Freshwater Biology

Freshwater Biology (2012) **57,** 993–1004 doi:10.1111/j.1365-2427.2012.02760.x

The relative contributions of diet and associated microbiota to the accumulation of UV-absorbing mycosporine-like amino acids in the freshwater copepod *Boeckella antiqua*

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SUMMARY

1. Mycosporine-like amino acids (MAAs) are ubiquitous compounds in aquatic organisms that are usually considered sunscreens that protect them from harmful ultraviolet radiation. Given that virtually all animals lack the metabolic pathways to synthesise MAAs *de novo*, they must acquire them either from their diet or from microorganisms living in close association. In freshwater copepods, accumulation of MAAs is stimulated by exposure to ultraviolet and/or visible radiation.
2. A 2 × 2 factorial experiment was performed to assess the contributions of dietary and microbial sources of MAAs in the freshwater copepod *Boeckella antiqua*. The treatments consisted of two different diets: an MAA-free diet, including only *Chlamydomonas reinhardtii*, and an MAA-rich diet, including both *C. reinhardtii* and *Peridinium inconspicuum*, crossed with two antibiotic treatments, with and without chloramphenicol. Treatment with chloramphenicol was intended to inhibit the development of bacteria associated with the copepods.
3. MAA concentration in *B. antiqua* was affected by the experimental conditions: (i) exposure to artificial PAR + UVR stimulated the accumulation of several MAAs (up to 62% increase in total MAA concentration with respect to the initial concentration); (ii) the presence of chloramphenicol in the culture media reduced the MAA concentration in copepods fed an MAA-free diet; (iii) in the absence of chloramphenicol, copepods fed the MAA-rich diet had significantly higher total MAA concentration than those fed the MAA-deficient diet; but (iv) dietary supplementation with an MAA-rich algae in the presence of chloramphenicol failed to significantly increase total MAA concentration.
4. Analysis of profiles from denaturing gradient gel electrophoresis (DGGE) showed that the prokaryotic community associated with the copepods was affected by chloramphenicol. Dendograms constructed from digitalised DGGE images consistently grouped the antibiotics treatments separately from the initial samples and the treatments without antibiotics. Two band positions were exclusive to treatments without antibiotics.
5. We conclude that when offered an MAA-rich diet, *B. antiqua* may accumulate a proportion of MAAs from diet. However, we suspect that in the absence of an MAA-rich dietary source (as in its natural habitat), virtually all MAAs present in *B. antiqua* are produced by copepod-associated prokaryotes.

Keywords: antibiotic, denaturing gradient gel electrophoresis, dinoflagellate, mycosporine-like amino acids, prokaryotes

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Introduction

Mycosporine-like amino acids (MAAs) are water-soluble, UV-absorbing (309–362 nm) compounds that are widely distributed among aquatic organisms (Shick & Dunlap, 2002; Sinha, Singh & Häder, 2007), particularly among freshwater copepods (Tartarotti *et al*., 2004; Pérez *et al*., 2006; Hansson, Hylander & Sommaruga, 2007; García *et al*., 2010). The photoprotective function of MAAs has been inferred from their efficient UV absorption (Cockell & Knowland, 1999; Conde, Churio & Previtali, 2000) and the correlation between MAA concentrations and the levels of UV radiation exposure. In copepods, it has been demonstrated that exposure to UVR stimulates the accumulation of MAAs (Moeller *et al*., 2005; Pérez *et al*., 2006; Hansson *et al*., 2007; García *et al*., 2010). Besides their photoprotective role, evidence is accumulating that MAAs may have additional functions, such as antioxidant activity, osmotic regulation and nitrogen storage (Shick & Dunlap, 2002). Thus, MAAs are increasingly regarded as 'multipurpose' secondary metabolites (Oren & Gunde-Cimerman, 2007) rather than as exclusively sunscreen compounds.

The MAA structure is a cyclohexenimine core conjugated with one or two amino acid substitutes. Several studies have reported evidence suggesting that MAAs are synthesised through a derivation of the shikimate pathway (Favre-Bonvin *et al*., 1987; Shick *et al*., 1999; Portwich & Garcia-Pichel, 2003). Recently, however, Balskus & Walsh (2010) reported the identification of a different MAA biosynthetic gene cluster in a cyanobacterium. Although for most organisms the biosynthetic pathway of MAAs is not fully understood, there is general consensus that animals are unable to synthesise MAAs *de novo* (Bentley & Haslam, 1990; Herrmann & Weaver, 1999). One exception may be the sea anemone *Nematostella vectensis* Stephenson. Using genomic mining approaches, Starcevic *et al*. (2008) found genes encoding shikimate pathway enzymes in *N. vectensis*, and Balskus & Walsh (2010) reported the presence of putative mycosporine gene clusters in the genome of this species. According to Starcevic *et al*. (2008), molecular evidence establishes horizontal transfer of ancestral genes of the shikimic acid pathway into the *N. vectensis* genome from both bacterial and eukaryotic (dinoflagellate) donors. All other animals so far studied are thought to acquire MAAs from either their food (Carefoot *et al*., 1998; Mason *et al*., 1998; Newman *et al*., 2000; Nagiller & Sommaruga, 2009) or their symbiotic partners (Banaszak & Trench, 1995; Sonntag, Summerer & Sommaruga, 2007).

A few calanoid copepods have been reported to accumulate MAAs when offered an MAA-rich diet [i.e. algal mixtures including the dynoflagellate, *Peridinium inconspicuum* Lemm (Moeller *et al*., 2005; Hylander *et al*., 2009)]. On the other hand, several centropagid copepods are able to accumulate MAAs even in the absence of a dietary source of these compounds (Pérez *et al*., 2006; García *et al*., 2010). Interestingly, additions of the bacteriostatic antibiotic chloramphenicol to the culture medium inhibited the accumulation of MAAs in the centropagid copepod *Boeckella antiqua* (García *et al*., 2010), pointing to symbiotic prokaryotes as a potential source of MAAs. So far, the relative contribution of dietary and symbiotic sources of MAAs has not been evaluated simultaneously in a single copepod species.

The existence of gut bacteria in zooplankton has long been recognised (Harris, 1993; Tang, 2005). Moreover, gut bacteria are known to provide important nutrients, such as essential amino acids and vitamins (Fong & Mann, 1980). However, our knowledge of the presence, composition and role of gut 'microflora' in freshwater zooplankton is still in its infancy. Most studies on gut 'microflora' have been conducted using culture-based techniques (Sochard *et al*., 1979; Hansen & Bech, 1996). However, given that culture media are generally very selective, only a small percentage of bacteria can be cultured and identified using traditional bacteriological approaches. Fortunately, the development of molecular techniques, such as fingerprinting methods, is providing promising tools to increase our understanding of zooplankton–microbes associations (Peter & Sommaruga, 2008).

In this study, we investigate the contribution of dietary and microbial sources of MAAs in freshwater copepods and perform an initial assessment of the prokaryotic flora associated with the copepods. An attempt was also made to identify bacterial entities that could potentially be sources of MAAs.

Methods

Sources of the experimental organisms

Samples of the copepod *B. antiqua* Menu Marque & Balseiro were collected from a shallow, turbid and fishless lake, Los Juncos (41°03′38″S 71°00′38″O, 907 m a.s.l.), using a 220-μm hand net. In the laboratory, the copepods were transferred to 20-L containers filled with 40 μm filtered water and stored overnight at room temperature in darkness. The two algae species used to feed the copepods were *Chlamydomonas reinhardtii* Dang (wild type, Carolina Biological Supplies, Burlington, NC, USA) and *P. inconspicuum* Lemm (UTEX 2255). The algae were grown under fluorescent cool-white light in modified (i.e.

without NaCl) Marine Biological Laboratory medium (*C. reinhardtii*) or MES volvox medium (*P. inconspicuum*). Cultures of *C. reinhardtii* support good copepod growth, but they lack MAAs. On the other hand, *P. inconspicuum* is a good source of MAAs for copepods but is a deficient food source unless supplemented with another alga species (Moeller *et al*., 2005).

Experimental set-up

A 2×2 factorial experiment was performed to assess the simultaneous effects of diet and the addition of an antibiotic on the accumulation of MAAs in the copepod *B. antiqua*. Each of the four treatments was run in three replicates. Groups of 65 adult (males and females) copepods were sorted randomly into twelve (i.e. four treatments × three replicates) 2.5-L, UVR-transparent acrylic cylinders (UVT Plasmatic, Malaga, Spain). Three additional groups of 60 adult individuals were preserved to assess the initial condition of the copepods (INI). The experiment lasted 10 days. The treatments consisted of two different diets: either *C. reinhardtii* (−PER) or *C. reinhardtii* plus *P. inconspicuum* (+PER), crossed with two bacteriostatic treatments (with and without antibiotic). The antibiotic treatments (+AB) included 10 mg L^{-1} of the bacteriostatic antibiotic chloramphenicol. The treatments without antibiotic were labelled −AB. Each of the four treatments (−AB−PER, −AB+PER, +AB−PER and +AB+PER) was run in three replicates. The experimental vessels were kept inside an environmental test chamber (Sanyo, MLR5, Moriguchi, Osaka, Japan) at 16 °C with a 12 : 12 dark/light cycle. They were exposed to visible and UV radiation to stimulate the accumulation of MAAs by the copepods. The radiation sources were ten Sanyo 40-W (PAR) and two Q-panel 340 (UVR) fluorescent tubes, placed vertically on the sides of the test chamber. Radiation was measured inside the environment chamber with a spectroradiometer (USB 2000; Ocean Optics, Dunedin, FL, USA). Daily fluences were 10.8 E m^{-2}, 166.5 KJ m^{-2} and 5.5 KJ m^{-2} for PAR (400–750 nm), UVA (400–320 nm) and UVB (320–280 nm), respectively.

The experimental units were cleaned every other day by pouring each vessel's content through a 220-μm mesh and gently rinsing the mesh with filtered water. During this procedure, immature stages (nauplii), faecal pellets and other debris were removed from the cultures. Dead adults were removed manually using a Pasteur pipette. The copepods were subsequently placed in clean acrylic cylinders previously filled with the corresponding fresh media (i.e. the antibiotic and algal combination prescribed for each treatment). This procedure (i.e. frequently replacing the whole copepod culture media) was adopted to minimise algal exposure to the antibiotic. The nominal algal concentration was chosen so that all treatments received a similar algal biovolume: for −PER 7000 *C. reinhardtii* cells mL^{-1} (*c.* 3.5 10^6 μm^3 mL^{-1}) and for +PER 5000 *C. reinhardtii* cell mL^{-1} plus 1000 *P. inconspicuum* cell mL^{-1} (*c.* 2.5×10^6 μm^3 mL^{-1} *C. reinhardtii* plus 0.72 10^6 μm^3 mL^{-1} *P. inconspicuum*). At the end of the experiment, all adult copepods from each replicate were harvested and washed twice with filtered water. Subsamples for HPLC analyses (30 individuals) were lyophilised and kept at −20 °C until extraction. Subsamples for DGGE analyses (10 whole individuals) were preserved in 96% EtOH and stored at room temperature. Subsamples for dry weight (the remaining individuals) were collected onto pre-burn weighed GF/F filters. In addition, samples of microorganisms present in the cultivation water for DGGE analyses were collected from each replicate. Hundred millilitre of the cultivation water was filtered through 0.2-μm polycarbonate filter (Millipore, Billerica, MA, USA). The filters were lyophilised and stored at −20 °C until DNA extraction.

Mycosporine-like amino acids

The HPLC analyses of MAAs were performed according to Tartarotti & Sommaruga (2002). HPLC analyses were performed on copepod samples and also on filtrates of fresh copepod culture media (including the corresponding algal species). The samples were extracted in 25% aqueous methanol, sonicated (1 min at 0.5 cycles and 20% amplitude; Sonics Vibra Cell, Newtown, CT, USA) and cleared by filtration (0.22 μm). Three hundred microlitre aliquots were injected into a chromatographer (Aktabasic; Amersham, Fairfield, CT, USA) equipped with a 5-μm-pore-size Phenosphere C-8 column (250 × 4.6 mm internal diameter; Phenomenex, Torrance, CA, USA) protected with an RP-8 guard column (Brownlee). Samples were run with a mobile phase of 0.1% acetic acid in 25% aqueous MeOH (vol : vol) and a flow rate of 0.79 mL min^{-1}. In the absence of certified standards, MAAs were identified by relative retention time, absorption peaks, co-chromatography with *Porphyra* extracts, co-chromatography with *B. antiqua* extracts (previously identified by Ruben Sommaruga at Innsbruck University) and comparison with *P. inconspicuum* (UTEX LB 2255) chromatographs (provided by Robert Moeller). The total content of MAAs in each sample was calculated from HPLC peak areas, using published molar extinction coefficients (Bandaranayake & Desrocher, 1999; Karentz, 2001; Conde *et al*., 2003). We used an average molar extinction coefficient (ξ = 40 000) for the unknown

MAA332 (Pérez *et al.*, 2006). The MAA content was normalised to the initial dry weight for copepods or to the chlorophyll concentration of the culture media and expressed as μg mg^{-1} DW or μg μg^{-1}Chl *a*. Chlorophyll *a* concentration was determined following the spectrophotometric method of Nusch (1980).

Molecular fingerprinting of copepod-associated microbiota

As a first step to characterise the microbial flora, we performed denaturing gradient gel electrophoresis (DGGE) on bacterial DNA extracted from the copepods, as well as the bacteria collected from the cultivation media. For DNA extraction, the samples were subsequently rehydrated with MQ water, ground after the addition of 0.75 ml CTAB lysis buffer (2% CTAB; 1.4 M NaCl, 100 mM Tris-Cl pH 8, 20 mM EDTA pH 8) (Ferrero *et al.*, 2010) and incubated at 60 °C for 30 min. DNA was extracted twice in chloroform–isoamilic alcohol (24 : 1). The extract was precipitated with isopropyl alcohol and resuspended in TE buffer. DNA extracts were stored at −20 °C until analysis.

For PCR amplification of 16S rRNA gene fragments, 1 μL of extracted DNA was used as template. PCR was carried out in 50 μL reaction mixtures using 1× Green Go Taq reaction Buffer (Promega, Madison, WI, USA), which contains 1.5 mM $MgCl_2$ (final concentration), 50 μM (each) deoxynucleotides (Invitrogen, Grand Island, NY, USA), 0.5 μM (each) bacterial primers GC-358f (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G- CCT ACG GGA GGC AGC AG-3′) (Muyzer, De Waal & Uitterlinden, 1993) and 907rM (5′-CCG TCA ATT C[A/C]T TTG AGT TT-3′) (Muyzer *et al.*, 1995), and 1.25U of Platinum Taq polymerase (Invitrogen). Thermocycling was preceded by a 5-min heating step at 94 °C, followed by 10 touchdown cycles, which consisted of denaturing at 94 °C for 1 min, annealing at 65 °C for 1 min and extension at 72 °C for 3 min (temperature decreased 1 °C on every cycle until the temperature reached 56 °C). After the touchdown cycles, 25 cycles of denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 3 min and a final extension cycle at 72 °C for 10 min were performed. An aliquot of the PCR product was analysed and quantified by comparison with a standard low-mass DNA ladder in a 1% agarose gel.

PCR products obtained were analysed on a 40–80% denaturant DGGE gel (Muyzer & Smalla, 1998; Schauer *et al.*, 2003). About 500–800 ng of the PCR products was applied to individual lanes in the gel. Electrophoresis was performed on a 0.75-mm-thick 6% polyacrylamide gels (37.5 : 1 acrylamide-to-bisacrylamide ratio), submersed in 1× TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 7.4) at 60 °C and run at 100 V for 16 h. After electrophoresis, the gels were stained for 45 min in 1× TAE buffer with Syber Gold nucleic acid stain (Molecular Probes, Eugene, OR, USA) and visualised under UV radiation using a FOTO/Convertible dual-light transilluminator and the FOTO/Analyst PC Image Application software (Photodyne, New Berlin, WI, U.S.A.).

DGGE bands were excised from the gel, resuspended in 30 μL of MQ water and stored at 4 °C overnight. Subsequently, 2 μL of the samples was used for reamplification with the original primer set (without a GC clamp), and the PCR products were purified with Qiaquick PCR Purification kit (QUIAGEN, Valencia, CA, USA) and sequenced with 357f primer. Direct sequencing of the amplification products was performed at the Facultad de Ciencias Exactas y Naturales (Universidad de Buenos Aires, Argentina). The resulting sequences (around 500 bases) were compared versus public DNA database, using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al.*, 1997).

Digitalised DGGE images were analysed using the TotalLab 100 software (Nonlinear Dynamics, Newcastle upon Tyne, UK). This software performs a density profile through each lane, detects bands and calculates the relative contribution of each band to the total band signal in the lane. We applied 'rolling ball' as the background subtraction method. Bands occupying the same position in the different lanes of the gel were matched automatically using the synthetic reference line matching method and then checked by visual inspection. A matrix was constructed taking into account the presence/absence of individual bands in all lanes and the relative intensity of each band compared to the total band intensity in each lane.

Statistical analyses

The statistical significance of the results was evaluated by one-way ANOVA, followed by the Tukey test of means comparisons. Homoscedasticity and normality were tested before the analyses. As regards DGGE profiles, the fingerprints consisted of banding patterns where each band was assumed to be an operational taxonomic unit (OTU). For the analysis of gel, Ward (1963) and neighbour-joining (Saitou & Nei, 1987) algorithms were performed, and corresponding dendograms showing the relationship between the DGGE profiles were constructed.

Results

We were able to identify eight different MAAs from *B. antiqua* extracts: shinorine (SH, 44%), porphyra 334

(PR, 16%), palythine (PI, 13%), mycosporine glycine (MG, 11%), an unknown compound (hereinafter MAA332, 10%), usujirene (US, 4%), palythene (PE, 1%) and asterina (AS, 0.2%).

During the experiment, the copepods were fed one of the two diets: (i) *C. reinhardtii* (−PER) and (ii) *C. reinhardtii* plus *P. inconspicuum* (+PER). The −PER diet contained no measurable amounts of MAAs. On the other hand, the +PER diet included eight different compounds: US (34%), SH (23%), PI (15%), MG (14%), MAA332 (4%), AS (1%), PE (8%) and an unidentified compound (∼1%). By the end of the experiment, the average survivorship of *B. antiqua* in all treatments was about 76%. No significant differences in survival were detected owing to the addition of antibiotic to the media (+AB=78% versus −AB=73%; two-way ANOVA, $P_{AB} = 0.468$) or the diet supplementation with *P. inconspicuum* (+PER = 78% versus −PER=73%; two-way ANOVA, $P_{PER}=0.470$).

The MAA composition of *B. antiqua* was affected by the experimental conditions and the different treatments assayed. First, as could be expected, the total MAA concentration in the −AB−PER treatment was higher (62%) than in copepods freshly collected from Laguna Los Juncos (INI) ($P = 0.029$, Tukey's test, Fig. 1). This increase was mostly attributable to increases in MG, SH and PR (Table 1 and Fig. 2).

Second, the presence of the antibiotic chloramphenicol in the copepod culture medium affected the concentration of MAAs. The copepods from the +AB−PER treatment had significantly lower total MAA concentration than those from the −AB−PER treatment ($P = 0.005$, Tukey's test). More specifically, the presence of the antibiotic resulted in lower concentrations of MG, SH and PR (Table 1 and Fig. 2).

Third, copepods fed the MAA-rich diet (−AB+PER) had significantly higher total MAA concentration than those fed the MAA-deficient diet −AB−PER treatments) ($P = 0.030$, Tukey's test). In particular, supplementation with *P. inconspicuum* increased the concentration of US, PE and AS, and to a lesser extent MG (Table 1 and Fig. 2).

Finally, dietary supplementation with an MAA-rich algae in the presence of the antibiotic chloramphenicol, (+AB+PER versus +AB−PER) failed to significantly increase total MAA concentration. Although the average concentrations of US and PE were significantly higher in the +AB+PER treatment than in the +AB−PER treatment (Table 1 and Fig. 2), these two compounds contributed little to the total MAA pool. The concentrations of more abundant MAAs, such as SH, tended to be higher in the +AB+PER treatment, but such differences were not statistically significant.

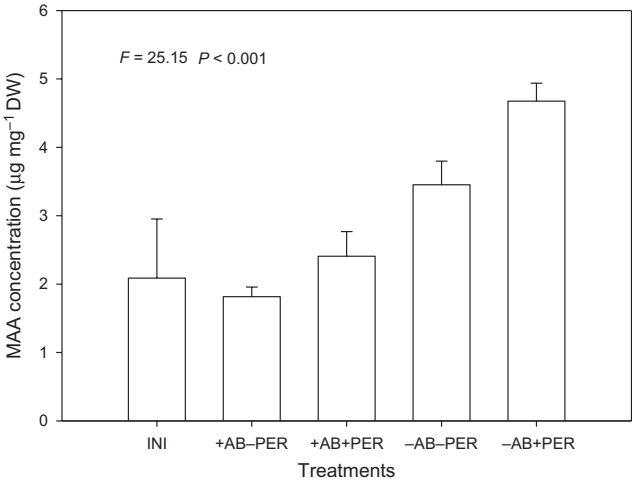

Fig. 1 Total MAA concentration in *Boeckella antiqua* before and after the experiment. INI: initial samples (i.e. individuals freshly collected from Laguna Los Juncos); +AB/−AB: with/without additions of the antibiotic, chloramphenicol, to the culture media; +PER/−PER: diet supplemented/not supplemented with the MAA-rich alga *Peridinium incospicuum*. The *F* and *P* values correspond to the overall significance of the ANOVA. The results of Tukey's tests for specific comparisons: (i) the effect of PAR + UVR exposure (INI versus −AB−PER), (ii) the effect of antibiotics (−AB−PER versus +AB−PER), (iii) the effect of dietary MAA supplementation in the absence of antibiotics (−AB−PER versus −AB+PER) and (iv) the effect of dietary MAA supplementation in the presence of antibiotics (+AB−PER versus +AB+PER) are listed in Table 1.

To evaluate whether the MAA sources were complementary or additive, we estimated the increase in MAA concentration as the sum of the increases from each separate source and compared this to the observed increase in MAA concentration when the copepods had simultaneous access to both sources. We found that the increase in MAA concentration estimated as the sum of the increased concentrations owing to endogenous and dietary sources separately was slightly (albeit non-significantly) lower than the observed increase owing to the simultaneous access to both sources ($P = 0.09$, Fig. 3a). This was evident for SH ($P = 0.96$), MG ($P = 0.06$) and PR ($P = 0.62$) (Fig. 3b–d).

From the DGGE gel image (Fig. 4), we were able to identify 157 bands, occupying 36 distinct positions. To analyse the DGGE profile, two similarity indexes (i.e. Ward and neighbour-joining algorithms) were performed. The dendograms resulting from both indexes consistently grouped the lanes corresponding to the antibiotic treatments (+AB+PER and +AB−PER) separately from the lanes corresponding to initial samples and the treatments without antibiotics (−AB+PER and −AB−PER) (Fig. 5a and b).

Table 1 Concentrations (mean ± SD) of MAAs in *Boeckella antiqua* (μg mg^{-1}DW) and comparisons between sample means

Comparing Sample Means	Total MAAs	SH	PR	MG	PI	MAA332	US*	PE*	AS
Effect of PAR ± UVR exposure									
INI	2.1 ± 0.86	0.9 ± 0.40	0.3 ± 0.10	0.2 ± 0.10	0.3 ± 0.15	0.2 ± 0.03	0.09 ± 0.06	0.02 ± 0.01	0.005 ± 0.002
−AB−PER	3.4 ± 0.34	1.5 ± 0.11	0.8 ± .11	0.8 ± 0.06	0.1 ± 0.06	0.2 ± 0.03	0.008 ± 0.005	0.008 ± 0.005	0.002 ± 0.0006
% MAA change	+62	+67	+167	+300	−67	0	−91	−60	−60
P (Tukey's test)	0.029	0.04	<0.001	0.002	n.s.	n.s.	0.02	n.s.	n.s.
Effect of antibiotics									
−AB−PER	3.4 ± 0.34	1.5 ± 0.11	0.8 ± 0.10	0.8 ± 0.06	0.1 ± 0.06	0.2 ± 0.02	0.008 ± 0.005	0.008 ± 0.005	0.002 ± 0.0007
+AB−PER	1.8 ± 0.14	0.7 ± 0.03	0.4 ± 0.02	0.3 ± 0.08	0.1 ± 0.03	0.3 ± 0.03	0.01 ± 0.004	0.003 ± 0.001	0.002 ± 0.0003
% MAA change	−47	−53	−50	−63	0	+50	+25	−63	0
P (Tukey's test)	0.005	0.003	<0.001	0.001	n.s.	n.s.	n.s.	n.s.	n.s.
Effect of dietary MAAs supplementation the absence of antibiotics									
−AB−PER	3.4 ± 0.34	1.5 ± 0.11	0.8 ± 0.10	0.8 ± 0.06	0.1 ± 0.06	0.2 ± 0.03	0.008 ± 0.005	0.008 ± 0.005	0.002 ± 0.0007
−AB+PER	4.7 ± 0.26	2.0 ± 0.13	0.9 ± 0.05	1.2 ± 0.13	0.2 ± 0.05	0.3 ± 0.04	0.1 ± 0.01	0.04 ± 0.0007	0.005 ± 0.0007
% MAA change	+38	+33	+13	+50	+100	+50	+1250	+500	+150
P (Tukey's test)	0.03	n.s.	n.s.	0.007	n.s.	n.s.	0.006	<0.001	0.05
Effect of dietary MAAs supplementation the presence of antibiotics									
+AB−PER	1.8 ± 0.14	0.7 ± 0.03	0.4 ± 0.02	0.3 ± 0.08	0.14 ± 0.03	0.3 ± 0.02	0.01 ± 0.004	0.003 ± 0.001	0.002 ± 0.0003
+AB+PER	2.4 ± 0.36	1.2 ± 0.18	0.4 ± 0.03	0.3 ± 0.12	0.16 ± 0.04	0.2 ± 0.02	0.1 ± 0.027	0.02 ± 0.003	0.004 ± 0.0012
% MAA change	+33	+71	0	0	+14	−33	+1000	+667	+100
P (Tukey's test)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.002	0.005	n.s.

SH, Shinorine; PR, porphyra-334; MG, mycosporine glycine; PI, palythine; unknown MAA332; US, usujirene; PE, palythene; AS, asterina.
n.s., not significant.
*A nonparametric Kruskal–Wallis one-way analysis of variance on ranks was preformed in lieu of parametric ANOVA.

The analysis of the occurrence of individual positions revealed the following:

1. Five positions (1, 14, 27, 31 and 36) occurred in at least one replicate of every treatment, as well as in the initial samples. Positions 1 and 36 were present in all replicates within every single treatment. We were able to sequence band 31, which resembled *Anabaena variabilis* (87% similarity), and bands 36A and 36B, which resembled *Bacillus* sp. (95% similarity).

2. Two positions (27 and 30) occurred in at least one replicate of the initial samples and the treatments that did not received antibiotics −AB−PER and −AB+PER). Position 27 was present in every single replicate. These two positions were absent from all replicates treated with antibiotics (+AB−PER and +AB+PER). The sequence of position 27 (bands 27A and 27B) resembled that of *Limnobacter* sp. (99% similarity).

3. Two positions (5 and 25) occurred only in replicates corresponding to the two antibiotics treatments (+AB−PER and +AB+PER). Position 5 was present in three of six replicates, while position 25 was present in 5 of six replicates. The sequence of position 5 (bands 5A and 5B) is similar to that of *Chryseobacterium gleum* (93% similarity).

4. Positions 3 (four of six replicates) and 29 (three of six replicates) were present in treatments fed a mixture of *C. reinhardtii* + *P. inconspicuum* (−AB+PER and +AB+-PER), but they were absent from all replicates corresponding to the initial samples and the treatments fed only *C. reinhardtii* (−AB−PER and +AB−PER). No band corresponding to these positions could be sequenced.

5. The sequence of a few additional bands could be retrieved: band 8 (*Cytophaga hutchinsonii*, 86% similarity), band 15 (*Methylotenera versatilis*, 99%), band 18 (*Acinetobacter johnsonii*, 100%), band 26 (*Polaromonas* sp., 98%) and band 34 (*Methyllocella silvestres*, 93%). These positions occurred haphazardly, that is, there was no relationship between their presence and treatments in which they occurred.

6. All the above-mentioned sequences are related to Gammaproteobacteria, Betaproteobacteria, Firmicutes, Alphaproteobacteria, Bacteroidetes or Cyanobacteria (Table 2).

The sequences retrieved from bands excised from the DGGE performed on DNA extracted from the cultivation media were related to the phylogenetic group β-proteobacteria: *Acidovorax radices* (98% similarity), *Rhodoferax ferrireducens* (98%), *Leptothrix cholodnii* (94%), *M. versatilis* (93%) and *Herbaspirillum seropedicae* (91%); Gammaproteobacteria: *Acinetobacter iwoffii* (100%) and *Acinetobacter* sp. (97%); Bacteroidetes: *Fluviicola*

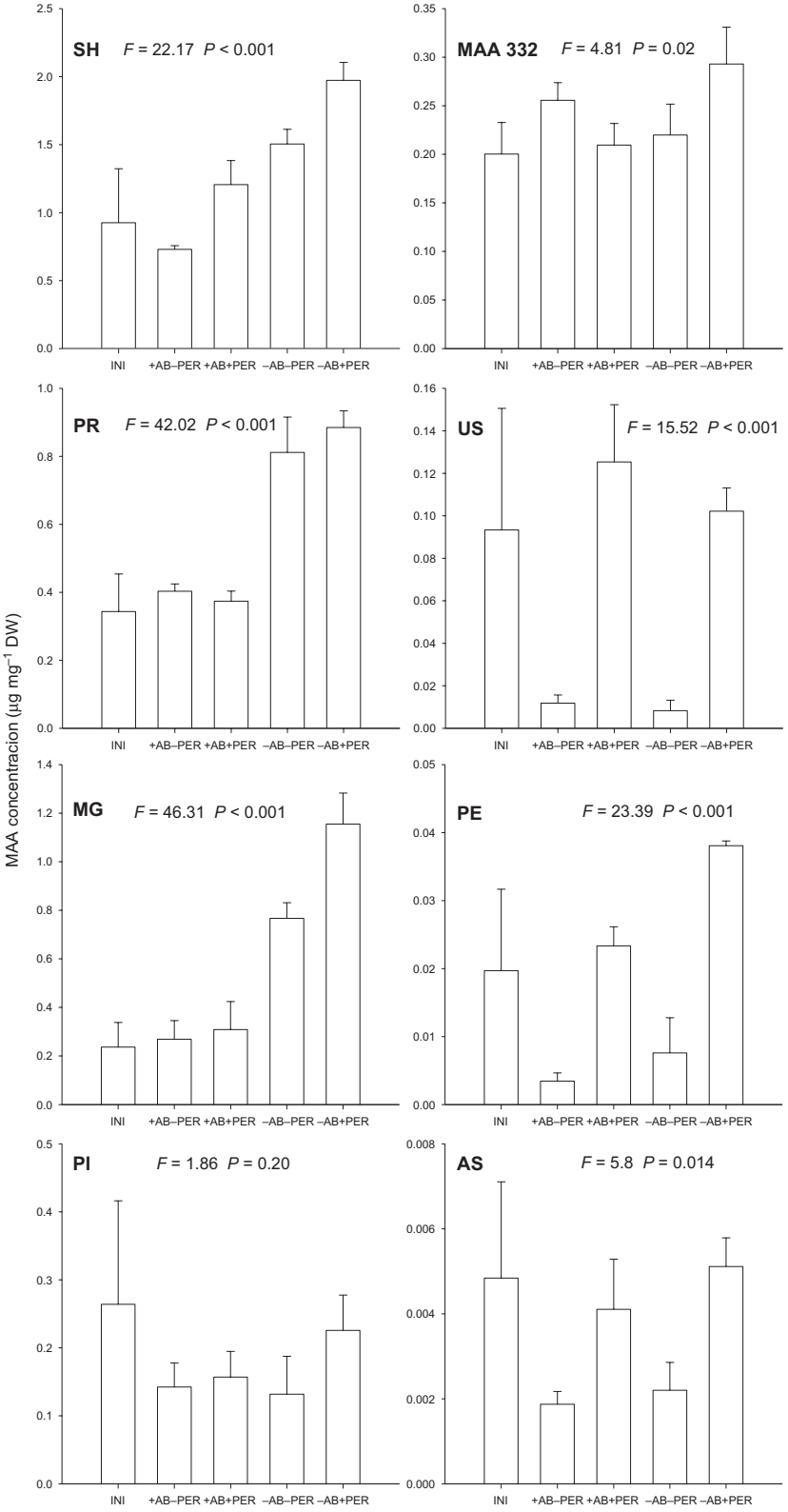

Fig. 2 Concentrations of individual MAAs in *Boeckella antiqua* before and after the experiment. Shinorine (SH), porphyra-334 (PR), mycosporine glycine (MG), palythine (PI), unknown MAA332, usujirene (US), palythene (PE) and asterina (AS). Treatment coding and statistical analyses as in Fig. 1.

taffensis (94%) and *Chryseobacterium gleum* (96%); Cyanobacteria: *Cyanothece* sp. (85%) and *Lyngbya majuscula* (80%); and Alphaproteobacteria: *Alpha proteobacterium* HIMB114 (94%).

Discussion

Boeckella antiqua individuals collected from Laguna Los Juncos had a total of eight different MAA compounds:

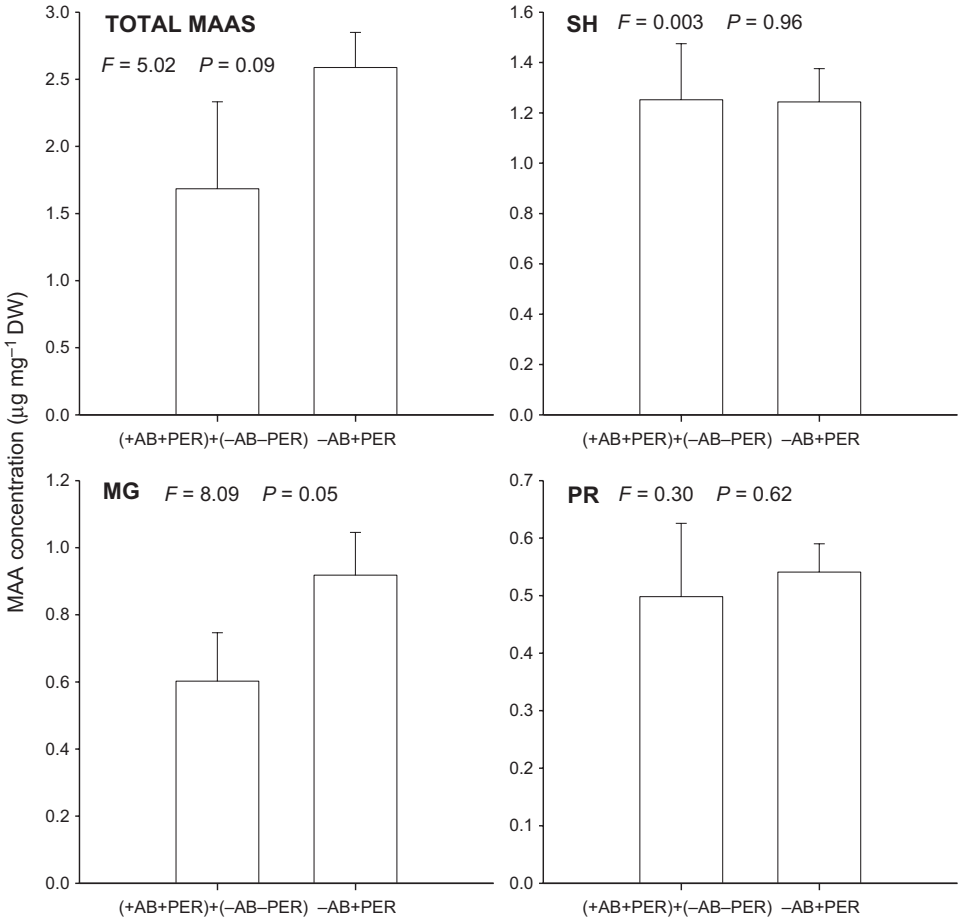

Fig. 3 Estimated increases in MAA concentration when the copepods had access to the two alternative sources of MAAs: diet and microorganisms. −AB+PER shows the observed increase when the copepods had simultaneous access to both sources; (+AB+PER)+(−AB−PER) shows the calculated increase assuming that the copepods had separated access to both sources (i.e. the sum of the increase attributable to diet alone plus the increase attributable to microorganisms alone). The *F* and *P* values correspond to the overall significance of the ANOVA for Total MAAs, (SH) shinorine, (MG) mycosporine glycine and (PR) porphyra-334.

shinorine, porphyra 334, palythine, mycosporine glycine, an unknown compound, usujirene, palythene and asterina. The first five compounds accounted for nearly 95% of the total MAA concentration. Four MAAs (SH, PR, MG and MAA332) have been reported previously in *B. antiqua* (Pérez *et al.*, 2006; García *et al.*, 2008).

During the experiment, the copepods were offered either an MAA-free diet or an MAA-rich diet. The latter included seven of the eight MAAs present in wild *B. antiqua* individuals (the exception being PR, which is absent from *P. inconspicuum* cultures), plus a minor (1% of total MAA concentration) unknown UV-absorbing compound. All eight compounds have been previously identified from *P. inconspicuum* cultures (Moeller *et al.*, 2005).

It is well established that exposure of *B. antiqua* individuals to UV and visible radiation (UVR + PAR) stimulates the accumulation of MAAs (Pérez *et al.*, 2006; García *et al.*, 2010). Moreover, we have previously demonstrated that the increase in MAA concentration occurring after several days of UVR+PAR irradiation does not require a dietary source of MAAs (*i.e.* it occurs even if the copepods are fed an MAA-free diet; Pérez *et al.*, 2006; García *et al.*, 2010). The results obtained in this study are fully consistent with our previous reports that average MAA concentration in the copepods fed an MAA-free diet (−AB−PER) and exposed to UVR + PAR resulted in significantly higher-than-average MAA concentration of individuals freshly collected from Laguna Los Juncos (INI).

In a previous study (García *et al.*, 2010), we had also shown that the addition of the bacteriostatic antibiotic chloramphenicol inhibited the bioaccumulation of MAAs in *B. antiqua*. This result was also confirmed in the present study: the average concentration of MAAs in copepods treated with antibiotic (+AB−PER) was significantly lower than that of untreated copepods (−AB−PER). This result

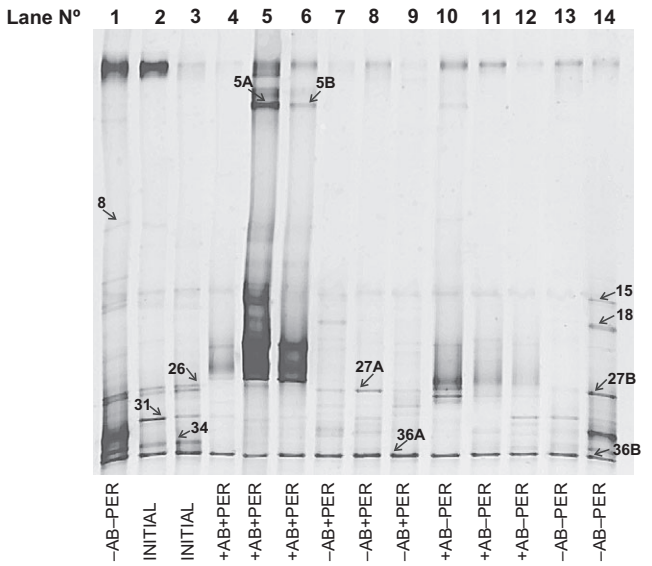

Fig. 4 DGGE profiles of the prokaryotic community associated with *Boeckella antiqua*. Numbers on the gel indicate the assigned positions of the bands that were excised and sequenced. Treatment coding as in Fig. 1.

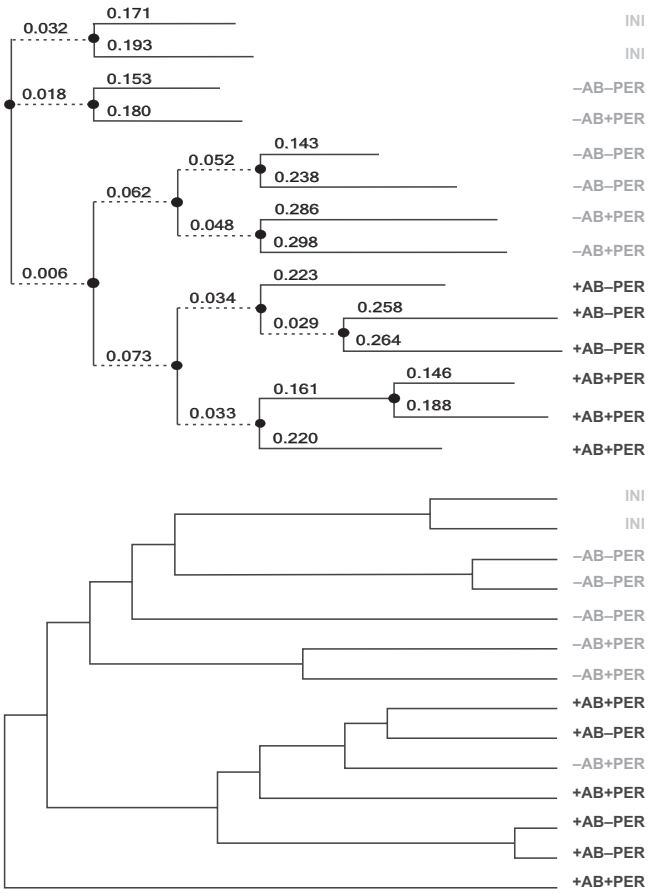

Fig. 5 Dendograms constructed from digitalised DGGE images of Fig. 4, using (a) the neighbour-joining algorithm (upper panel) and (b) Ward's hierarchical clustering method (lower panel). Treatment coding as in Fig. 1.

holds for total MAA concentration as well as for the concentrations of the most abundant individual compounds. It is worth noting that the addition of the antibiotic had no effect on the survival of the copepods.

The findings that MAA accumulation could occur in the absence of a dietary source (Pérez *et al*., 2006; García *et al*., 2008) and the evidence demonstrating that MAA accumulation is inhibited by the bacteriostatic antibiotic chloramphenicol (García *et al*., 2010) led us to propose that the source of MAAs in these copepods could be prokaryotic organisms living in close association with them. Here, we have explored this possibility in greater detail by studying the effect of the antibiotic on the prokaryotic community associated with the copepods. The analysis of the DGGE profiles showed that the prokaryotic community is, in fact, affected by the antibiotic treatment. The dendograms resulting from two different similarity indexes consistently grouped the lanes corresponding to the antibiotic treatments (+AB+PER and +AB−PER) separately from the lanes corresponding to initial samples (INI) and the treatments without antibiotic (−AB+PER and −AB−PER). In addition, the analysis of individual bands showed that two positions occurred exclusively in initial samples as well as in the two treatments that did not received antibiotic, but were absent from all replicates treated with chloramphenicol. Two bands (27A and 27B) corresponding to one of these positions could be sequenced. They resemble the betaproteobacteria *Limnobacter* (99% similarity), which is known to have genes encoding for the shikimic acid pathway (http://www.uniprot.org/uniprot/A6GR94#section_ref).

Overall, the microbial community retrieved from the copepods did not reflect the community in the surrounding medium. Interestingly, no sequences resembling *Limnobacter* sp. could be retrieved from the cultivation media. Differences between the microbiota associated with pelagic organisms and the microbiota in the surrounding medium have been reported for *Daphnia magna* Straus (Freese & Schink, 2011) and *Bosmina coregoni* Baird and *Thermocyclops oithonoides* (Sars) (Grossart *et al*., 2010).

Animals may accumulate metabolites from one or more sources. Moeller *et al.* (2005) presented convincing evidence suggesting that a diet including the MAA-rich dinoflagellate *P. inconspicuum* induced the accumulation of MAAs in *Leptodiaptomus minutus* (Lilljeborg). The results of our experiment are consistent with the findings of Moeller *et al.* (2005): the copepod fed a diet supplemented with *P. inconspicuum* (−AB+PER) had average

Table 2 Bacterial community composition and similarity indexes of the sequenced bands (see Fig. 4)

Band No.	Access Number of band sequence	Closest match	Percentage of similarity	Access number of closest mach	Phylogenetic group
5.A	JQ415972	*Chryseobacterium gleum*	93	ACKQ02000003.1	Bacteroidetes
5.B	JQ415973	*Chryseobacterium gleum*	93	ACKQ02000003.1	Bacteroidetes
8	JQ415968	*Cytophaga hutchinsonii*	86	NC008255.1	Bacteroidetes
15	JQ392565	*Methylotenera versatilis*	99	NC014207.1	Betaproteobacteria
18	JQ361694	*Acinetobacter lwoffii*	100	AFQY01000001.1	Gammaproteobacteria
26	JQ415969	*Polaromonas* sp.	98	NC007948.1	Betaproteobacteria
27.A	JQ415966	*Limnobacter* sp.	99	ABCT01000008.1	Betaproteobacteria
27.B	JQ415967	*Limnobacter* sp.	99	ABCT01000008.1	Betaproteobacteria
31	JQ415970	*Anabaena variabilis*	87	NC007413.1	Cyanobacteria
34	JQ415971	*Methylocella silvestris*	93	NC011666.1	Alphaproteobacteria
36.A	JQ415964	*Bacillus coagulans*	95	NC016023.1	Firmicutes
36.B	JQ415965	*Bacillus* sp.	94	ACWC01000034.1	Firmicutes

Similarities between 5.A and 5.B, 27.A and 27.B, and 36.A and 36.B are 100%, 100% and 99%, respectively.

total MAA concentration significantly higher than those fed exclusively *C. reinhardtii* (−AB−PER). This increase was, however, comparatively modest (38%) and was due mostly to increases in the least abundant compounds (MG, US, PE and AS). In contrast, the increases in the most abundant MAAs (SH and PR) were not statistically significant. The failure to increase the concentration of PR was expected, as the alga *P. inconspicuum* lacks this compound. But the modest increase in SH (33%) was unexpected given that SH is one of the most abundant MAAs in *P. inconspicuum* (27%).

In the experiment reported here, the copepods had access, either separately or simultaneously, to two alternative sources of MAAs: (i) individuals fed exclusively *C. reinhardtii* and not receiving chloramphenicol (−AB−PER) had only access to an alternative (probably prokaryotic symbiotic) source, (ii) individuals fed a mixture of *C. reinhardtii* and *P. inconspicuum* and treated with antibiotic (+AB+PER) had only (or at least mostly) access to a dietary source and (iii) individuals fed a mixture of *C. reinhardtii* and *P. inconspicuum* and not treated with antibiotic (−AB+PER) had simultaneous access to both sources of MAAs. We found that the increase in MAA concentration estimated as the sum of the increased concentrations owing to dietary and non-dietary sources separately was slightly (albeit non-significantly) lower than the observed increase owing to the simultaneous access to both sources. These results suggest that under the stimulatory conditions of PAR + UVR exposure, the copepods accumulate as much MAAs as possible from either source, rather than adjusting the MAA concentration to the prevailing irradiance conditions. In other words, we suspect that the processes of MAA assimilation from the two sources are independent. This evidence is in line with the previous suggestion that MAAs may be accumulated in excess at places or during periods that favour their accumulation (García *et al.*, 2008; Orfeo *et al.*, 2011).

In our experiment, exposure to UVR in the absence of a dietary source of MAAs resulted in a 62% increase in MAA concentration. Access to an MAA-rich diet further increased the average MAA concentration by 38%. The latter is a relatively modest increase, particularly considering that the MAA concentration in the diet was high (*c.* 0.1 μg total MAAs μg^{-1} Chl *a*). In most natural lakes, the concentration of MAAs in seston samples is much lower and most often undetectable (Laurion, Lami & Sommaruga, 2002; García *et al.*, 2008). We therefore suspect that the contribution of dietary MAAs to the total MAA pool of wild *B. antiqua* (and perhaps other copepods) is low, if not negligible. It is also worth noting that the addition of chloramphenicol to the culture medium impeded the accumulation of dietary MAAs, suggesting that the prokaryote microflora may not only be a source of these compounds but also play a role in the process of MAA assimilation.

This study, as well as our previous investigations of the source of MAAs in copepods, has been performed on freshwater, centropagid copepods. As far as we know, the prokaryotic origin of MAAs has not been investigated in other copepod groups. It may be that MAAs are consistently absent from other planktonic crustaceans, such as cladocerans, and it is certainly tempting to speculate whether this may be due to a failure of assimilation or to different microbial flora.

Acknowledgments

We thank Ruben Sommaruga for his critical reading of a previous version. This work was supported by ANPCyT (PICT-2007 02035 and 01205) and Universidad Nacional del Comahue (UNC 04/B001).

This study is dedicated to the memory of Dr. Robert E. Moeller.

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(Manuscript accepted 26 January 2012)