5	3.4 × 10^{10}

[a]ND: not detected.

9.4 to 6.9 × 10^3 ng L^{-1} and 3.4 to 1.8 × 10^5 ng L^{-1}, respectively (Table 2). The levels of RAL and β-carotene in Taihu Lake were found to be significantly higher than levels of RAs (up to 2.0 × 10 ng L^{-1} in lake water and 0.4 to 4.2 × 10^2 ng L^{-1} in blooms) reported in our previous paper.[12]

During the sampling season, the dominant cyanobacteria genus in Taihu Lake was *Microcystis* (Figure S4, Supporting Information), consisting primarily of three species: *Microcystis aeruginosa*, *Microcystis wesenbergii* and *Microcystis flos-aquae* (Figure S5, Supporting Information), in which *Microcystis aeruginosa* and *Microcystis flos-aquae* were mainly responsible for the production of RAs by cyanobacteria blooms.[12] The cell density of *Microcystis* at each sampling site is listed in Table 2. Similar to the result in our previous study in which intracellular concentrations of RAs in blooms correlated with the cell densities of *Microcystis* (R^2 = 0.29, p < 0.01),[12] the intracellular concentrations of RAL in blooms also showed a correlation with the cell densities of *Microcystis* (R^2 = 0.32, p < 0.01). Accordingly, it is reasonable to hypothesize that *Microcystis* in blooms would also be capable of producing RAL in addition to RAs.

It has been reported that RAL can be synthesized in cyanobacteria such as *Synechocystis* and *Nostoc* through an asymmetric cleavage of β-apo-carotenals catalyzed by carotenoid oxygenases.[32,33] In this study, the excellent correlation between the intracellular RAL and β-carotene (R^2 = 0.83, p < 0.01, Figure 3a) further suggested that RAL would be transformed from β-carotene or other kinds of carotenoids in blooms. While RAs in vertebrates were directly converted from RAL which was metabolized from either ROH by dehydrogenation or carotenoids (e.g., β-carotene) via oxidative cleavage,[34] little information is now available on the biosynthetic pathway of RAs in cyanobacteria and algae although RAs and their metabolites have been detected in cyanobacteria.[12,35] Here the detection of RAL in cyanobacteria blooms and the good correlation between the intracellular RAL and RAs (R^2 = 0.94, p < 0.01, Figure 3b) provide a clue to support a hypothesis that RAL could be involved in the production of RAs in cyanobacteria and algae, and further studies on the biotransformation from RAL to RAs in these phytoplankton are needed.

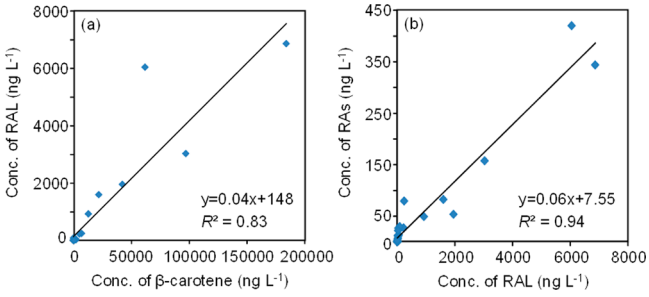

Figure 3. Linear correlation between (a) intracellular concentrations of RAL and β-carotene and (b) intracellular RAs and RAL in cyanobacteria blooms in Taihu Lake, China. RAL: retinal; RAs: retinoic acids.

RAL and β-Carotene in Cultures of Cyanobacteria and Algae. To further determine which cyanobacteria species were mainly responsible for the production of RAL in Taihu Lake, 39 species of freshwater cyanobacteria and algae (Table 3), including those observed in Taihu Lake and some other species commonly observed in aquatic environments, were studied to determine their potential of producing RA precursors. Similar to the results in Taihu Lake, only RAL and β-carotene were detected, while ROH and RPT were not found in any cyanobacteria/algae species. Nearly all species except *Synedra* sp. (FACHB-1131; Table 3) investigated in this study could produce RAL. The greatest amount of intracellular RAL was found in *Chlamydomonas* sp. (FACHB-715; 1.9 × 10^3 ng g^{-1} DW), followed by *Microcystis flos-aquae* (FACHB-1028; 9.7 × 10^2 ng g^{-1} DW), *Microcystis* sp. (FACHB-1009; 3.3 × 10^2 ng g^{-1} DW), *Microcystis ichthyoblabe* (FACHB-1294; 2.8 × 10^2 ng g^{-1} DW), *Microcystis viridis* (FACHB-1284; 2.5 × 10^2 ng g^{-1} DW), *Ankistrodesmus* sp. (FACHB-1044; 2.5 × 10^2 ng g^{-1} DW), and *Microcystis aeruginosa* (FACHB-912; 2.2 × 10^2 ng g^{-1} DW). Because all cultures obtained from FACHB were unialgal, an additional analysis of axenic cyanobacterial cultures was conducted. The result demonstrated that the axenic strains of the cyanobacteria *Microcystis aeruginosa* (PCC 7806) and *Synechococcus* sp. (PCC 7002) also produced RAL, and the concentration was 28.6 and 21.8 ng g^{-1} DW, respectively. For β-carotene, it could be detected in all cyanobacteria and algae species with concentrations from 1.5 × 10 (*Navicula* sp. FACHB-1054) to 1.8 × 10^4 (*Euglena gracilis* FACHB-848) ng g^{-1} DW (Table 3). Among these species, the concentration of β-carotene in *Microcystis aeruginosa* was 1.4 × 10^3 to 6.3 × 10^3 ng g^{-1} DW (Table 3), which was lower than those reported previously in *Microcystis aeruginosa* (7.4 × 10^3 ng g^{-1} DW).[36] When the results of the laboratory and field studies are considered together, it can be concluded that the dominant cyanobacteria species in Taihu Lake, including *Microcystis aeruginosa*, *Microcystis wesenbergii*, *Microcystis flos-aquae*, and *Microcystis ichthyoblabe*, can make great contributions to the production of RAL measured in blooms, considering the predominant abundance of *Microcystis* in cyanobacteria blooms (Figure S4, Supporting Information) and high production of RAL by laboratory-cultured *Microcystis* species (Table 3).

The production of RAL by cyanobacteria was dependent on the species, even within the same genus. For instance, the concentration of RAL in *Microcystis flos-aquae* (FACHB-1028) was 3- to 14-fold greater than those in other six *Microcystis* species (Table 3). The origin of the cyanobacteria was also found to have great effect on the production of RAL. For example, FACHB-1039 from Dianchi Lake and FACHB-1171 from Taihu Lake were both *Aphanizomenon flos-aquae*, but the intracellular concentration of RAL in FACHB-1039 (1.4 × 10^2 ng g^{-1} DW) was nearly 6.5-fold greater than that in FACHB-1171 (2.1 × 10 ng g^{-1} DW). Also, productions of RAL and β-carotene were found to be associated with the growth phase of cyanobacteria. As shown in Figure 4, the intracellular concentrations of RAL and β-carotene in *Microcystis flos-aquae* (FACHB-1028) increased as a function of culture time and reached the highest at 53 days. Although this growth-dependent production was also observed in RAs,[12] different trends for RAL and β-carotene were found: while the production of RAs remained constant after reaching the concentration peak at 60 days, the concentrations of RAL and β-carotene dropped abruptly after 53 days and then became constant after 70 days.

Widely occurring cyanobacteria/algae blooms in aquatic environments have been recognized as an important health risk for both humans and ecosystems due to their productions of various toxins.[37] Recently, teratogenic RAs were found to exist in a eutrophic lake in China and were mainly produced by *Microcystis* species in cyanobacteria blooms. In this study we found that two RA precursors, RAL and β-carotene, also existed in cyanobacteria blooms in the same lake and that *Microcystis* species were also able to produce high amounts of RAL in

Table 3. Intracellular Concentrations of RAL and β-Carotene in Cultured Cyanobacteria and Algae Species

				concentration (ng g^{-1} DW)	
phylum	species	origin	density (g DW L^{-1})	RAL	β-carotene
Cyanophyta	*Merismopedia* sp. FACHB-286	United States	1.10	1.7×10^2	3.2×10^3
	Synechocystis sp. FACHB-898	France	1.68	7.6×10	4.4×10^3
	Microcystis sp. FACHB-1009	Dianchi Lake (China)	1.82	3.3×10^2	1.4×10^3
	Microcystis flos-aquae FACHB-1028	Taihu Lake (China)	1.85	9.7×10^2	4.3×10^3
	Microcystis aeruginosa FACHB-905	Dianchi Lake	1.90	8.4×10	1.4×10^3
	Microcystis aeruginosa FACHB-912	Taihu Lake	2.06	2.2×10^2	6.3×10^3
	Microcystis wesenbergii FACHB-908	Dianchi Lake	1.83	6.8×10	2.4×10^3
	Microcystis viridis FACHB-1284	Dianchi Lake	1.90	2.5×10^2	4.8×10^3
	Microcystis ichthyoblabe FACHB-1294	Taihu Lake	1.62	2.8×10^2	9.0×10^3
	Chroococcus sp. FACHB-193	Donghu Lake (China)	2.33	1.3×10	3.7×10^2
	Nostoc sp. FACHB-1042	China	2.38	7.9×10	1.7×10^3
	Nostoc sp. FACHB-1043	China	2.60	8.6	2.0×10^2
	Anabaena cylindrical FACHB-1038	Dianchi Lake	1.77	7.2×10	7.2×10^2
	Anabaena sp. FACHB-1088	Nanhu Lake (China)	1.82	1.4×10^2	6.0×10^2
	Anabaena flos-aquae FACHB-1092	Dianchi Lake	1.74	3.7×10	1.8×10^3
	Pseudanabaena sp. FACHB-1277	Xionghe Reservoir (China)	1.78	1.0×10^2	8.3×10^3
	Oscillatoria tennuis FACHB-247	United States	2.22	7.3	8.4×10^2
	Oscillatoria planctonica FACHB-708	Donghu Lake	2.00	5.7×10	1.7×10^3
	Oscillatoria raciborskii FACHB-881	Dianchi Lake	1.79	7.5×10	1.6×10^3
	Phormidium mucicola FACHB-723	China	2.41	6.5×10	1.0×10^3
	Phormidium sp. FACHB-1099	Dianchi Lake	2.02	7.9×10	8.6×10^2
	Aphanizomenon flos-aquae FACHB-1039	Dianchi Lake	1.80	1.4×10^2	1.0×10^3
	Aphanizomenon flos-aquae FACHB-1171	Taihu Lake	1.78	2.1×10	2.2×10^3
	Aphanizomenon issatschenkoi FACHB-1247	Donghu Lake	2.16	1.7×10^2	7.2×10^3
Chlorophyta	*Chlamydomonas* sp. FACHB-715	Donghu Lake	1.14	1.9×10^3	2.5×10^3
	Chlorella sp. FACHB-1067	China	2.15	2.6×10^2	1.2×10^3
	Pediastrum sp. FACHB-704	China	1.53	9.3×10	7.5×10^2
	Scenedesmus sp. FACHB-933	Dianchi Lake	2.62	4.5×10	9.3×10
	Selenastrum capricornutum FACHB-271	United States	1.85	7.9×10	3.3×10^3
	Ankistrodesmus sp. FACHB-1044	Dianchi Lake	2.27	2.5×10^2	2.6×10^3
	Oedogonium sp. FACHB-686	China	1.73	1.9×10^2	1.4×10^3
	Staurastrum sp. FACHB-719	Taiping Lake (China)	2.10	1.0×10^2	3.9×10^3
Bacillariophyta	*Cyclotella hebeiana* FACHB-1030	Guanqiao Pond (China)	1.13	1.3×10^2	1.8×10^2
	Nitzschia sp. FACHB-510	China	1.54	2.9×10	2.8×10^2
	Navicula sp. FACHB-1054	Taihu Lake	1.42	1.7×10	1.5×10
	Melosira varians FACHB-1034	Guangzhou (China)	1.38	1.4×10^2	4.1×10
	Synedra sp. FACHB-1131	Donghu Lake	0.90	ND	3.2×10^3
	Cymbella sp. FACHB-844	China	38.39	2.6	1.5×10^2
Euglenophyta	*Euglena gracilis* FACHB-848	Japan	2.26	1.4×10^2	1.8×10^4

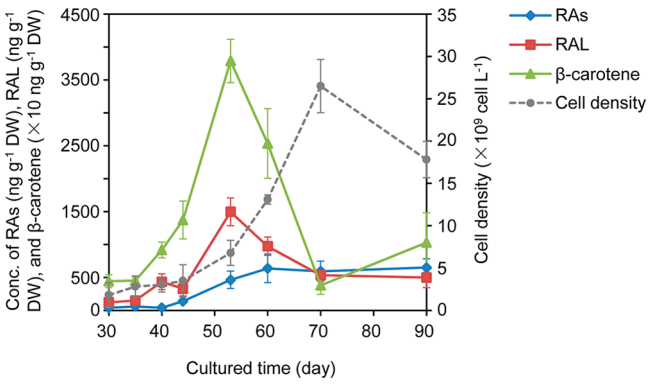

Figure 4. Changes in intracellular productions of RAs, RAL, β-carotene, and cell density with the growth of *Microcystis flos-aquae* (FACHB-1028). RAL: retinal; RAs: retinoic acids.

blooms. Further investigation showed that RAL widely existed in various kinds of laboratory-cultured cyanobacteria and algae species which were commonly observed in blooms and aquatic environments. RAL in eutrophic waterbodies may pose a risk to animals living in the water and feeding on algae. It was reported that RAL could induce malformations such as edema, brain deformities, duplication of the otic placodes and otoliths, and a shortened and bent tail in the early development of zebrafish with a maximal acceptable toxicant concentration (MATC) of about 70 nM (20 μg L^{-1}).[11] Because some eutrophic lakes and reservoirs are used as sources of drinking water and food, such as freshwater clams and snails, RAL may also pose a risk to the health of humans. Also, the results in the present study imply that the biotransformation from RAL to RAs exist in cyanobacteria and algae, which provides an important clue for further investigating the mechanism underlying the biosynthetic pathway of RAs in cyanobacteria and algae.

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■ ASSOCIATED CONTENT

Supporting Information

Additional information include Figures S1, S2, S3, S4, and S5 and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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