

The effect of different methanol concentrations and temperatures on the extraction of mycosporine-like amino acids (MAAs) in algae and zooplankton

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With 4 figures and 2 tables

Abstract: Mycosporine-like amino acids (MAAs) are a family of intracellular UV-absorbing compounds involved in the protection of aquatic organisms against solar UV radiation. These compounds have been identified and quantified in many marine and freshwater organisms, however, no standard method to analyze these compounds is available. Consequently, protocols to extract MAAs largely differ, for example, in the type of solvent, concentrations, temperatures, and times used. In this study, we tested whether the concentrations of MAAs are affected by extraction at different temperatures and methanol (MeOH) concentrations. Natural assemblages of freshwater phytoplankton, the cyclopoid copepod *Cyclops abyssorum taticus*, and the marine alga *Porphyra* sp. were used as test organisms. The MAAs shinorine, palythine, MAA 331, asterina-330, and porphyra-334 present in the organisms examined were generally best extracted at 45 °C in 25 % aqueous MeOH. In *Porphyra* sp. and natural freshwater phytoplankton, the mean total MAA concentrations obtained with this protocol were respectively ~13 and ~3 times higher than when extracted in 100 % MeOH at 4 °C. In *Cyclops*, concentrations of MAAs were also highest when extracted in 25 % MeOH at 45 °C, but there was no statistically significant difference among the different protocols ($P = 0.079$). Depending on the organism examined, both MeOH concentration and temperature affected extraction efficiency and final MAA concentration. Our results stress the need for *a priori* testing the influence of these variables to assure that the highest concentration is obtained without altering the qualitative MAA composition. Based on these results, direct comparison of MAA concentrations reported in the scientific literature should be done with caution.

Key words: Intracellular UV-absorbing compounds, copepods, phytoplankton, HPLC, ultraviolet radiation.

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Introduction

Mycosporine-like amino acids (MAAs) are a family of UV-absorbing compounds described for a wide variety of marine (see reviews by KARENTZ 2001, SHICK & DUNLAP 2002) and freshwater (SOMMARUGA & GARCIA-PICHEL 1999, XIONG et al. 1999, TARTAROTTI et al. 2001, LAURION et al. 2002) organisms, which potentially protect cellular targets from UV damage. Although no standard method exists to quantify MAAs by HPLC, most authors have used the analytical procedure described by DUNLAP & CHALKER (1986). However, protocols to extract MAAs from fresh or freeze-dried aquatic organisms largely differ among studies. For example, ethanol (50 % to 80 %) or most commonly methanol (MeOH) in different concentrations (20 % to 100 %) have been used (e.g., DUNLAP et al. 1989, CARRETO et al. 1990, SHICK et al. 1992, GARCIA-PICHEL & CASTENHOLZ 1993, BANASZAK & TRENCH 1995, ADAMS & SHICK 1996, WHITEHEAD et al. 2001). In some cases, extraction has been facilitated by cell or tissue rupture, i.e., sonication, or by adding chemicals such as tetrahydrofuran to the solvent (e.g., DUNLAP et al. 1986, BANASZAK & TRENCH 1995, JEFFREY et al. 1999, TARTAROTTI et al. 2001). In addition to the different solvents and concentrations used, extraction temperatures and times reported in the scientific literature range from -20°C to 50°C (e.g., MARCHANT et al. 1991, GARCIA-PICHEL & CASTENHOLZ 1993, BANASZAK & TRENCH 1995, JEFFREY et al. 1999, KUFFNER 2001, WHITEHEAD et al. 2001) and from 20 min to 3 d (e.g., SHICK et al. 1992, KARSTEN et al. 1998, JEFFREY et al. 1999, BANDARANAYAKE & DES ROCHER 1999, WHITEHEAD & VERNET 2000), respectively. A summary of the different extraction protocols found in the literature can be downloaded from the following URL: http://zoology.uibk.ac.at/limno/MAAs_methods.pdf.

SOMMARUGA & GARCIA-PICHEL (1999) observed that extractions of phytoplankton with 20 % aqueous MeOH at 45°C for 2 h apparently resulted in higher MAA concentration than in 90 % aqueous MeOH for 24 h at 4°C . However, the effect of different MeOH concentrations and temperatures were not explicitly tested. PÉREZ-RODRÍGUEZ et al. (1998) observed that extraction of UV-absorbing compounds with different solvents in a chlorophyte did not cause major changes in absorption patterns, however, quantitative information was only reported for the extraction in MeOH.

To our best knowledge, the efficiency of different extraction protocols of MAAs has not been previously assessed. Consequently, in this study, we tested whether different temperatures and MeOH concentrations affect the total and individual MAA concentration in different types of biological materials. To address this objective, four different protocols were evaluated on samples of natural freshwater phytoplankton, the copepod *Cyclops abyssorum taticus*, and the red marine macroalga *Porphyra* sp. These organisms are

known to have several, commonly found MAAs (KARSTEN et al. 1998, TARTAROTTI et al. 2001, LAURION et al. 2002). Moreover, *Porphyra* sp. is often used as a secondary standard (e.g., SHICK et al. 1992, KARSTEN et al. 1998, JEFFREY et al. 1999).

Materials and methods

Source of test organisms and sample processing

Samples of phytoplankton and zooplankton were taken on 20 July 2000 in Gossenköllesee, a lake located in the Central Alps (47° 13' N, 11° 01' E, Austria) at 2417 m above sea level. Water samples for phytoplankton were collected with a horizontal Van Dorn sampler (5 L) at 9 m depth (i.e., chlorophyll maximum at that time of the year) and pooled in large opaque carboys. After gentle mixing, subsamples for phytoplankton analyses (1.5 L) were first passed through a net of 100 µm mesh size to exclude zooplankton and then filtered onto Whatman GF/F filters at low vacuum pressure (0.3–0.4 atm). Dominant phytoplankton groups at this depth and time of the year are: Dinophyceae (e.g., *Gymnodinium* sp.), Chrysophyceae, and Cryptophyceae (e.g., *Cryptomonas* sp. and *Rhodomonas minuta*). Zooplankton were sampled by vertical net (55 µm mesh size) tows made at the center of the lake around noon. In the laboratory, the organisms were kept at 4 °C and dark conditions until further processing within 24 h. Zooplankton were concentrated onto a net sieve of 100 µm mesh size and washed several times with tap water to remove phytoplankton. Under a stereo microscope, five CO₂-narcotized adult females of the cyclopoid copepod *Cyclops abyssorum taticus* were carefully transferred into Eppendorf microcentrifugation vials (2 ml) kept on ice. The GF/F filters and vials were kept at –80 °C until posterior analysis within one week. Freeze-dried *Porphyra* sp. (Nori) was obtained from a foodstore and kept at –80 °C until its extraction, for which small pieces (~20 mg) were placed in Eppendorf vials.

Extraction and HPLC analyses

Four different protocols were used to extract MAAs with gradient grade MeOH for liquid chromatography: I) 100 % MeOH at 4 °C for 24 h; II) 100 % MeOH at 45 °C for 2 h; III) 25 % aqueous MeOH at 4 °C for 24 h, and IV) 25 % aqueous MeOH for 2 h at 45 °C (Fig. 1). Extractions were done in the dark with five (natural phytoplankton and freeze-dried *Porphyra*) or four (zooplankton) replicates. Extraction volumes were 1.4 ml for *Porphyra* sp. and *C. abyssorum taticus*, and 7.5 ml for phytoplankton samples. In total, three consecutive extractions were made for each sample and protocol. At the beginning of the first extraction, samples were placed on ice and treated with a tip sonicator (diameter: 2 mm) for 1 min at 0.5 cycles and 20 % amplitude (UP 200S, Dr. Hielscher GmbH, Germany). The extracts were cleared using Whatman GF/F filters and subsequently evaporated to dryness under vacuum in 2 ml Eppendorf microcentrifugation vials with 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 and quantification of MAAs, the dried extracts were

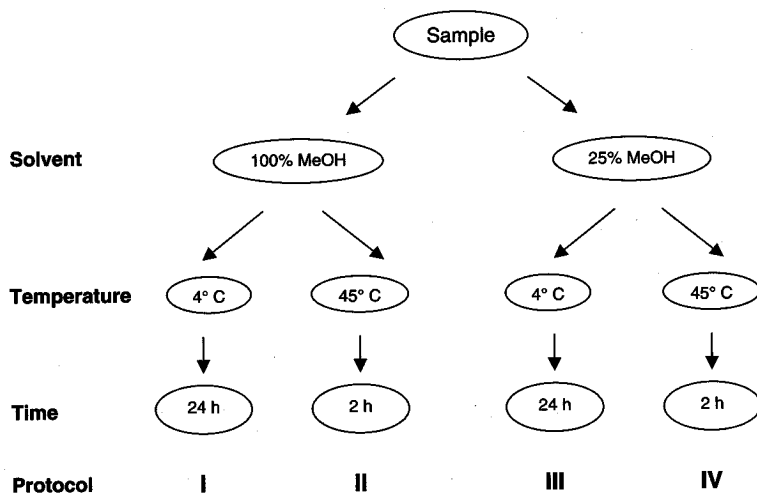


Fig. 1. Methanol concentration, temperature, and duration used in the different MAA extraction protocols (I-IV).

resuspended in 50 to 100 μl of 25 % MeOH (v : v), and 20–70 μl aliquots were injected in a Phenosphere 5 μm pore size C8 column (250 \times 4.6 mm, Phenomenex) protected with a RP-8 (Brownlee) guard column, for isocratic reverse-phase HPLC analysis. During the analysis, samples in the autosampler were kept at 15 $^{\circ}\text{C}$, while the column was maintained at 20 $^{\circ}\text{C}$. The mobile phase consisted of 0.1 % acetic acid in 25 % aqueous MeOH (v : v) running at a flow rate of 0.79 ml/min. The MAAs in the eluate were detected with a diode array detector (Dionex UVD340S) using four preselected channels (310, 320, 334, and 360 nm). The MAAs were identified by comparison with published retention times and by co-chromatography with purified standards obtained from Dr. FERRAN GARCIA-PICHEL, or with standards prepared from marine alga extracts (*Porphyra yezoensis* obtained from Dr. ULF KARSTEN). Peak purity was checked by analyzing the spectrum over the entire wavelength range. The total content of specific MAAs in each sample was calculated from HPLC peak areas, using published molar extinction coefficients (see TARTAROTTI et al. 2001). For quantification of one unknown compound with a maximum absorption at 331.4 nm (hereafter designated as MAA 331) found in phytoplankton, an average molar extinction coefficient of 40,000 was used. Concentrations of MAAs were expressed as $\mu\text{g/L}$ in the case of phytoplankton or normalized to the dry weight (DW) of *Porphyra* and *Cyclops*. MAAs found in trace amounts were not further considered for the statistical analysis. Extraction coefficients and efficiencies of MAAs were calculated with the equations derived by DUNLAP & CHALKER (1986).

Effect of different sonication times on final MAA concentrations

For this objective, samples of *Porphyra* (~20 mg) were sonicated as described above for 1, 2, 4, and 6 min at the beginning of the extraction in 25 % aqueous MeOH at 45 $^{\circ}\text{C}$

for 2 h. Samples without sonication served as control. Treatments and control consisted of 3 replicates each. Further processing of the samples and HPLC analyses were as described above.

Statistics

The results were analyzed by one-way ANOVA with a significance level of $P < 0.05$ to determine differences among treatments. Post-hoc comparisons were made with the Tukey test (GLANTZ 1997).

Results

Extraction efficiencies and relative proportion of MAAs

Efficiencies after three serial extractions ranged from 44.7% to 99.9% (Table 1). In all cases, the same main MAAs were detected with the different protocols (Fig. 2). The relative proportions of the individual MAAs in *Porphyra* sp. did not differ significantly when comparing protocols III and IV (Table 2).

Table 1. Extraction coefficients and efficiencies after three extractions for the most abundant MAAs in the marine red alga *Porphyra* sp., freshwater phytoplankton, and the cyclopoid copepod *Cyclops abyssorum taticus* derived with four different extraction protocols (see Fig. 1). PR: porphyra-334; SH: shinorine; PI: palythine; MAA 331: unknown MAA; AS: asterina-330; *: MAA was not detectable after the second extraction. Negative values indicate that concentrations were higher after the first extraction.

Protocol/ MAA	<i>Porphyra</i> sp.				Phytoplankton			<i>C. abyssorum taticus</i>		
	PR	SH	PI	AS	SH	MAA 331	AS	SH	PR	AS
I										
Extr. Coef.	0.551	0.523	0.509	0.541	-0.336	0.430	-0.632	0.761	0.860	0.770
Ex. Eff. (%)	90.9	89.1	88.2	90.3	-138.5	81.5	-334.7	98.6	99.7	98.8
r ²	0.939	0.945	0.909	0.917	0.506	1.000	0.824	0.928	0.983	0.973
II										
Extr. Coef.	0.332	0.320	0.179	0.212	0.381	0.642	0.520	0.885	0.785	0.901
Ex. Eff. (%)	70.2	68.6	44.7	51.0	76.3	95.4	89.0	99.8	99.0	99.9
r ²	0.984	0.980	0.819	0.843	0.950	0.807	0.790	0.995	0.962	0.987
III										
Extr. Coef.	0.841	0.839	0.794	0.801	0.339	0.545	0.207	0.904	*	*
Ex. Eff. (%)	99.6	99.6	99.1	99.2	71.2	90.6	50.0	99.9		
r ²	0.974	0.974	0.981	0.982	0.991	0.831	0.999	0.982		
IV										
Extr. Coef.	0.831	0.834	0.793	0.812	0.780	*	0.762	0.919	*	0.896
Ex. Eff. (%)	99.5	99.5	99.1	99.3	98.9		98.6	99.9		99.9
r ²	0.998	0.999	1.000	0.998	0.997		0.997	0.953		0.951

Table 2. Results of the post-hoc all pairwise multiple comparison tests for the relative proportion of the most abundant MAAs among protocols using different methanol concentrations and temperatures. Different letters indicate protocols that differed significantly for the respective MAA (Tukey test, $p < 0.05$). Abbreviations for the different MAAs as in Table 1.

Protocol/MAA	<i>Porphyra</i> sp.				Phytoplankton			<i>Cyclops</i>		
	PR	SH	PI	AS	SH	MAA 331	AS	SH	PR	AS
I: 100%, 4°C	a	a	a	a	a	a	a	a	a	a
II: 100%, 45°C	b	b	b	b	ab	ab	a	a	a	a
III: 25%, 4°C	cd	bc	cd	cd	ac	c	a	a	a	a
IV: 25%, 45°C	d	abc	d	d	cd	cd	a	a	a	a

However, the ratios of the distinct MAAs differed significantly when comparing protocols I and II (Fig. 2 A, Table 2). In natural phytoplankton assemblages, there was no significant difference in the relative proportions of the distinct MAAs obtained with protocols I and II, or with protocols III and IV (Fig. 2 B, Table 2). However, the relative proportion of MAA 331 was higher (~33%) in protocols I and II than in protocols III and IV (~9%). Accordingly, the relative proportion of shinorine changed, while the proportion of asterina-330 remained constant (~8%), regardless of the extraction protocol used (Fig. 2 B). The relative proportions of the individual MAAs in *C. abyssorum tatricus* were not significantly different ($p > 0.05$) among the different protocols (Fig. 2 C, Table 2).

MAA concentrations in *Porphyra* sp.

The main MAAs detected in *Porphyra* sp. were porphyra-334, shinorine, palythine, and asterina-330. Mean total MAA concentrations obtained with protocols I and II (Fig. 3 A inset) were lower than those obtained with protocols III and IV. Although the highest concentration was observed in protocol IV, no significant difference was found with protocol III (Fig. 3 A inset).

MAA concentrations in phytoplankton

Shinorine, MAA 331, and asterina-330 were the most abundant MAAs present in the phytoplankton samples examined. The lowest mean total MAA concentration was found when extracting the samples with protocol I (Fig. 3 B inset). Among the protocols II, III and IV, the mean total MAA concentration was not significantly different (Fig. 3 B inset).

MAA concentrations in *Cyclops abyssorum tatricus*

The most abundant MAAs in adult females of this copepod were shinorine, asterina-330, and porphyra-334. A slightly higher mean total MAA concentra-

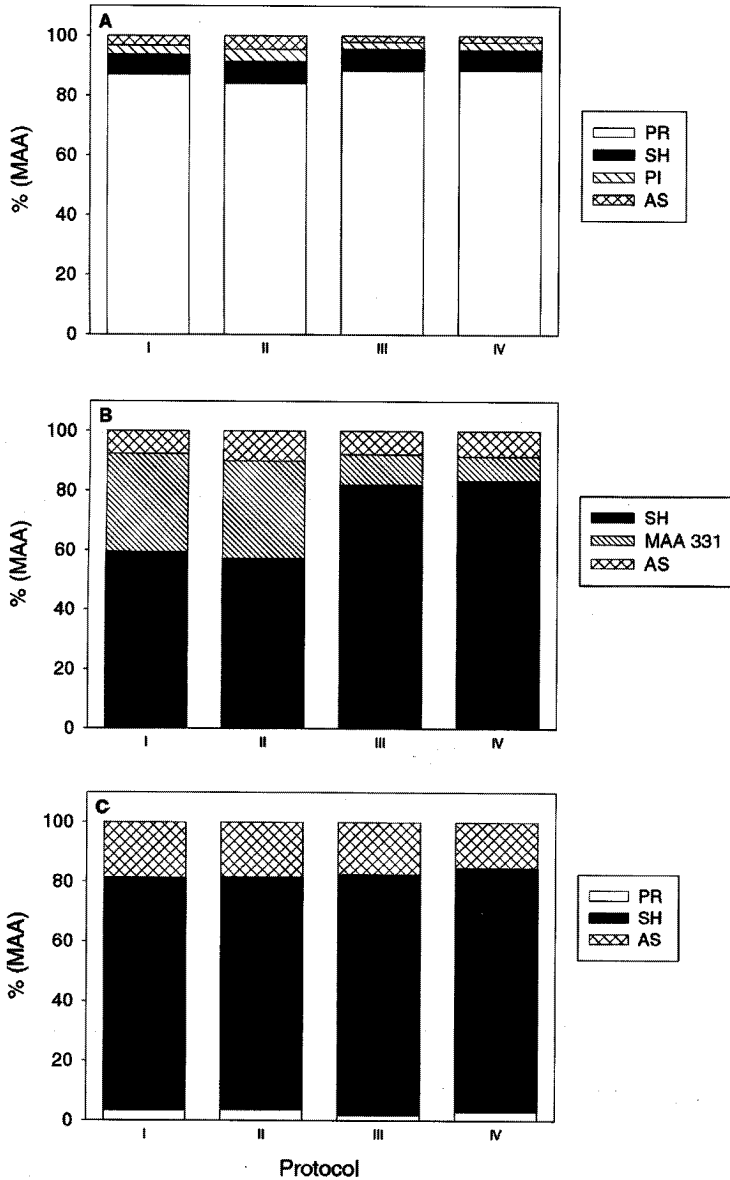


Fig. 2. Relative proportions of the distinct main MAAs resulting from the different extraction protocols (I-IV) for the marine alga *Porphyra* sp. (A), natural freshwater phytoplankton (B), and the cyclopoid copepod *Cyclops abyssorum taticus* (C). Abbreviations for the MAAs as in Table 1.

tion was measured with the extraction at 45 °C in 25 % MeOH (protocol IV), however, differences among the four protocols were not significantly different (Fig. 3 C inset).

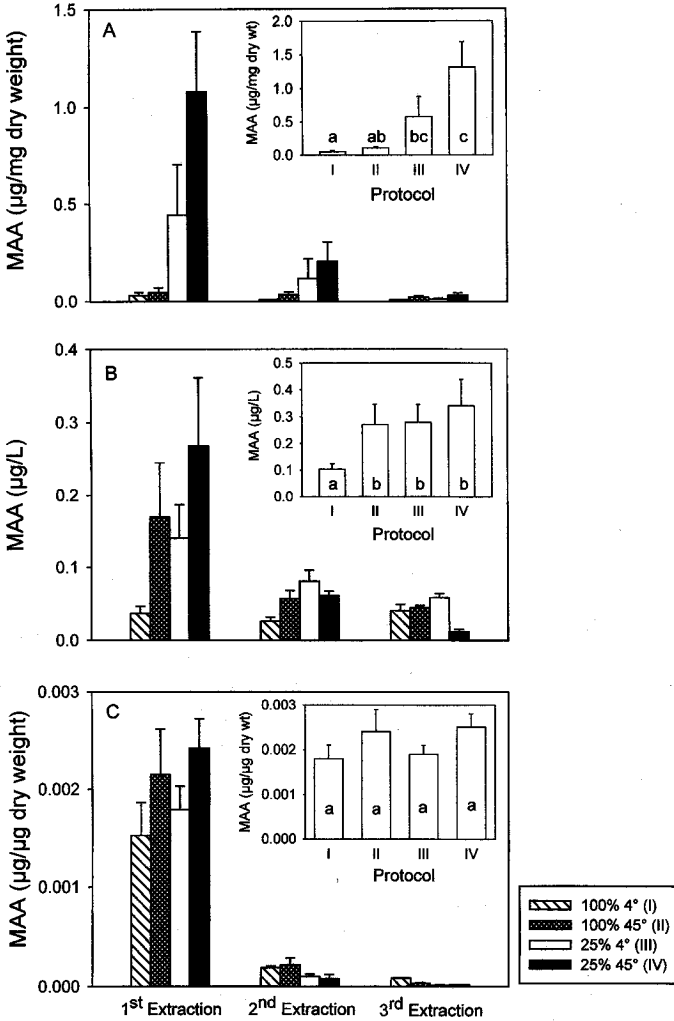


Fig. 3. Mean concentrations \pm 1 SD of total MAAs for the four extraction protocols (I–IV) and the three consecutive extractions made in the marine alga *Porphyra sp.* (A), natural phytoplankton assemblages (B), and the cyclopoid copepod *Cyclops abyssorum taticus* (C). Insets show the sum of MAA concentrations of the three extractions for the four different extraction protocols. Different letters inside the bars indicate protocols that differed significantly ($p < 0.05$, Tukey test). Protocol codes as shown in Fig. 1.

Effect of different sonication times on MAA concentrations in *Porphyra sp.*

Mean total concentrations of MAAs significantly increased in all treatments when compared to the control (Fig. 4). However, concentrations were similar

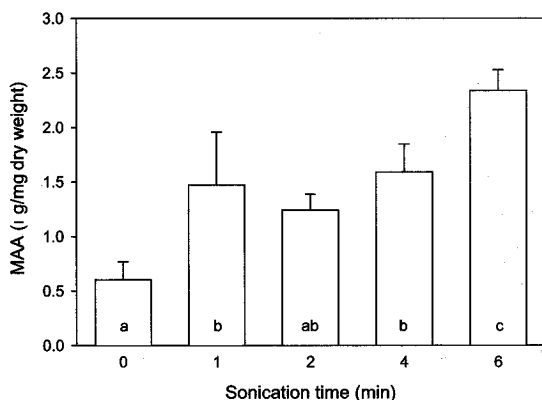


Fig. 4. Mean concentrations \pm 1 SD of total MAAs in *Porphyra* sp. after different sonication times in samples extracted in 25 % aqueous MeOH at 45 °C for 2 h. Different letters inside the bars indicate treatments that differed significantly ($p < 0.05$, Tukey test).

when the samples were sonicated for 1, 2, or 4 min, but significantly higher after 6 min.

Discussion

The results from the present study show that different extraction protocols affect total MAA concentrations recovered from different biological materials. The MAAs shinorine, palythine, MAA 331, asterina-330, and porphyra-334 were generally best extracted in 25 % aqueous MeOH at 45 °C (Fig. 3). These findings suggest that concentrations of MAAs obtained from extractions in either high MeOH concentrations or at low temperatures in similar biological material may be underestimated. However, the effect seems to differ depending on the type of organisms considered. Thus, for example, in *Porphyra* and in natural freshwater phytoplankton assemblages, the mean total concentrations of MAAs obtained in 25 % aqueous MeOH at 45 °C were respectively ~13 and ~3 times higher than in extractions made with 100 % MeOH at 4 °C. On the other hand, there were no significant differences among protocols in the case of *C. abyssorum tatricus*. Although we tested the effect of extraction on four MAAs that are frequently found in many marine and freshwater organisms (JEFFREY et al. 1999, KARENTZ 2001, TARTAROTTI et al. 2001) there are ~19 different MAAs described to date. Thus, it is not possible to assure that the most efficient extraction protocol found in this study can be used for all MAAs and biological materials. MAAs such as shinorine or porphyra-334 are chemically stable at 45 °C (CONDE et al. 2000, SINHA et al. 2000), there-

fore, extractions made at this temperature will not alter their molecular structure. However, potential modifications of other more labile MAAs cannot be ruled out and need to be tested.

In *Porphyra* sp., the concentration of MeOH had a stronger effect on the final concentration of MAAs than temperature, while in *C. abyssorum taticus*, the opposite was true (Fig. 3 A, C insets). On the other hand, in phytoplankton, both temperature and MeOH concentration seem to affect the final MAA concentration (Fig. 3 B inset). Moreover, when extracting *Porphyra* in 100 % MeOH, up to 21 % of the total MAA concentration was still found after the third extraction, while in 25 % aqueous MeOH it was ~2 % (Fig. 3 A). This finding suggests that in certain organisms extraction of MAAs may be insufficient without serial extractions. In fact, in many studies MAAs have been extracted only once.

Although the same main MAAs were extracted independently of the protocol used (Fig. 2), the relative proportions of the different MAAs changed, but again in an organism-specific manner (Table 2). For example, in natural freshwater phytoplankton, the relative proportion of shinorine was higher when extracting in 25 % aqueous MeOH, regardless of the extraction temperature (Fig. 2 B). At present we have no explanation for these differences mainly because there is no information where MAAs are located and to which other compounds they can be bound.

For the different organisms tested, extraction efficiencies were high (>98 %; Table 1) when using 25 % aqueous MeOH and high temperature (45 °C). In *Cyclops*, extraction efficiencies exceeded 98 % regardless of the protocol used, while in *Porphyra* these values were low (≤ 70 %, Table 1) when extracting in 100 % MeOH at 45 °C (≥ 88 % in protocols I, III, and IV). In addition, in phytoplankton, extraction efficiencies for shinorine and asterina-330 were low in protocol III (Table 1). Although the sonication time needed for an efficient extraction has to be tested as shown in *Porphyra* (Fig. 4), all samples were sonicated in the same way. Nevertheless, high MeOH concentrations seem to be less efficient to extract MAAs in cells like those of marine macroalgae. WHITEHEAD et al. (2001) reported that in the pteropod *Clione antarctica*, extraction efficiencies are 32 % after exhaustive serial extractions in 80 % aqueous MeOH at -14 °C for 12–16 h. These authors suggested that the low efficiency is due to the elastic nature of the lyophilized organisms. WHITEHEAD & VERNET (2000) hypothesized that incomplete extraction of MAAs in coastal phytoplankton with 100 % MeOH at 4 °C for ~12 h was responsible for the lack of correspondence between pigment-specific in vivo particle absorption and MAA concentrations. The same observation was done by SOMMARUGA & GARCIA-PICHEL (1999) for phytoplankton from Gosenköllesee. WHITEHEAD & VERNET (2000) suggested that there might be two classes of MAAs within a cell. One class of MAAs that is easily extractable in

high MeOH at low temperatures and another one that needs a more rigorous extraction procedure. DUNLAP & CHALKER (1986) pointed out that MAAs can be extracted in tetrahydrofuran-MeOH, ethanol, or MeOH with extraction efficiencies after three extractions typically exceeding 90%. Such high extraction efficiencies (>98%) were confirmed only for *C. abyssorum taticus*, however, in the other groups examined, efficiencies ranged from 45 to 91% when using 100% MeOH (Table 1). In *Porphyra*, extraction efficiencies for palythine and asterina were $\leq 51\%$, and in natural phytoplankton assemblages, the efficiency for shinorine was 76% when extracting three times in 100% MeOH at 45°C (Table 1).

In conclusion, while a general protocol to extract MAAs from aquatic organisms probably does not exist, the present data indicate that selection of the methanol concentration and temperature is important in estimating the concentration of these substances. Without a proper test of the most efficient protocol to assure the highest MAA recovery without altering their qualitative composition, it will be difficult to compare concentrations among different taxa or populations reported in the scientific literature. Moreover, a low extraction efficiency of MAAs will change the interpretation of ecological relationships or mask their importance as photoprotective compounds.

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