

NOTE

Packaging of mycosporine-like amino acids in dinoflagellates

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ABSTRACT: Synthesis of mycosporine-like amino acids (MAAs) can significantly protect phytoplankton cells against damaging ultraviolet (UV) radiation, depending on the concentration, type and cellular distribution of these UV sunscreens. We addressed the hypothesis that MAAs are concentrated around UV-sensitive organelles for improved efficiency, thereby increasing their 'package effect'. This was investigated for 2 species of MAA-producing dinoflagellates, with comparative analyses of a MAA-free diatom. Spectral absorbance of dinoflagellates suspended in their growth medium was relatively small throughout the MAA-absorbing region despite high concentrations of these compounds (determined by HPLC), suggesting that MAAs are highly packaged in intact cells. The measured *in vivo* absorbance of suspended and filtered phytoplankton cells revealed an extra-cellular release of water-soluble MAAs during freezing. The release of MAAs upon thawing enabled the calculation of an MAA packaging index based on the comparison between absorption characteristics before (MAAs inside cells in suspension) and after freezing (MAAs released from cells on thawed filters). Additionally, MAA packaging was evaluated from the reconstruction of absorption spectra from the individual MAA concentration. Consistent with our hypothesis, the results showed that UV absorption was up to 80% lower in the intact cells relative to the MAAs dissolved in solution. These results imply a high degree of MAA packaging in these dinoflagellates, that may increase the protection efficiency for specific cellular targets.

KEY WORDS: Package effect · Photoprotection · MAAs · UV-absorbing compounds · Bio-optics · *Alexandrium tamarense*

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INTRODUCTION

Light absorption by phytoplankton cells is a key process in photosynthesis. *In vivo* light absorption is highly variable, being affected mostly by photoacclimation (Stramski & Morel 1990), nutrient status (Sosik & Mitchell 1995) and cell size (Ohi et al. 2002). UV radiation is also absorbed by phytoplankton and can cause damage to several metabolic pathways (Vincent & Neale 2000). Cells possess protective mechanisms

against UV radiation (Roy 2000), which influence their specific absorption in this waveband. For instance, the accumulation of mycosporine-like amino acids (MAAs) can affect the sensitivity of microalgae to UV radiation (Garcia-Pichel & Castenholz 1993, Neale et al. 1998). Hence, measurement of cell absorption in the UV range is a factor of interest in photobiology, a field of increasing importance in view of the decline in the stratospheric ozone levels and the climate-induced changes in water UV transparency (Williamson et al. 1996).

Pigment packaging has a major influence on the light harvesting efficiency of algal cells (Kirk 1994 and references therein). The absorption efficiency of phytoplankton is a non-linear function of pigment concentration, thylakoid arrangement and cell size (Berner et al. 1989, Nelson et al. 1993). A similar packaging effect for UV sunscreens could be of importance to MAA-producing phytoplankton. For UV sunscreens however, packaging can be viewed as effectiveness, since with equal investments, a large organism would benefit more than a small one. The bio-optical model developed by Garcia-Pichel (1994) indicates that MAAs should only be marginally effective at reducing UV radiation due to the short pathlength characteristic of relatively small phytoplankton cells (assuming a homogeneous cytoplasmic distribution of MAAs). However, the localization of MAAs around UV-sensitive organelles (packaged MAAs) might increase their efficiency, although this has not yet been assessed. MAA packaging has been suggested previously for the colonial prymnesiophyte *Phaeocystis antarctica* (Davidson & Marchant 1994, Moisan & Mitchell 2001). However, MAA concentrations observed in colonial prymnesiophytes appeared too high to be contained intracellularly, suggesting their association with the extracellular colonial matrix (Marchant et al. 1991, Riegger & Robinson 1997).

In the present study, we addressed the hypothesis that MAAs are packaged, likely around UV-sensitive organelles, in 2 MAA-rich dinoflagellate species. Two approaches were taken to test this: (1) by comparing the *in vivo* UV absorption of suspended dinoflagellate cells with that of filtered and frozen cells (causing a freezing-related release of MAAs). (2) by reconstructing the *in vivo* UV absorption from the concentration of individual MAAs, as measured by HPLC, following the approach described for pigments in Bidigare et al. (1990). This reconstruction should reproduce the *in vivo* UV absorption of suspended cells only if no packaging is present (Nelson et al. 1993).

MATERIALS AND METHODS

Mono-specific cultures included 3 dinoflagellates, 2 strains of *Alexandrium tamarense* (Provasoli-Guillard National Center for Culture of Marine Phytoplankton CCMP116, isolated from Ría de Vigo, Spain, and CCMP1771, isolated from the Tamar Estuary, England; hereafter Ate and At), 1 of *Heterocapsa triquetra* (CCMP449; Ht), and 1 diatom, *Thalassiosira pseudonana* (CCMP1335; Tp). Cells were grown under ca. 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of incident PAR enriched with UVA radiation (identified with '+') and under reduced irradiance (ca. 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of incident PAR) without supplemented UVA (identified

with '-'), to generate different concentrations of MAAs (see Laurion et al. 2003 for details).

For chl *a* and MAA measurements, 8 to 25 ml of the cultures were filtered onto glass fiber filters and kept at -80°C until subsequent extraction. Chl *a* concentration was measured by HPLC following the method of Zapata et al. (2000; mobile phase B1). MAAs were extracted in 25% aqueous methanol (vol:vol) following a 2-step procedure, and separated by reverse-phase isocratic HPLC (Shick et al. 1999). Details are given in Laurion et al. (2003). A 20 ml subsample was fixed with acidified Lugol's solution and cells were counted under an inverted microscope.

The absorption spectra of phytoplankton suspensions were obtained using a Perkin-Elmer Lambda 2 spectrophotometer equipped with an integrating sphere (Labsphere RSA-PE-20). Absorbance was measured between 280 and 850 nm (OD_s). The absorbance of the dissolved organic matter naturally released in the media during growth (OD_{fil}) was also measured (except for Tp; this was checked a few weeks later and the absorbance by DOM remained low for this strain). For this, cells were removed by low-pressure filtration. For measurements of particle absorbance from filter pads, 1.86 ml aliquots were filtered onto glass fibre filters. One set of duplicate filters was frozen immediately in liquid nitrogen and stored at -80°C for 2 d ($\text{OD}_{\text{f frozen}}$). A second set of filters was stored frozen as above, but upon thawing the filters were rinsed with 5 ml filtered sea water (0.2 μm) before they were scanned ($\text{OD}_{\text{f frozen rinsed}}$). This step accounted for the water-soluble material that might have exuded from the algal cells or been released upon cell breakage. The filtrate of this rinsing step was kept for further analysis ($\text{OD}_{\text{rinsing}}$). Details on baseline and blank corrections, and null point adjustment are given in Laurion et al. (2003).

RESULTS

Both dinoflagellate species had high concentrations of MAAs, ranging from 5.6 to 14.5 $\mu\text{g MAAs } \mu\text{g chl } a^{-1}$ (35.0 to 329.5 pg MAAs per cell) depending on species and growth conditions (Table 1). This table also presents concentrations in MAAs per cell volume (assuming ellipsoidal cells). These concentrations fall in the range of values found in cultured and natural marine dinoflagellates (e.g. Neale et al. 1998, Whitehead & Vernet 2000; see Laurion et al. 2002 for a comparison table). Carreto et al. (2001) found a lower MA concentration in cultures of *A. tamarense* (6.18 pg cell $^{-1}$), likely related to the low growth irradiance used (65 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). A total of 7 MAAs were identified, with 1 additional abundant MAA absorbing at 333 nm (M333; Table 1). Unidentified MAAs (excluding M333) re-

Table 1. Cell abundance and the concentration of chlorophyll *a* (chl *a*) and mycosporine-like amino acids (MAAs), with the percentage of individual MAAs (with wavelength at maximum absorption) as determined by HPLC (MG: mycosporine-glycine; SH: shinorine; PR: porphyra-334; PA: palythene; M333: unidentified; PI: palythene; US: usujirene; PE: palythene) and the chl *a*-specific *in vivo* phytoplankton absorption coefficient at 675 nm for cells in suspension (a_s^*). nd = not detected; na = not available

Species	Cells ($10^6 l^{-1}$)	chl <i>a</i> (pg cell $^{-1}$)	a_s^* (m 2 mg chl a^{-1})	MAAs (μ g chl a^{-1})	MAAs (pg cell $^{-1}$)	MAAs (pg μ m $^{-3}$)	MG (%)	SH (%)	PR (%)	PA (%)	M333 (%)	PI (%)	US (%)	PE (%)	Other MAAs (%)
Ate+	4.35	18.4	0.014	14.5	266.5	0.0080	24.0	7.3	8.4	8.3	1.0	19.1	0.9	28.7	2.3
Ate-	4.68	37.8	0.015	7.9	297.5	0.0089	17.7	5.2	10.8	4.4	0.9	44.2	0.4	15.2	1.3
At+	3.83	42.2	0.017	7.8	329.5	0.0115	8.6	7.3	2.8	1.9	22.1	1.7	5.8	49.2	0.5
At-	3.54	35.9	0.015	5.6	201.8	0.0070	6.5	4.6	4.6	0.8	12.7	2.3	3.2	64.2	1.1
Ht+	26.34	5.2	0.020	6.8	35.0	0.0156	3.0	41.5	0.8	nd	39.7	2.5	0.5	6.5	5.5
Ht-	43.47	3.5	0.027	na	-	-	-	-	-	-	-	-	-	-	-
Tp	145.9	0.16	0.021	nd	-	-	-	-	-	-	-	-	-	-	-

^aSome of these data were taken from Laurion et al. (2003)

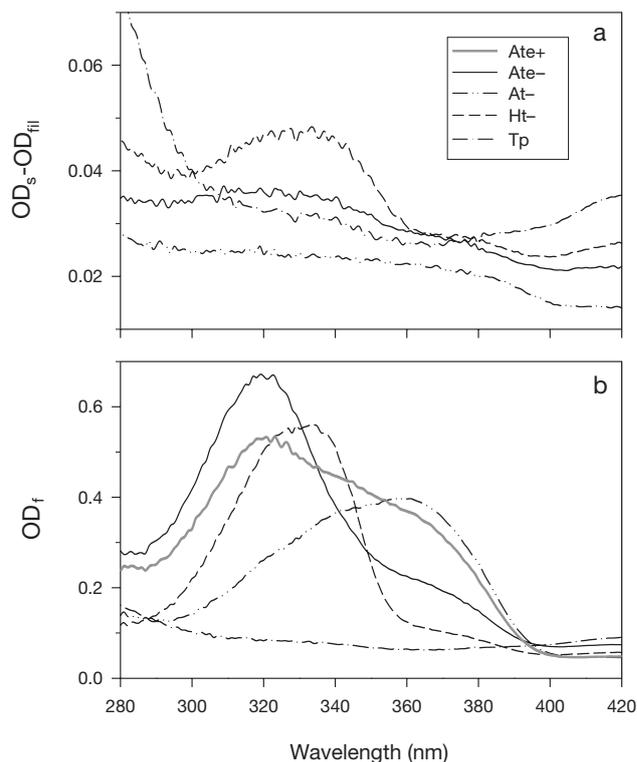


Fig. 1. Absorbance spectra in the UV waveband of (a) suspended cells ($OD_s - OD_{fil}$) and (b) frozen filters for Ate+, Ate-, At-, Ht- and Tp ($OD_{f \text{ frozen}}$). OD_{fil} was subtracted from OD_s except for Tp (explaining the increase of OD toward lower wavelengths)

mained below 6% of total MAAs eluted. No MAAs appeared in the chromatograms of Tp.

The *in vivo* absorbance spectra of suspended cells are presented in Fig. 1a (spectra were selected to optimize the presentation). Despite high MAA concentrations, the UV absorbance of suspended cells of the dinoflagellates is relatively flat compared to scans of filtered cells. These spectra were corrected for the absorbance of dissolved compounds in the culture media. Cultures of Ht had considerable amounts of dissolved organic compounds, the shape of the spectra indicating a strong contribution of dissolved MAAs (see Laurion et al. 2003, their Figs. 3 & 4).

The MAA composition (Table 1) corresponded closely to the shape of the spectral absorbance of filtered cells (Fig. 1b). The diverse assemblage of MAAs in Ate gave a wide peak in the *in vivo* absorbance spectrum, whilst the dominance of M333 and palythene in At shifted the UV absorbance peak toward higher wavelengths. The dominance of 2 MAAs absorbing at roughly the same wavelength in Ht gave a narrower absorbance peak. Such large UV absorbance peaks for filtered cells have been reported in some other studies (Neale et al. 1998, Sosik 1999) and is

related to the effect of handling (at least for these dinoflagellates; Laurion et al. 2003). These results suggest that a gradual release of MAAs started immediately after or during filtration, while freezing greatly increased this release. When thawed filters were rinsed with filtered seawater prior to scanning, this large UV peak found with frozen filters decreased considerably (more than 98% of the MAA peak disappeared for Ate and At and more than 90% for Ht) and appeared in the rinsing water.

The freezing-related release of MAAs allowed us to calculate an index of MAA packaging ($\pi_1 = 1 - [\text{OD}_{\text{s-fil}} \times \beta / \text{OD}_{\text{f frozen}}]$), assuming that MAAs were unpackaged in thawed cells on filters. The absorbance of suspended cells (OD_{s}) was corrected for the absorbance of the medium (OD_{fil}) and for pathlength amplification (β) caused by multiple scattering of light through the filter (Kiefer & SooHoo 1982). β values were obtained empirically from the ratio of filtered to suspended absorption averaged over the visible waveband (the 'true' pathlength amplification) i.e. where there was no artifact caused by the presence of MAAs. Highly packaged MAAs should cause low values of $\text{OD}_{\text{s-fil}}$ (corrected for β) compared to $\text{OD}_{\text{f frozen}}$. The π_1 values calculated varied between 0 and 0.94, depending on species and growth light conditions, and were maximal at the same wavelengths as for the absorbance spectra. Values of π_1 averaged over 280 to 380 nm are given in Table 2. This index of MAA packaging can be considered a minimum in the case of Ht, since an unknown fraction of MAAs was not released out of thawed cells.

Fig. 2 shows the reconstruction of spectral absorption in Ate cultures grown with and without additional UVA, following the approach of Bidigare et al.

Table 2. Averaged packaging index (π) of MAAs calculated over 280 to 380 nm: $\pi_1 = 1 - (\text{OD}_{\text{s-fil}} \times \beta / \text{OD}_{\text{f frozen}})$, $\pi_2 = 1 - (\text{OD}_{\text{s-fil}} / \text{OD}_{\text{sum+det}})$. The β values used are the averages obtained over the visible waveband, i.e. where there is no artifact caused by the presence of MAAs. π_2 is calculated from spectral reconstruction based on the concentration and spectral characteristics of the various MAAs present; no π_2 values are available for Ht- and Tp because there are no MAAs available or present (cf. Table 1)

Species	π_1	π_2
Ate+	0.88	0.80
Ate-	0.65	0.56
At+	0.77	0.62
At-	0.69	0.51
Ht+	0.62	0.41
Ht-	0.63	-
Tp	0.02	-

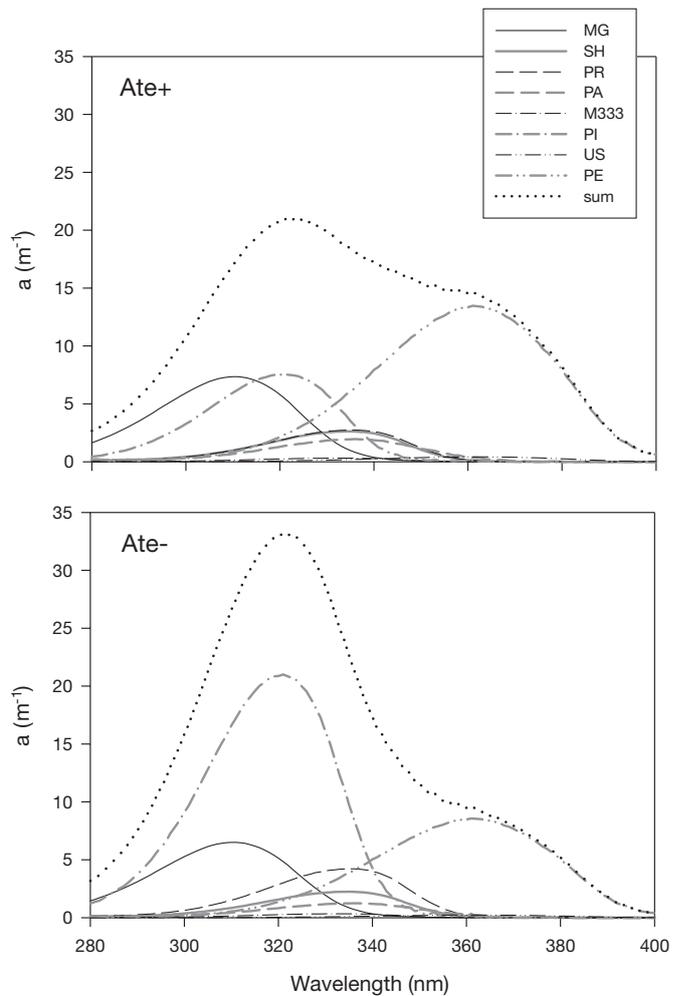


Fig. 2. Reconstruction of spectral absorption from individual MAA concentrations (HPLC) in Ate cultures grown with (Ate+) and without (Ate-) additional UVA

(1990) for pigments (unidentified MAAs were not included). The difference between light treatments is particularly striking at 360 nm, where the abundance of PE affects the shape of the spectra. The summed MAA absorbance, OD_{sum} , was highly correlated with values obtained from the frozen filter rinsing water, $\text{OD}_{\text{rinsing}}$ (Pearson Product Moment correlations for the dinoflagellates; $r > 0.97$ and $p < 0.0001$; absorption coefficients for Ate+ shown in Fig. 3). OD_{sum} was added to a spectrum of detrital particles (OD_{det} ; estimated by fitting an exponential curve under the suspended cell spectra of each culture) and was then compared to the absorbance of suspended cells to estimate an MAA packaging index $\pi_2 = 1 - (\text{OD}_{\text{s-fil}} / \text{OD}_{\text{sum+det}})$. Values of π_2 are lower than π_1 (Table 2), but both indexes are highly correlated (Pearson Product Moment correlation, $r = 0.95$, $p = 0.0136$).

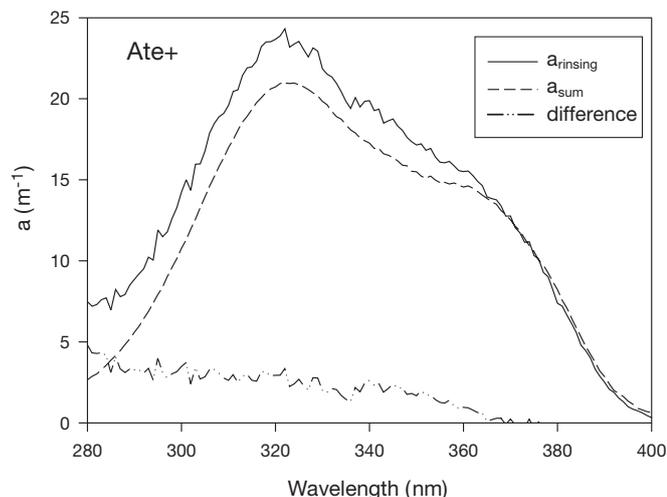


Fig. 3. Comparison between the absorption coefficients of water-soluble material exuded out of cells upon freezing (collected by a rinsing step) and the absorption reconstructed from the MAA concentrations obtained by HPLC for Ate+

DISCUSSION

The flattening of the absorbance spectra for cells in suspension (Fig. 1) can be explained by the package effect (or internal self-shading) of MAAs. Duysens (1956) demonstrated theoretically that the absorption spectrum of a substance contained in particles appears flattened when compared with the absorption of the same substance (same concentration) in solution. The flattening in the UV waveband likely depends on the shape and size of cells, but also on the composition and internal distribution of the UV absorbing compounds within the cells (as seen for pigments in the visible range; e.g. Sathyendranath et al. 1987). A non-homogenous distribution of MAAs in the cells, targeted around UV-sensitive organelles for increased protection efficiency, has been suggested by Neale et al. (1998) and could be a major cause of this UV flattening. Even if measured cell absorption of UV radiation is reduced when MAAs are packaged, the protection efficiency of specific targets would increase, affecting the estimation of sunscreen photoprotection, i.e. the proportion of damaging photons prevented from reaching sensitive targets. The sunscreen factor developed by Garcia-Pichel (1994) corresponds to the fractional reduction of UV exposure to a centrally located cellular target when MAAs are synthesized as a response of light conditions (Riegger & Robinson 1997, Bracher & Wienke 2000). However, this factor cannot be calculated here, since the dinoflagellate cultures grown under both light treatments contained significant MAA concentrations (MAA-free back-

ground efficiency factor for internal self-shading cannot be calculated).

Both methods used in this study to evaluate MAA packaging gave essentially the same results, except that pi_1 was slightly higher than pi_2 . pi_1 could possibly be overestimated if β values were underestimated or other optical effects associated with the filter pad technique were occurring. Spectral reconstruction of dissolved absorption from individual MAA absorbance spectra and concentration (Bidigare et al. 1990) appeared to be accurate, since when converted into comparable units, the reconstructed absorption was only slightly lower than the absorption obtained from the rinsing water (i.e. what was released from cells upon thawing; Fig. 3).

Both strains of *Alexandrium tamarense* grown under enhanced UVA irradiance ('+' cultures) had a higher index of MAA packaging compared to cultures grown without additional UVA ('-' cultures). This was not the case for Ht (comparison of pi_1 only), but the packaging index for this species was biased by the incomplete release of MAAs during freezing (full extraction of MAAs for HPLC analysis required sonication for Ht, but not for At, possibly because they have more resistant membranes). The highest index of packaging was found in Ate+ (average $pi_1 = 0.80$), with an 80% decrease in UV absorption in cells relative to solution. This culture had indeed the highest concentration of MAAs relative to chl *a* (Table 1). It is also interesting to note that these 2 strains of the same species, *A. tamarense*, responded slightly differently to the same light treatment, likely indicating differences in photoadaptation and genetic characteristics (Ate was isolated from Spain, while At came from England). The lower packaging index found in Ht is consistent with the smaller cell size of this dinoflagellate (diameter of ca. 18 μm , compared to ca. 38 μm for *A. tamarense*). Outdoor cultures of these dinoflagellates increased their production of MAAs under enhanced UVB doses as compared to natural sunlight conditions, Ate being more successful than At and Ht, since it was able to maintain similar growth rates under both UVB treatments (Laurion & Roy unpubl.).

Interestingly, higher pi values corresponded to higher concentrations of MAAs normalized to chl *a*, but not always when normalized to cell number or cell volume (Tables 1 & 2), suggesting that MAAs are associated with chloroplasts. It is worthwhile noting that packaging of pigments also occurs in the MAA-rich *Alexandrium tamarense* cells, as seen from the lower value of the chl *a*-specific absorption coefficient for cells in suspension (a_s^* at 675 nm; Table 1) compared to the specific absorption coefficient of chl *a* in solution (equal to 0.0207 $\text{m}^2 \text{mg chl } a^{-1}$ at 675 nm; Bricaud et al. 1983, Nelson et al. 1993). MAAs may associate somehow with the pigment packets inside chloroplasts.

Considering the high cost of MAA synthesis (Raven 1991, Shick & Dunlap 2002) and their restricted sun-screen efficiency in small cells (2 to 20 μm ; Garcia-Pichel 1994), packaging of MAAs around sensitive sites (e.g. nucleus, chloroplasts) may increase the efficiency of photoprotection for small phytoplankton cells, as compared to a homogeneous cytoplasmic distribution of these sunscreens (such as found in cyanobacteria; Garcia-Pichel & Castenholz 1993). Since MAAs are formed via the shikimate pathway (Shick et al. 1999), they are likely produced in the chloroplasts, which may be connected to the nucleus (Falkowski & Raven 1997). A package index of 0.8 would correspond to MAAs concentrated in $\frac{1}{5}$ of the total cell volume (assuming that Beer's law applies). This seems possible if MAAs are enclosed within the chloroplasts, since chloroplasts often represent a fairly large fraction of the cell volume. One may question the physiological limit of such high concentrations of MAAs (e.g. 330 pg in one cell of *At* corresponds to an average cellular concentration of 40 mmol l^{-1}) if they were restricted to a small fraction of the cell volume. For example, the MAAs restricted to $\frac{1}{5}$ of the total volume would give a concentration of 0.2 mol l^{-1} , which corresponds to the estimated maximal content of UV sunscreens considering the osmotic pressure (Garcia-Pichel 1994). The above considerations hold for freely diffusing MAAs, which may not be the case for all MAAs, as some do not seem to be readily extractable (see Tartarotti & Sommaruga 2002). The above issues need further investigation to fully understand the cellular distribution of MAAs in different species and to assess their photoprotective efficiency.

Acknowledgements. We thank D. Karentz for providing secondary standards of MAAs, K. Whitehead for LC-MS analyses that were performed to confirm the identification of most MAAs, M. Shick for fruitful discussions, D. Mueller, W. Vincent, A. Morel, A. Weidemann and an anonymous reviewer for insightful comments and careful reviews and G. Ouellet for microscopic counts. Funds from the Natural Sciences and Engineering Research Council of Canada (I.L. and S.R.) supported this research.

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Editorial responsibility: Otto Kinne (Editor), Oldendorf/Luhe, Germany

*Submitted: January 19, 2004; Accepted: July 4, 2004
Proofs received from author(s): September 6, 2004*