Cyanobacteria blooms produce teratogenic retinoic acids

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Deformed amphibians have been observed in eutrophic habitats, and some clues point to the retinoic acids (RAs) or RA mimics. However, RAs are generally thought of as vertebrate-specific hormones, and there was no evidence that RAs exist in cyanobacteria or algae blooms. By analyzing RAs and their analogs 4-oxo-RAs in natural cyanobacteria blooms and cultures of cyanobacteria and algae, we showed that cyanobacteria blooms could produce RAs, which were powerful animal teratogens. Intracellular RAs and 4-oxo-RAs with concentrations between 0.4 and 4.2×10^2 ng/L were detected in all bloom materials, and extracellular concentrations measured in water from Taihu Lake, China, were as great as 2.0×10 ng/L, which might pose a risk to wildlife through chronic exposure. Further examination of 39 cyanobacteria and algae species revealed that 32 species could produce RAs and 4-oxo-RAs ($1.6–1.4 \times 10^3$ ng/g dry weight), and the dominant cyanobacteria species in Taihu Lake, *Microcystis flos-aquae* and *Microcystis aeruginosa*, produced high amounts of RAs and 4-oxo-RAs with concentrations of 1.4×10^3 and 3.7×10^2 ng/g dry weight, respectively. Most genera of cyanobacteria that could produce RAs and 4-oxo-RAs, such as *Microcystis*, *Anabaena*, and *Aphanizomenon*, often occur dominantly in blooms. Production of RAs and 4-oxo-RAs by cyanobacteria was associated with species, origin location, and growth stage. These results represent a conclusive demonstration of endogenous production of RAs in freshwater cyanobacteria blooms. The observation of teratogenic RAs in cyanobacteria is evolutionarily and ecologically significant because RAs are vertebrate-specific hormones, and cyanobacteria form extensive and highly visible blooms in many aquatic ecosystems.

eutrophication | phytoplankton | deformities | Asia

Retinoic acids (RAs) are essential for physiological processes such as reproduction, cell proliferation and differentiation, vision, and embryonic development (1). However, extensive research has proved that RAs are among the most potent known animal teratogens (2). Exposure of embryos to exogenous RAs causes a spectrum of malformations, including defects of the neural tube and central nervous system; skeleton, palate, ear, and other craniofacial malformations; defects in the heart, thymus, and urogenital system; as well as missing or duplicate limbs and digits in offspring of humans, rodents, chickens, the African clawed frog (*Xenopus laevis*), and fish (3–10). When frogs were exposed to RAs during metamorphosis, a suite of abnormalities, such as reductions and deletions of the hind limb, bony triangles, and eye deformities, could occur (11–15). These abnormalities observed in the laboratory are similar to those found in wild frogs (16, 17).

Deformed amphibians have been observed in eutrophic habitats (18). Although the infections with parasites that were related to eutrophication were thought to be the mechanism of malformation (19, 20), the causes for frog deformities still remain controversial (21–23). Some clues point to RAs, including all-*trans*-RA (atRA), 13-*cis*-RA (13cRA), and 9-*cis*-RA (9cRA), or RA mimics (16, 17). These substances could be the cause of malformations through regulation of RA receptor-mediated signaling if they occurred in the surface waters or algae and

vegetation eaten by tadpoles (24). However, because they were one of the first morphogens identified in vertebrates, RAs were generally thought of as vertebrate-specific hormones (25), and there was no evidence that RAs were found in the eutrophic environment.

Under normal physiological conditions, atRA and 13cRA are present in human urine (26). In addition to the endogenous origin, atRA and 13cRA are also synthesized artificially for clinical treatment of dermatitis, such as acne and psoriasis (27). RAs and their metabolites can enter into the aquatic environments through discharge of domestic sewage. Concentrations of RAs and their bioactive metabolites 4-oxo-RAs in treated wastewater have generally been found to be less than 1.0 ng/L (28, 29). Recently, anomalous concentrations of RAs and 4-oxo-RAs (up to 20 ng/L) were measured in some protected drinking water sources (30), which suggested that there might be unidentified sources of RAs in aquatic systems. There are some reports on the existence of retinal, one of the precursors of RAs, in algae, and isomerization of the all-*trans*-retinal chromophore into 13-*cis*-retinal activated the photoreceptor channel, whereas RAs were not associated with photoreceptors in algae (31–33). The nonanimal hydroxylase enzyme (CYP120A1) in the cyanobacteria *Synechocystis* was reported to be capable of converting RAs to corresponding hydroxyl derivatives (34), and a hydroxylated RA, 7-OH-RA, has recently been observed in several species of cyanobacteria (35). These results led us to postulate that cyanobacteria or algae might have the potential to synthesize RAs, and such autogenously produced RAs could cause adverse effects on wildlife in eutrophic environments.

To demonstrate this hypothesis, the production of RAs and 4-oxo-RAs in cyanobacteria bloom materials from a eutrophic lake in China was studied by using a unique analytical method described in this paper. Further examination of 39 representative cyanobacteria and algae species was carried out to determine the specific species that could produce teratogenic RAs and 4-oxo-RAs.

Results and Discussion

RAs and 4-Oxo-RAs in Cyanobacteria Blooms. Taihu Lake, the third-largest freshwater lake in China, is hypereutrophic and has experienced blooms of cyanobacteria that have drawn worldwide attention (36, 37). The northern part of Taihu Lake, which is the most hypertrophic area, was chosen for this study (Fig. S1), and details of the study area are described in *SI Materials and Methods*. RAs and 4-oxo-RAs were detected in 20 of 24 water

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Table 1. Concentrations of intracellular and extracellular RAs and 4-oxo-RAs in cyanobacteria blooms collected from Taihu Lake, China

| Sampling sites	Intracellular concentrations, ng/L						Extracellular concentrations, ng/L					
	at-4-oxo-RA	13c-4-oxo-RA	9c-4-oxo-RA	atRA	13cRA	9cRA	at-4-oxo-RA	13c-4-oxo-RA	9c-4-oxo-RA	atRA	13cRA	9cRA
1	0.4	0.1	0.2	ND	ND	ND	ND	ND	ND	1.5	3.7	ND
2	0.2	0.1	0.1	ND	ND	ND	ND	ND	ND	1.9	1.9	ND
3	0.2	0.1	0.1	1.1	ND	ND	ND	ND	ND	1.5	5.8	3.2
4	0.1	0.2	0.1	ND	ND	ND	ND	ND	ND	1.0	ND	ND
5	0.2	ND	ND	0.8	0.3	ND	ND	ND	ND	1.5	0.7	1.7
6	2.2	0.4	0.7	7.2	3.6	ND	ND	ND	ND	1.4	4.3	ND
7	2.2	0.4	0.3	2.9	1.5	ND	ND	ND	ND	ND	ND	ND
8	2.8	0.7	0.6	6.3	1.4	ND	ND	ND	ND	2.6	4.5	ND
9	9.8	1.7	2.7	4.4×10	2.1×10	ND	ND	ND	ND	ND	ND	ND
10	2.1	0.2	0.5	6.4	3.2	ND	ND	ND	ND	1.0	0.8	0.7
11	2.2×10	4.5	4.7	9.0×10	3.7×10	ND	6.0	ND	1.0	5.9	4.6	2.3
12	1.3	0.2	0.4	7.6	2.0	ND	ND	ND	ND	ND	ND	ND
13	1.5	0.2	0.3	1.6×10	9.8	ND	2.0	ND	0.4	2.9	1.6	0.5
14	1.3	0.2	0.3	1.8×10	6.4	ND	ND	ND	ND	1.0	2.2	ND
15	1.7	0.2	0.2	2.5	1.4	ND	ND	ND	ND	3.3	2.0	2.3
16	3.6	0.5	0.9	1.9×10	6.0	ND	1.0	ND	0.4	ND	3.2	ND
17	2.1	0.3	0.6	1.2×10	6.8	ND	ND	ND	ND	0.7	1.0	ND
18	0.1	ND	ND	0.7	0.5	ND	ND	ND	ND	0.9	0.7	0.8
19	0.1	ND	ND	2.2	0.7	ND	ND	ND	ND	2.5	ND	ND
20	2.7	0.3	0.5	3.1×10	1.9×10	ND	0.6	ND	0.1	ND	ND	ND
21	2.7×10	ND	4.9	3.7×10	1.3×10	ND	2.0	ND	0.6	ND	ND	ND
22	1.6×10^2	2.1×10	2.1×10	9.8×10	1.2×10^2	ND	0.8	ND	0.2	ND	ND	ND
23	3.7	1.9	1.6	2.6×10	1.6×10	ND	ND	ND	ND	ND	ND	ND
24	6.0×10	1.5×10	1.1×10	2.2×10^2	4.1×10	ND	1.1	ND	0.3	ND	ND	ND

ND, not detected.

samples with an average concentration of 3.9 ± 4.4 ng/L (Table 1, Fig. 1, and Fig. S2), which was greater than those (0.7 ± 0.8 ng/L) reported previously in a river basin, Liaodong Bay, China, where untreated wastewaters are discharged directly into the rivers (29). The greatest total concentration of RAs and 4-oxo-RAs in water from Taihu Lake was 2.0×10 ng/L, which was comparable to concentrations in raw influents of sewage treatment plants (28). Taihu Lake is an important drinking water source for local residents, and domestic wastewaters without treatment are not directly discharged into the lake. Thus, such high concentration of RAs and 4-oxo-RAs were hypothesized to come from other sources. Of the RAs and 4-oxo-RAs, atRA/all-*trans*-4-oxo-RA (at-4-oxo-RA) and 13cRA/13-*cis*-4-oxo-RA (13c-4-oxo-RA) were the predominant compounds and accounted for $53\% \pm 26\%$ and $33\% \pm 28\%$, respectively, of total concentrations in detected samples (Fig. S2). Compound 9cRA/9-*cis*-4-oxo-RA (9c-4-oxo-RA) was detected in 12 water samples at concentrations ranging from 0.1 to 3.3 ng/L and accounted for $14\% \pm 14\%$ of total concentrations in detected samples. This

report of 9cRA, a natural retinoid X receptor ligand in animal and human, in the environment is significant.

To determine potential sources of RAs and 4-oxo-RAs in Taihu Lake, these compounds were further investigated in samples of blooms. The target compounds were detected in all bloom samples from Taihu Lake at concentrations ranging from 0.4 to 4.2×10^2 ng/L (Table 1, Fig. 1, and Fig. S2). The predominance of atRA/at-4-oxo-RA and 13cRA/13c-4-oxo-RA levels observed in bloom samples were similar to those in water (Fig. S2). Because concentrations of RAs and 4-oxo-RAs in blooms were greater than those in water (Fig. S2), the blooms were considered to be the main source of RAs and 4-oxo-RAs in lake water. Although 9cRA was not detected in samples of blooms, its metabolite 9c-4-oxo-RA was detected in 21 of 24 bloom samples at concentrations from 0.1 to 2.1×10 ng/L in detected samples, suggesting that 9cRA occurred in blooms at relatively low concentrations during the period of sampling.

The biomass of phytoplankton at each sampling location in Taihu Lake ranged from 6.7×10^6 to 9.7×10^{10} cells per liter.
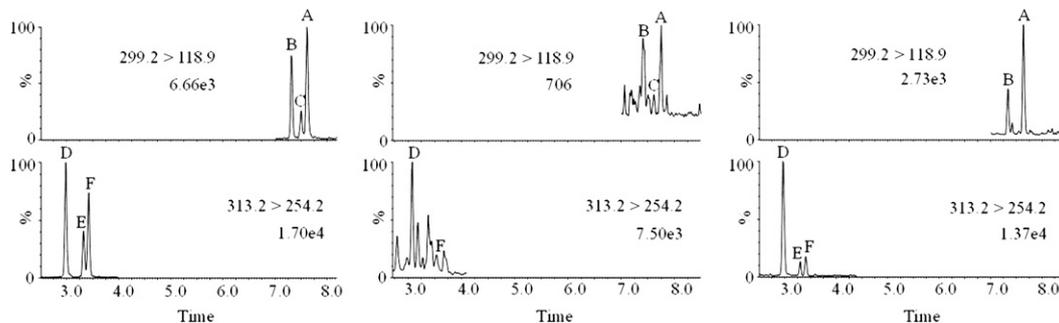
Fig. 1. UPLC-MS/MS chromatograms of RAs and 4-oxo-RAs in no. 11 sample from Taihu Lake. (*Left*) Standards (10 parts per billion). (*Center*) Water sample. (*Right*) Bloom sample. A, atRA; B, 13cRA; C, 9cRA; D, at-4-oxo-RA; E, 13c-4-oxo-RA; F, 9c-4-oxo-RA.

The phytoplankton community in Taihu Lake was dominated by cyanobacteria (Fig. S3), comprising primarily the genera *Microcystis*, *Aphanizomenon*, *Anabaena*, *Pseudanabaena*, and *Planktothricoides* (Fig. S4). In most samples, *Microcystis* accounted for more than 90% of the total abundance of cyanobacteria cells (Fig. S4). *Microcystis* in blooms consisted primarily of three species: *Microcystis aeruginosa*, *Microcystis wesenbergii*, and *Microcystis flos-aquae* (Fig. S5). A statistically significant correlation was observed between intracellular concentrations of RAs/4-oxo-RAs and numbers of *Microcystis* cells ($P = 0.006$, $r^2 = 0.2938$), which further suggested that *Microcystis* in blooms was capable of producing the observed concentrations of RAs and 4-oxo-RAs.

RAs and 4-Oxo-RAs in Cultures of Cyanobacteria and Algae. To further determine which cyanobacteria species were mainly responsible for the production of RAs and 4-oxo-RAs by blooms in Taihu Lake, 39 species of freshwater cyanobacteria and algae (Table S1), including those observed in Taihu Lake and some other species commonly observed in aquatic environments, were studied to determine their potential for producing RAs and 4-oxo-RAs. Cultures were purchased from the Freshwater Algae Culture

Collection of the Institute of Hydrobiology (FACHB), Chinese Academy of Sciences. Intracellular RAs and 4-oxo-RAs were detected in 32 species studied, including 22 Cyanophyta, 6 Chlorophyta, 3 Bacillariophyta, and 1 Euglenophyta (Table 2, Fig. 2, and Fig. S6). The greatest amount of intracellular RAs and 4-oxo-RAs was found in *M. flos-aquae* [FACHB-1028; 1.4×10^3 ng/g dry weight (DW)]. Other species, including *Anabaena* sp. (FACHB-1088; 5.7×10^2 ng/g DW), *Chlamydomonas* sp. (FACHB-715; 3.8×10^2 ng/g DW), *M. aeruginosa* (FACHB-912; 3.7×10^2 ng/g DW), *M. ichthyoblabe* (FACHB-1294; 3.4×10^2 ng/g DW), and *Ankistrodesmus* sp. (FACHB-1044; 3.2×10^2 ng/g DW), also produced measureable quantities of RAs and 4-oxo-RAs. These compounds were also detected in the culture media of 12 cyanophyta and 1 chlorophyta (Table 2, Fig. 2, and Fig. S6). The greatest extracellular concentrations of RAs and 4-oxo-RAs were detected in the culture medium of *Aphanizomenon flos-aquae* (FACHB-1039; 2.4×10^3 ng/L), followed by *M. flos-aquae* (FACHB-1028; 1.2×10^3 ng/L) and *Phormidium* sp. (FACHB-1099; 3.0×10^2 ng/L). Other than cyanobacteria, only green algae *Ankistrodesmus* sp. (FACHB-1044) produced detectable extracellular at-4-oxo-RA (2.5×10 ng/L) in culture medium. Because all cultures obtained from FACHB were unialgal, an additional

Table 2. Concentrations of intracellular and extracellular RAs and 4-oxo-RAs in cultured cyanobacteria and algae

Sample	Density, g DW/L	Intracellular concentration, ng/g DW						Extracellular concentration, ng/L					
		at-4-oxo-RA	13c-4-oxo-RA	9c-4-oxo-RA	atRA	13cRA	9cRA	at-4-oxo-RA	13c-4-oxo-RA	9c-4-oxo-RA	atRA	13cRA	9cRA
FACHB-286	1.10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-898	1.68	5.0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-1009	1.82	2.4×10	1.1×10	4.5	ND	ND	ND	8.9	ND	ND	8.1×10	3.8×10	ND
FACHB-1028	1.85	1.2×10^2	5.1×10	4.0×10	2.5×10^2	4.3×10^2	5.2×10^2	1.4×10^2	2.7×10	3.2×10	4.2×10^2	2.0×10^2	3.9×10^2
FACHB-905	1.90	5.6	7.9	2.7	2.5×10	2.5×10	ND	ND	ND	ND	ND	ND	ND
FACHB-912	2.06	3.8×10	2.2×10	1.1×10	9.0×10	7.0×10	1.4×10^2	6.7×10	ND	ND	ND	ND	ND
FACHB-908	1.83	3.4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-1284	1.90	5.6	1.5	2.0	3.6×10	3.6×10	1.0×10^2	ND	ND	ND	ND	ND	ND
FACHB-1294	1.62	2.0×10	5.8	5.5	6.1×10	8.6×10	1.6×10^2	ND	ND	ND	ND	ND	ND
FACHB-193	2.33	3.4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-1042	2.38	4.2×10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-1043	2.60	1.2×10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-1038	1.77	5.4×10	3.7	7.4	ND	ND	ND	1.2×10	ND	ND	ND	ND	ND
FACHB-1088	1.82	1.4×10^2	4.8×10	3.0×10	1.5×10^2	9.0×10	1.1×10^2	9.0	ND	ND	ND	ND	ND
FACHB-1092	1.74	ND	ND	ND	ND	ND	ND	ND	ND	ND	7.0×10	ND	ND
FACHB-1277	1.78	2.6	ND	ND	ND	ND	ND	3.2	ND	ND	ND	ND	ND
FACHB-247	2.22	8.4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-708	2.00	5.6	1.2	1.4	8.0×10	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-881	1.79	3.8	ND	ND	ND	ND	ND	ND	ND	ND	1.1×10^2	ND	ND
FACHB-723	2.41	2.5	ND	ND	1.9×10	1.4×10	ND	ND	ND	ND	ND	ND	ND
FACHB-1099	2.02	1.6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.0×10^2
FACHB-1039	1.80	1.2×10^2	2.0×10	2.2×10	ND	ND	ND	6.9×10^2	1.4×10^2	1.6×10^2	6.1×10^2	2.9×10^2	5.0×10^2
FACHB-1171	1.78	7.5	4.9	2.3	ND	ND	ND	3.4	ND	ND	ND	ND	ND
FACHB-1247	2.16	6.4×10	1.2×10	7.2	ND	3.9×10	ND	1.2×10	4.3	1.9×10	ND	ND	ND
FACHB-715	1.14	3.1	ND	ND	1.1×10^2	2.7×10^2	ND	ND	ND	ND	ND	ND	ND
FACHB-1067	2.15	2.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-704	1.53	1.0×10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-933	2.62	2.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-271	1.85	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-1044	2.27	2.0×10	8.0	5.0	4.3×10	1.5×10^2	9.5×10	2.5×10	ND	ND	ND	ND	ND
FACHB-686	1.73	ND	ND	ND	ND	1.3×10^2	ND	ND	ND	ND	ND	ND	ND
FACHB-719	2.10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-1030	1.13	2.4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-510	1.54	1.4×10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-1054	1.42	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-1034	1.38	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-1131	0.90	4.8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-844	38.39	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FACHB-848	2.26	9.5	1.1×10	3.0	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND, not detected.

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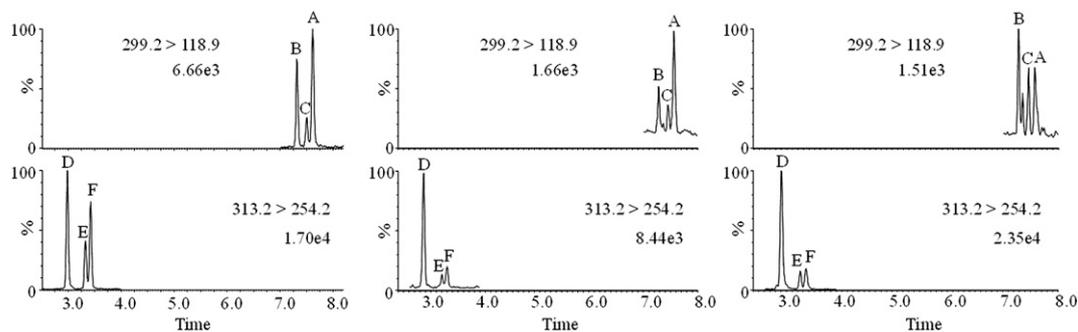
Fig. 2. UPLC-MS/MS chromatograms of RAs and 4-oxo-RAs in culture of *M. flos-aquae* (FACHB-1028). (*Left*) Standards (10 parts per billion). (*Center*) Extracellular sample. (*Right*) Intracellular sample. A, atRA; B, 13cRA; C, 9cRA; D, at-4-oxo-RA; E, 13c-4-oxo-RA; F, 9c-4-oxo-RA.

analysis of axenic cyanobacterial cultures was conducted. The axenic strains were obtained from the Pasteur Culture Collection of Cyanobacteria (PCC), France. The result demonstrated that the axenic strains of the cyanobacteria *M. aeruginosa* (PCC 7806) and *Synechococcus* sp. (PCC 7002), also produced RAs and 4-oxo-RAs (Fig. S7), and the concentrations were 1.8–2.5 ng/g DW.

The profiles of RAs and 4-oxo-RAs observed in the cultured cyanobacteria and algae were similar to those in blooms and water samples collected from Taihu Lake. atRA/at-4-oxo-RA were the predominant compounds, accounting for 71% ± 33% of intracellular concentrations of total RAs/4-oxo-RAs in detected samples, followed by 13cRA/13c-4-oxo-RA (19% ± 25%). Of the 39 cyanobacteria and algae species, 9cRA/9c-4-oxo-RA were detected in 14 species with intracellular concentrations from 1.4 to 5.6×10^2 ng/g DW (Table 2). Although the physiological function for the normal existence and role of 9cRA is of great interest in vertebrates (25), 9cRA was only identified in *Xenopus* embryos (38), in humans, or in mice after excessive amounts of retinol or atRA (39, 40). A recent study determined 9cRA to be a naturally occurring RA with concentrations of 3–12 ng/g (10–40 pmol/g) in mouse pancreas, which confirms that 9cRA contributes to the biological activity of vitamin A in vivo (41). Our findings of relatively frequent detections and relatively great concentrations of 9cRA (up to 5.2×10^2 ng/g DW) and 9c-4-oxo-RA (up to 4.0×10 ng/g DW) in cultured cyanobacteria and algae suggested that more studies about RAs should focus on cyanobacteria and algae in the future. Generally, 4-oxo-RAs were considered to be metabolites of RAs formed in vertebrates by cytochrome P450 enzymes such as CYP26A1 and CYP26B1, which have been cloned in human, mouse, rat, and chicken (25). The appearances of RAs and 4-oxo-RAs in natural blooms and cultured cyanobacteria and algae at relatively high concentrations first indicated that cyanobacteria and algae were able to transform RAs into their 4-oxo metabolites. It was inferred that corresponding biotransformation enzymes should exist in cyanobacteria and algae, especially those making up blooms. Based on biotransformation pathways in vertebrates, RAs would also undergo biotransformation to form hydroxylated transformation products (OH-RAs). The OH-RAs are less potent than atRA in binding to RA receptors (42). Recently, 7-OH-RA was identified in cultured cyanobacteria (35), but the estimated concentrations of 7-OH-RA were as much as 35-fold less than those of RAs and 4-oxo-RAs observed in this study.

When results of the laboratory and field studies are considered together, it can be concluded that the dominant cyanobacteria species, *M. flos-aquae* and *M. aeruginosa*, were primarily responsible for production of RAs and 4-oxo-RAs measured in blooms from Taihu Lake. Absolute and relative concentrations of RAs and 4-oxo-RAs depended on the species, even within the same genus. For instance, concentrations of RAs and 4-oxo-RAs in *M. flos-aquae* (FACHB-1028) were 3- to 420-fold greater than

those in the other six *Microcystis* species (Table 2). The origin of cyanobacteria was also found to have great effects on the production of RAs and 4-oxo-RAs. For example, FACHB-912, from Taihu Lake, and FACHB-905, from Dianchi Lake, were both *M. aeruginosa*, but the intracellular concentrations of RAs and 4-oxo-RAs in FACHB-912 (3.7×10^2 ng/g DW) were nearly five-fold greater than those in FACHB-905 (6.6×10 ng/g DW). Besides the differences among species and origin locations, the concentrations and profiles of RAs and 4-oxo-RAs were also associated with the phase of cyanobacterial growth. As shown in Fig. 3, whereas concentrations of 4-oxo-RAs in *M. flos-aquae* (FACHB-1028) remained constant from 40 to 90 d, concentrations of atRA, 13cRA, and 9cRA all increased as a function of time, especially after 50 d, when 9cRA appeared and the concentrations of all RA isomers abruptly increased, then became constant. The bloom season in Taihu Lake is from May to October, and the density of cyanobacteria cells reaches a maximum in July. Based on previously reported (43) and present results, the natural blooms in Taihu Lake were dominated by cyanobacteria (over 90%), and production of RAs and 4-oxo-RAs depended on cyanobacteria cell numbers (Fig. 3); therefore, concentrations of RAs and 4-oxo-RAs in Taihu Lake might increase to their highest in July, and the lowest concentrations would appear in wintertime.

Blooms, especially cyanobacteria blooms, occur in freshwaters or marine environment all over the world and have been recognized as an important health risk for both humans and ecosystems because of their production of various toxins such as hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and irritant toxins (44, 45). The results reported here demonstrate that natural cyanobacteria blooms can produce teratogenic RAs and 4-oxo-RAs. On the basis of controlled laboratory studies with individual species,
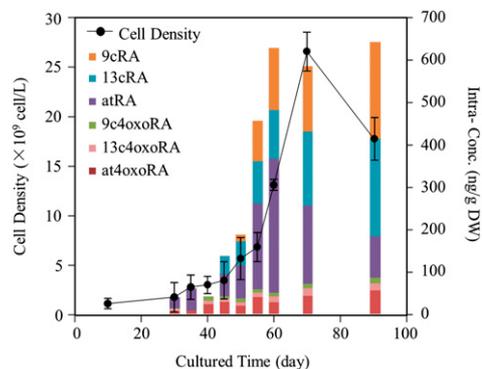
Fig. 3. Changes in intracellular production of RAs and 4-oxo-RAs with the growth of *M. flos-aquae* (FACHB-1028).

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we determined the species responsible for production of RAs and 4-oxo-RAs in Taihu Lake, China. The results also show that some cyanobacteria species, especially those that are common in blooms and sometimes are dominant species, such as *M. flos-aquae*, *M. aeruginosa*, *Anabaena*, and *Ap. flos-aquae*, can produce significant amounts of RAs and 4-oxo-RAs. Because RAs are generally thought of as vertebrate-specific hormones that signal via nuclear receptors cascades (25), the signal-transduction pathway and function of RAs in cyanobacteria and algae remain to be elucidated. Exposure to RA through both diet and/or directly from water can cause mortality and deformities in tadpoles of *X. laevis* (14, 15). Extracts of cyanobacteria can cause developmental toxicity, including mortality, growth inhibition, and malformations in *X. laevis*, and these effects have been shown not to be caused by microcystins (46). Altogether, the results of these studies indicate that RAs and 4-oxo-RAs found in Taihu Lake might pose a risk for animals living in the water and feeding on algae. Because eutrophic lakes and reservoirs are used as sources of drinking water and food, such as freshwater clams and snails, RAs could also pose a risk to the health of humans.

Materials and Methods

Field Collections. Twenty-four surface (0–0.5 m) water samples containing blooms of cyanobacteria were collected in October 2010 from Taihu Lake. Samples were stored in amber bottles and were treated immediately after arrival at laboratory. A portion (30–200 mL) of each sample was preserved in Lugol's solution for algae identification and enumeration.

Cultured Cyanobacteria and Algae. Thirty-nine unialgal cultures of freshwater cyanobacteria and algae, including 24 Cyanophyta, 8 Chlorophyta, 6 Bacillariophyta, and 1 Euglenophyta (Table S1), were purchased from the FACHB, Chinese Academy of Sciences. Two axenic cultures of cyanobacteria, *M. aeruginosa* (PCC 7806) and *Synechococcus* sp. (PCC 7002), were obtained from the PCC, France. The cultures were used for analysis when the cyanobacteria and algae that cultured under axenic condition were in a logarithmic growth phase and the number of cells was $>10^8$ cells per liter.

M. flos-aquae (FACHB-1028) was further used to assess the influence of growth stage on the production of RAs and 4-oxo-RAs. The cyanobacteria was grown in BG11 medium at 25 °C on a 14-h light/10-h dark cycle. During the culture time, the culture was shaken three times every day. The cyanobacteria and medium were taken for analysis of RAs and 4-oxo-RAs on days 10, 30, 35, 40, 45, 50, 55, 60, 70, and 90. The cell counting was carried out ($n = 6$), and the number of cells (cells per liter) was calculated at each sampling time.

Preparation of Samples from Taihu Lake. Water was vacuum-filtered through GF/C filters (1.2 μm; Whatman) for analysis of extracellular RAs and 4-oxo-RAs, and cyanobacteria cells remaining on filters were used for the measurement of intracellular RAs and 4-oxo-RAs. The details of the chemicals and standards used are provided in *SI Materials and Methods*. The method used to quantify the extracellular RAs and 4-oxo-RAs was based on previously published methods (29). The methods for analyzing all target RAs and 4-oxo-RAs in cyanobacteria blooms and cultured species were initially developed and validated.

The GF/C filters were placed into a 50-mL centrifuge tube containing 15–20 mL of acetone and sonicated for 10 min. After disruption of cells in an ice bath by use of an ultrasonic cell disrupter system (10- to 20-s pulse, repeat 10 times, 600–800 W) and centrifugation at 3,000 × g for 10 min, the supernatant was transferred to another centrifuge tube and then 15 mL of acetone was added to the residues. The mixture was mechanically shaken for 5 min and subsequently centrifuged (3,000 × g for 10 min). The combined supernatant was concentrated to a final volume of 5 mL by evaporation with nitrogen and then diluted with ultrapure water to 50 mL after surrogate standards (acitretin and atRA-d_5) were added. The mixture was loaded onto the preconditioned Oasis HLB cartridge (6 mL, 200 mg; Waters), which was

then dried with nitrogen. 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 and then purified by use of previously described methods (29) before injection to the ultraperformance liquid chromatography/electrospray ionization–tandem mass spectrometry (UPLC-ESI-MS/MS) for the analysis of RAs and 4-oxo-RAs.

Preparation of Cultured Cyanobacteria and Algae. Cultures were centrifuged at 6,000 × g for 10 min. The supernatant was used to analyze the extracellular RAs and 4-oxo-RAs, and the filtered cells were used for the quantification of intracellular RAs and 4-oxo-RAs.

An aliquot (15 mL) of the supernatant was added with 3.75 mL of acetone and spiked with surrogate standards, and then the mixture was loaded under gravity onto the Oasis HLB cartridge, which had been preconditioned with 6 mL of ethyl acetate, 6 mL of methanol and 12 mL of 20% (vol/vol) aqueous acetone. The cartridge was then dried with nitrogen, and analytes were eluted with 6 mL of ethyl acetate followed by 3 mL of acetone. Extracts were evaporated to dryness under a gentle stream of nitrogen and reconstituted with 150 μL of acetonitrile.

For identification and quantification of intracellular RAs and 4-oxo-RAs, cells were suspended in 2–3 mL of phosphate buffer (0.2 M, pH = 6.0) before performing cell disruption as described above. After being spiked with surrogate standards, the sample was extracted three times with 5 mL of hexane/chloroform (3:1; vol/vol) by mechanically shaking for 5 min, and the combined organic layer was evaporated to dryness under a gentle stream of nitrogen. The residue was purified by use of previously described methods (29) and dissolved in 350 μL of acetonitrile before injection to the ultraperformance liquid chromatography-tandem mass spectrometry system for quantification of RAs and 4-oxo-RAs.

Identification and Quantification of RAs and 4-Oxo-RAs. For natural samples, the absolute recoveries of target analytes were 54–66% and 51–60% in bloom samples and water samples, respectively (Table S2). Recoveries of surrogate standards were as follows: 45% ± 14% (blooms) and 54% ± 23% (waters) for atRA-d_5 and 74% ± 21% (blooms) and 56% ± 16% (waters) for acitretin. Limits of quantification (LOQs) of RAs and 4-oxo-RAs were 0.1–0.8 ng/L in blooms and 0.06–0.4 ng/L in waters, respectively (Table S2). For cultured samples from FACHB, the absolute recoveries of target analytes were 50–70% and 52–76% in intracellular and extracellular samples, respectively (Table S2). Recoveries of surrogate standards in intracellular samples were 44% ± 14% for atRA-d_5 and 56% ± 17% for acitretin, and recoveries from extracellular samples were 49% ± 16% for atRA-d_5 and 53% ± 18% for acitretin. The limits of quantification for RAs and 4-oxo-RAs ranged from 0.5 to 6.0 ng/g DW in intracellular samples and from 3.0 to 20 ng/L in extracellular samples (Table S2). In this study, atRA-d_5 and acitretin served as surrogate standards to quantify RAs (atRA, 13cRA, and 9cRA) and 4-oxo-RAs (at-4-oxo-RA, 13c-4-oxo-RA, and 9c-4-oxo-RA), respectively. Surrogate standards were used to compensate for signal suppression caused by suppression of ionization by cellular matrix, losses of analytes during preparation, and variations in the instrument response from injection to injection in ultraperformance liquid chromatography-tandem mass spectrometry analysis. The relative recoveries for all target analytes (corrected for surrogate standards) were 83% ± 9% to 129% ± 3%. All analyses were quantified within the range of limits of quantification or were reported as not detected.

Statistical Analysis. Pearson correlation analysis was conducted with SPSS software, version 19 for Windows.

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Supporting Information

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SI Materials and Methods

Chemicals and Standards. All-*trans*-retinoic acid (atRA), 13-*cis*-RA (13cRA), 9-*cis*-RA (9cRA), and acitretin were purchased from Sigma. All-*trans*-4-oxo-RA (at-4-oxo-RA), 13-*cis*-4-oxo-RA (13c-4-oxo-RA), 9-*cis*-4-oxo-RA (9c-4-oxo-RA), and atRA-d_5 were obtained from Toronto Research Chemicals.

Methanol, acetonitrile, ethyl acetate, hexane, methylene chloride, and chloroform were HPLC grade and purchased from Fisher Chemical. HPLC-grade formic acid and acetic acid were provided by Dima Technology. Ultrapure water was obtained by a Milli-Q Synthesis water purification system (Millipore) under a conductivity of 18.2 $\Omega \cdot cm^{-1}$.

Study Area. Taihu Lake (119°54′–120°36′ N, 30°56′–31°33′ E), the third-largest freshwater lake in China (surface area: 2,338 km^2, maximum depth: 2.6 m, average depth: 1.9 m, mean water residence time: 264 d), is located in the highly developed and densely populated Yangtze Delta and serves as an important source of water for irrigation, aquaculture, and industrial waters as well as the primary drinking water source for 30 million residents in the lake basin and Shanghai. Since the 1980s, eutrophication as a consequence of human activities has become an increasingly serious issue in Taihu Lake, and annual lake-wide cyanobacteria blooms have occurred during warm seasons every year.
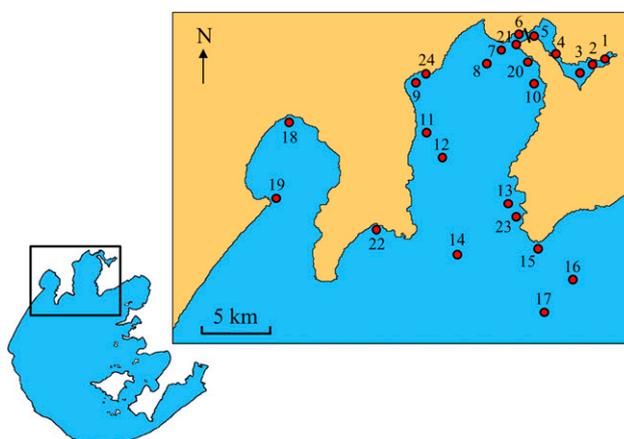
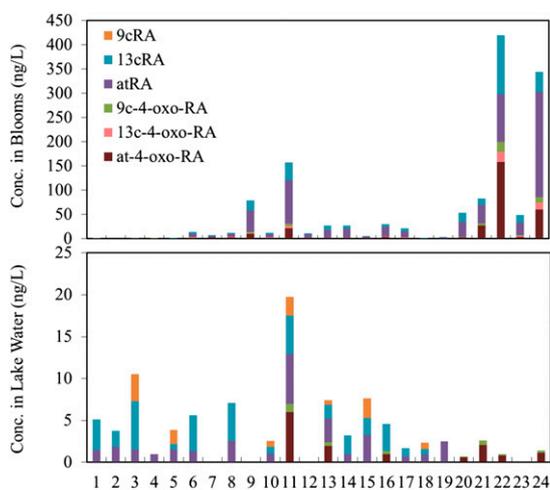**Fig. S1.** Sampling sites in Taihu Lake, China.

Fig. S2. Concentrations of RAs and 4-oxo-RAs in bloom samples (*Upper*) and water samples (*Lower*) collected from Taihu Lake, China. The *x* axis represents the sampling sites.

Fig. S3. Composition of phytoplankton biomass in Taihu Lake, China.

Fig. S4. Composition of cyanobacteria biomass in Taihu Lake, China.

Fig. S5. Composition of *Microcystis* colonies in Taihu Lake, China.

Fig. S6. Intracellular (*Upper*) and extracellular (*Lower*) concentrations of RAs and 4-oxo-RAs in cultured cyanobacteria and algae.

Fig. S7. Ultraperformance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) chromatograms of intracellular retinoids in axenic strains of *Microcystis aeruginosa* (PCC 7806). *A*, atRA [1.9 ng/g dry weight (DW)]; *B*, at-4-oxo-RA (0.3 ng/g DW); and *C*, 9c-4-oxo-RA (0.3 ng/g DW).

Table S1. Information on the cyanobacteria and algae surveyed

Phylum	Genus	Species	Strain no.	Origin
Cyanophyta	*Merismopedia*	*Merismopedia* sp.	FACHB-286	United States
	Synechocystis	*Synechocystis* sp.	FACHB-898	France
	Microcystis	*Microcystis* sp.	FACHB-1009	Dianchi Lake (China)
		M. flos-aquae	FACHB-1028	Taihu Lake (China)
		M. aeruginosa	FACHB-905	Dianchi Lake
		M. aeruginosa	FACHB-912	Taihu Lake
		M. wesenbergii	FACHB-908	Dianchi Lake
		M. viridis	FACHB-1284	Dianchi Lake
		M. ichthyoblabe	FACHB-1294	Taihu Lake
	Chroococcus	*Chroococcus* sp.	FACHB-193	Donghu Lake (China)
	Nostoc	*Nostoc* sp.	FACHB-1042	China
		Nostoc sp.	FACHB-1043	China
	Anabaena	*A. cylindrica*	FACHB-1038	Dianchi Lake
		Anabaena sp.	FACHB-1088	Nanhu Lake (China)
		A. flos-aquae	FACHB-1092	Dianchi Lake
	Pseudanabaena	*Pseudanabaena* sp.	FACHB-1277	Xionghe Reservoir (China)
	Oscillatoria	*O. tenuis*	FACHB-247	United States
		O. planctonica	FACHB-708	Donghu Lake
		O. raciborskii	FACHB-881	Dianchi Lake
	Phormidium	*P. mucicola*	FACHB-723	China
		Phormidium sp.	FACHB-1099	Dianchi Lake
	Aphanizomenon	*Ap. flos-aquae*	FACHB-1039	Dianchi Lake
		Ap. flos-aquae	FACHB-1171	Taihu Lake
		Ap. issatschenkoi	FACHB-1247	Donghu Lake
Chlorophyta	*Chlamydomonas*	*Chlamydomonas* sp.	FACHB-715	Donghu Lake
	Chlorella	*Chlorella* sp.	FACHB-1067	China
	Pediastrum	*Pediastrum* sp.	FACHB-704	China
	Scenedesmus	*Scenedesmus* sp.	FACHB-933	Dianchi Lake
	Selenastrum	*S. capricornutum*	FACHB-271	United States
	Ankistrodesmus	*Ankistrodesmus* sp.	FACHB-1044	Dianchi Lake
	Oedogonium	*Oedogonium* sp.	FACHB-686	China
	Staurastrum	*Staurastrum* sp.	FACHB-719	Taiping Lake (China)
Bacillariophyta	*Cyclotella*	*C. hebeiana*	FACHB-1030	Guanqiao Pond (China)
	Nitzschia	*Nitzschia* sp.	FACHB-510	China
	Navicula	*Navicula* sp.	FACHB-1054	Taihu Lake
	Melosira	*M. varians*	FACHB-1034	Guangzhou (China)
	Synedra	*Synedra* sp.	FACHB-1131	Donghu Lake
	Cymbella	*Cymbella* sp.	FACHB-844	China
Euglenophyta	*Euglena*	*E. gracilis*	FACHB-848	Japan

FACHB, Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences.

Table S2. Recoveries and limits of quantification (LOQs) for RAs and 4-oxo-RAs in different matrices

Parameter	Sample	Matrix	at-4-oxo-RA	13c-4-oxo-RA	9c-4-oxo-RA	atRA	13cRA	9cRA
Percentage recovery	Natural blooms	Intracellular	62 ± 5	53 ± 9	54 ± 3	61 ± 4	66 ± 7	54 ± 8
		Extracellular	55 ± 6	60 ± 7	59 ± 7	51 ± 9	56 ± 8	59 ± 4
	Cultured algae	Intracellular	70 ± 1	61 ± 7	69 ± 3	56 ± 9	61 ± 7	50 ± 3
		Extracellular	76 ± 3	68 ± 2	59 ± 8	58 ± 5	52 ± 6	63 ± 2
LOQ	Natural blooms	Intracellular	0.1	0.1	0.1	0.3	0.3	0.8
		Extracellular	0.06	0.1	0.1	0.2	0.2	0.4
	Cultured algae	Intracellular	0.5	1.0	1.0	3.0	4.0	6.0
		Extracellular	3.0	4.0	10	8.0	8.0	20

LOQ concentrations are expressed in ng/L except for intracellular cultured algae, which is expressed in ng/g DW.