Depth-Related Alkaloid Variation in Mediterranean *Aplysina* Sponges

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Total amounts and patterns of bromoisoxazoline alkaloids of *Aplysina* sponges from Croatia (Mediterranean Sea) were analyzed along an underwater slope ranging from 1.8 to 38.5 m. Total amounts of alkaloids varied from sample to sample and showed no correlation with depth. In contrast, striking differences of alkaloid patterns were found between sponges from shallow sites (1.8–11.8 m) and those collected from deeper sites (11.8–38.5 m). Sponges from shallow depths consistently exhibited alkaloid patterns typical for *Aplysina aerophoba* with aerophobin-2 (**2**) and isofistularin-3 (**3**) as main constituents. Sponges from deeper sites (below 11.8 m) resembled *Aplysina cavernicola* with aerothionin (**4**) and aplysinamisin-1 (**1**) as major compounds. The typical *A. cavernicola* pigment 3,4-dihydroxyquinoline-2-carboxylic acid (**6**), however, could not be detected in *A. aerophoba* sponges but was replaced by the *A. aerophoba* pigment uranidine (**5**) which appeared to be present in all sponge samples analyzed. During transplantation experiments sponges from sites below 30 m featuring the *A. cavernicola* chemotype of bromoisoxazoline alkaloids were translocated to shallower habitats (10 m). The alkaloid patterns in transplanted sponges were found to be stable over a period of 12 months and unaffected by this change in depth. In a further experiment, clones of *Aplysina* sponges from shallow depths of 5–6 m resembling the *A. aerophoba* chemotype were either kept *in situ* under natural light conditions or artificially shaded by excluding photosynthetically active radiation (PAR). Neither **4** nor **1** were detected in artificially shaded sponges over an observation period of 12 months. In summary, two chemically distinct types of *Aplysina* sponges were discovered in this study that proved to be remarkably stable with regard to the bromoisoxazoline patterns and unaffected either by changing the light conditions or depth. It is not clear presently whether the *Aplysina* sponges collected from depths < 11.8 m represent a new chemotype of *A. cavernicola* lacking the pigment **6** or whether we have incidentally come across a so far undescribed species of the genus *Aplysina*.

Key words: Aplysina Sponges, Bromoisoxazoline Alkaloids, Depth Profile, Chemical Variation

Introduction

Sponges of the genus *Aplysina* are known for their structurally unique bromoisoxazoline alkaloids (Fig. 1) (Cimino *et al.*, 1983; Ciminiello *et al.*, 1997; Thoms *et al.*, 2003a) which act as potent chemical defense against predators and microorganisms (Teeyapant *et al.*, 1993; Weiss *et al.*, 1996; Kelly *et al.*, 2003; Encarnación-Dimayuga *et al.*, 2003; Thoms *et al.*, 2004). The Mediterranean Sea is home to two *Aplysina* species: *Aplysina aerophoba* which occurs in water depths as low as 1 m (Riedl, 1983; Pansini, 1997; Thoms *et al.*, 2003b), and *Aplysina cavernicola* which prefers shaded caves and deeper habitats (40 m or lower) (Wilkinson and Vacelet, 1979; Thoms *et al.*, 2003b). Both *Aplysina* species accumulate aerophobin-2 (**2**) and aplysinamisin-1 (**1**) as major alkaloids, with the latter compound not always being found in *A. aerophoba* (Teeyapant *et al.*, 1993; Ciminiello *et al.*, 1997), but differ with regard to the occurrence of aerothionin (**4**), isofistularin-3 (**3**) and their yellowish pigments. *A. aerophoba* typically contains isofistularin-3 (**3**) and lacks detectable amounts of aerothionin (**4**) (Cimino *et al.*, 1983; Ciminiello *et al.*, 1997). Its highly unstable yellow

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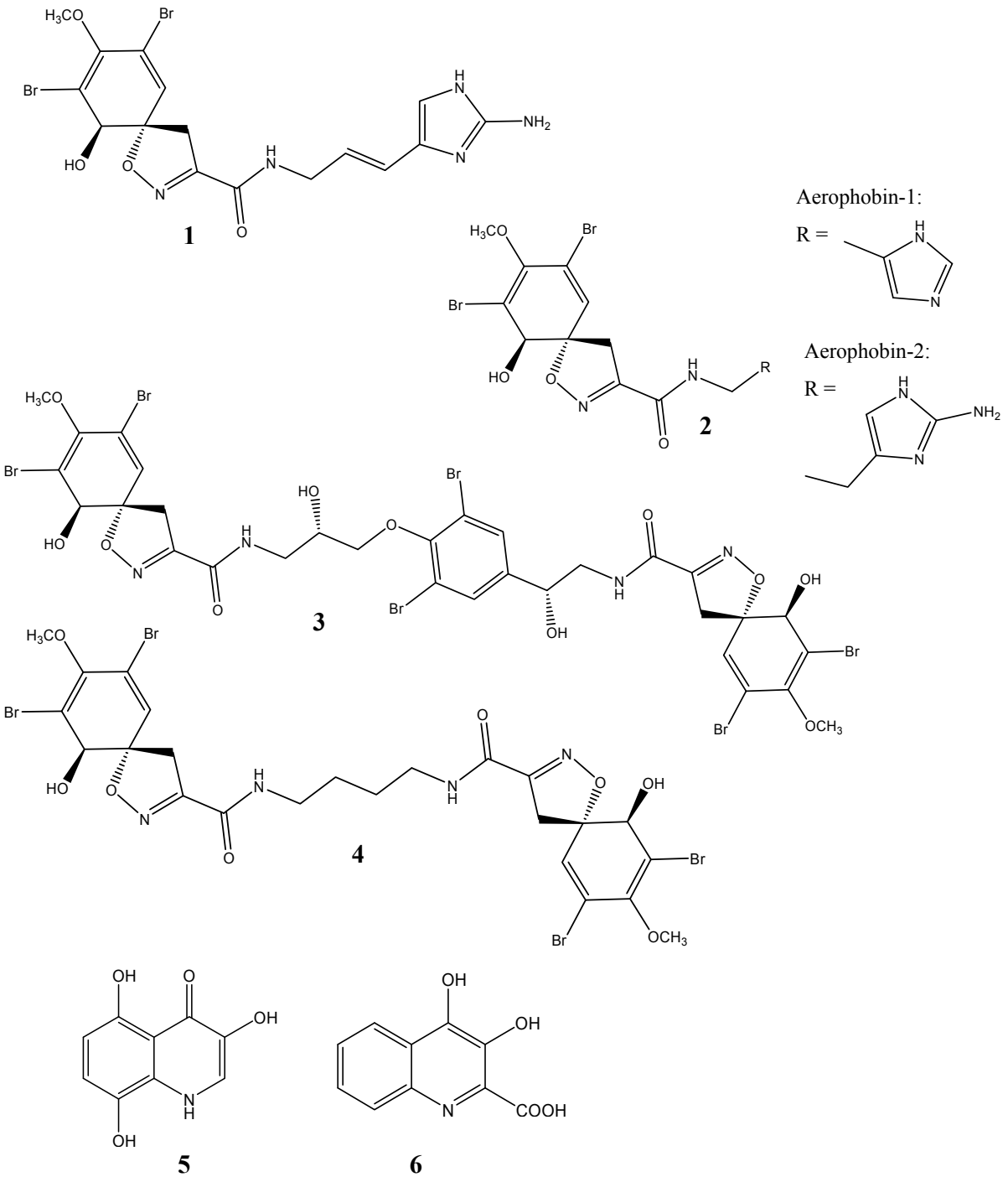

Fig. 1. Chemical structures of *Aplysina* metabolites: aplysinamisin-1 (**1**), aerophobin-1 and -2 (**2**), isofistularin-3 (**3**), aerothionin (**4**), uranidine (**5**), and 3,4-dihydroxyquinoline-2-carboxylic acid (**6**).

pigment uranidine (**5**) polymerizes rapidly when exposed to air yielding a black polymer (Cimino *et al.*, 1984). In contrast, *A. cavernicola* accumulates aerothionin (**4**) as a major isoxazoline alkaloid and, instead of uranidine (**5**), the chemically rather stable pigment 3,4-dihydroxyquinoline-2-carboxylic acid (**6**) (Thoms *et al.*, 2003a, 2004).

Like other sessile marine organisms sponges usually live along steep environmental gradients and hence face spatially variable selection. Aquatic littoral habitats such as depth gradients generate heterogeneity in several ways, involving, *e.g.*, light quality, temperature, sea surge, nutrient availability and changing risk of predation. However, evidence for the adaptation of secondary metabolite production of sponges to changing biotic and/or abiotic environmental factors is so far scarce (Thompson *et al.*, 1987; Page *et al.*, 2005; Abdo *et al.*, 2007) and further studies are clearly needed. We now report for the first time on depth-related variations in alkaloid patterns of Mediterranean *Aplysina* sponges.

Material and Methods

Collection and extraction of sponges

Aplysina sponges were sampled in April 2006 (22 individuals in total) and in August 2006 (13 individuals in total) along an underwater slope at Sveta marina (East coast of the Istrian Peninsula, Croatia) ranging from a depth of 1.8 to 38.5 m. Specimens of approximately the same size were collected in special Kautex sampling vials by self-contained underwater breathing apparatus (SCUBA) diving. In order to avoid damage of the sponge tissue, the substrate to which the sponges were attached was removed together with the sponges.

During transport in ambient seawater using cooling boxes, contact of samples with air was avoided. Approximately 5 cm^3 sponge tissue was cut off from each individual and frozen (–20 °C) immediately after transfer. Cutting of sponge tissue with a scalpel and immediate freezing do not affect the isoxazoline alkaloids content (Thoms *et al.*, 2006). The frozen sponge tissue was then lyophilized, ground with a mortar, and extracted exhaustively over night with methanol (50 ml per 100 mg sponge tissue). After centrifugation, 1.5 ml of the extract were transferred into 2-ml reaction tubes. Methanol was evaporated via vacuum centrifugation (Savant Speedvac SPD111V, Thermo Scientific, Langenselbold, Germany). The dried samples were redissolved in 450 μl HPLC methanol and directly submitted to HPLC analysis.

HPLC analysis of sponge alkaloids

Extracts were analyzed using a HPLC system coupled to a photodiode-array detector (Dionex, Germering, Germany; column prefilled with Eurosphere C-18, 5 μm, 125 × 4 mm i.d.; Knauer, Germany). Routine detection was at 254 and 280 nm. A solvent system consisting of 0.02% phosphoric acid at pH 2 (A) and methanol (B) at a gradient increasing linearly from 10% to 100% B within 25 min and a hold for 10 min at 100% B was used for compound separation. Identification of substances was based on their online ultraviolet (UV) spectra and on direct comparison with previously isolated standards. All compounds were quantified by HPLC using calibration curves obtained for the respective isolated substances.

Quantification of alkaloids

Since alkaloid contents were calculated on a weight (in μmol per g dry weight) and not on a volume basis, data do not directly take into account any skeletal density changes. However, measurement of the volume (recorded as replacement of water in ml) and dry weight (in g) of ten pieces of varying size cut from different *Aplysina* individuals revealed a linear correlation between volume and dry weight ($R^2 = 0.981$). These data show that differences in the skeletal density, if present, have a negligible impact on the dry weight of samples. The dry weight thus appears to be a suitable reference value to present the alkaloid contents in *Aplysina*.

Assessment of environmental parameters

All parameters were measured in the Adriatic Sea close to Sveta marina in the vicinity of Rovinj (Croatia) in July 2006. Temperature, conductivity, pH value and oxygen content were gauged by a multi-parameter probe (MPP 350; WTW GmbH, Weilheim, Germany) provided with a 25-m cable from the surface to a depth of 25 m in 1 m steps. Light (photosynthetically active radiation, PAR) was quantified, from the surface to a depth of 15 m in steps of 5 m, with a quantum sensor connected to a microvolt integrator (Delta-T Devices Ltd, Cambridge, England). For the wa-

ter tightness the testing probe was deposited in a waterproof box (Lexan Utility Box, GSI Outdoors, Spokane, USA). Measuring faults caused by the Plexiglas plate were eliminated by raising a factor (f = 1.3636) under a defined light source. Current velocities were gathered at depths of 5 and 15 m using tracer droplets, which were injected with a syringe in front of a black benchmark. Time was taken with a waterproof microchronometer.

Transplantation to different depths

Aplysina sponges involved in the transplantation experiment consisted of four individuals of similar size originating from depths of 31.3 to 38.5 m at the underwater slope in Sveta marina. Whole specimens were attached to a grid by using cable ties and transplanted to a depth of 10 m in the Limski Kanal (in the vicinity of Rovinj) in April 2006. Different cable tie colour codes allowed recognition of individual samples. Approximately 5 cm³ sponge tissue was cut off from each individual and directly frozen (to prevent the bioconversion of isoxazoline alkaloids) for HPLC analysis at the beginning of the experiment in April 2006 (t_0), after 4 months in August 2006 (t_1), and after 12 months in April 2007 (t_2). Crude extracts were prepared by lyophilizing sponge tissue and extraction with methanol as described above. Analysis of alkaloid patterns was achieved by HPLC.

Transplantation to different light conditions

All 18 *Aplysina* sponges involved in this experiment originated from depths of 5 to 6 m in the Limski Kanal (Rovinj) and were sampled in April 2006. To test for the influence of light on the alkaloid contents and patterns, the sponges were cut into two pieces. One fragment was allowed to grow *in situ* under natural illumination, the other one was artificially shaded by a basket covered with black plastic foil, thereby causing a complete erasement of the PAR. Sponges were attached to a grid by using cable ties and maintained at a depth of 6 m in the Limski Kanal starting in April 2006. Different cable tie colour codes allowed recognition of individual samples. Approximately 5 cm³ sponge tissue was cut off from each individual and directly frozen for HPLC analysis at the beginning of the experiment in April 2006 (t_0), after 4 months in August 2006 (t_1), and after 12 months in April 2007 (t_2). Crude extracts were prepared as described for the depth transplantation experiment.

Data analysis

For all data analyses, SPSS v. 13.0 for Windows was used. The Spearman rank correlation was employed in order to test for a correlation between secondary metabolite content and depth. Secondary metabolite contents of samples involved in the transplantation experiments were analyzed using a 2-way repeated measures analysis of variance (ANOVAR). If normal distribution and homogeneity of variances of the data analyzed could not be assumed, the Friedman test (for repeated measures) was employed. Means ± standard deviation are shown. In bar graphs error bars depict standard deviations.

Results and Discussion

Mediterranean *Aplysina* sponges were collected in spring/summer 2006 along a depth gradient ranging from 1.8 to 38.5 m in the Adriatic Sea close to Rovinj (Croatia). Changes in environmental parameters that were likewise assessed for different stations of the depth gradient include temperature (Fig. 2a) (decrease from 24 °C at the surface to 18 °C at a depth of 25 m) and salinity (Fig. 2b) (increase from 44.4 to 46.3‰ along the gradient) whereas pH value and amount of oxygen remained stable irrespective of changing depths (Fig. 2c and d). On the other hand, light decreased drastically from 100% at the surface to less than 20% at a depth of 15 m (Fig. 2e). The water current velocity was reduced from a speed of 1.6 cm/s at a depth of 5 m to 0.8 cm/s at 15 m depth (Fig. 2f).

The absolute amounts of bromoisoxazoline alkaloids varied greatly between different sponge samples and showed no correlation with depth (Fig. 3a, Spearman correlation; April 2006: N = 22, r_s = –0.333, P = 0.230; August 2006: N = 13, r_s = 0.429, P = 0.144). This is in accordance with earlier studies on *Aplysina* sponges that also had reported large variations of total alkaloid amounts between different sponge individuals sampled in the Adriatic Sea (Thoms *et al.*, 2006). The alkaloid profiles on the other hand proved to be far more consistent for individual sponges from similar depth regimes (Fig. 3b, c).

For sponges collected in April 2006, the alkaloid patterns were significantly correlated with depth [Fig. 3b, Spearman correlation, $N = 22$; aplysinamisin-1 (**1**): $r_s = 0.525$, $P = 0.012$; aerophobin-2 (**2**): $r_s = -0.620$, $P = 0.002$; isofistularin-3 (**3**): $r_s = -0.566$, $P = 0.006$; aerothionin (**4**): $r_s = 0.669$, $P = 0.001$]. Two distinctly different types of alkaloid patterns were detected: samples originating from less than

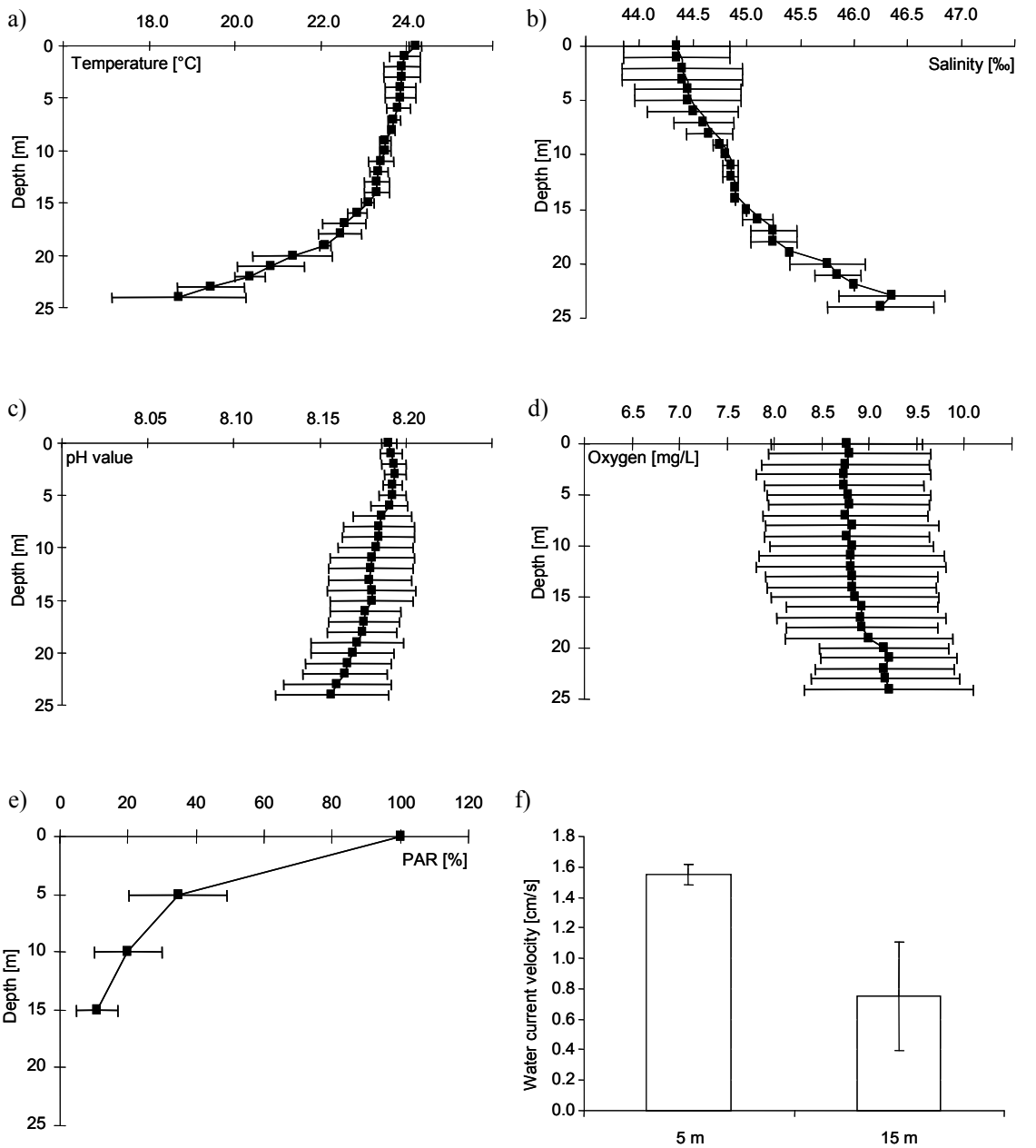

Fig. 2. Environmental parameters measured in the Adriatic Sea in depths of 0 to 25 m close to Rovinj, Croatia in July 2006: (a) temperature; (b) salinity; (c) pH value; (d) oxygen concentration; (e) light (photosynthetically active radiation, PAR) down to 15 m; (f) current velocity in 5 and 15 m depth. Error bars depict standard deviation.

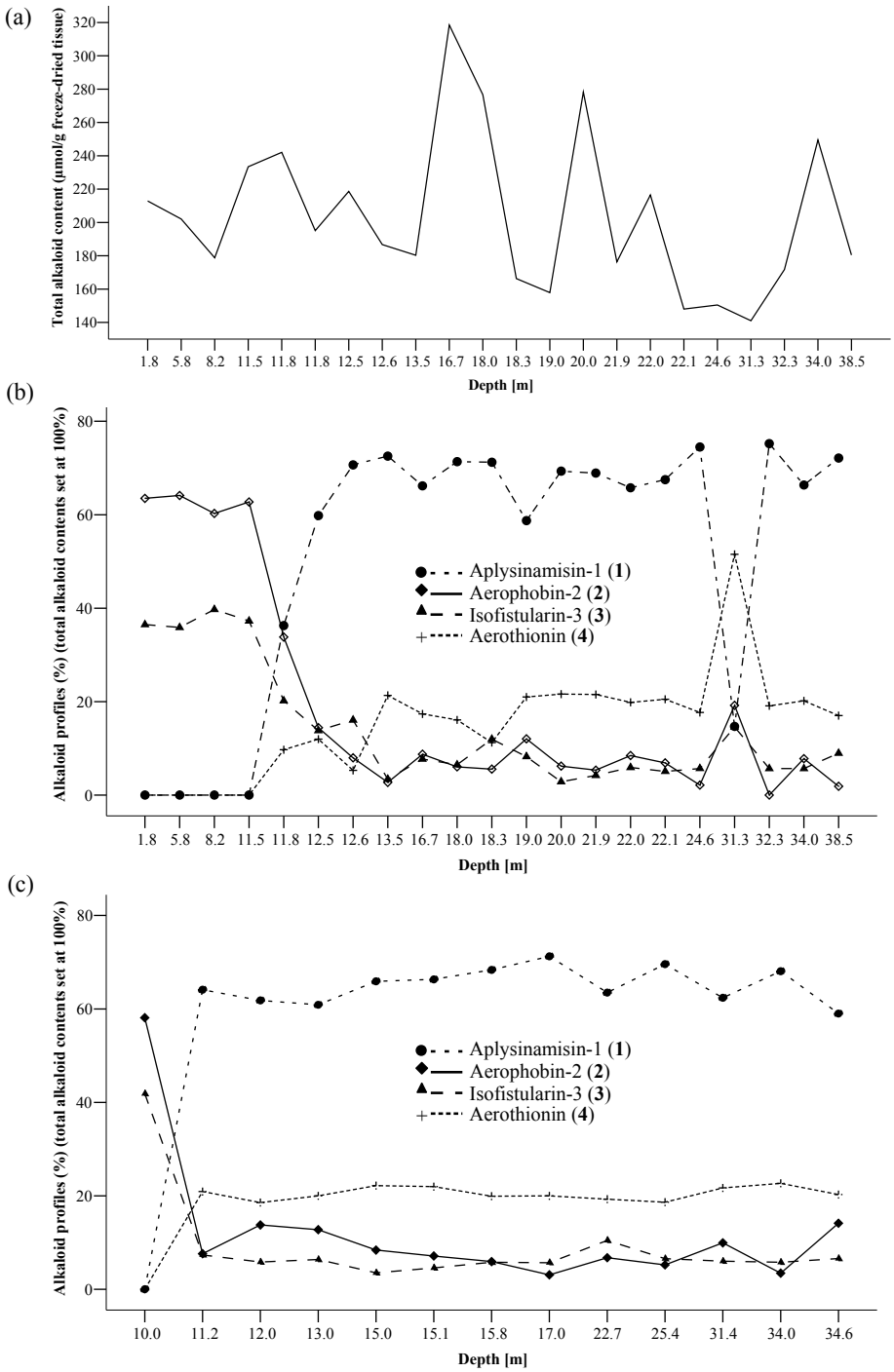

Fig. 3. (a) Total alkaloid content (in μmol/g freeze-dried sponge tissue) in *Aplysina* individuals sampled along a underwater slope ranging from 1.8 to 38.5 m. (b, c) Relative amounts (in %) of aplysinamisin-1 (**1**), aerophobin-2 (**2**), isofistularin-3 (**3**) and aerothionin (**4**) in sponges collected (b) in April 2006 (N = 22) or (c) in August 2006 (N = 13). Neither the *A. aerophoba* pigment uranidine (**5**) nor the *A. cavernicola* pigment 3,4-dihydroxyquinoline-2-carboxylic acid (**6**) were detectable.

11.8 m featured the *A. aerophoba* chemotype and were characterized by aerophobin-2 (**2**) and isofistularin-3 (**3**) as major constituents. In contrast, sponges collected at 11.8 m or below contained aplysinamisin-1 (**1**) and aerothionin (**4**) as dominant bromoisoxazoline alkaloids and thus resembled the *A. cavernicola* chemotype. Uranidine (**5**) which is characteristic for *A. aerophoba* could not be detected in any of the sponges analyzed due to its known chemical lability (Cimino *et al.*, 1984). The pigment 3,4-hydroxyquinoline-2-carboxylic acid (**6**) that is typical for *A. cavernicola* (Thoms *et al.*, 2003a, 2004) was likewise not observed although it is chemically far more stable than uranidine (**5**). Instead, all sponge samples in this study, irrespective of the depth at which they had been collected, developed a black or blue colouration when exposed to air which is typical for polymerization products of uranidine (**5**) (Cimino *et al.*, 1984). Thus, sponges from depths below 12 m resemble *A. cavernicola* with regard to bromoisoxazoline alkaloids but differ clearly from it with regard to their pigment. These latter specimens thus resemble neither *A. aerophoba* nor *A. cavernicola* but rather hold an intermediate position between the two species. Sponges collected in August 2006 showed a homogenous alkaloid pattern that resembled the alkaloid patterns detected in the first series of experiments (April 2006), even though for this series of samples no significant correlation between alkaloid patterns and depth was observed [Fig. 3c, Spearman correlation, $N = 13$; aplysinamisin-1 (**1**): $r_s = 0.286$, $P = 0.344$; aerophobin-2 (**2**): $r_s = -0.390$, $P = 0.188$; isofistularin-3 (**3**): $r_s = -0.126$, $P = 0.681$; aerothionin (**4**): $r_s = 0.335$, $P = 0.144$] due to the fact that only a single specimen originating from a depth <10 m had been included.

Aplysina samples originating from depths of 31.3 to 38.5 m and resembling *A. cavernicola* with regard to their alkaloid composition were selected for transplantation to shallower habitats (9–10 m). Alkaloid patterns remained stable and unaffected by transplantation during the whole period of observation (one year) (ANOVAR with time as the within-subject factor and compound [aerophobin-2 (**2**), aplysinamisin-1 (**1**), isofistularin-3 (**3**) and aerothionin (**4**)] as the between-subject factor: *d.f.* = 3, $F = 0.316$, $P = 0.813$). Our data are in agreement with an earlier study on *A. cavernicola* at the island of Elba (Italy) that had likewise reported no changes in the alkaloid composition of sponges transplanted from a depth of 40 m to a depth of 15 m over an observation period of 3 months (Thoms *et al.*, 2003b).

In a further experiment the effect of light on the alkaloid accumulation was analyzed. Sponges from a depth of 5–6 m were cut in half and the resulting clones were subjected to different light conditions: natural illumination and artificial shading. All clonal explants showed an alkaloid pattern characteristic for *A. aerophoba* at the onset of the experiment with neither aerothionin (**4**) nor aplysinamisin-1 (**1**) being detectable. During the course of the experiment (one year) the alkaloid patterns did not change significantly [Fig. 4, ANOVAR with time as the within-subject factor and treatment ("light" for natural illumination or "dark" for artificial shading) as the between-subject factor, *d.f.* = 1; aerophobin-1 (**2**): $F = 0.578$, $P = 0.476$; aerophobin-2 (**2**): $F = 0.025$, $P = 0.875$; isofistularin-3 (**3**): $F = 0.376$, $P = 0.562$]. From the 18 clones that had been recruited for the experiment in April 2006 (t_0), seven survived during the

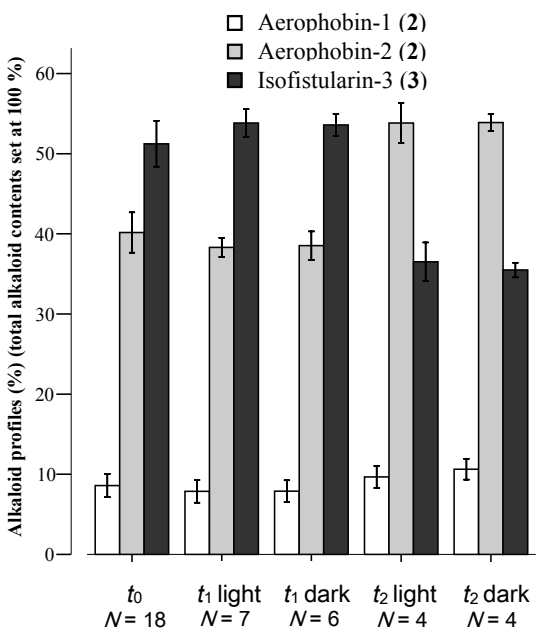

Fig. 4. Alkaloid patterns (relative amounts in %) of aerophobin-1 (**2**), aerophobin-2 (**2**), and isofistularin-3 (**3**) of clonal transplants maintained under natural light conditions or subjected to artificial shading before transplantation (t_0), after 4 months (t_1) and after 12 months following transplantation (t_2). Bars ± standard deviation are shown.

artificially shaded and six during the naturally illuminated treatment until August 2006 (t_1). This sample size further decreased until April 2007 (t_2) to four clones in each of the two treatments (Fig. 4). In t_0 and t_1 samples isofistularin-3 (**3**) represented the major alkaloid and dominated over aerophobin-2 (**2**) in both treatments. Sponges that were analyzed after one year (t_2) showed a reversed ratio with aerophobin-2 (**2**) as major alkaloid irrespective of the light regime chosen.

In summary, neither transplantation of sponges to different depth regimes nor maintaining sponges under differing light conditions affected the alkaloid patterns in a significant manner. On the contrary, alkaloid patterns proved to be remarkably stable and unaffected by changing the environmental conditions. Since chemical and physical environmental factors that are likely to have an influence on alkaloid composition such as light, temperature or salinity changed gradually rather than abruptly along the analyzed depth gradient (Fig. 2), an area of co-occurring *A. aerophoba* and *A. cavernicola*-like chemotypes would have been expected. Instead we found a clear boundary between the two well-defined chemotypes of sponges ("shallow" and "deep" specimens) at a depth of approximately 12 m which at present cannot be explained. Since *A. aerophoba* and *A. cavernicola* only differ by a series of phenotypically quite variable characters such as colour, shape and surface texture, sponge taxonomists have debated whether the two taxa should be considered as distinct species, or, due to their different ecological requirements, rather as ecotypes (Ciminiello *et al.*, 1997). Analysis of the secondary metabolites produced by these two *Aplysina* species seemed to offer further characteristics to cope with this question (Ciminiello *et al.*, 1997). However, since chemical characteristics distinguishing between both *Aplysina* species seem to merge at least with regard to the sponge pigment in our dataset, the question remains whether Mediterranean *Aplysina* sponges represent in fact only one species albeit with two different chemotypes. Future studies are required to systematically investigate the correlation between habitat, morphology and secondary metabolites of *Aplysina* sponges from different locations in order to eventually answer this question.

Acknowledgements

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