UV-absorbing mycosporine-like compounds in planktonic and benthic organisms from a high-mountain lake

Ruben Sommaruga¹* and Ferran Garcia-Pichel²

With 7 figures in the text

Abstract: We investigated the occurrence, concentration and composition of mycosporines (mycosporine-like amino acids, MAAs) in planktonic organisms and epilithic cyanobacteria from a clear water high-mountain lake situated in the Central Alps, Austria. Two bi-substituted MAAs were identified by HPLC in extracts made of 1996 plankton samples with 90% aqueous methanol, i.e. asterina-330 (λ_max = 330 nm) and shinorine (λ_max = 334 nm). Extracts with 20% aqueous methanol for 2 h at 45 °C revealed the additional presence of another MAA tentatively identified as palythine (λ_max = 320 nm) in the 1998 planktonic assemblage. In the upper 3 m of the water column the total concentration of MAAs decreased exponentially with depth, but the maxima for both absolute and chlorophyll-a specific concentrations were observed close to the bottom at 8.5 m depth. This was explained by the accumulation of MAAs in the copepod Cyclops abyssorum tatricus that stays in deep water, during daytime. The copepodite III stage contained the 3 MAAs found in phytoplankton but also the mono-substituted compound, mycosporine-glycine (mycosporine-gly: λ_max = 310 nm). The concentration of MAAs in C. abyssorum tatricus was highest for shinorine (1.45% of the dry weight) and lowest for mycosporine-gly (0.02% of the dry weight). Epilithic cyanobacteria had a more diverse MAA spectrum than plankton, and produced not only asterina-330 and shinorine but also palythinol (λ_max = 332 nm), mycosporine-gly and two unidentified compounds with λ_max = 330 and 340 nm. The composition and also the relative abundance of the cyanobacterial MAAs changed with depth. Mycosporine-gly was found at the lakeshore where Gloeocapsa sp. dominates, but it was absent at 0.5 and 2.5 m depth dominated by Schizothrix sp. and Tolypothrix sp., respectively. We could not detect any MAAs in the cysts of the red snow alga Chlamydomonas nivalis, which develops on top of the winter cover shortly be-

¹ Authors’ addresses: University of Innsbruck, Institute of Zoology and Limnology, Technikerstr. 25, A-6020 Innsbruck, Austria. E-mail: ruben.sommaruga@uibk.ac.at.
² Max-Planck Institute for Marine Microbiology, Celsiusstr. 1, 28359 Bremen, Germany.
* Author for correspondence.
fore ice-melt. These results expand to alpine lakes the range of ecosystems in which these compounds may play a significant biological role.

Introduction

Alpine lakes experience high UV irradiances due to the natural increase of UV radiation with elevation. In the Alps, for example, solar UV-B (290–320 nm) radiation was found to increase by about 20% per 1,000 m of elevation (Blumthaler et al. 1993). Therefore, high altitude lakes receive considerably more UV-B radiation than lowland lakes. Additionally, lakes situated above the tree line generally have a very high water transparency for UV due to their low concentration of chromophoric or coloured dissolved organic matter (Morris et al. 1995, Sommaruga & Penner 1997). Consequently, highly transparent lakes deserve special attention, particularly considering a scenario of increasing UV-B fluxes caused by stratospheric ozone depletion.

Organisms living in such ecosystems, however, must have developed adaptive mechanisms to cope with high-UV irradiances. One possible strategy is the synthesis of UV-absorbing compounds that may act as natural sunscreens. One group of compounds, most commonly known as mycosporines, mycosporine-like amino acids or MAAs, is widely distributed in marine organisms (Karentz et al. 1991) and is also present in terrestrial microorganisms like fungi (Favre-Bonvin et al. 1987), cyanobacteria (Garcia-Pichel & Castenholz 1993) and lichens (Büdel et al. 1997). However, almost nothing is known about their occurrence, concentration and composition in freshwater organisms.

MAAs are water-soluble, low molecular weight compounds having high molar extinction coefficients and absorption maxima ranging from 310 to 360 nm. Chemically, they consist of a cyclohexenone or cyclohexenimine substituted with amino compounds (amino, amine, amino acid, amino alcohol) in carbon 1 (mono-substituted) or in carbons 1 and 3 (bi-substituted). Approximately 17 aminocyclohexenimine and 15 aminocyclohexenone derivatives have been identified in different taxa of marine and terrestrial organisms (Karentz et al. 1991, Garcia-Pichel & Castenholz 1993, Bandaranayake et al. 1997, Dunlap & Shick 1998). Laboratory studies have demonstrated that MAAs can play a moderate sunscreen role in cyanobacteria (Garcia-Pichel & Castenholz 1993) and metazoans (Adams & Schick 1996). The frequently observed correlation between their concentration and solar exposure found in nature, suggests that they play an important role as UV sunscreens in general. In addition, MAAs may function as moderate antioxidants (Dunlap & Yamamoto 1995) and may be related to reproductive processes (Bandaranayake et al. 1997). A biosynthetic origin for mycosporines
through the early shikimic acid pathway has been suggested for fungi (Favre-Bonvin et al. 1987). Whereas this pathway is also found in algae and bacteria, it is lacking in invertebrates and vertebrates (Herman 1983). Marine metazoans, however, were found to obtain MAAs either from their food or in symbiotic association with unicellular autotrophs (Shick et al. 1991, Bana- zak & Trench 1995, Carrol & Shick 1996).

MAA composition can vary widely within large taxonomic groups, but it seems to be conserved within lower phylogenetic clades. Thus, for example cyanobacteria, may contain as many as 13 different MAAs (Garcia-Pichel et al. 1993) but closely related clusters of a strain show considerable resemblance in MAA complement (Karsten & Garcia-Pichel 1996, Garcia-Pichel et al. 1998). In the marine dinoflagellate Prorocentrum micans, porphyra-334, mycosporine-glycine, shinorine and asterina-330 were found (Lesser 1996), whereas the red-tide dinoflagellate Lingulodinium polyedra, contained porphyra-334, mycosporine-glycine: valine, palythine, palytholin and palythene (Vernet & Whitehead 1996). In another red-tide dinoflagellate Alexandrium excavatum, porphyra-334, mycosporine-glycine, palythine, asterina-330, shinorine, palythenic acid and the isomeric mixtures of usujirene and palythene were found (Carreto et al. 1990).

Here we report for the first time the occurrence, concentration and composition of MAAs in planktonic organisms and benthic cyanobacteria of a transparent high-mountain lake in the Alps.

**Materials and methods**

The study was carried out during summer 1996 in Gossenköllesee, situated above the tree line at 2,417 m above sea level in the Central Alps, Tyrol, Austria (47° 13’ N, 11° 01’ E). The lake (area: 1.7 ha, maximum depth: 9.9 m) has a high UV transparency with 10 % of the surface UV-B (nominal λ: 305 nm) and 33 % of the UV-A radiation (nominal λ: 340 nm), as measured with a PUV500 underwater radiometer, reaching the bottom after ice break-up (Sommaruga & Psenner 1997). A field station on the shore facilitated the laboratory work. Additional sampling was carried out in the early 1998 summer.

For the analysis of MAAs in plankton samples, 2 to 3 liters of water were collected around noon at 0, 1.5, 3, 5 and 8.5 m depth with an opaque sampler and immediately filtered through a Whatman GF/F filter. The filters were frozen at −20 °C until extraction within 1 week. Samples of epilithic cyanobacteria were collected between 0 and 2.5 m depth by SCUBA diving following the zonation described in Rott & Perneger (1994). The only copepod present in this lake, Cyclops abyssorum tetricus, was collected during daytime from 8.5 m depth with a 5 liter sampler and concentrated on a net sieve of 100 μm mesh size. The sieve was washed several times with filtered (<0.45 μm) lake water to remove phytoplankton and 160 individuals were carefully
picked-up and placed on a wet Whatman GF/F filter kept on an ice-pack. The biomass of the copepodites was estimated based on the relationship between length and dry weight calculated for this species by Praptokardiyo (1979). Cysts of the red snow alga *Chlamydomonas nivalis* were collected directly from the snow in late spring. After melting at 4 °C, the sample was filtered onto a Whatman GF/F filter.

In most cases the extraction of MAAs was done with 90 % aqueous methanol for 24 h in the dark at 4 °C. Occasionally, extractions with 20 % aqueous methanol at 45 °C for 2 h were also performed (Garcia-Pichel & Castenholz 1993). Then the samples were briefly sonicated (2 min) on an ice bath and the extracts cleared using a 0.1 μm pore size Whatman Anodisc filter. The extracts were scanned between 250 and 750 nm in a double beam Hitachi U-2000 spectrophotometer using quartz cuvettes of 5 cm pathlength against a 90 or 20 % aqueous methanol reference. Then the extracts were evaporated until dryness under vacuum using a SpeedVac concentrator (Savant) at 45 °C. The samples were stored at −80 °C for further characterization using high performance liquid chromatography (HPLC). For separation of MAAs, the concentrated extracts were resuspended in 100–200 μl of 20 % methanol and 20–100 μl aliquots were injected in a Hypersil 5 μm pore size C₁₈ column for isocratic reverse-phase HPLC analysis (Garcia-Pichel & Castenholz 1993). The mobile phase was 25 % aqueous methanol plus 0.2 % acetic acid. The MAAs in the eluate were detected by online UV absorption spectroscopy (220–450 nm, peak measurement was carried out at 320 nm). The MAAs were identified by co-chromatography with authenticated primary standards (obtained from Dr. Deneb Karentz and originally prepared by Dr. Walter Dunlap) or with secondary standards prepared from cyanobacterial cultures. The concentration of MAAs was quantified by UV spectroscopy using published molar extinction coefficients (Ito & Hirata 1977, Takano et al. 1978, Chioccarà et al. 1980). Since mycosporine-glycine is unstable, the molar extinction coefficient used was that for the stable methyl ester form. The extinction coefficient for asterina-330 has not been reported, consequently we used a generic coefficient of 120 1 g⁻¹ cm⁻¹ (Garcia-Pichel 1994). Concentrations of the different MAAs were normalized to the concentration of chlorophyll-a (Chl-a), which was analyzed in parallel samples after the same extraction procedure but using acetone as solvent. The equations of Jeffrey & Humphrey (1975) were used to calculate the concentration of pigments.

Particle absorption (aₚ) measurements of parallel plankton samples (zooplankton excluded) were made by the quantitative filter technique with pathlength corrections (Mitchell 1990). Whatman GF/F filters from a single batch were used for all measurements. The samples were kept at 4 °C in the dark until analysis within 24 h. The absorbance was measured in the same spectrophotometer described above with a wetted GF/F filter as a reference. A smoothing function (Savitsky-Golay) was applied to the scan obtained. Pigment-specific particle absorption (a*ₚ) was calculated dividing aₚ by the concentration of chlorophyll-a plus phacocytin.

**Results and discussion**

Fig. 1 shows the characteristic absorbance of MAAs in plankton samples extracted with 90 % aqueous methanol. Two MAAs, shinorine (λ_max = 334 nm)
Fig. 1. Example of absorption spectra of aqueous methanol (90%) extracts of plankton for different depths in the water column of Gossenköllesee on 22 July 1996. Samples collected at noon on a sunny day.

and asterina-330 ($\lambda_{\text{max}} = 330$ nm) were found in 1996 plankton samples extracted with aqueous methanol 90%. From the surface to 3 m depth both absolute and chlorophyll-a specific concentrations of asterina-330 were higher than that of shinorine, while at 5 and 8.5 m depth, concentrations of shinorine were higher (Fig. 2 b, c). Total concentrations of MAAs ranged from 21.6 to 139.4 ng l$^{-1}$ or from 0.015 to 0.053 $\mu$g $\mu$g$^{-1}$ Chl-a (Fig. 2 d). In the upper 3 m, absolute concentrations of MAAs decreased exponentially with depth, but this pattern was reversed at 5 m depth and the highest total MAA concentration was found at 8.5 m depth. Although the chlorophyll-a concentration was highest at 8.5 m depth (Fig. 2 a), this pattern was also observed when the concentration of MAAs was normalized to the chlorophyll-a content.

The summer phytoplankton of this lake is dominated by nanoplancktonic forms, mainly dinoflagellates such as Woloszynskia sp., Gymnodinium uberrium, G. cecoides, Gymnodinium sp., Amphilidium elenkinii, the chrysophytes Ochromonas sp. and Chromulina sp., and the diatoms Fragilaria deli-
catissima, Cyclotella aff. gordonensis and C. distinguenda var. unipunctata (NAUWERCK 1966, HALAC et al. 1997). A deep maximum of chlorophyll-a is typical for transparent alpine lakes during summer (PECHLANER 1971). However, we expected to find a decrease in MAAs concentration with depth if they were to play a role as sunscreens. In contrast to the pattern described above, our particle absorption measurements from parallel samples, where only phytoplankton was concentrated, indicated a higher pigment-specific particle absorption (a*ₚ) at 334 nm at the surface and a decrease with depth (Fig. 3). This discrepancy prompted us to search for other possible sources of MAAs in the plankton that may have masked the expected pattern.

The copepod Cyclops abyssorum tatricus characteristically found in deep water layers during daytime (EPPACHER 1968) was a potential candidate to explain the maximum MAA concentration found at 8.5 m depth. Although at present, there is no evidence that invertebrates can synthesize MAAs de novo, they can accumulate them through the diet (CARROLL & SHICK 1996). In addition, bacteria present in the gut may be an additional source of these compounds (ARAI et al. 1992). As expected, the methanolic extracts of C. abyssorum tatricus (copepodite stage III) clearly showed a very high absorption at 334 nm that even exceeded the carotenoid peak at 474 nm (Fig. 4). The very low absorption at the red chlorophyll-a peak (665 nm) indicated that the occurrence of MAAs in this species was not associated with an occasional presence of phytoplankton in their gut. Analysis of the extract by HPLC revealed the
Fig. 3. Pigment-specific spectral absorption (a*<sub>p</sub>) of particles at different depths in Gossenköllesee on 22 July 1996.

Fig. 4. Methanolic extract (90 %) of the copepod *Cyclops abyssorum taticus* showing the peak of the carotenoid absorption maximum at 474 nm and of UV-absorbing compounds at 334 nm. The inset shows the chemical structure of the mycosporine-like amino acid shinorine, the most abundant compound in this species.
presence of shinorine, asterina-330, mycosporine-glycine ($\lambda_{\text{max}} = 310$ nm) and another MAA tentatively identified as palythine ($\lambda_{\text{max}} = 320$ nm). The highest MAA concentration was found for shinorine (0.046 $\mu$g ind$^{-1}$ or 1.45 % of the dry weight) followed by palythine (0.020 $\mu$g ind$^{-1}$ or 0.63 % of the dry weight), asterina-330 (0.008 $\mu$g ind$^{-1}$ or 0.24 % of the dry weight) and mycosporine-glycine (0.0005 $\mu$g ind$^{-1}$ or 0.02 % of the dry weight). To our knowledge, the work of KARENTZ et al. (1991) is the only one that allows comparison of MAAs with another copepod species, *Calanus propinquus*. In this marine species the peak of absorption was found at 328 nm and the MAAs identified included palythine, porphyra-334, shinorine, palythene and mycosporine-glycine; the last being the most abundant. The distinct MAA composition between these species is likely to be a reflection of dietary differences. The total concentration of MAAs in *C. propinquus*, however, was $\sim$16 times lower than in *C. abyssorum tatricus* (0.00146 and 0.0234 $\mu$g $\mu$g$^{-1}$ dry weight, respectively).

An important difference between the MAA composition of the phytoplankton and *C. abyssorum tatricus* was the unique presence of mycosporine-glycine in the latter. As discussed above, de novo synthesis of MAAs is not known in metazoa, thus the presence of mycosporine-glycine in this copepod may indicate a transformation from another MAA or another different source for this compound like bacteria. Recently, DUNLAP & SHICK (1998) reported that shinorine can be converted into mycosporine-glycine by bacteria, and suggested that this type of transformation may occur in the gut of a sea urchin species. The hydrolytic conversion of some bi-substituted to mono-substituted MAAs occurs also under mildly acidic conditions (PORTWICH & GARCIA-PICHEL unpubl.).

Although in Gossenköllesee, the adult stage of *C. abyssorum tatricus* is never exposed to a maximal UV dose because it avoids shallow waters at midday, GLIWICZ (1986) reported its occurrence also in shallow ponds. The accumulation of these compounds by the female adults of *C. abyssorum tatricus* may be an important adaptive strategy for the survival of the first life stages that thrive close to the surface. In the Antarctic limpet *Nacella concinna*, concentrations of MAAs were higher in ovary and eggs than in the gut, testis or in the body tissue (KARENTZ et al. 1992). Furthermore, ADAMS & SHICK (1996) demonstrated that MAAs play a photoprotective role in eggs of the sea urchin *Strongylocentrotus droebachiensis*. These authors found an inverse relationship between the UV-induced cleavage delay of the embryos and the MAA concentration (mostly shinorine). Future studies should determine the concentration of MAAs in the different stages of *C. abyssorum tatricus* and their photoprotective capacity in eggs of this species.

Another surprising result of our study was the low concentration of MAAs found in the plankton samples (Fig. 2). For example, total MAAs concentration in natural phytoplankton assemblages from Antarctica ranged from $\sim$0.36 to 1.67 $\mu$g $\mu$g$^{-1}$ Chl-a during a two-week exposure to UV radiation + photosyn-
Mycosporine-like compounds

Fig. 5. Absorbance from extracts made with 90% and 20% aqueous methanol in parallel plankton samples collected from 8 m depth (see text for the methods).

Theoretically active radiation (VILLAFÁNE et al. 1995). In our case, total MAA concentrations in the water column were only between 0.015 to 0.053 μg μg⁻¹ Chl-a. The low concentration was coincident with the relatively low absorption at the MAAs peak when compared, for example, with the maximum red absorption of Chl-a at 665 nm (Fig. 1). However, the pigment-specific absorption of particles (a⁎p) indicated again an opposite pattern (Fig. 3). Moreover, in several occasions no distinct MAAs peak was observed with our routine extraction procedure (90% aqueous methanol). One possible explanation for this discrepancy is the low extraction efficiency of MAAs in phytoplankton samples with 90% aqueous methanol. Extractions in parallel with 20 and 90% aqueous methanol indicated that the efficiency of the latter was much lower (Fig. 5). The HPLC analysis of the 20% extract gave concentrations of MAAs ranging between 0.30 and 0.69 μg μg⁻¹ Chl-a. Our results are preliminary, but we have serious grounds to caution against the use of high methanol concentrations for MAAs extraction in phytoplankton.

The methanolic extracts of the cysts of the red snow alga Chlamydomonas nivalis had no peak in the UV range suggesting the absence of MAAs, at least in measurable amounts (data not shown). THOMAS & DUVAL (1995) also found no peak or just a modest one in methanolic extracts of the same species collected from Sierra Nevada, USA. As suggested by several authors, the extraordinary concentration of secondary carotenoids, in particular astaxanthin and its esters, in snow algae plays an important role as photoprotective compounds (BIDIGARE et al. 1993, KOBAYASHI et al. 1997, MÜLLER et al. 1998). Indeed the
sunscreen factor provided by carotenoids in these cysts is among the highest measured for microorganisms (GARCIA-PICHEL 1994).

Benthic cyanobacteria had more diverse MAAs than plankton, including not only shinorine and asterina-330, but also palythinol ($\lambda_{\text{max}} = 332$ nm), mycosporine-glycine ($\lambda_{\text{max}} = 310$ nm), and two unidentified UV-absorbing compounds with absorption maximum at 330 and 340 nm. A typical spectrum of the methanolic extracts from benthic cyanobacteria collected at different depths is shown in Fig. 6. The composition and relative concentration of MAAs differed depending on the depth zones characterized by the dominant cyanobacteria (Fig. 7). The samples obtained from the lakeshore in the zone defined by ROTT & PERNEGGER (1994) as dominated by Gloeocapsa sp. was characterized by the absence of shinorine and the presence of mycosporine-glycine, a compound not found in the other zones. The highest concentration was found for an unknown compound with absorption maximum at 330 nm (Fig. 7a). The samples collected at 0.5 and 2.5 m depth, characterized by the
Fig. 7. Relative concentrations of different MAAs in benthic cyanobacteria collected at the lake shore (A), 0.5 m depth (B) and 2.5 m depth (C) in Gossenköllesee.

dominance of *Schizothrix* sp. and *Tolipothrix* sp., respectively, presented the same qualitative composition with palythiol as the most abundant MAA (Fig. 7b,c).

The fact that mycosporine-glycine was only found in epilithic cyanobacteria exposed to high solar UV radiation is interesting because besides its absorption in the UV-B range ($\lambda_{\text{max}} = 310\,\text{nm}$), the shortest for any MAA, this
compound has also an antioxidant function (DUNLAP & YAMAMOTO 1995) thus providing protection against photooxidative stress.

In conclusion, the present study reports for the first time the presence and characterization of MAAs in different organisms from a freshwater lake. These results expand to alpine lakes the range of ecosystems in which these compounds may play a significant biological role. Further investigations should address several open questions, for instance, which factors regulate the synthesis of these substances, particularly the ability of different wavelengths in the solar spectrum to induce MAAs synthesis, and their potential for UV attenuation in the water column (SOMMARUGA & PSENNER 1997).

Acknowledgements

We thank LEO FUREDER and HARALD PHEOFER for SCUBA diving in Gossenköllesee, RAMONA APPLE for help with the HPLC analyses, BARBARA TARTAROTTI for separating the copepodites and estimating the dry weight, DENEK KARENTZ for supplying the primary standards, and MALCOLM SHICK, ROLAND PSENNER and two anonymous reviewers for improving the clarity of a previous version of this manuscript. This work was supported by a project from the Austrian Science Foundation (FWF, P11856-BIO).

References


Mycosporine-like compounds

