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Distribution of mycosporine-like amino acids and photoprotective carotenoids among freshwater phytoplankton assemblages

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ABSTRACT: Phytoplankton have evolved different strategies to minimize the potential damage caused by solar ultraviolet radiation (UVR), including the synthesis of UV-absorbing compounds that act as sunscreens and carotenoids that provide protection against photooxidative stress. The concentration and qualitative composition of mycosporine-like amino acids (MAAs) and photoprotective carotenoids (PPCs) were investigated in natural phytoplankton assemblages of 26 lakes located below and above the treeline in the Alps and the Pyrenees. Among these lakes, exposure to UV varied because of differences in the incident spectral dose, water column transparency, stratification, and maximum depth. Seven MAAs were identified, but the most abundant were shinorine (λ_{max} = 334 nm) and palythine (λ_{max} = 320 nm). The highest concentrations of MAAs (up to 9.6 µg [µg chl *a*]$^{-1}$) and PPCs (up to 0.6 µg [µg chl *a*]$^{-1}$) were found in the clearest and shallowest lakes, while phytoplankton from lakes with low UV transparency generally presented low values. However, phytoplankton of some clear lakes located at high altitude did not have high concentrations of these compounds. Consequently, underwater downwelling UVR, UV water transparency, or lake altitude explained only a low percentage (<26%) of the variability among lakes in MAA and PPC concentration. Within the water column, the concentration of MAAs decreased in most cases with depth, suggesting their photoprotective role. Our results indicate that MAAs and PPCs are widespread among lake phytoplankton assemblages and suggest that other environmental factors besides UV exposure are important in regulating their synthesis.

KEY WORDS: MAA · Sunscreens · Ultraviolet radiation · UV · Solar radiation · Photooxidative stress · Alpine lakes

INTRODUCTION

Phytoplankton have a range of photoprotective mechanisms to minimize the potential damage caused by solar ultraviolet radiation (UVR; 290 to 400 nm) that include, for example, the synthesis of UV-absorbing compounds and of carotenoids that act as direct sunscreens or against photooxidative stress, respectively (Roy 2000). Examples of UV-absorbing compounds are scytonemin, an indol alkaloid with a maximum *in vivo* absorption at ca 370 nm found only in the sheath of mat-forming and epilithic cyanobacteria (Garcia-Pichel & Castenholz 1991), and mycosporine-like amino acids (MAAs). MAAs are a family of water-soluble, low molecular weight compounds with high molar extinction coefficients (ε: 28 100 to 50 000 M^{-1} cm^{-1}) and absorption maxima between 309 and 360 nm. MAAs have been found in phytoplankton cultures (Carreto et al. 1990, Marchant et al. 1991, Davidson et al. 1994, Helbling et al. 1996, Riegger & Robinson 1997, Neale et al. 1998, Jeffrey et al. 1999, Xiong et al. 1999), natural marine phytoplankton assemblages (Karentz et al. 1991, Negri et al. 1992, Bidigare et al.

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1996, Bracher & Wiencke 2000, Whitehead & Vernet 2000), and zoozanthellae living in symbiosis with corals (Shick et al. 1999). Information on these compounds in natural freshwater phytoplankton and their photoprotective role is scarce. Sommaruga & Garcia-Pichel (1999) measured the abundance and composition of MAAs in planktonic and benthic algal communities of a clear high mountain lake in the Central Alps. They found higher pigment-specific phytoplankton *in vivo* absorption (at 334 nm) at the surface than in deeper layers in the water column. They also found high concentrations of MAAs in epilithic cyanobacteria. Benthic mat communities of a high altitude tropical lake also synthesized MAAs; however, phytoplankton did not appear to have such protection (Kinzie et al. 1998).

The frequently observed correlation between MAA concentration and solar exposure found in nature suggests that these compounds play an important role in UV protection. Moreover, experiments with the dinoflagellate *Gymnodinium sanguineum* have shown that MAAs indeed protect against inhibition of photosynthesis (Neale et al. 1998).

Besides the potential photodamage directly caused by UVR to cell targets, the interaction of this radiation with oxygen and various cell chromophores can result in the production of damaging reactive oxygen species. Algal cells, however, appear to have quenching mechanisms that allow them to detoxify these photochemical reaction products. Certain carotenoids have antioxidant activity while others participate in the xanthophyll cycle, which prevents photoinhibition by dissipating the excess light energy (see Roy 2000 and references therein). Accumulation of carotenoids specifically in response to UVR has been reported for cyanobacteria and chlorophytes (Buckley & Houghton 1976, Bidigare et al. 1993, Goes et al. 1994), but there is little information about their distribution among natural freshwater phytoplankton assemblages.

In this study, our objectives were to assess (1) how widespread photoprotective compounds (MAAs and carotenoids) are among phytoplankton assemblages of lakes located across an altitude gradient and (2) the importance of UV exposure for the variability in the concentrations of these compounds. We addressed these objectives by means of profiles in a broad suite of lakes in several locations in Europe that revealed a large variability in MAA and carotenoid concentrations.

MATERIALS AND METHODS

Study sites. Twenty-six lakes in the Tyrolean Alps (Austria and Italy, n = 19) and in the Pyrenees (Spain, n = 7) were sampled in 1998 (see Laurion et al. 2000 for details). Four lakes (Mondsee [MON], Traunsee [TRA], Achensee [ACH], and Piburgersee [PIB]) are located in the pre-alpine region of the Alps at altitudes <930 m above sea level (a.s.l.), while all others are situated above 1480 m a.s.l. Lake elevation ranges from 422 to 2799 m a.s.l. in the Alps and from 1900 to 2400 m a.s.l. in the Pyrenees (Table 1). The treeline is situated approximately at 2000 m in this region of the Alps and at 2200 m in the Pyrenees. Most lakes in the Alps were sampled in July and in October (MON and TRA sampled only in November), whereas lakes in the Pyrenees were sampled once in September. The ice-cover of some of the highest located lakes had recently melted when they were sampled in July, while in October, the ice-cover had started to build over Rotfelssee (ROT) and was already formed over Oberer Plenderlesee (OPL). Following Laurion et al. (2000), the lakes were separated into 3 categories according to the dominant catchment vegetation: lakes above the treeline with a poor soil cover or with less than 5% of sparse pine trees (hereafter 'exposed rock' category; n = 11), lakes at high altitudes but surrounded by alpine meadows (hereafter 'meadows'; n = 5), and lakes with forested catchments (hereafter 'trees'; n = 10).

Attenuation coefficient measurements. A PUV-500A radiometer (Biospherical Instruments Inc, San Diego, CA, USA) provided a measure of downwelling irradiance at 305, 320, 340 and 380 nm (full bandwidth at half maximum is 8 to 10 nm), and of downwelling photosynthetically available radiation (PAR; 400 to 700 nm). The instrument also recorded depth, temperature, and upwelling radiance centered at 683 nm (naturally induced fluorescence). Details on the calculation of the diffuse attenuation coefficients of downwelling UVR (K_d) are given in Laurion et al. (2000). The fraction of the water column to which 1% of the surface UVR at 320 nm penetrated ($Z_{1\%}$:Z_{max}) was calculated from K_d (at 320 nm) and the maximum lake depth (Table 1).

Chlorophyll *a* (chl *a*), UV-absorbing compounds, and carotenoids. The lake water was sampled at 1 to 5 discrete depths with a modified 5 l Schindler-Patalas sampler in the Alps or a Ruttner bottle in the Pyrenees and samples were kept in the dark (and cold when possible) until processed in the laboratory (within ~6 h). The selection of sampling depths was based on profiles of PAR and induced chl *a* fluorescence as measured with a Backscat I-Fluorometer (Haardt, Kleinbarkau, Germany, model 1101.1; excitation 380 to 540 nm, emission 685 nm). A sub-sample of lake water (250 ml) was fixed with acidified Lugol's solution and maintained in the dark at 4°C for further qualitative microscopic analysis. Three separate filters were prepared for chl *a*, MAAs, and carotenoid analyses. The

Table 1. Total concentration of mycosporine-like amino acids (MAAs) (A_{MAA} calculated from the absorption spectra and MAAs by HPLC), percentages of individual MAAs determined by HPLC (MG: mycosporine-glycine; SH: shinorine; PR: porphyra-334; PI: palythine; AS: asterina; MAA-357: tentatively identified as usujirene; and PE: palythene), and xanthophylls and cyanobacterial pigments (DIA: diadinoxanthin; ZEA: zeaxanthin; MYX: myxoxanthophyll; ECH: echinenone). The lake altitude, the fraction of the water column to which 1% of the surface UV radiation at 320 nm penetrated ($Z_{1\%}$:Z_{max}), and chlorophyll *a* concentration (chl *a*) are also given. Lakes were classified into 3 categories based on Laurion et al. (2000). Lakes located in the Pyrenees are identified with the letter p; all others are located in the Alps. a.s.l.: above sea level; t: traces. ACH: Achensee; ANT: Antholzer See; BAR: Estany Barbs; DRA: Drachensee; DUR: Durnholzer See; GKS: Gossenköllesee; GRA: Estany Gran; KLA: Klammsee; LIC: Lichtsee; LLA: Estany de Llauset; MON: Mondsee; MPL: Mittlere Plenderlesee; NEG: Estany Negre; OBB: Obernbergersee; OBR: Obersee; OPL: Oberer Plenderlesee; PIB: Piburger See; PJO: Pfitscher Joch See; PRA: Pragser Wildsee; RED: Estany Redo; ROT: Rotfelssee; SEE: Seebensee; SMA: Estany Sant Maurici; SOS: Schwarzsee ob Sölden; TOR: Estany Tort; TRA: Traunsee

Lake	Altitude (m a.s.l.)	Date (1998)	Sampling depth (m)	$Z_{1\%}$:Z_{max}	Chl *a* (µg l^{-1})	A_{MAA} (nm m^{-1})	MAAs (µg l^{-1})	MG (%)	SH (%)	PR (%)	PI (%)	AS (%)	MAA-357 (%)	PE (%)	DIA (µg l^{-1})	ZEA (µg l^{-1})	MYX (µg l^{-1})	ECH (µg l^{-1})
Exposed rock																		
BAR p	2360	10 Sep	2	0.38	0.77	0.17	0.49	12	41	t	28	t	13	6			0.04	0.05
DRA	1874	22 Jul	1	0.83	0.39	0.36	0.36	0	93	0	t	7	0	0		0.10 (18[a])	0.06 (6)	0.03
GKS	2417	23 Jul	1	1.33	1.03	3.49	2.50	0	84	0	1	15	0	0		0.04	0.15	0.04
		06 Oct	2	1.94	1.96	0.91	1.31	2	53	3	33	7	2	0			0.17	0.08
GRA p	2345	10 Sep	2	0.34	0.87	0.38	0.71	13	34	2	36	0	13	2			0.04	0.06
MPL	2317	21 Jul	1	1.25	2.4	13.76	22.92	5	55	0	28	7	5	t	0.14	0.28	1.05	0.15
		10 Oct	5[b]	1.52	2.72	0.85	0.97	0	85	0	t	15	0	0			0.07	0.17
NEG p	2320	09 Sep	2	0.17	0.3	0.33	0.43	6	49	t	34	3	6	2			0.09 (20)	0.05
OPL	2344	21 Jul	1	3.22	0.75	2.82	2.74	0	87	0	3	10	0	0				0.04[c]
		10 Oct	2	3.41	1.38	0.12	0.17	0	93	0	7	t	0	0				0.07
RED p	2240	08 Sep	2	0.23	1.08	4.98	5.56	2	45	0	46	4	2	1			0.21	0.05
ROT	2485	23 Jul	2.5	3.81	2.43	0.68	0.92	0	55	0	39	7	0	0			0.20	0.09
		06 Oct	2	3.49	2.5	5.93	5.79	0	94	0	0	6	0	0		0.08	1.44	0.04
SOS	2799	30 Jul	2.5	0.78	1.36	4.71	4.69	t	71	0	22	t	7	t		0.12 (5)	0.08	0.05
TOR p	2310	09 Sep	2	0.08	1.35	2.63	3.00	t	75	0	22	t	3	t			0.20	0.07
Meadows																		
KLA	2258	18 Jul	1.5	0.93	1.21	0.32	0.50	5	89	0	t	6	0	0	0.09		0.22	0.08
		14 Oct	2	1.02	0.34	0	0.20	0	59	0	33	t	8	t				0.04
LIC	2104	15 Jul	1	0.33	2.69	0.87	2.00	10	43	0	13	t	21	14			0.15	0.14
		09 Oct	2	0.16	3.61	0.06	0.24	34	52	0	t	0	t	14				0.25
LLA p	2125	12 Sep	2	0.10	0.58	0.01	0.04	22	48	0	30	0	t	t				0.04
OBR	2016	17 Jul	1	0.20	0.59	0.25	0.57	4	46	0	35	3	6	6		0.05 (21)	0.05 (13)	0.03
		13 Oct	2	0.16	1.09	1.58	2.15	9	40	0	18	t	24	10			0.46	0.16
PJO	2231	24 Jul	1.5	0.18	7.97	14.63	13.35	5	40	0	55	t	0	0		0.15	0.19	0.28
Trees																		
ACH	929	11 Jul	3	0.01	1.1	0.13	0.33	12	56	0	4	0	19	9		0.13 (18)	0.07 (9)	0.10
		12 Oct	2	0.02	0.49	0.01	0.03	0	100	0	0	0	0	0			0.06 (5)	0.06
ANT	1640	17 Jul	1	0.06	1.54	0.02	0.07	0	83	0	17	0	0	0			0.13 (7)	0.08
		13 Oct	2	0.13	3.3	0.08	0.52	3	59	0	22	3	10	3			0.56	0.31
DUR	1560	19 Jul	1	0.21	0.38	0	0.00	0	t	0	0	0	0	0		0.22 (7.5)		0.03
		15 Oct	2	0.26	0.25	0	0.00	0	0	0	0	0	0	0				0.03
MON	481	05 Nov	2	0.02	3.52	0.02	0.05	0	100	0	0	0	0	0			0.32	0.35
OBB	1590	15 Jul	1	0.19	1.08	0	0.03	t	100	0	0	0	0	0			0.09	0.07
		09 Oct	2	0.37	0.6	0.01	0.06	0	85	0	15	0	0	0				0.05
PIB	913	11 Jul	1	0.07	1.74	0.47	1.23	10	35	t	25	t	24	6		0.09	0.15	0.10
		16 Oct	2	0.06	2.03	0.07	0.37	4	28	t	21	0	47	0			0.28	0.26
PRA	1489	18 Jul	1	0.09	1.31	0.98	1.09	0	48	0	39	4	5	4			0.24	0.16
		14 Oct	2	0.06	2.65	0.22	0.23	21	71	0	8	t	0	0			0.34	0.42
SEE	1650	08 Oct	2	0.55	1.6	0.39	0.44	0	85	0	12	3	0	0		0.05	0.30	0.32
SMA p	1905	14 Sep	2	0.08	1	0.08	0.22	8	37	0	36	0	13	6				0.07
TRA	422	06 Nov	2	0.00	1.05	0	0.00	0	t	0	0	0	0	0			0.05	0.14

[a]Values measured deeper are given (with depth in parentheses) when not detected at the surface; [b]Value at surface not available; [c]Value for 4.5 m (not available at 1 m)

lake water was pre-screened by a 100 µm mesh to remove large zooplankton that bioaccumulate carotenoids and MAAs in high concentrations (Sommaruga & Garcia-Pichel 1999). However, a bias may have been introduced from MAAs present in small rotifers (Tartarotti et al. 2001) and other microzooplankton.

For chl *a* measurements, 0.6 to 3.5 l of lake water was filtered onto glass fiber filters (Whatman GF/F) and frozen at –20°C until subsequent extraction (within 3 wk). Pigments were extracted in 12.5 ml of 90% alkaline acetone (v:v) for 24 h in the dark at 4°C. Filters were briefly sonicated with a tip sonicator (1 min) on an ice bath and the extracts cleared using a 0.1 µm pore size Anodisc filter (Whatman). The extracts were scanned in a spectrophotometer (double beam Hitachi U-2000; scans between 400 and 750 nm) against an acetone reference and using 5 cm glass cuvettes. The optical density measured at 664 nm always remained above 0.03 and in most cases was more than 1 order of magnitude higher than the optical density at 750 nm. The equations of Jeffrey & Humphrey (1975) were used to calculate the concentration of pigments.

For MAA measurements, 0.8 to 4.85 l of lake water was filtered onto glass fiber filters (Whatman GF/F) and frozen at –80°C until subsequent extraction (within 4 mo). MAAs were extracted in 12.5 or 15 ml of 20% aqueous methanol (v:v) in the dark for 24 h at 4°C, followed by a 2 h extraction in a water bath at 45°C (Sommaruga & Garcia-Pichel 1999). Filters were sonicated, and the extracts were clarified and scanned as above (scans between 250 and 750 nm; 5 cm SUPRASIL I quartz cuvette), to obtain a general view on the combined UV-absorbing compounds. The extracts were subsequently dried under vacuum in 2 ml cryovials, using a SpeedVac concentrator (Savant) at 45°C. The air in the cryovials was replaced with argon, and the samples were stored at –80°C for further characterization using high performance liquid chromatography (HPLC). The concentrated extracts were resuspended in 0.5 to 2 ml of 55% aqueous methanol (v:v). When the concentration in this solution was too high, a small portion was subsampled for further dilution. MAAs were separated by reverse-phase isocratic HPLC, injecting 10 µl aliquots in a Brownlee (Perkin Elmer Corp., Shelton, CT, USA) RP-8 column (Spheri-5; 4.6 mm inner diameter [i.d.] × 250 mm) protected by an RP-8 guard (4.6 mm i.d. × 30 mm), in a mobile phase of 0.1% acetic acid in 55% aqueous methanol (Carefoot et al. 1998). The MAAs in the eluate were detected by online UV spectroscopy. Peaks were measured at 313 and 340 nm (Karentz et al. 1991). The MAAs were identified by comparison with retention times (order of appearance; see Fig. 6A), by the ratio of peak areas determined at 313 and 340 nm and by co-chromatography with purified standards (obtained from Dr F. Garcia-Pichel) or with secondary standards prepared from invertebrate extracts (*Aplysia dactylomela* eggs obtained from Dr D. Karentz, and *Anthopleura* sp. and *Palythoa* sp. obtained from Dr M. Shick). Additionally, several samples were cross-checked with another HPLC system equipped with a diode array detector (Dionex Corp., Sunnyvale, CA, USA) to confirm the identity of the different MAAs. The total content of specific MAAs in each sample was calculated from HPLC peak areas, using published extinction coefficients (see Tartarotti et al. 2001). The extinction coefficient for asterina-330 (AS) has not been reported in the literature but that of palythinol, a nearly identical chromophore, was consequently adopted (Dunlap et al. 1989).

For carotenoid measurements, 0.6 to 3.4 l of lake water was filtered onto glass fiber filters (Whatman GF/F) and frozen at –20°C until subsequent extraction (within 8 mo). The carotenoids were extracted for ca 10 h with 2 to 6 ml of 90% aqueous acetone (v:v), and the extracts were cleared by centrifugation. The HPLC (System Gold Beckman) comprised a double pump module for gradient elution, a visible UV (UV/VIS) detector, and a diode array detector. The acetone extract was first read in the UV/VIS spectrophotometer, with absorbance spectra recorded between 350 and 800 nm. Pigments were then separated by reverse-phase HPLC, injecting 300 µl aliquots in a Beckman Hypersil ODS C-18 column (5 µm; 4.6 mm i.d. × 250 mm) protected by a Hypersil guard column (4.6 mm i.d. × 45 mm). The elution mixture was based on Mantoura & Llewellyn (1983) with some modifications. Solvent mixtures that gave the best separation were as follows: solvent A, 80:20 methanol:water (propionic acid and butyl-ammonium phosphate buffer were added to the water as ion pairing agents with a final concentration of 1 mM of each); and solvent B, 60:40 acetone:methanol. The initial condition was a flow rate of 1 ml min^{-1} and 15% solvent B followed by a linear gradient to 95% B over 45 min with a parallel flow rate gradient from 1 to 2 ml min^{-1} and subsequent isocratic elution to 55 min. Pigments were identified by co-chromatography with commercially available primary standards (Water Quality Institute, VKI, Hørsholm, Denmark) or with culture material purified by thin-layer chromatography (donated by Hoffman La Roche, Vitamin Research and Technical Development Laboratory, Basel, Switzerland).

Data analysis. All statistical analyses were performed with the Sigma-Stat 2.03 software package (SPSS Inc., Chicago, IL, USA). The Shannon-Wiener diversity index (Zar 1999) was used to calculate the diversity of MAAs.

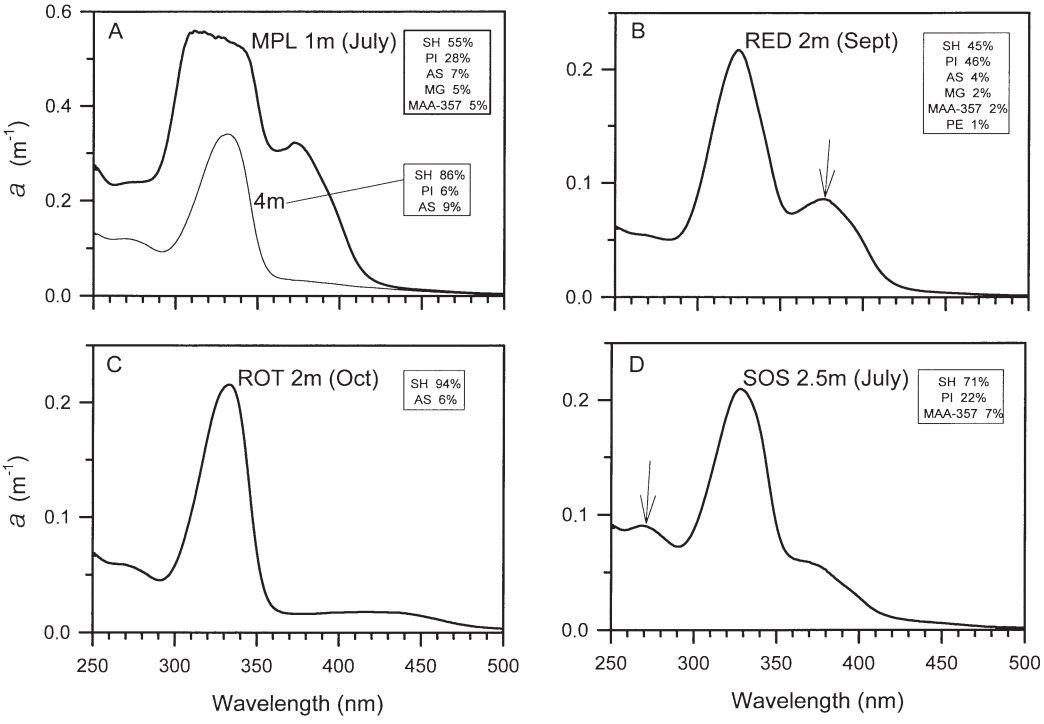

Fig. 1. Absorption (*a*) spectra of aqueous methanol extracts (20%) for (A) Mittlere Plenderlesee (MPL) in July at 1 and 4 m (thin line), (B) Estany Redo (RED) at 2 m, (C) Rotfelssee (ROT) in October at 2 m, and (D) Schwarzsee ob Sölden (SOS) at 2.5 m. The mycosporine-like amino acids (MAAs) identified by HPLC are indicated as a percentage of the total MAA concentration (SH: shinorine; PI: palythine; AS: asterina; MG: mycosporine-glycine; MAA-357: tentatively identified as usujirene; PE: palythene). Arrows indicate the absorption peaks at 270 and 375 nm

RESULTS

Total concentration of UV-absorbing compounds

The phytoplankton assemblages in this lake series showed diverse combinations of UV-absorbing compounds (Fig. 1). The spectral shapes shown in Fig. 1 result from the varying composition of MAAs and other compounds absorbing from ca 285 nm to >400 nm. In several cases, peaks around 270 nm (e.g., Fig. 1D), and 375 to 380 nm (e.g., Figs 1B & 2) were apparent. The latter peak likely corresponds to scytonemin, but as it was not eluted on the chromatograms and no standard was available, it will be referred as to UAC_{375} hereafter.

An indirect estimation of total concentration of MAAs (A_{MAA} in nm m^{-1}; Table 1) was first obtained by integrating the area under the absorption maximum centered around 330 nm on the methanol extracts (the integration from ca 285 to 360 nm ranged from 0 to 18.8 nm m^{-1}). This estimation compared well to the total concentration of MAAs obtained by HPLC (MAAs = 0.995 A_{MAA} + 0.1742, r^2 = 0.90, $p < 0.001$, n = 83). Moreover, the wavelength at peak maximum was coincident with the absorption maximum of the most abundant MAA.

Total MAA concentration for surface values ranged from undetectable to 22.9 µg l^{-1} (average of 1.96 µg l^{-1}, n = 39) or when normalized to the chl *a* content (hereafter chl *a*-specific concentration), from undetectable to 9.6 µg [µg chl *a*]$^{-1}$ (average of 1.13 µg [µg chl *a*]$^{-1}$, n = 39). One of the highest MAA concentration was found in Pfitscher Joch See (PJO) with 17.9 µg l^{-1} at the surface; however, the chl *a*-specific concentration remained only slightly above the average (2.3 µg [µg chl *a*]$^{-1}$). The highest MAA concentration (absolute and chl *a*-specific) was found in Mittlere Plenderlesee (MPL). Estany Redo (RED) also presented one of the highest chl *a*-specific concentrations of MAAs in the epilimnion. The highest chl *a*-specific concentration of UAC_{375} was also found in MPL and RED (data not shown). In general, the MAA concentration among the lakes appeared to be higher in July than in October (averages for July and October are 1.18 and 0.44 µg [µg chl *a*]$^{-1}$, respectively), but the difference for the whole data set was not significant (Wilcoxon Signed rank test, p = 0.3529, n = 18).

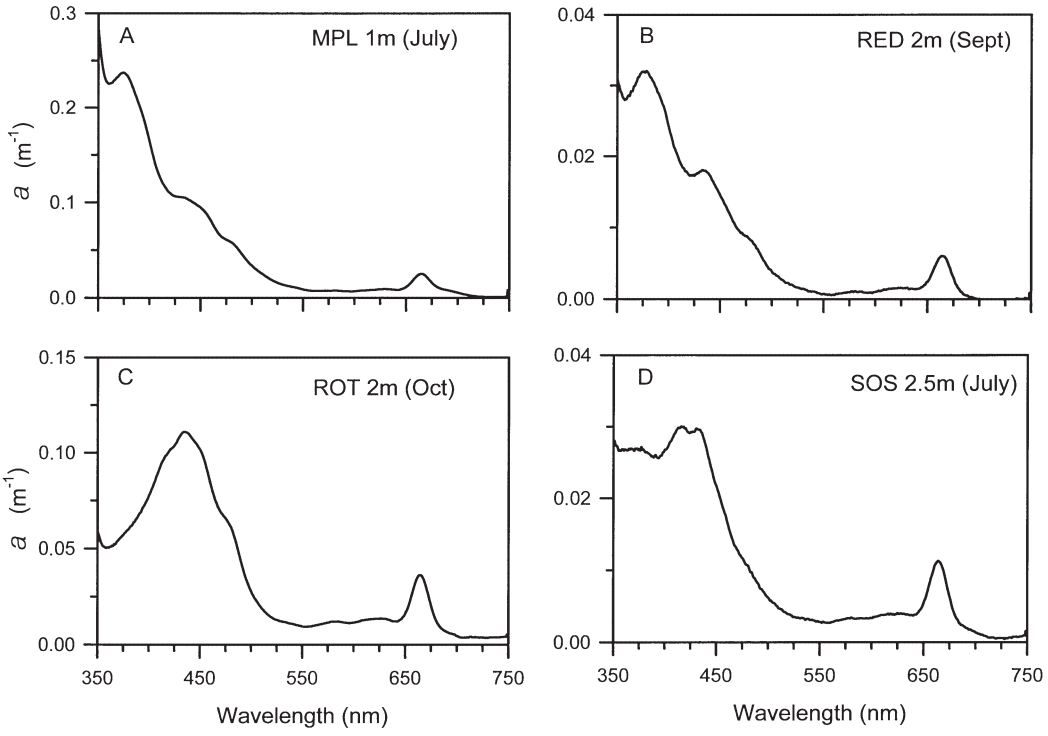

Fig. 2. Absorption (*a*) spectra of acetone extracts for (A) MPL in July at 1 m, (B) RED at 2 m, (C) ROT in October at 2 m, and (D) SOS at 2.5 m

MAA composition

The most abundant MAAs found in the lake series were shinorine (SH) and palythine (PI), representing on average 61 and 22% of the total MAAs, respectively (Table 1). Mycosporine-glycine (MG), porphyra-334 (PR), AS, palythene (PE), and 1 unknown UV-absorbing compound with a maximum absorption at 357 nm, tentatively identified as usujirene (Tsujino et al. 1979) and referred as to MAA-357 hereafter, were present from trace levels up to 47% of the total identified MAA pool. In the transparent Gossenköllesee (GKS), for example, SH was the most abundant MAA (water column average of 82%), followed by AS (14%) and PI (5%). Lichtsee (LIC) in July, Obersee (OBR) in October, and PIB in July contained the highest diversity of MAAs (Shannon-Wiener diversity index = 0.63; average for surface waters of all lakes = 0.36). The phytoplankton of several lakes presented a low diversity of MAAs, with more than 93% of the total identified MAAs composed of SH (Drachensee [DRA], OPL in October, ROT in October, ACH in October, MON, and Obernbergersee [OBB]). The contribution of PR was always low (<3%). Lake LIC had especially high concentrations of MG and PE (34 and 14%, respectively, in October), 2 MAAs that were normally found in lower proportions (on average 5 and 2%, respectively).

Depth distribution of MAAs

In most lakes, the chl *a*-specific concentration of MAAs was highest at the surface and decreased with depth (Fig. 3A), with surface values significantly higher than values at the bottom (or at the deepest sampled point; Wilcoxon signed rank test, $p < 0.001$, $n = 26$). However, in some cases (e.g., in DRA, GKS, and Schwarzsee ob Sölden [SOS]) a sub-surface maximum was present (Fig. 3B). Only in OBB (July) and PIB (October) did the chl *a*-specific MAA concentration increase with depth (16.5 and 1.4 times more concentrated at 6 and 11 m than at the surface, respectively). The composition was generally similar throughout the water column, with the exception of MPL, where the proportion of SH increased with depth while PI and other MAAs decreased (see Fig. 1A), OBR, where the proportion of MG, MAA-357, and PE increased with depth (only significant for MAA-357; $r^2 = 0.90$, $p = 0.0477$), and ACH, where the MAA composition in October completely differed between 2 and 5 m depth (SH and MG + PI, respectively).

Carotenoids

The analysis of the acetone extracts revealed the presence of pigments characteristic of major algal

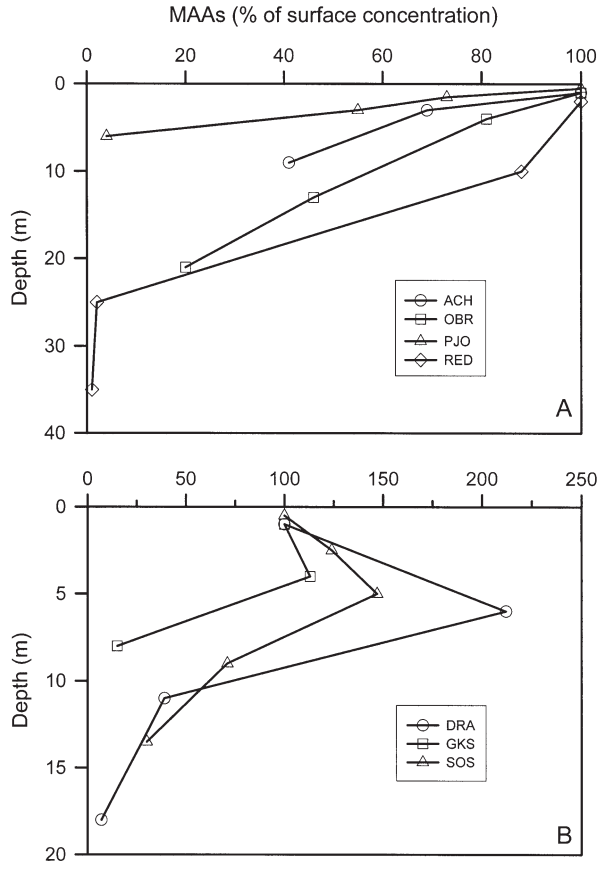

Fig. 3. Depth distribution of chl *a*-specific MAA concentrations expressed as a percentage of surface value in (A) Achensee (ACH), Obersee (OBR), Pfitscher Joch See (PJO), and RED and (B) Drachensee (DRA), Gossenköllesee (GKS) and SOS during July (except for RED in September). Other lake abbreviations as in previous figures

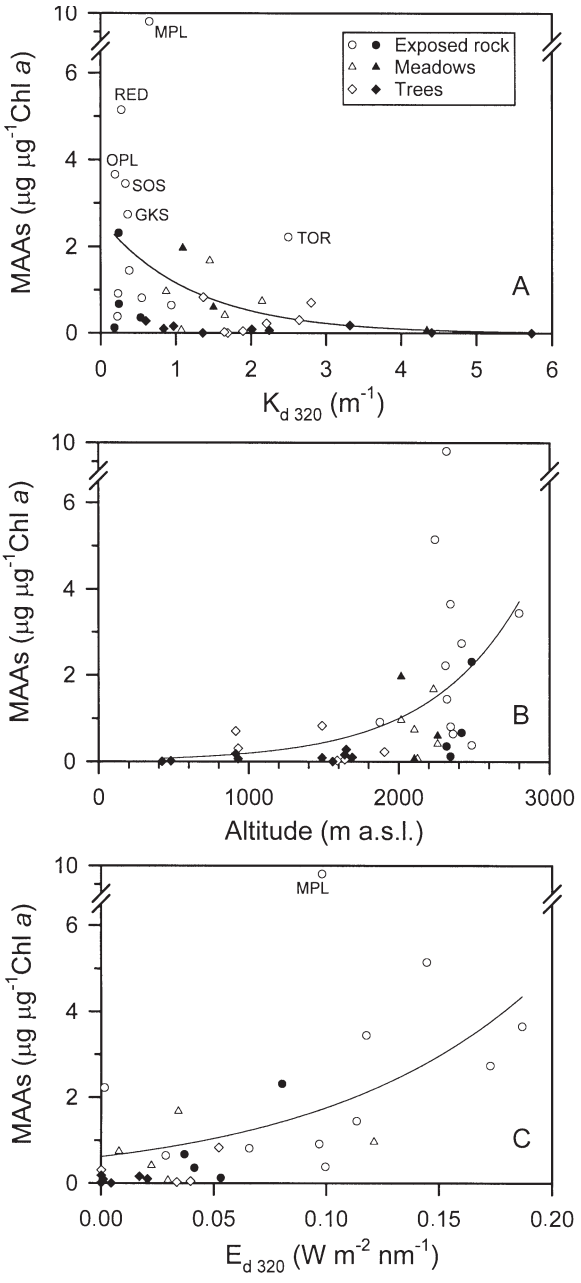

Fig. 4. Relationships between the chl *a*-specific concentration of MAAs and (A) the attenuation coefficient (K_d) at 320 nm, (B) the lake altitude, and (C) the underwater downwelling irradiance at sampling depth (E_d) at 320 nm (only data at the lake surface were used). Open symbols are for July or September sampling; filled symbols, for October or November. a.s.l.: above sea level; OPL: Oberer Plenderlesee; TOR: Estany Tort. Other lake abbreviations as in previous figures

groups such as dinoflagellates, chrysophytes, chlorophytes, and cyanobacteria. For example, the presence of peridinin (in deeper waters of Antholzer See [ANT] and ROT), alloxanthin (in deeper waters of GKS and in ROT in October), and lutein (in MPL) indicated the presence of dinoflagellates, cryptophytes, and chlorophytes, respectively. Three photoprotective carotenoids (PPCs) were identified in the chromatograms: diadinoxanthin (DIA), zeaxanthin (ZEA), and myxoxanthophyll (MYX). DIA was only detected in MPL and Klammsee (KLA), while varying concentrations of ZEA and MYX (generally associated with cyanobacteria) were found in several lakes (Table 1). The secondary carotenoid, echinenone (ECH), was detected in all lakes (range 0.03 to 0.82 µg l^{-1}), indicating the importance of cyanobacteria in mountain lakes. Concentrations of this pigment normalized to the chl *a* content were not significantly different between surface and deep layers (paired *t*-test, p = 0.1559, n = 26).

Variability among lakes

A negative exponential relationship was found between surface values of chl *a*-specific MAA concentrations and the K_d at 320 nm (r^2 = 0.15, p = 0.0156, n = 39, Fig. 4A). Generally, the highest MAA concentra-

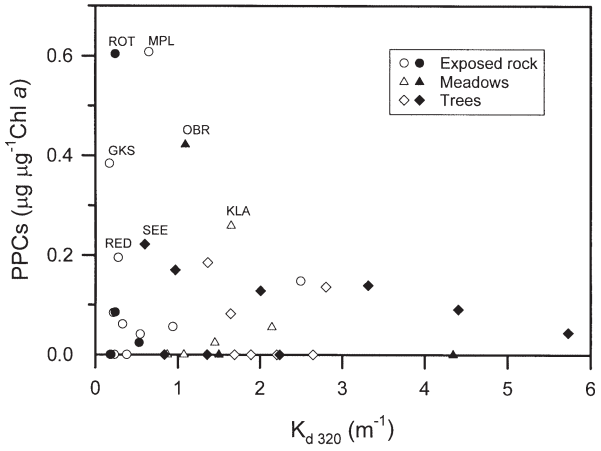

Fig. 5. Relationship between the chl *a*-specific concentration of photoprotective carotenoids (PPCs; diadinoxanthin, zeaxanthin, and myxoxanthophyll) and K_d from 320 nm (only data at the lake surface were used). Open symbols are for July or September sampling; filled symbols, for October or November. KLA: Klammsee; SEE: Seebensee. Other lake abbreviations as in previous figures

tions were found in the clearest lakes (MPL, RED, OPL, SOS, and GKS), but there were some exceptions; for example, ROT in July, and GKS and OPL in October. On the other hand, lakes with low UV transparency generally presented low MAA concentrations (e.g., ACH, LIC, MON, PIB, Pragser Wildsee [PRA], and TRA), especially when sampled in October. Thus, lakes PIB and PRA had relatively high MAA concentrations in July, but only in surface waters (more than an order of magnitude higher at the surface than at depth). The high-altitude Estany Tort (TOR) (2310 m a.s.l.) presented particularly high concentrations of MAAs despite its low transparency to UV (K_d at 320 = 2.50 m^{-1} compared to an average of 0.53 m^{-1} for the exposed rock lake category), but only in surface waters (2.21 and 0.19 µg MAAs [µg chl *a*]$^{-1}$ at 2 and 8 m, respectively). An exponential relationship was also obtained for chl *a*-specific MAA concentrations versus lake altitude (r^2 = 0.21, p = 0.0147, n = 39; Fig. 4B) or against the absolute downwelling UV irradiance at sampling depth (E_d at 320 nm, r^2 = 0.26, p = 0.0027, n = 32; Fig. 4C). Only measurements of downwelling irradiance under a sunny sky or scattered clouds were used in this regression.

Total chl *a*-specific carotenoid concentration showed no clear relationship with UV transparency, but when PPCs were plotted against K_d (at 320 nm), the relationship, although not significant, resembled the one found for MAAs (p > 0.05; Fig. 5). However, several compounds detected by HPLC (see Fig. 6B) displaying typical carotenoid absorption spectra could not be identified. Some of these compounds could have been photoprotective, yet there were no noticeable altitude- or depth-related changes in the major unidentified carotenoids.

DISCUSSION

Concentration of MAAs and comparison with other studies

The MAA concentration in this series of lakes was generally comparable to or exceeded the chl *a* concentration (average of 1.13 µg MAA [µg chl *a*]$^{-1}$) and reached up to 9.5 µg MAA [µg chl *a*]$^{-1}$ in MPL. The MAA concentrations were comparable to those reported in other studies with cultured phytoplankton species as well as with natural marine and freshwater phytoplankton assemblages (Table 2). However, the comparison must be done with caution because, as suggested by Sommaruga & Garcia-Pichel (1999), different extraction protocols may give contrasting results. For example, chl *a*-specific MAA concentrations measured in GKS during summer 1996 (Sommaruga & Garcia-Pichel 1999) were within the same range as values measured in 1998 (this study), but only for the extractions made with 20% MeOH.

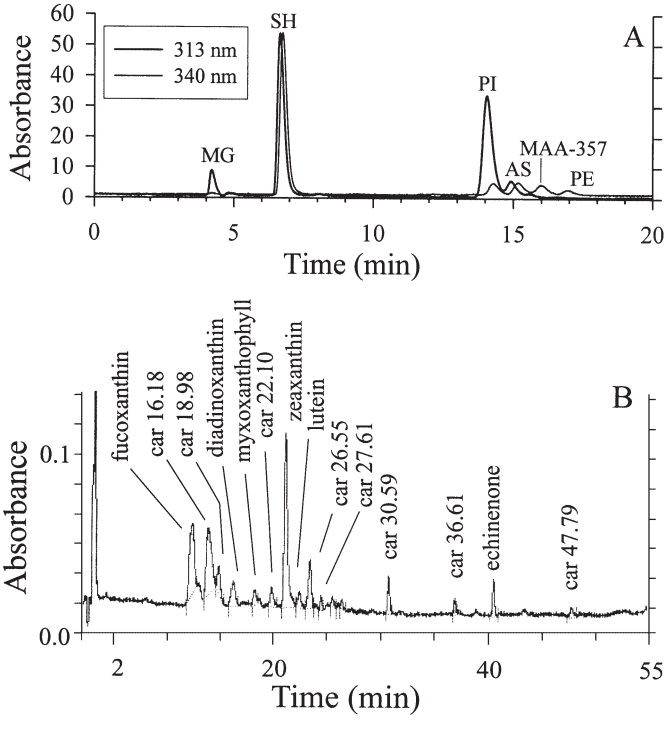

Fig. 6. Chromatograms for MPL in July at 1 m for (A) MAAs (see Table 1 for abbreviations) and (B) carotenoids (detection wavelength: 460 nm). car: unidentified carotenoid followed by its retention time

Table 2. Comparison of MAA concentrations in different marine and freshwater phytoplankton assemblages. MW: molecular weight

Material studied	MAA conc. µg [µg chl *a*]$^{-1}$	Original data (when different) and comments	Extraction method	Source
Natural lake phytoplankton				
GKS only	0.3 to 2.7		20% MeOH	This study
All 26 lakes	0 to 9.5		24 h at 4ºC + 2 h at 45ºC	
Natural lake phytoplankton (GKS)	0.3 to 0.7	0.015 to 0.053 µg [µg chl *a*]$^{-1}$ when extracted in 90% MeOH at 4°C	20% MeOH 2 h at 45ºC	Sommaruga & Garcia-Pichel (1999)
Natural marine phytoplankton	Up to ~1.3	Up to ~1 µg l^{-1} Chl *a* range given: 0.8 to 2.43 µg l^{-1}	25% MeOH 4 h at 45ºC	Bracher & Wiencke (2000)
Marine dinoflagellate cultures	0.7 and 14	Algae grown under 15 and 76 W m^{-2}	100% MeOH overnight at 4ºC	Neale et al. (1998)
Natural marine phytoplankton	~0.4 to 2		100% MeOH 6 h at 4ºC	Villafañe et al. (1995)
Marine dinoflagellate cultures and natural communities	Up to ~21	Up to 69.6 nmol MAAs [µg chl a]$^{-1}$ generic MAA MW = 300 g mol^{-1}	100% MeOH overnight at 4ºC	Whitehead & Vernet (2000)[a]

[a]Published values need to be corrected by multiplying by 10^{-4} (K. Whitehead pers. comm.)

The close fit between MAA concentrations determined from the integration of the absorption peak in the UV band (A_{MAA}) and those obtained from HPLC may be of interest for studies that were solely based on absorption measurements. However, self-absorption by very high MAA concentrations or interference by other UV-absorbing material should be considered and explicitly checked by HPLC before this empirical relationship can be used.

Depth distribution of MAAs

The chl *a*-specific concentration of MAAs decreased with depth in most lakes (Fig. 3), even in cases where the water column was well mixed (such as in Estany Barbs [BAR], MPL, OBR in October, and in the epilimnion of PJO and RED), supporting the idea that MAAs were synthesized in response to UV exposure. In ACH, MPL (see Fig. 1A), and OBR, the MAA composition notably changed with depth, probably reflecting differences in community composition. In GKS, Sommaruga & Garcia-Pichel (1999) found a decrease in MAA concentrations from the surface to 3 m, followed by an increase to maximum values at the lake bottom. The proportion of SH and AS also changed with depth, SH being most abundant at the bottom. The increase in total MAAs and SH concentrations at depth, however, was caused by the presence of cyclopoid copepods that bioaccumulate high MAA concentrations, particularly of SH (Sommaruga & Garcia-Pichel 1999). When measuring the pigment-specific *in vivo* absorption of phytoplankton (334 nm), these authors found the highest values at the surface and a decrease with depth. Daily migration of phytoplankton most likely has a great impact on what is found in the water at the time of sampling. In the present study, we did not detect significant vertical differences in the composition of MAAs in GKS, suggesting that bias due to other members of the plankton, like small rotifers and ciliates that may have passed through the 100 µm mesh net, was not significant. Moreover, profiles of MAA concentration in GKS normalized to the protein or chl *a* content gave the same distribution pattern (Sommaruga unpubl.).

MAA variability among lakes

Lakes at high altitude are exposed to high incident UVR (Blumthaler et al. 1993) and are generally highly UV-transparent, as they possess low concentrations of colored dissolved organic matter (Sommaruga et al. 1999, Laurion et al. 2000). Consequently, phytoplankton from those lakes must have different strategies to minimize the potential damage caused by UV. Our series of lakes presented a large variety of UV-absorbing compounds and underwater light conditions. Many of the lakes sampled offered reduced UV refuges with $Z_{1\%}$:Z_{max} (at 320 nm) averaging 0.75 (range of 0.004 to 3.8). The relationship between MAAs and UV water transparency clearly shows that phytoplankton from the most transparent lakes possessed the highest concentration of MAAs (Fig. 4A). The variability explained was slightly higher when the MAA concentration was related to absolute values of downwelling UVR at the respective sampling depths. This variable takes into account the differ-

ences in incident UVR in addition to differences in water column transparencies (although an average of UV irradiance over 1 to 2 d before sampling would have been more appropriate to reflect the previous history of light exposure). Nevertheless, the variability in MAA concentrations explained by UV water transparency or downwelling UVR was low (<26%). These results are in contrast to those found for zooplankton from freshwater lakes where the UV transparency (K_d) and the $Z_{1\%}$:Z_{max} ratio explained 74 and 86% of the variability in MAA concentrations among lakes, respectively (Tartarotti et al. 2001). Although normalization of MAA concentration by the chl *a* content might have introduced a bias caused by the presence of algal groups having different MAA/chl *a* ratios (or of MAA-containing microzooplankton), our results suggest that the synthesis of MAAs by phytoplankton is affected by factors other than UV transparency. For example, Estany Gran (GRA) and Estany Negre (NEG) had high transparency but low MAA concentrations. These lakes are deep (maximum depth 25 and 70 m, respectively) and had a deep mixed layer at the time of sampling (12 m for both lakes), suggesting that phytoplankton were exposed to lower daily UV doses than shallower or more stratified lakes with similar UV transparency. Other transparent lakes presenting low MAA concentrations were sampled in October (GKS, MPL, OPL covered by 2 to 3 cm of ice, and Seebensee [SEE]). At this time of the year, the synthesis of MAAs might not be an advantageous strategy, as incident UVR is relatively low compared to summer values (shorter day length, lower solar angle). Moreover, the variability in MAA concentration among phytoplankton assemblages may be affected by nutrient availability in those lakes. For example, nitrogen is essential for the synthesis of MAAs (Litchman et al. 2002), but the consequences of limitation by phosphorus, which is more typical for freshwaters, has not yet been assessed. In addition, studies have demonstrated that the ability to produce significant amounts of MAAs through photoinduction in phytoplankton is limited to certain species or taxa (Helbling et al. 1996, Hannach & Sigleo 1998). The model of Garcia-Pichel (1994) suggests that among nanoplankters (cell radii of 1 to 10 µm, a size-class abundant in many lakes sampled in this study), MAAs can afford benefits only at the expense of relatively important investments and with restricted efficiencies. Finally, repair mechanisms for UV damage is an additional important strategy used by phytoplankton, and the efficiency of repair may influence the algal composition in UV-stressed environments. This strategy might have been especially important in those UV-transparent lakes where sunscreens were less abundant.

Photoprotective carotenoids

PPCs were detected in several lakes. Three xanthophylls were identified: DIA was found in KLA and MPL, ZEA was present in several lakes, and MYX was detected in almost all lakes (Table 1). DIA is associated with diatoms, chrysophytes, or dinoflagellates, while ZEA and MYX are signature pigments of cyanobacteria. The accumulation of carotenoids under conditions of excessive light is known to play a photoprotective role and is typically observed in surface populations of cyanobacteria, chlorophytes, and red-tide dinoflagellates (Pearl et al. 1983, Ben-Amotz et al. 1989, Vernet et al. 1989). Specifically, the xanthophyll cycle plays a photoprotective role by promoting heat dissipation of excess light energy in the light-harvesting centers of chloroplast membranes (Demers et al. 1991). Moreover, Götz et al. (1999) showed that increasing amounts of ZEA resulted in higher protection of photosynthesis against UVB damage in the cyanobacterium *Synechococcus* PCC7942.

The presence of DIA in MPL and KLA (with $Z_{1\%}$:Z_{max} close to 1) likely indicates another photoprotective strategy used by the algal community of these lakes. Likewise, the highest chl *a*-specific concentration of ZEA was found in MPL and SOS, 2 lakes with high exposure to UVR (attenuation depth $Z_{1\%}$ at 320 nm of 7 and 14 m, respectively). Similarly, the concentration of MYX was particularly high in clear lakes such as MPL, ROT, RED, and GKS. Nevertheless, many carotenoids remained unidentified in our study; thus, the importance of carotenoids as an additional photoprotective mechanism needs to be further assessed in these communities.

Diversity of photoprotective compounds

Although all 7 MAAs were detected in some cases (e.g., in BAR, NEG, and PIB), the most abundant MAAs found in our lake series were SH and PI. This is similar to results on MAA distribution among several marine taxa reviewed by Karentz (2001), where the most frequently found compound was PI, followed by SH. A large diversity of PPCs and the highest concentration of MAAs were found in MPL in July (Table 1; Fig. 6). This lake, located at 2317 m a.s.l., is one of the most UV-exposed lakes in this series ($Z_{1\%}$:Z_{max} of 1.25). This lake had a distinct phytoplankton community, with a dominance of chrysophytes (*Ochromonas* sp.), dinoflagellates (*Gymnodinium* sp. and *Peridinium* sp.), and other small flagellates, and relatively high biomass (2.4 µg chl *a* l^{-1}). Dinoflagellates have been observed to synthesize MAAs and hence tolerate high levels of UVR (Carreto et al. 1990, Negri et al. 1992, Neale et al.

1998, Jeffrey et al. 1999). This lake also showed a relatively high concentration of MG (1.2 µg l^{-1}), known for its antioxidant function (Dunlap & Yamamoto 1995) and also large amounts of 3 PPCs, DIA, ZEA, and MYX. The high concentration of MAAs in this lake might have, in turn, influenced the UV transparency, which was relatively high for such a high altitude lake ($K_{d\ 320}$ = 0.65 m^{-1}; see Laurion et al. 2000). Lake RED was also one of the clearest lakes sampled, but much deeper than MPL (maximum depth of 73 m). The thermocline was well established at 14 m in September, and the complete epilimnion was exposed to more than 1% of surface UVR (at 320 nm). This lake also had high concentrations of MYX and MAAs (and a large diversity, including also MG) but only in the epilimnion (5.2 compared to 0.1 µg MAAs [µg chl *a*]$^{-1}$ at 2 and 25 m, respectively), suggesting again the photoprotective role of these compounds. Other lakes also possessed high MAA diversity (e.g., BAR, GRA, LIC, OBR, and PIB), providing a wide range of wavelengths screened across the UVB and UVA bands.

The absorption peaks around 380 nm detected in several acetone extracts (see Fig. 2) suggest the presence of scytonemin. This pigment, however, is known to be present only in the sheath of cyanobacterial mats or colonies (Garcia-Pichel & Castenholz 1991). While filamentous or colonies of cyanobacteria were only observed in some lakes and in low abundance (OBR, PIB, PJO, PRA, TOR), the volume used for microscopic observation was much smaller than that filtered for pigment analyses (60 ml sedimented compared to several liters filtered). On the other hand, peaks at 370 to 378 nm have also been reported for cultures of the dinoflagellate *Gymnodinium catenatum* (Jeffrey et al. 1999). These authors suggested that this unknown compound could be a new class of UV-absorbing compounds or the *cis*-peak form of a carotenoid. Nevertheless, the presence of this UVA-absorbing compound found in lakes with the highest UV exposure suggests a photoprotective role.

CONCLUSIONS

Our results show the widespread occurrence of photoprotective compounds (MAAs and carotenoids) among freshwater phytoplankton assemblages in lakes of very different characteristics. While the highest concentrations of these compounds were found in the clearest lakes, the relationship between UV exposure and concentration among these lakes was not straightforward. For instance, the phytoplankton of some UV-transparent lakes did not synthesize significant amounts of MAAs. Variables other than UV exposure that may affect phytoplankton community composition such as temperature, N:P ratios, and pH probably added variability to the data set analyzed. Consequently, to identify the environmental factors regulating the synthesis of photoprotective compounds in freshwater phytoplankton assemblages, it will be necessary to do experiments considering, for example, nutrient enrichment and exposure to specific wavelengths of the solar spectrum.

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