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Sources of mycosporine-like amino acids in planktonic *Chlorella*-bearing ciliates (Ciliophora)

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SUMMARY

1. Mycosporine-like amino acids (MAAs) are a family of secondary metabolites known to protect organisms exposed to solar UV radiation. We tested their distribution among several planktonic ciliates bearing *Chlorella* isolated from an oligo-mesotrophic lake in Tyrol, Austria. In order to test the origin of these compounds, the MAAs were assessed by high performance liquid chromatography in both the ciliates and their symbiotic algae.
2. Considering all *Chlorella*-bearing ciliates, we found: (i) seven different MAAs (mycosporine-glycine, palythine, asterina-330, shinorine, porphyra-334, usujirene, palythene); (ii) one to several MAAs per species and (iii) qualitative and quantitative seasonal changes in the MAAs (e.g. in *Pelagodileptus trachelioides*). In all species tested, concentrations of MAAs were always <1% of ciliate dry weight.
3. Several MAAs were also identified in the *Chlorella* isolated from the ciliates, thus providing initial evidence for their symbiotic origin. In *Uroleptus* sp., however, we found evidence for a dietary source of MAAs.
4. Our results suggest that accumulation of MAAs in *Chlorella*-bearing ciliates represents an additional benefit of this symbiosis and an adaptation for survival in sunlit, UV-exposed waters.

Keywords: mixotrophy, mutualism, mycosporine-like amino acids, natural sunscreens, symbiosis

Introduction

Ciliates are among the most important members of microbial food webs in oceans and lakes (Pierce & Turner, 1992; Weisse & Müller, 1998; Foissner, Berger & Schaumburg, 1999; Dolan & Pérez, 2000). Many ciliates live as mixotrophs. That is, in addition to heterotrophic nutrition, they either sequester chloroplasts from their algal food (kleptoplasts) or they live mutualistically with green algae of the genera *Chlorella* (Dolan, 1992; Jones, 1994) or *Symbiodinium* (Lobban *et al*., 2002). Such mutualisms are common and provide a close coupling between hosts and symbionts, with inorganic nutrients passing from host to algae and photosynthate (e.g. maltose) from algae to host (Muscatine, 1973, 1990; Reisser, 1992). This relationship offers mainly nutritional advantages, especially in oligotrophic systems (Dolan, 1992; Jones, 1994). In freshwater lakes, ciliates bearing *Chlorella* are common and seasonally numerous in sunlit waters (Sonntag *et al*., 2006), and have also been found associated with micro-oxic layers (Berninger, Finlay & Canter, 1986).

Solar ultraviolet radiation (UVR, 290–400 nm) has damaging effects on planktonic organisms, particularly on their DNA and other cellular components (Harm, 1980; Karentz, Cleaver & Mitchell, 1991a; Karentz *et al*., 1991b; Sommaruga & Buma, 2000). However, organisms can cope with potentially harm-

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ful ambient UVR by: (i) avoidance, e.g. through phototaxis; (ii) repair, as in DNA photoreactivation or (iii) protection, by synthesising or accumulating a series of photoprotective compounds, such as pigments (melanin, carotenoids) or mycosporine-like amino acids (MAAs), that directly or indirectly absorb the energy of the solar radiation (Sommaruga, 2001). The latter are intracellular, colourless water-soluble compounds, having their maximum absorption between 309 and 360 nm, which is in the range of the damaging UV-B and UV-A wavelengths (Karentz *et al*., 1991b; Dunlap & Shick, 1998).

The MAAs tested hitherto are photochemically stable and have high molar extinction coefficients (Conde, Churio & Previtali, 2000; Karentz, 2001; Shick & Dunlap, 2002; Conde *et al*., 2003). The basic chromophores responsible for the UVR absorbance in MAAs are apparently derived from the early stages of the shikimic pathway (Hirata *et al*., 1979; Favre-Bonvin *et al*., 1987), present in bacteria and algae, but not in metazoans. For ciliates, the shikimic pathway is not known. Many metazoans, however, obtain MAAs through their diet (Carroll & Shick, 1996; Newman *et al*., 2000; Moeller *et al*., 2005) or from symbiotic partnerships (Dunlap & Shick, 1998). Mycosporine-like amino acids are widespread in freshwater organisms, such as, cyanobacteria (Sommaruga & Garcia-Pichel, 1999; Liu, Häder & Sommaruga, 2004), natural phytoplankton assemblages (Sommaruga & Garcia-Pichel, 1999; Laurion, Lami & Sommaruga, 2002), rotifers and copepods (Sommaruga & Garcia-Pichel, 1999; Moeller *et al*., 2005; Tartarotti & Sommaruga, 2006). Mycosporine-like amino acids have also been reported in the freshwater ciliate *Stentor amethystinus* Leidy, 1880 that hosts *Chlorella* (Tartarotti *et al*., 2004) and, just recently, in the marine *Symbiodinium*-bearing ciliate *Maristentor dinoferus* Lobban *et al*., 2002 (Sommaruga *et al*., 2006). These reports, however, only included analyses of the ciliates *in toto* and thus did not assess whether the MAAs originated from their symbiotic algae or from their algal diet.

In this study, we first assessed the distribution of MAAs among different *Chlorella*-bearing ciliates from an oligo-mesotrophic lake. Secondly, we analysed changes in the qualitative and quantitative composition of MAAs in those species and, thirdly, we investigated the source of MAAs by testing the ability of cultured symbiotic *Chlorella* to synthesise MAAs when exposed to simulated solar radiation in the laboratory.

Methods

Ciliate collection and processing

In the ice-free seasons of 2004 and 2005, we collected ciliates from the upper 2 m of Piburger See. This is a small (area: 13.4 ha), deep (maximum depth: 24.6 m), oligo-mesotrophic lake located at 913-m above sea level, in the Central Alps in Tyrol, Austria (47°11′N, 10°53′E). The lake is meromictic and usually ice-covered from December to April. Detailed limnological information on Piburger See can be found elsewhere (Sommaruga & Psenner, 1995) and on UV transparency in Laurion *et al.* (2000). Samples were collected by vertical and horizontal net hauls (10-μm mesh size) and gathered in clean 1-L plastic bottles (HCl-washed and thoroughly rinsed with tap and lake water).

In the laboratory, the samples were kept at ambient lake temperature until further processing. Depending on their occurrence and abundance, the following eu- and epiplanktonic (i.e. attached) *Chlorella*-bearing species were analysed for MAAs: *Vorticella chlorellata*, *Uroleptus* sp., *Pelagodileptus trachelioides*, *Stokesia vernalis* and *Teuthophrys trisulca trisulca* (Table 1). They were collected on seven occasions between June and September 2004 and on five occasions between July and October 2005. Ciliates were identified based on their morphology from observations of living individuals after Foissner *et al.* (1999) and references therein.

Individual ciliates assigned to one species were picked out of the sample with a micropipette (Fig. 1). Each individual ciliate was transferred consecutively to over five drops of sterile filtered (0.2 μm) lake water on a clean glass slide and inspected under the microscope to assure they were free of any adhered phytoplankton. Subsequently, the ciliates were placed in well-plates filled with sterile filtered lake water and left for at least 1 h or overnight to ensure the complete digestion of algae in their food vacuoles. The digestive cycle, for example, in *Paramecium caudatum* Ehrenberg, 1833 feeding on yeast lasts between 21 and 60 min (Fok, Lee & Allen, 1982). The individuals were then again cleaned as described above and finally collected in a 2-mL vial (Eppendorf, Hamburg, Germany). The vial was stored at −80 °C until MAAs analysis. We collected as many individuals of one species as possible and their number per vial ranged from 4 to 84, depending on their density in the original water sample.

Table 1 Characteristics of the *Chlorella*-bearing ciliates tested for mycosporine-like amino acids and numbers of symbiotic algae within ciliates

Ciliate species & taxonomic affiliation	Size (μm)	*Chlorella* cells ciliate^{-1}
Pelagodileptus trachelioides (Zacharias, 1894) Haptoria	Total length (= trunk + proboscis) 300–600 (230–800 × 100–300)*	Numerous (2–4 μm across)*,‡ Approximately 500*,‡
	Trunk 225 × 76 (105–420 × 45–120), proboscis 338 × 11 (50–900 × 10–13)†	
Teuthophrys trisulca trisulca (Chatton & De Beauchamp, 1923) Haptoria	200 (150–300 × 50–150)*	Numerous (4–6 μm across)*,‡
	263 × 112 (230–310 × 90–140)†	Approximately 500†,‡
Uroleptus sp.§ Stichotrichia	106 × 29 (78–144 × 22–36)†	Approximately 100†,‡
Stokesia vernalis Wenrich, 1929 Peniculia	150 (100–220) in diameter*	Often in low number*
	171 × 156 (150–211 × 130–200)†	Densely crowded in several isolated 'packages'; approximately 500†
Vorticella chlorellata Stiller, 1940 Peritrichia	53 × 40 (44–64 × 34–48)*	Not specified (5–6 μm across)*,‡
	31 × 29 (25–39 × 21–41)†	Approximately 100†,‡

Sizes are presented as mean length/width with minimum and maximum given in brackets.
*Data from Foissner *et al.* (1999).
†Own measurements.
‡*Chlorella* evenly distributed within the ciliate.
§Description in preparation.

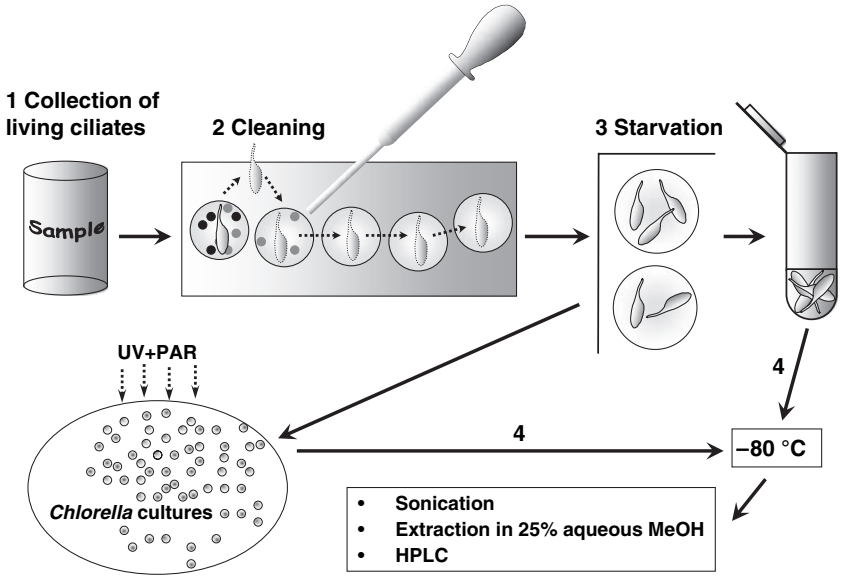

Fig. 1 Flow chart of mycosporine-like amino acids (MAAs) analysis in ciliates and isolated *Chlorella*. For details see text.

Chlorella cultures

From the ciliates investigated, we established cultures of their symbiotic *Chlorella*, except for *V. chlorellata*. First, single ciliates were cleaned as described (Fig. 1, steps 1–3) and then starved in Woods Hole MBL medium (Guillard & Lorenzen, 1972) for some days. To isolate the *Chlorella*, the ciliates were disrupted by gentle sonication for 20 s at 7 W (Sonoplus, HD 2070; Bandelin, Berlin, Germany). Subsequently, the *Chlorella* were concentrated by centrifugation (8000 *g* for 5 min), washed three times with sterile Woods Hole MBL medium, and precultivated in the same medium. In the case of *T. trisulca trisulca*, we cultivated the *Chlorella* after the ciliates had died during starvation. Growing *Chlorella* cultures were transferred onto agar plates prepared with Woods Hole MBL medium in addition of Rifampicin (Sigma-Aldrich, Vienna, Austria) to prevent bacterial growth. After several re-inoculation steps, algal cultures were grown axenically in liquid Woods Hole MBL medium. The algae were grown at 17–21 °C in an environmental growth chamber with a

16 : 8 h light : dark cycle. Photosynthetically active radiation (PAR) was provided by five cool white lamps (L36/W20; Osram, Vienna, Austria) delivering 180 μmol m^{-2} s^{-1}. During the light period, the cultures were additionally exposed to UV-B (1.38 W m^{-2}) and UV-A radiation (5.21 W m^{-2}) for 1 h. Ultraviolet radiation was provided by one A-340 Q-panel lamp (Q-Panel, Bolton, England). Cultures were regularly checked microscopically for bacterial contamination.

To stimulate the synthesis of MAAs, *Chlorella* cultures were grown to the early stationary phase in quartz tubes (100 mL; Helios Italquartz, Milan, Italy) under the same radiation conditions, but additionally exposed for 1–2 h day^{-1} to higher UV radiation (four Q-panel lamps A-340 delivering 2.47 W m^{-2} UV-B, 8.60 W m^{-2} UV-A). The *Chlorella* were concentrated by centrifugation (8000 ***g*** for 5 min) and the pellet stored at −80 °C until MAAs were analysed.

MAAs analysis

Mycosporine-like amino acids were extracted after Tartarotti & Sommaruga (2002) with slight modifications. Briefly, the ciliates and *Chlorella* cultures were consecutively extracted with 25% aqueous methanol (v : v, MeOH; Merck, Darmstadt, Germany) for 2 h at 45 °C and for 12 h at 4 °C. Before extraction, we added 100–300 μL of precooled (4 °C) 25% aqueous MeOH (v : v) to the frozen sample and immediately sonicated on ice for 3 min at 42 W, to ensure the rupture of the ciliates and particularly of the resistant *Chlorella* cell walls. When further concentration of the extracts was necessary (because of a low number of individuals or too high initial extraction volume), MeOH extracts were dried by vacuum centrifugation at room temperature (Savant, SC 110; Thermo Fischer Scientific, Waltham, MA, U.S.A.) and re-suspended in 100 μL of 25% aqueous MeOH (v : v). Finally, the extracts were cleared by centrifugation at 16 100 ***g*** for 10 min and analysed by high performance liquid chromatography (HPLC). Aliquots of 50–80 μL were injected in a Phenosphere C8 column (250 × 4.6 mm, 5-μm pore size, Phenomenex, Aschaffenburg, Germany) protected with a RP-8 guard column (Brownlee, PerkinElmer; Waltham, MA, U.S.A.) for isocratic reverse-phase HPLC analysis for 15–25 min. Samples were run with a mobile phase of 0.1% acetic acid in 25% aqueous MeOH (v : v) and at a flow rate of 0.7 mL min^{-1}. The MAAs in the eluate were detected by online UV spectroscopy. Peak measurement was carried out at 310, 320, 334 and 360 nm in a Dionex system (Dionex, Vienna, Austria) with a diode array detector (scanning from 200–595 nm). Individual absorption spectra were identified by their relative retention time and co-chromatographic analysis with reference MAAs extracted from *Porphyra yezoensis* Ueda, 1932 and *Palythoa* sp. (courtesy of U. Karsten and J.M. Shick to R.S.).

The total content of the specific MAAs in each sample was calculated from HPLC peak area, using published molar extinction coefficients (see Karentz, 2001). The molar extinction coefficient for asterina-330 was assumed to be the same as that of palythinol (Dunlap *et al*., 1989) and that of usujirene was supposed to be the same as that of palythene, as they are chemical isomers. Concentrations of MAAs in ciliates were normalised to the dry weight (DW) of each species (expressed as μg μg^{-1} DW) using a conversion factor of 0.15 × fresh weight and assuming a density value of 1 (Foissner, Berger & Kohmann, 1992).

Results

Seven known MAAs were identified by HPLC from the methanolic extracts of the ciliates and their respective *Chlorella* (Figs 2 & 3). These were (i) mycosporine-glycine (MG, $\lambda_{max} = 310$); (ii) palythine (PI, $\lambda_{max} = 320$); (iii) asterina (AS, $\lambda_{max} = 330$); (iv) shinorine (SH, $\lambda_{max} = 334$); (v) porphyra (PR, $\lambda_{max} = 334$); (vi) usujirene (US, $\lambda_{max} = 357$) and (vii) palythene (PE, $\lambda_{max} = 360$). However, MAAs were not always detected. Further, two unknown compounds absorbing in the UV-range characteristic for MAAs were found, one in *T. trisulca trisulca* and another in the *Chlorella* of *Uroleptus* sp. (see below). All ciliate species examined contained at least one MAAs at certain times in detectable amounts (Figs 2 & 3). Overall, SH, PI and AS were the most common MAAs present in the samples. The concentration of MAAs was <1% of ciliate DW in all cases.

In non-starved *V. chlorellata*, we found three different MAAs in approximately similar proportions (35% SH, 28% PI, 36% AS) as well as a relatively high total MAAs concentration per individual compared with other ciliates (Figs 2 & 3).

The hypotrich *Uroleptus* sp. was found on two occasions in 2004 (Figs 2 & 3). In July, we detected 100% PI in non-starved individuals, which resulted in

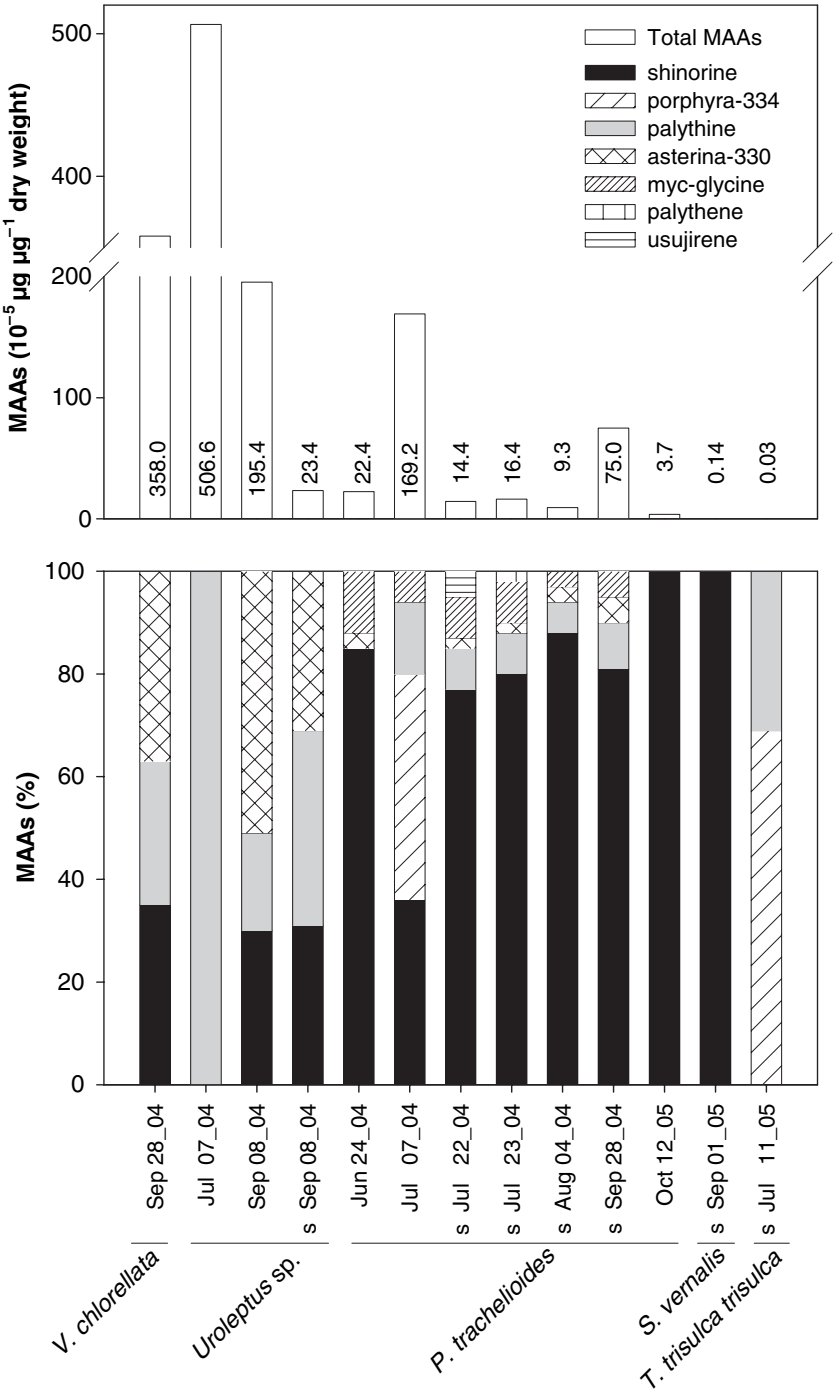

Fig. 2 Total mycosporine-like amino acids (MAAs) concentrations (10^{-5} µg µg^{-1} DW, upper panel) and their relative contribution (%, lower panel) from the MAAs-positive observations of five *Chlorella*-bearing ciliates from Piburger See. s, denotes ciliates starved prior to MAAs analysis.

the highest MAAs concentration per individual (506.6 10^{-5} µg µg^{-1} DW) of any ciliate species investigated. In September, individuals of *Uroleptus* sp. were analysed before and after starvation. On both occasions, we detected the same suite of MAAs (SH, PI, AS), though after starvation the total amount of MAAs decreased by a factor of 10 (0.20–0.02% DW). In detail, the amount of SH decreased by 88%, of PI by 76%, and that of AS by 93% in the starved individuals. Further, in the *Chlorella* of *Uroleptus* sp., we found an unknown UV-absorbing compound with a maximum absorption at around 310 nm and a retention time of 8 min. However, this compound was not detected in the extracts of the ciliate.

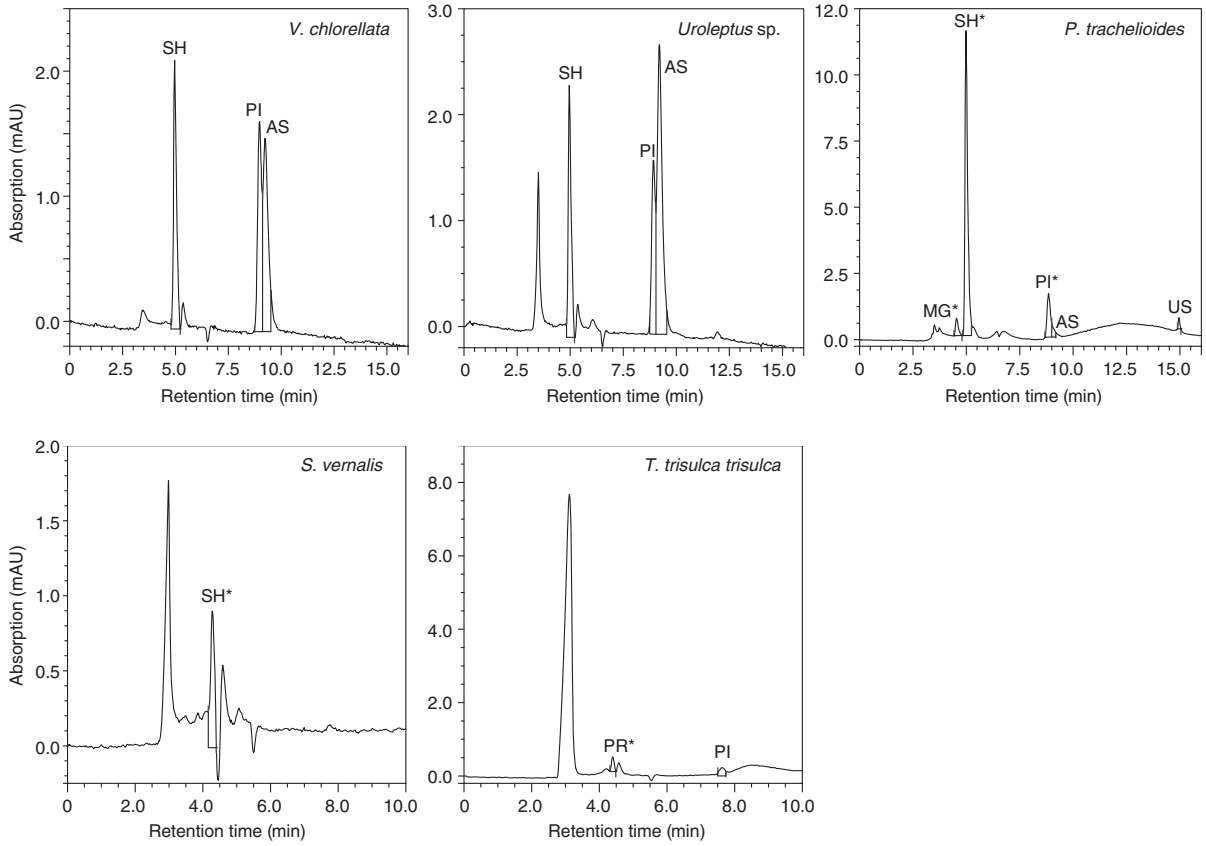

Fig. 3 HPLC chromatograms (detection at 320 nm) of aqueous methanolic extracts (25%) of five *Chlorella*-bearing ciliate species from Piburger See. AS, asterina-330; MG, mycosporine-glycine; PI, palythine; PR, porphyra-334; SH, shinorine; US, usujirene; *denotes MAAs verified in cultured *Chlorella* of the respective ciliate. Note different scales on the axes.

In *P. trachelioides*, we observed seven distinct MAAs in different concentrations and compositions over the time (Figs 2 & 3). Overall, SH was the predominant compound comprising 36–100% in all MAAs-positive observations of this species, except for July 2004, when PR (43%) dominated over SH (36%), PI (14%) and MG (6%). Apart from the July 2004 samples we observed, in addition to SH, PI (0–14%), MG (0–12%), AS (0–5%), US (0–5%) and PE (0–2%). In the respective *Chlorella*, we found MG, PI and SH. At the beginning of July 2004, we detected the highest concentrations of MAAs in non-starved *P. trachelioides*. Furthermore, MAAs from two sampling occasions on 22 and 23 July 2004 were similar in *P. trachelioides* except for US and PE, respectively. In October 2005, 100% SH was found. The MAAs concentrations in *P. trachelioides* were always <1% of the ciliate DW, i.e. 0.01–0.2%.

Stokesia vernalis was found on two occasions and only once with MAAs (Figs 2 & 3). Shinorine was the only compound detected and in low concentration (0.00014% of the ciliate DW). In the cultured *Chlorella* from this species, SH and MG were present.

The large ciliate *T. trisulca trisulca* (Table 1) was observed twice and had the lowest MAAs concentrations of any species investigated, 0.00003% DW (Figs 2 & 3). Porphyra and a further unknown compound, absorbing at approximately 328 nm and with a retention time of 3.8 min, were found in the respective *Chlorella.*

Discussion

In this study, we have shown that *in toto* analyses of five *Chlorella*-bearing ciliate species revealed the presence of MAAs. Mycosporine-like amino acids have been reported before in two heterotrich ciliates: the freshwater species *S. amethystinus* (Tartarotti *et al.*, 2004) and the marine *M. dinoferus* (Sommaruga *et al.*, 2006), but their origin has not been previously

analysed. There are two probable sources of MAAs found in mixotrophic ciliates: first, via *de novo* synthesis by the *Chlorella* symbionts (Fig. 3) and, secondly, via dietary accumulation through feeding on phytoplankton (Fig. 2). One important result of our study was that we were able to confirm the synthesis of MAAs by the symbiotic *Chlorella* isolated in culture and, thus, to provide the first evidence for the symbiotic origin of these metabolites. In some cases, we observed discrepancies between the suite of MAAs detected in cultures and in the host, similar to previous reports from marine organisms, such as corals or sea anemones (Banaszak & Trench, 1995; Shick *et al*., 1999). These discrepancies probably resulted from axenic growing conditions of the algae in culture, as well as from bacterial transformations and interconversions of MAAs that can occur in the host (Dunlap & Shick, 1998; Portwich & Garcia-Pichel, 2003). In the *Chlorella* of *S. vernalis*, for example, we detected not only SH but also MG, while only SH has been verified from the ciliate analysed *in toto*. One possible explanation for these findings is the conversion of MG into SH, as observed by Portwich & Garcia-Pichel (2003) in a cyanobacterium. Further, the dietary accumulation of MAAs was supported by our findings in non-starved and starved *Uroleptus* sp., when we observed a conspicuous decrease after starvation in the concentrations of the same suite of compounds (Fig. 2). The MAAs detected in this ciliate were also coincident with the main MAAs present in phytoplankton of Piburger See (Laurion *et al*., 2002). Thus, we conclude that the MAAs in *Uroleptus* sp. have a dietary origin.

In agreement with other studies, we observed that the concentrations, composition and occurrence of MAAs varied among or even within species and sampling occasions (Fig. 2). For example, in *P. trachelioides* collected on two consecutive days (22 & 23 July 2004), the dominant MAAs were the same, whereas US and PE were found only once (Fig. 2). Changes in the suite of MAAs may have different explanations, although palythene is known to be a cis–trans isomer of US that is more photostable than the latter and preferentially accumulated (Conde *et al*., 2003). Another example of a different suite of MAAs was observed, in *P. trachelioides*. Only in this ciliate did we find MG in significant amounts, an MAAs known to have a moderately antioxidant activity (Dunlap & Chalker, 1986; Dunlap & Yamamoto, 1995). This compound seems to be important in symbiotic relationships where photosynthesising algae cause photooxidative stress in their hosts, as observed in corals and other marine symbiotic organisms (Dunlap & Chalker, 1986; Dunlap & Yamamoto, 1995). Thus, we also expected to find it in other *Chlorella*-bearing ciliates. In *T. trisulca trisulca*, however, that bears about the same number of symbionts as *P. trachelioides*, MG was never detected (Table 1; Figs 2 & 3). Mycosporine-glycine is considered a primary compound from which others are derived, and thus its absence may result from precursor–product interconversions (Portwich & Garcia-Pichel, 2003).

Generally, concentrations of MAAs in diverse algae, cyanobacteria and metazoans account for <1% of the DW and it is assumed that they are distributed homogeneously within the cytoplasm (Karentz *et al*., 1991b; Garcia-Pichel & Castenholz, 1993). However, MAAs concentrations of up to 3.1% of DW have been reported for copepods (Tartarotti, Laurion & Sommaruga, 2001). Depending on the organism studied, concentrations of MAAs have usually been normalised to the DW, chlorophyll-*a* or protein content (Tartarotti & Sommaruga, 2002). Our estimates of the concentrations of MAAs in specific ciliates can be considered as a first approximation and are probably underestimates. It would be more accurate to refer the MAAs concentrations to the biovolume or biomass of *Chlorella*. However, it is difficult to estimate properly the number of *Chlorella* within ciliates (Table 1). Moreover, many more cell compartments exist in such symbiotic organisms rendering the assumption of a homogeneous distribution uncertain. Nevertheless, based on the model of Garcia-Pichel (1994), even a small investment of an organism's DW into MAAs synthesis increases the UV absorption considerably, especially in the UV-B range. This is particularly the case for cell radii larger than 10 μm. Thus, the presence of several layers of absorbing cell matter from *Chlorella* further increases the protection factor for important ciliate cell components, such as the DNA-containing nuclei (Garcia-Pichel, 1994). For example, in *V. chlorellata* or *Uroleptus* sp. that have several *Chlorella* layers evenly distributed in the ciliate, the UV-screening efficiency is expected to be larger than in the case of *S. vernalis*, which has low numbers of algae present in a few 'packages'

(Table 1). In *P. trachelioides* and *T. trisulca trisulca*, the *Chlorella* algae are mainly distributed in their trunks where the nuclei are located and probably better protected (Foissner *et al*., 1999).

Ultraviolet radiation may not only harm the cell organelles of a ciliate, but also inhibit the photosynthesis of the algae or even damage their photosynthetic pigments (Villafañe *et al*., 2003). Thus, the internal self-shading caused by several layers of *Chlorella* is also probably essential for the algae themselves, because the efficiency factor for UV-screening decreases with cell size (Garcia-Pichel, 1994). Considering both MAAs and cell matter, the model of Garcia-Pichel (1994) estimates an absorption efficiency of 20–80% for the ciliates with cell widths between 20 and 300 μm (= smallest distance between cell surface and nuclei, Table 1), but only <10% for small single cells such as *Chlorella* (3–5 μm).

Mycosporine-like amino acids are effective photoprotectants (Banaszak, 2003; Moeller *et al*., 2005) and the presence of several of those compounds provide aquatic organisms with a 'broad-band' UV-filter (Sommaruga & Garcia-Pichel, 1999; Karentz, 2001; Shick & Dunlap, 2002). The *Chlorella*-bearing ciliates investigated were almost exclusively found in the surface waters of Piburger See in summer where UVR penetration is still significant (1% attenuation depth at 320 nm = 1.5 m, Laurion *et al*., 2000). Thus, the presence of MAAs might offer both ciliates and algae protection from harmful UVR. However, the protective role of MAAs as an additional benefit in the symbiosis between *Chlorella* and ciliates requires further investigation.

Acknowledgments

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