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Can light intensity cause shifts in natural product and bacterial profiles of the sponge *Aplysina aerophoba*?

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Abstract

The potential role of microbial consortia on sponge chemistry is well known. However, how environmental factors affect microbial and chemical profiles and how these shifts affect the sponge holobiont are far from being understood. This study experimentally investigated the effect of light on both the concentration of secondary metabolites and the bacterial assemblages of the sponge *Aplysina aerophoba*. We quantified major brominated alkaloids (BAs) using a high-performance liquid chromatography system coupled with a UV-detector and analysed sponge-associated bacteria using denaturing gradient gel electrophoresis of 16S rRNA gene amplicons. We identified distinct chemical and bacterial profiles between the ectosome and the choanosome of *A. aerophoba*. The abundance of most secondary metabolites increased regardless of the illumination regime. We found that the probability of occurrence of three microbial phylotypes (operational taxonomic units 84, 86 and 87) was strongly associated with increasing concentrations of three brominated compounds (aerophobin-1, aplysinamisin-1 and isofistularin-3). Although the role of these bacteria remains uninvestigated, these associations between natural products and specific microbial phylotypes outline further hypotheses that will improve our understanding of the organization and functioning of these complex host–symbiont interactions.

Introduction

Sponges are benthic invertebrates often characterized by prolific production of chemical defenses with ecological and biotechnological relevance (Paul *et al.* 2006; Erwin *et al.* 2010). Sponges have been found to harbor a diverse microbiota that may be involved in secondary metabolite biosynthesis (Unson *et al.* 1994; Flatt *et al.* 2005) and other aspects of host metabolism (Erwin & Thacker 2008; Ribes *et al.* 2012), because of this association these sessile invertebrates are referred with the term sponge holobiont. The role of these microbial symbionts has been well investigated in the last few years and has led to the development of sponge microbiology as a research discipline (Grozdanov & Hentschel 2007; Taylor *et al.* 2007; Egan *et al.* 2008; Hentschel *et al.* 2012).

The huge number of sponge natural products discovered (Erwin *et al.* 2010; Blunt *et al.* 2012), and the remarkable diversity of microbial taxa reported in sponges (Lee *et al.* 2011; Schmitt *et al.* 2012) have presented an excellent opportunity to study the relationship between secondary metabolites and sponge-associated microbes and to understand the function of microbial consortia in sponge hosts. To date, sponge chemical ecology research has mainly focused on chemical defenses against predators (Pawlik *et al.* 2002, 2013). Studies of secondary metabolites with activities against competitors or fouling came later (Paul *et al.* 2011). The mystery of the origin of these compounds led to studies that concentrated their attention on sponge-associated microbial diversity as a source of potential bioactive compounds (Piel 2004; Moore 2006). Although sponge–cyanobacteria

symbioses are quite well studied (Thacker 2005; Erwin & Thacker 2007, 2008), for most microbial symbionts their persistence in the host and their function are completely unknown. Understanding the processes that control the natural variation of chemical and bacterial diversity in sponges may help shed light on the roles of their symbionts. The current poor comprehension of these processes is hindering the development of marine chemical ecology, particularly in sponges (Paul *et al.* 2006).

Considerable effort has been undertaken to elucidate the potential biotic and abiotic factors that cause variation in the concentration of natural products in sponges. Among the biotic factors, changes in secondary chemistry have been observed due to sponge size, competition for space, predation and fouling (Becerro *et al.* 1995; Turon *et al.* 1996; Duckworth & Battershill 2001). Among the abiotic factors, depth, illumination, hydrodynamism and water temperature appear to affect compound abundances (Thompson *et al.* 1987; Becerro *et al.* 1995; Page *et al.* 2005; Abdo *et al.* 2007; Ferretti *et al.* 2009). Intraspecific variation (*i.e.* intra-individual, geographic, temporal variability) must also be considered in order to distinguish between natural variation and external modulation of the production of secondary metabolites (Kreuter *et al.* 1992; Turon *et al.* 1996; Duckworth & Battershill 2001; Page *et al.* 2005; Abdo *et al.* 2007; Ferretti *et al.* 2009; Freeman & Gleason 2010; Noyer *et al.* 2011; Sacristán-Soriano *et al.* 2011a,b, 2012; Sacristán-Soriano 2013).

The variability of sponge-associated microbes has received comparable research efforts. To date, the majority of studies has been restricted to evaluating intra-specific variation [*i.e.* within and between specimens of the same host (Althoff *et al.* 1998; Taylor *et al.* 2004, 2005; Thiel *et al.* 2007b) over a number of spatial and temporal scales (Friedrich *et al.* 2001; Webster & Hill 2001; Webster *et al.* 2004; Hoffmann *et al.* 2006; Erwin *et al.* 2012b)] and inter-specific variation [*i.e.* between host species (Hentschel *et al.* 2002; Taylor *et al.* 2004; Usher *et al.* 2004; Noyer *et al.* 2010)]. However, microbial ecology is a step behind chemical ecology in sponges. Little is known so far about the susceptibility of this symbiotic microbial consortium to biotic (*e.g.* disease) and abiotic (*e.g.* temperature) factors (Lemoine *et al.* 2007; Webster *et al.* 2008a,b; Erwin *et al.* 2012b; Simister *et al.* 2012).

In the present study, we tested whether light can cause fluctuations in natural products and symbionts in the sponge *Aplysina aerophoba* Nardo, 1833. Are chemical and microbial profiles affected by different levels of light exposure? Are changes in secondary metabolites and associated bacteria correlated? Studies that explore links between bacteria and natural products are scarce (Sacristán-Soriano *et al.* 2011b; Sacristán-Soriano 2013) and would benefit tremendously our understanding of the links between sponge-associated bacteria and the production of natural products. The sponge *A. aerophoba* has both well-known secondary chemistry and microbial diversity that have received considerable attention, making it a suitable organism with which to explore host/symbiont relationships. We used high-performance liquid chromatography (HPLC) and denaturing gradient gel electrophoresis (DGGE) to investigate the abundance of major secondary metabolites and the complex bacterial consortium found in external and internal regions of *A. aerophoba*.

Material and Methods

Sample collection and experimental set-up

In spring 2006, we collected 18 specimens of the sponge *Aplysina aerophoba* Nardo, 1833 by scuba diving between 3 and 10 m deep at Portbou (Northwestern Mediterranean) to set up an experiment to investigate the role of light in the sponge-associated bacterial community and secondary metabolite production. Specimens were carefully removed from the natural substrate to minimize damage, placed in sealed bags underwater and, once at the surface, immediately transferred to two 60-l barrels. Sponges were then transported (2–3 h) to the Marine and Environmental Research Mediterranean Centre in Barcelona (CMIMA, CSIC). The experiment was carried out in two 125-l tanks with running seawater at a flow rate of 1.74×10^{-5} $m^3 \cdot s^{-1}$ and a pumping pipe at mid-depth. We put nine specimens in each tank and created a light gradient by combining two floodlights and a black mesh (Fig. 1). We measured light intensity in the field during a sunny spring day with *in situ* data loggers (model HOBO Pendant UA-002-08; Onset Computer

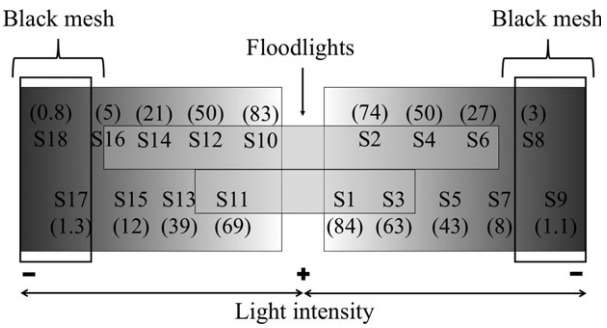

Fig. 1. Experimental design. We put 18 specimens of *Aplysina aerophoba* into two tanks with running seawater (S1–S18). We created a light gradient with high intensities in bright zones (+) and low intensities in dark zones (−). We show light measurements, expressed in microeinsteins ($\mu E \cdot m^{-2} \cdot s^{-1}$), of each individual in parentheses (see Material and Methods for details).

Corporation, Bourne, MA, USA) placed from 14:00 to 15:00 h at 0, 1 and 3 m depths in Blanes (NW Mediterranean, around 100 km from Portbou) to get a rough idea of the maximum light conditions to which sponges could be exposed. Experimental light conditions mimicked the maximum light intensity encountered by sponges in our study area at a depth of 1 m to total darkness, but following a natural light cycle (see Fig. 1 for details of actual light data).

Sponges were maintained *ex situ* under *in situ* conditions with the exception of light availability. Specimens were acclimated to water tanks with running seawater for 1 week before the beginning of the experiment. The experiment lasted 4 months (April–July 2006) and we sampled specimens at the beginning and at the end of the experiment. All sponge sampling and manipulation occurred underwater to prevent the degradation of compounds that this sponge experiences when in contact with air. After the initial sampling, sponges were covered with the black mesh to create the experimental light gradient and remained untouched until the end of the experiment. Samples consisted of small pieces (<1 ml in volume) from the ectosome and choanosome of each sponge. We used an EtOH-sterilized scalpel to prevent bacterial contamination of our samples. Samples were immediately frozen with liquid nitrogen and stored at −80 °C until processed. These samples were used to characterize the chemical and bacterial profiles of *A. aerophoba* as described below.

HPLC analysis and compound quantification

The major BAs of *Aplysina aerophoba* were quantified following the methodology described in Sacristán-Soriano *et al.* (2011a,b, 2012). With this methodology, we have never detected the biotransformation process that converts the high molecular weight BAs into low molecular weight (LMW) BAs described for this species (Ebel *et al.* 1997). To avoid changes in the chemical profiles due to manipulation, we minimized the time of manipulation, avoided air exposure of fresh samples, froze sponges immediately after sampling, freeze-dried material and kept it at −20 °C. With our methods, LMW BAs were either absent from our chromatograms or at concentrations below our detection threshold.

High-performance liquid chromatography analyses were performed as described in Sacristán-Soriano *et al.* (2011a,b, 2012). The four major compounds (aerophobin-1, aerophobin-2, aplysinamisin-1 and isofistularin-3) observed in the HPLC chromatograms had previously been isolated and characterized by classic spectrometric techniques (^{1}H and ^{13}C nuclear magnetic resonance; liquid chromatography-mass spectrometry; UV-spectroscopy) and comparison of spectroscopy data with published values from the literature (Cimino *et al.* 1983; Ciminiello *et al.* 1997; Lira *et al.* 2011). Purified compounds were used for the quantification process. Full details on the chemical methods can be found in Sacristán-Soriano *et al.* (2011b).

For quantification of the natural products, approximately 30 mg of freeze-dried sponge tissue from ectosomal and choanosomal samples was extracted with methanol (MeOH) following the methodology described in Sacristán-Soriano *et al.* (2011a,b; 2012). After the injection of the crude extract into the HPLC system, BAs were detected at 245 nm from data collected across the 210–800 nm wavelength range. Peak areas were integrated and quantified with calibration curves based on the four major compounds purified that were used as external standards (Sacristán-Soriano *et al.* 2011b). The final amounts of the natural compounds were calculated by averaging three replicate injections. We corrected compound abundances to account for the exact mass of sponge tissue extracted. Concentrations of BAs were expressed in $mg \cdot g^{-1}$ of dry mass of sponge tissue.

DNA extraction and PCR amplification

We took a small piece of each frozen sample and preserved them with ethanol (EtOH; 100% final concentration) until they were processed. DNA was extracted from approximately 2 mg of EtOH-preserved wet mass sponge tissue using a DNeasy Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and as described in Sacristán-Soriano *et al.* (2011b). The effectiveness of the method has been tested for environmental samples (Simonelli *et al.* 2009). DNA extracts were run in an agarose gel to check integrity and concentration using a standard mass ladder (DNA Smart Ladder; Eurogentec, Seraing, Belgium). Small differences in yield extraction were visualized among samples. However, such differences were not expected to produce qualitative changes in the DNA mixtures.

PCR amplification of a fragment of the bacterial 16S rRNA gene suitable for subsequent genetic fingerprinting analysis was carried out using the universal bacterial primer combination of BAC358F with a GC-clamp (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′) and BAC907RM, which amplify a fragment approximately 568 bp long as described elsewhere (Casamayor *et al.* 2000; Sacristán-Soriano *et al.* 2011b). The cycling conditions were as follows: one initial denaturing step for 5 min at 94 °C; 10 touchdown cycles of 1 min at 94 °C, 1 min at 70 °C (with a 1 °C decrease every cycle) and 3 min at 72 °C; 20 cycles of 1 min at 94 °C, 1 min at 60 °C and 3 min at 72 °C; and one final

elongation step for 5 min at 72 °C. The PCR mix consisted of 34 μl sterilized MilliQ water, 5 μl 10× reaction buffer, 1.5 μl $MgCl_2$ (50 mM), 1 μl deoxyribonucleoside triphosphates (10 mM each), 2.5 μl of each primer (10 μM), 2.25 μl bovine serum albumin (6 $mg \cdot ml^{-1}$), 0.25 μl EcoTaq polymerase (5 $U \cdot \mu l^{-1}$; ECOGEN, Barcelona, Spain) and 1 μl DNA template. The amounts of DNA template ranged between 10 and 100 ng of DNA for the different samples. We did not observe any remarkably loss/gain of DGGE bands in the fingerprinting analysis within this range of DNA concentrations. PCR products were run in an agarose gel using a standard mass ladder (DNA Smart Ladder; Eurogentec) to quantify the PCR product obtained in each case (Casamayor *et al.* 2000; Demergasso *et al.* 2008).

DGGE analysis of PCR products and sequencing

Denaturing gradient gel electrophoresis (DGGE) was performed as described in Sacristán-Soriano *et al.* (2011b). A comparable amplicon mass for each sample (*c.* 600 ng of PCR product) was added to the DGGE, and gels were run for 4 h at a constant voltage of 200 V and at 60 °C in a 40–70% vertical denaturant gradient (100% denaturant agent is 7 M urea and 40% deionized formamide; Casamayor *et al.* 2000; Sacristán-Soriano *et al.* 2011b). Gel image files were processed with NIH IMAGE software (National Institutes of Health, Bethesda, MD, USA) and the presence/absence data were recorded. Absolute quantitative data on the abundance of specific bacteria require the use of other techniques (Casamayor *et al.* 2000, and references therein). Although we did not expect differences in presence/absence data between DGGE gels, we avoided comparisons between gels by loading samples for direct comparison on a single gel.

Prominent bands were excised from the gel with a sterilized scalpel, re-suspended in 25 μl MilliQ water, and stored at 4 °C overnight. An aliquot (2–5 μl) of the supernatant was used for PCR re-amplification with the original primer set, and the PCR product was sequenced using external sequencing facilities (Macrogen, Seoul, Korea). Sequences were aligned and edited with SEAVIEW 4 software (Gouy *et al.* 2010). Sequences were then sent for BLAST search (March 2013; http://www.ncbi.nlm.nih.gov/BLAST/) to get an indication of what sequences were retrieved. Sequences with >97% sequence identity to a cultured nearest phylogenetic neighbor in the GenBank database were named at the species level. A band (operational taxonomic unit, OTU) was defined as a stained signal whose intensity was >0.2% of the total intensity for each lane. We used the Ribosomal Database Project II (Cole *et al.* 2007) sequence classifier to assess taxonomic affiliations.

Phylogenetic analysis of associated bacteria

Phylogenetic analysis of 16S rRNA gene sequences was conducted to determine the affiliations between the sequences recovered from *Aplysina aerophoba* herein and those previously reported from this species (Hentschel *et al.* 2002; Pabel *et al.* 2003; Erwin & Thacker 2007, 2008; Bayer *et al.* 2008; Webster *et al.* 2008b; Ahn *et al.* 2009; Abdelmohsen *et al.* 2010; Off *et al.* 2010; Pimentel-Elardo *et al.* 2010; Sacristán-Soriano *et al.* 2011b), which were retrieved from GenBank. Sequences previously published from *A. aerophoba* (n = 154), best hits from BLAST searches (n = 13) and 16S rRNA-DGGE band sequences (n = 27) from this study were aligned using MAFFT (Katoh *et al.* 2002) with two outgroup sequences from Archaea (*Haloarcula vallismortis* and uncultured Crenarchaeota, GenBank accession nos D50851 and EF529650, respectively). Maximum likelihood (ML) phylogenetic trees were constructed in RAxML (Stamatakis *et al.* 2008) using the general time reversible model with a gamma distribution of variable substitution rates among sites (GTR + G). Data were re-sampled using 100 bootstrap replicates, and a thorough ML search was conducted to optimize the topology and retrieve the best-scoring tree. Due to the variable length of the 16S rRNA gene sequences being compared, a binary backbone constraint tree was constructed from long (>1000 bp) sequences and used to restrict topology changes when introducing short (<1000 bp) sequences into the phylogeny (Erwin *et al.* 2012a).

Data analysis

We used multivariate statistical methods available in the PRIMER 6 software (Clarke & Warwick 2001) to analyse the data on secondary metabolites and bacteria of *Aplysina aerophoba* as a function of tissue layer (ectosome and choanosome). Untransformed data were used to calculate Bray–Curtis similarity and permutational analysis of multivariate dispersion (PERMDISP) was used to check for homogeneity of variances between groups. Permutational multivariate analysis of variance (PERMANOVA) was then used to test for differences in secondary metabolite and bacterial data across tissue layers. Taking into account the PCR bias and the limitations of DGGE as a quantitative technique (Muyzer & Smalla 1998), we used presence/absence bacterial data as a conservative approach to test for qualitative changes in bacteria. Individual ANOVAs were used to analyse each compound and each OTU across tissue type. We used square-root or rank transformation when raw data failed to meet parametric assumptions.

We carried out repeated measures analyses of co-variance (ANCOVAs) in the SYSTAT 12 software (SPSS

1999a,b) to analyse separately each secondary metabolite and each OTU from both tissue layers. We used ANCOVAs on the chemical and bacterial data to test for the effect of light over the course of the experiment (with time as a factor and light as a co-variate). We used square-root or rank transformation when raw data failed to meet parametric assumptions. We also used presence/absence OTU data to test for qualitative changes in bacteria.

Owing to the large number of variables (*i.e.* secondary metabolites and OTUs) analysed in the present study, we used factor analysis to establish groups (*i.e.* factors) of compounds and OTUs that were correlated with one another within groups but largely independent among groups (Tabachnick & Fidell 2001; Sacristán-Soriano *et al.* 2011b). Specifically, we used a principal component analysis extraction with a minimum eigenvalue of 1 to estimate the number of factors. To facilitate interpretation, we used varimax rotation as it minimizes the number of variables that load highly on a factor and maximizes the loading variance across factors. The independent factors that we obtained (*i.e.* scores of factors) were used as variables in a canonical correlation analysis to test for any correlation between the chemical and bacterial factors. We then used logistic regression (1) to uncover the relationship between the secondary metabolites and OTUs within the correlated factors. We calculated the probability of occurrence of an OTU plotting the natural logarithm (ln) of the ratio between the probability of presence (p) and the probability of absence (1−p) as a function of compound abundance (X), where b is the regression slope and a is the y-axis intercept:

$$\ln\left(\frac{p}{1-p}\right) = a + bX \quad (1)$$

Each logistic model was then assessed by McFadden's R^2 and P-value. The odds ratio (e^b) provided a more intuitively meaningful quantity for the regression coefficient (b). This parameter is the multiplicative factor by which the probability of occurrence of an OTU changes when the compound concentration increases by one unit.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences were deposited in GenBank under accession nos KC143239–KC143286.

Results

Natural product variation

We analysed a total of 72 samples (18 individual sponges × two tissue layers × two sampling times) to characterize the chemical profile of the sponge *Aplysina aerophoba*. We quantified the four major BAs of this species, previously described in several studies (Cimino *et al.* 1983; Ciminiello *et al.* 1997; Ebel *et al.* 1997; Sacristán-Soriano *et al.* 2011b): aerophobin-1 (Aero1), aerophobin-2 (Aero2), aplysinamisin-1 (Aply1) and isofistularin-3 (Iso3).

To compare the abundance of BAs between the ectosome and the choanosome, 36 samples from the 18 specimens were analysed at the beginning of the experiment. We found that the two tissue layers significantly differed in their BA content (one-way PERMANOVA, $P = 0.003$). The choanosome was significantly enriched in Aero2, Aply1 and Iso3 (one-way ANOVA, $P < 0.001$, $P = 0.035$, $P = 0.003$, respectively), while Aero1 presented a uniform distribution between the ectosomal and choanosomal layers (one-way ANOVA, $P = 0.051$). We therefore treated the two sponge tissue layers separately in the analysis of the effect of light over time.

When we compared the 18 specimens at the beginning and at the end of the experiment, we found that most of the compounds significantly increased their abundances in both tissue layers after 4 months. In the ectosome, the concentration of Aero1, Aero2 and Iso3 was greater after the experiment (ANCOVA, $P = 0.006$, $P = 0.007$, $P = 0.044$, respectively; Fig. 2), while the abundance of Aply1 remained unchanged (ANCOVA, $P = 0.234$). In the choanosome, however, the abundance of Aero1 and Aply1 significantly increased over time (ANCOVA, $P = 0.003$, $P = 0.037$, respectively; Fig. 2), while the amounts of Aero2 and Iso3 remained statistically invariable (ANCOVA, $P = 0.308$, $P = 0.070$, respectively). We failed to detect a direct effect of light on BA abundances in either the ectosomal or the choanosomal layers (ANCOVA, $P > 0.05$ in all cases). Only the concentration of Aero1 in the outer part of the sponge (*i.e.* the ectosome) was significantly affected by light (ANCOVA, $P = 0.027$; Fig. 2). We also failed to detect any effect of different light exposures upon the concentrations of compounds over the course of the experiment, as shown by the nonsignificant interaction between light and time (ANCOVA, $P > 0.05$ in all cases).

Bacterial community variation

We analysed different specimens depending on what we wanted to examine for direct comparison on a single DGGE gel. To compare the presence of bacteria between the ectosome and the choanosome, we analysed 18 samples from nine specimens randomly chosen at the beginning of the experiment. We identified a total of 18 different band positions, and we assigned each position to an OTU (Fig. 3). We found highly significant differ-

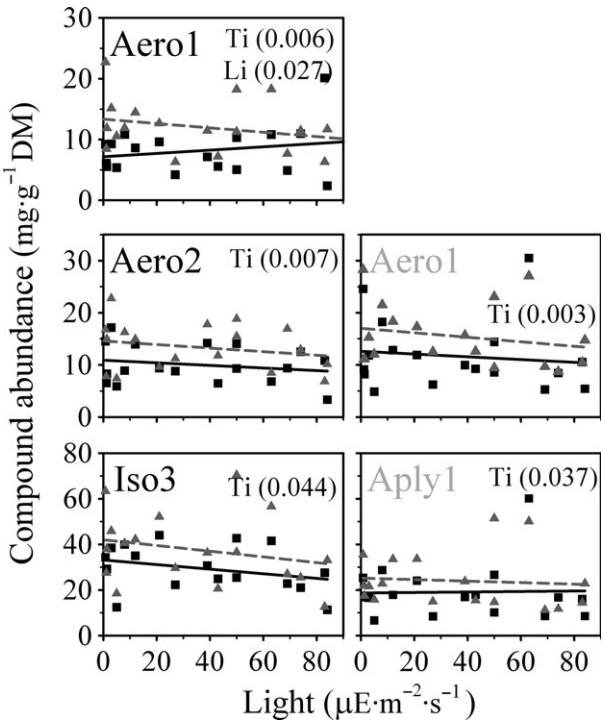

Fig. 2. Repeated measures analysis of co-variance on secondary metabolite concentrations [mg·g^{-1} (dry mass) of sponge tissue ±1 SE]. Black squares represent measures at the beginning of the experiment and dark gray triangles represent measures at the end. Compounds in black lettering symbolize the ectosome and compounds in gray lettering symbolize the choanosome. Significant factors are shown with P-values in parentheses ($P \leq 0.05$). Ti, time; Li, light; Aero1, aerophobin-1; Aero2, aerophobin-2; Aply1, aplysinamisin-1; Iso3, isofistularin-3.

ences in the bacterial assemblage between the ectosome and choanosome of the sponge (one-way PERMANOVA, $P = 0.001$). From those 18 different OTUs, nine were responsible for the differences found between the two parts of the sponge. OTUs 3, 15 and 17 were restricted to the ectosome, while OTUs 1, 4, 5, 6, 16 and 18 were exclusively found in the choanosome. The remaining nine OTUs were either distributed over both tissue layers (OTUs 7, 8, 9 and 10) or were uncommon and restricted to a particular sample (OTUs 2, 11, 12, 13 and 14).

To assess the effect of light over time, we analysed nine specimens at the beginning and at the end of the experiment, treating the two tissue layers separately (*i.e.* a total of 18 samples in each gel; Fig. 3). In the ectosome, we identified a total of 16 OTUs (OTUs 59–74; Fig. 3). Although almost all of the OTUs were not affected by light (ANCOVA, $P > 0.05$ in all cases), we detected a significant effect of light in one of them, OTU 62 (ANCOVA, $P = 0.035$). In the choanosome, however, we identified 15 OTUs (OTUs 75–89; Fig. 3), most of which

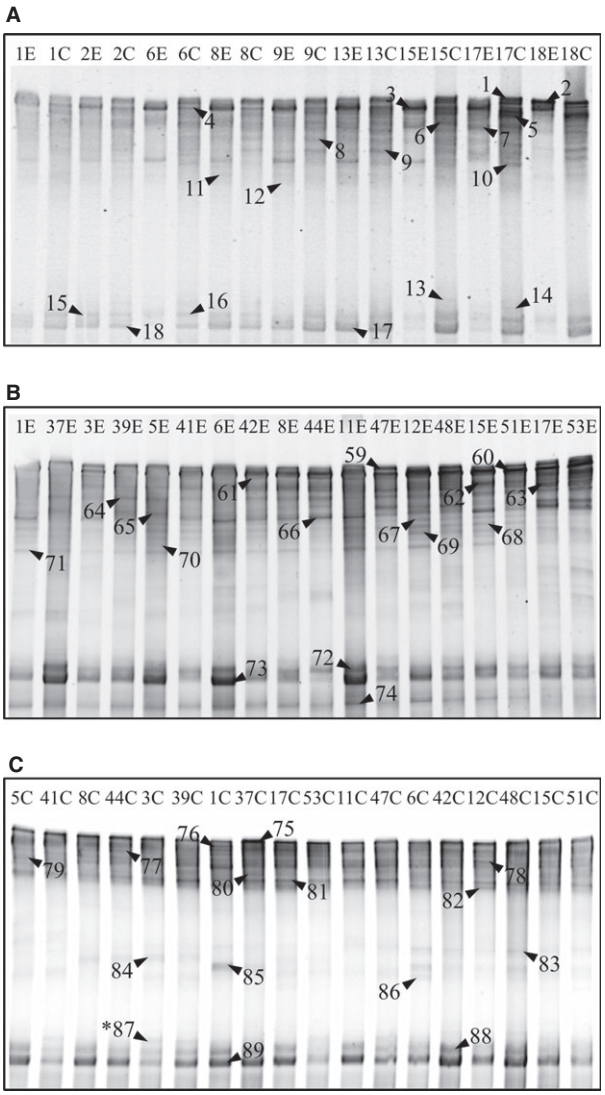

Fig. 3. 16S rDNA-denaturing gradient gel electrophoresis gels of *Aplysina aerophoba* samples. (A): We compared the bacterial community between the ectosome (E) and the choanosome (C) of nine specimens (1, 2, 6, 8, 9, 13, 15, 17 and 18) at the beginning of the experiment. We tested the effect of light over time in (B) the ectosome and in (C) the choanosome. We compared the measurements at the beginning of the experiment (1, 3, 5, 6, 8, 11, 12, 15 and 17) and at the end (37, 39, 41, 42, 44, 47, 48, 51 and 53) of nine individuals covering the whole light gradient. Numbers (1–18; 59–89) show the operational taxonomic units (OTUs) that were identified and sequenced. The asterisk (*) shows an unclassified OTU.

showed non-significant differences over time (ANCOVA, $P > 0.05$ in all cases). However, we found that OTU 77 varied after 4 months of the experiment (ANCOVA, $P = 0.039$). We also found a significant effect of light in the same OTU (77) (ANCOVA interaction time × light, $P = 0.017$).

Relationship between natural products and bacterial community

We used factor analysis to reduce the number of individual compounds and bacterial strains to a few consistent groups of compounds and OTUs that were highly correlated with one another within groups and independent among groups. The two tissue layers were analysed separately. In the ectosome, factor analysis resulted in one chemical (CF) and four bacterial factors (BFs) that explained 73.98% and 70.46% of the total variance, respectively (Tables 1 and 2). In the choanosome, however, factor analysis resulted in two CFs and four BFs that explained 98.80% and 81.74% of the total variance, respectively (Tables 1 and 2). Canonical correlation analysis (CCA) on the factor analysis scores resulted in two significant correlations in the choanosome, between CF2 and BF5, and BF6 (Table 3). Logistic regression analyses between the compounds and bacterial types present in the correlated factors resulted in seven significant correlations in the choanosome (out of 18 possible correlations; Fig. 4), which is an unlikely event to be explained by chance (binomial distribution, $P = 1.41E^{-5}$). OTUs 84, 86 and 87 were positively related to compounds Aero1, Aply1 and Iso3 (Fig. 4). Odds ratios ranged from 1.074 to 1.384 (Fig. 4); *i.e.* the probability of occurrence of these particular OTUs was from 1.074 to 1.384 times as likely as the compound abundance was increased one unit.

Table 1. Chemical factors (CF) obtained from the factor analysis of chemical data from the ectosome and choanosome of *Aplysina aerophoba*[a].

compound	loading value obtained with CF CF1	
ectosome		
Iso3	0.99	
Aero1	0.98	
Aply1	0.88	
Aero2	0.50	
	CF2	CF3
choanosome		
Aero1	0.99	–
Iso3	0.99	–
Aply1	0.98	–
Aero2	–	0.99

Aero1 = aerophobin-1; Aero2 = aerophobin-2; Aply1 = aplysinamisin-1; Iso3 = isofistularin-3.

[a]To facilitate interpretation, we only show compound loadings that highly load on a factor. The closer the absolute loading values to 1.0, the stronger the association between the variables (compounds) and the factors.

Phylogenetic analysis of excised 16S rRNA-DGGE bands

We excised from the gels and sequenced eighty-two 16S ribosomal RNA-DGGE bands that belonged to 48 different OTUs (Fig. 3). This meant an average of almost two sequenced bands per OTU with identical sequence, although we selected only one sequence representing each OTU (Table 4). Comparison of the 16S rRNA gene sequences with the GenBank database showed a large range of bacterial taxa present in *Aplysina aerophoba*. After assessing taxonomic affiliations, we discarded the sequences with poor taxonomic resolution and low identity percentages in the BLAST search (Table 4) from the subsequent analyses and interpretation of results. The phylogenetic analysis revealed that most of the sequences recovered from *A. aerophoba* in this study were closely related to other sequences previously reported from the same species (Fig. 5). OTUs 62, 66, 73 and 82, however,

Table 2. Bacterial factors (BFs) obtained from the factor analysis of bacterial data from the ectosome and choanosome of *Aplysina aerophoba*[a].

OTU	loading value obtained with BF BF1	BF2	BF3	BF4
ectosome				
69	−0.85	–	–	–
61	0.80	–	–	–
70	−0.62	–	–	–
67	–	0.82	–	–
63	–	0.73	–	–
64	–	0.67	–	–
66	–	–	−0.77	–
60	–	–	−0.77	–
59	–	–	0.70	–
65	–	–	–	−0.76
62	–	–	–	0.76
	BF5	BF6	BF7	BF8
choanosome				
83	−0.91	–	–	–
85	−0.89	–	–	–
84	0.68	–	–	–
87	0.63	–	–	–
82	−0.61	–	–	–
86	–	0.91	–	–
80	–	–	0.93	–
77	–	–	−0.70	–
78	–	–	–	0.98

OTU = operational taxonomic unit.

[a]To facilitate interpretation, we only show OTU loadings that highly load on a factor. The closer the absolute loading values to 1.0, the stronger the association between the variables (OTUs) and the factors. The sign of the values represents the positive or negative nature of their association.

Table 3. Canonical correlation analyses between chemical (CFs) and bacterial factors (BFs) from the ectosome and choanosome of *Aplysina aerophoba*.

	R (P)[a]		R (P)[a]	
ectosome	CF1	choanosome	CF2	CF3
BF1	n.s.	BF5	0.445 (0.037)	n.s.
BF2	n.s.	BF6	0.542 (0.014)	n.s.
BF3	n.s.	BF7	n.s.	n.s.
BF4	n.s.	BF8	n.s.	n.s.

n.s. = non-significant correlations.
[a]The correlation coefficient (R) and P-value of significant correlations ($P \leq 0.05$) are given.

were related to sequences derived from a non-sponge source (Fig. 5). Phylogenetic analysis was also used to qualitatively compare the bacterial community recovered from the two tissue layers. The ectosome included representatives of Cyanobacteria (n = 12) as an exclusive group, representatives of Alphaproteobacteria (n = 5), Deltaproteobacteria (n = 1) and Gammaproteobacteria (n = 1), and one sequence affiliated to Actinobacteria (n = 1). In the choanosome, however, sequences recovered belonged only to Gammaproteobacteria (n = 3), Alphaproteobacteria (n = 2) and Deltaproteobacteria (n = 2).

Discussion

Natural products: intra-individual and *ex situ* variability

A preliminary comparison of external and internal tissue layers of *Aplysina aerophoba* showed an enriched core in BAs, which have been reported to possess a markedly deterrent activity (Thoms *et al.* 2004). These intra-specimen differences support those reported previously in other studies of the same species (Sacristán-Soriano *et al.* 2011b, 2012). *Ircinia* spp. showed the same distribution pattern, with higher concentrations of secondary compounds in inner as opposed to outer tissue regions (Freeman & Gleason 2010). However, other studies detected no differences in chemical defenses between the sponge surface and the inner tissues (Swearingen & Pawlik 1998; Burns *et al.* 2003; Rohde & Schupp 2011). Another pattern has also been found in sponges with an opposite allocation of secondary compounds where external layers were in principle better defended (Kubanek *et al.* 2002; Furrow *et al.* 2003; Richelle-Maurer *et al.* 2003; Peters *et al.* 2009; Freeman & Gleason 2010). All of these patterns can be explained by the optimal defense theory, under which areas more prone to predatory attack or more critical for species survival allocate more defensive metabolites under the assumption that resources used in the production of chemical defenses are limited (McKey 1974, 1979; Rhoades 1979). So, predatory feeding types (*i.e.* predators making deep or superficial bites) may drive the distribution of secondary compounds (Freeman & Gleason 2010). The predator of *A. aerophoba*, *Tylodina perversa*, is known to feed preferentially on the outer ectosome layer (Becerro *et al.* 2003). The larger concentration of chemical defenses in the inner choanosome, where basic sponge physiological processes such as reproduction and water pumping occur, may deter *T. perversa* that could be left to feed on a tissue more prone to regenerate after predation. In fact, artificial wounds made in other species that emulate bite marks left by a predator

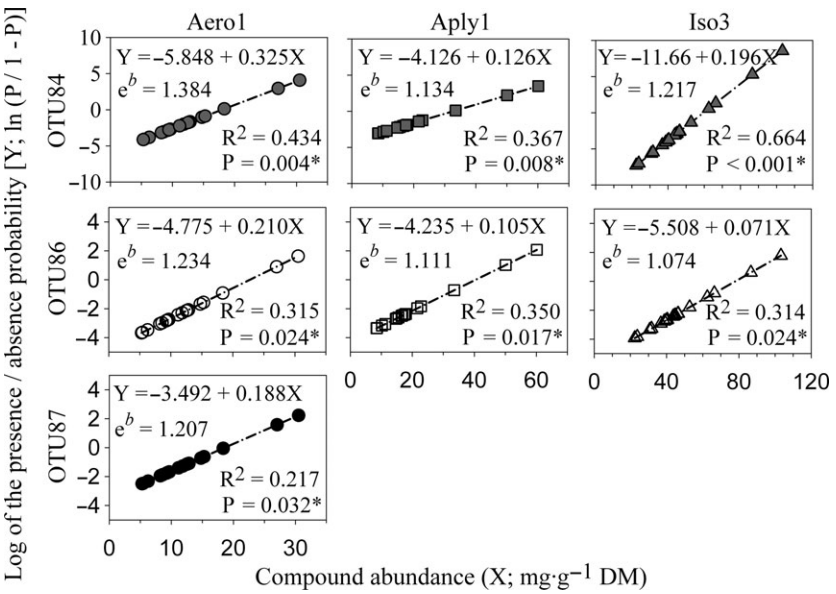

Fig. 4. Logistic regressions between concentrations of compounds, expressed in $mg \cdot g^{-1}$ (dry mass, DM) of sponge tissue, and operational taxonomic units (OTUs), expressed as the natural logarithm of the ratio between probabilities of presence (p) and absence (1−p) of an OTU. For each regression, we show the logistic equation, odds ratio (e^b), R^2 and P-value. *All logistic regressions were significant ($P \leq 0.05$) after Benjamini–Yekutieli adjustment. Aero1, aerophobin-1 (circles); Aply1, aplysinamisin-1 (squares); Iso3, isofistularin-3 (triangles). OTU 84 (in gray); OTU 86 (in white); OTU 87 (in black) (see Material and Methods for details).

Table 4. 16S rDNA sequence identities of sequenced bands excised from denaturing gradient gel electrophoresis gels.

OTU	putative division	closest relative in database (isolation source; GI[a])	% identity
1	Firmicutes	*Thermanaeromonas toyohensis* strain ToBE (subterranean water; 219857149)	83.5
2	Cyanobacteria	*Prochlorococcus marinus* subsp. *pastoris* strain PCC 9511 (seawater; 265678459)	96.0
3	Cyanobacteria	*Prochlorococcus marinus* subsp. *pastoris* strain PCC 9511 (seawater; 265678459)	95.7
4	Firmicutes	*Thermanaeromonas toyohensis* strain ToBE (subterranean water; 219857149)	83.5
5	Firmicutes	*Thermanaeromonas toyohensis* strain ToBE (subterranean water; 219857149)	83.5
6	Deltaproteobacteria	*Geobacter sulfurreducens* PCA (freshwater sediments; 265678873)	83.9
7	Alphaproteobacteria	*Thalassobaculum litoreum* strain CL-GR58 (seawater; 343205758)	87.6
8	Deltaproteobacteria	*Desulfobulbus rhabdoformis* strain M16 (seawater oil platform; 265678870)	83.5
9	Gammaproteobacteria	*Thiohalophilus thiocyanatoxydans* strain HRhD 2 (hypersaline waters; 343198845)	89.9
10	Deltaproteobacteria	*Desulfurivibrio alkaliphilus* strain AHT2 (freshwater; 343198972)	83.0
11	Cyanobacteria	*Prochlorococcus marinus* subsp. *pastoris* strain PCC 9511 (seawater; 265678459)	96.0
12	Cyanobacteria	*Prochlorococcus marinus* subsp. *pastoris* strain PCC 9511 (seawater; 265678459)	96.0
13	Nitrospira	*Thermodesulfovibrio hydrogeniphilus* strain Hbr5 (terrestrial hot spring; 343205695)	80.3
14	Firmicutes	*Thermacetogenium phaeum* strain PB (thermal wastewater; 219856869)	81.4
15	Cyanobacteria	*Prochlorococcus marinus* subsp. *pastoris* strain PCC 9511 (seawater; 265678459)	96.0
16	Alphaproteobacteria	*Oceanibaculum indicum* strain P24 (deep seawater; 343206001)	85.5
17	Cyanobacteria	*Prochlorococcus marinus* subsp. *pastoris* strain PCC 9511 (seawater; 265678459)	95.8
18	Chloroflexi	*Dehalogenimonas lykanthroporepellens* strain BL-DC-9 (subterranean water; 343199099)	85.0
59	Firmicutes	*Thermanaeromonas toyohensis* strain ToBE (subterranean water; 219857149)	82.8
60	Cyanobacteria	*Prochlorococcus marinus* subsp. *pastoris* strain PCC 9511 (seawater; 265678459)	95.9
61	Actinobacteria	*Iamia majanohamensis* strain NBRC 102561 (*Holothuria edulis*; 343200947)	90.6
62	Alphaproteobacteria	*Nitratireductor aquibiodomus* strain NL21 (seawater treatment reactor; 219878123)	88.8
63	Alphaproteobacteria	*Thalassobaculum litoreum* strain CL-GR58 (seawater; 343205758)	90.5
64	Deltaproteobacteria	*Desulfoglaeba alkanexedens* strain ALDC (oily wastewater; 343203773)	84.4
65	Gammaproteobacteria	*Thioalkalivibrio paradoxus* strain ARh1 (freshwater sediments; 219857426)	91.4
66	Alphaproteobacteria	*Rhizobium oryzae* strain Alt 505 (*Oryza alta*; 343205905)	90.9
67	Cyanobacteria	*Prochlorococcus marinus* subsp. *pastoris* strain PCC 9511 (seawater; 265678459)	95.8
68	Cyanobacteria	*Prochlorococcus marinus* subsp. *pastoris* strain PCC 9511 (seawater; 265678459)	96.1
69	Cyanobacteria	*Prochlorococcus marinus* subsp. *pastoris* strain PCC 9511 (seawater; 265678459)	96.1
70	Firmicutes	*Thermacetogenium phaeum* strain PB (thermal wastewater; 219856869)	85.9
71	Cyanobacteria	*Prochlorococcus marinus* subsp. *pastoris* strain PCC 9511 (seawater; 265678459)	96.1
72	Deltaproteobacteria	*Pelobacter acetylenicus* strain WoAcy1 (seawater sediments; 265678930)	83.7
73	Alphaproteobacteria	*Filomicrobium insigne* strain SLG5B-19 (oil-polluted saline sediment; 343205707)	87.6
74	Cyanobacteria	*Prochlorococcus marinus* subsp. *pastoris* strain PCC 9511 (seawater; 265678459)	95.8
75	Firmicutes	*Thermanaeromonas toyohensis* strain ToBE (subterranean water; 219857149)	82.0
76	Firmicutes	*Clostridium litorale* strain W6 (seawater sediments; 265678962)	80.8
77	Alphaproteobacteria	*Oceanibaculum indicum* strain P24 (deep seawater; 343206001)	88.9
78	Alphaproteobacteria	*Thalassobaculum litoreum* strain CL-GR58 (seawater; 343205758)	91.7
79	Deltaproteobacteria	*Desulfobulbus rhabdoformis* strain M16 (seawater oil platform; 265678870)	85.9
80	Deltaproteobacteria	*Thermodesulforhabdus norvegica* strain A8444 (seawater oil platform; 219846379)	85.7
81	Gammaproteobacteria	*Thioalkalivibrio paradoxus* strain ARh1 (freshwater sediments; 219857426)	91.6
82	Gammaproteobacteria	*Ectothiorhodosinus mongolicus* strain M9 (freshwater; 343198539)	90.8
83	Actinobacteria	*Mycobacterium intermedium* (*Homo sapiens* disease; 343206248)	82.8
84	Chloroflexi	*Caldilinea aerophila* DSM 14535 strain STL-6-O1 (sludge wastewater; 343200191)	82.4
85	Firmicutes	*Clostridium sporogenes* strain McClung 2004 (soil; 265678923)	82.6
86	Deltaproteobacteria	*Geoalkalibacter subterraneus* strain Red1 (seawater oil platform; 343199051)	82.5
88	Deltaproteobacteria	*Geobacter sulfurreducens* PCA (freshwater sediments; 265678873)	83.9
89	Firmicutes	*Clostridium hiranonis* DSM 13275 strain TO-931 (*Homo sapiens*; 265678309)	79.0

[a]GI = GenInfo Identifier number accessible through the National Center for Biotechnology Information website.

have been shown to regenerate within days or weeks (Wulff 2010) but these hypotheses remain to be tested in *A. aerophoba*.

The *ex situ* experiment testing the effect of light on *A. aerophoba* individuals under the given cultivation conditions revealed that the relative compositions of the four main BAs (Aero1, Aero2, Aply1 and Iso3) remained almost the same under all conditions. However, most of the BAs increased in abundance in both sponge layers after 4 months of *ex situ* cultivation. Both Klöppel *et al.*

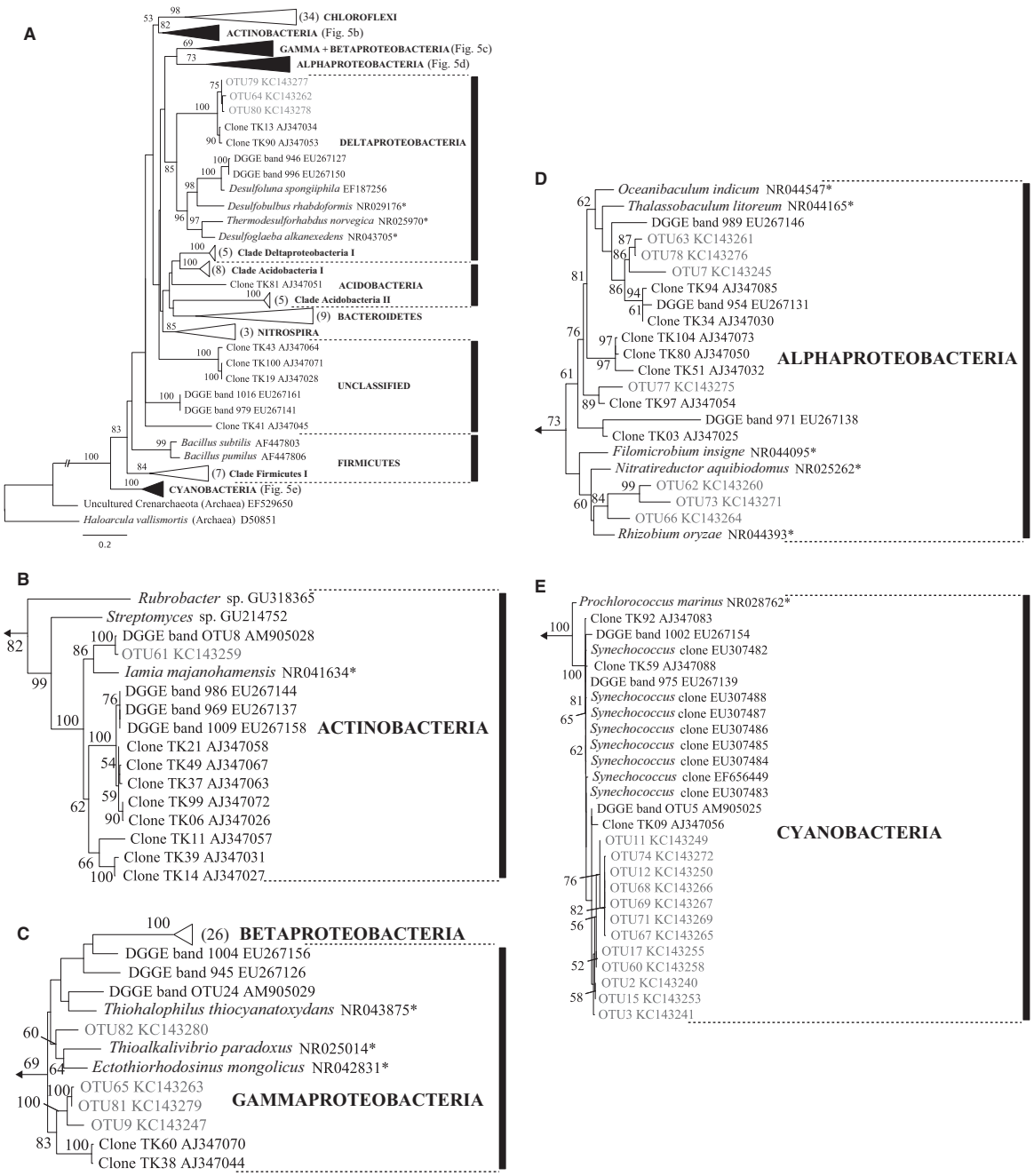

Fig. 5. (A): Phylogeny of bacterial 16S rRNA gene sequences from *Aplysina aerophoba*. Tree topology was constructed using maximum likelihood criteria, and numbers on nodes represent bootstrap support (<50% not shown). Terminal nodes denote the sequence source and GenBank accession numbers; gray values correspond to sequences from this study. For condensed clades (white triangles), the total number of sequences (in parentheses) is shown. Asterisks (*) indicate sequences from non-sponge sources. See (B), (C), (D) and (E) for condensed black clades. The accession numbers of the sequences from condensed white clades are: Chloroflexi (AJ347033, AJ347035–AJ347038, AJ347040–AJ347043, AJ347055, AJ347059–AJ347062, AJ347065, AJ347066, AJ347068, AJ347069, AJ347074–AJ347077, AJ347079–AJ347082, AJ347084, AJ347086, AJ347087, AM905025-AM905027, EU267143, EU267162); Clade Deltaproteobacteria I (EU267130, EU267136, EU267152, EU267153, EU267167); Clade Acidobacteria I (EU267129, EU267140, EU267155, EU267160, AJ347029, AJ347049, AJ347052, AJ347078); Clade Acidobacteria II (AJ347047, EU267132, EU267145, EU267159, EU267163); Bacteroidetes (EU267124, EU267125, EU267128, EU267135, EU267149, EU267151, EU267166, AM905030, AJ347046); Nitrospira (AJ347039, AJ347048, EU055608); Clade Firmicutes I (EU267123, EU267133, EU267134, EU267147, EU267148, EU267164, EU267165); Betaproteobacteria (EF529668–EF529682, EF529684–EF529686, EF529688–EF529695).

(2008) and Gerçe *et al.* (2009) also documented an increase of alkaloid content in *A. aerophoba* after several months of *ex situ* maintenance under natural and artificial conditions, respectively. The factors that caused the increase in BA concentration have not yet been determined, except for Aero1 in the outer part of the sponge, the concentration of which seemed to be directly affected by different illumination regimes. Other studies on *Aplysina* species have reported that neither transplanting sponges to different depth regimes nor cultivating them under different light conditions affected alkaloid concentrations (Thoms *et al.* 2003; Putz *et al.* 2009). By contrast, several sponge species have been found to be more toxic in shaded than in well-illuminated habitats, accumulating more bioactive compounds (Becerro *et al.* 1995; Ferretti *et al.* 2009). Furthermore, when the effect of light was tested in *Dysidea granulosa* by transplanting specimens to different illumination regimes, the concentration of secondary metabolites decreased without light (Becerro & Paul 2004). The diterpene content of the sponge *Rhopaloeides odorabile* may also experience light-induced production (Thompson *et al.* 1987). It is also possible that other factors associated with light (*e.g.* competition, predation) may determine the abundance of bioactive compounds rather than a direct effect of light.

In conclusion, the production of bioactive compounds in sponges appears to be species-specific and responds to site-specific ecological factors (*e.g.* light, predation, fouling), as suggested by Page *et al.* (2005). Multiple arguments may explain the increase in the amount of sponge secondary metabolites after *ex situ* cultivation. The ectosome of *A. aerophoba* is known to seasonally increase the concentration of brominated metabolites during the months when our experiment was carried out (Sacristán-Soriano *et al.* 2012). We used running seawater from the ocean at the naturally occurring temperature and so it is highly likely that our trial did not disrupt seasonality in our sponge specimens. Alternatively, experimental conditions might have caused a stress reaction leading to increasing abundances of natural products. Another potential explanation is a loss of sponge tissue under maintenance conditions (Klöppel *et al.* 2008; Gerçe *et al.* 2009) that may possibly have led to an accumulation of secondary metabolites in the remaining tissue. However, we did not observe evident changes in sponge tissue and we believe that seasonality is a plausible explanation for the increase in brominated compounds in our samples.

Associated bacteria: intra-individual and *ex situ* variability

Denaturing gradient gel electrophoresis has been found to be useful to assess changes in the microbial consortia inhabiting sponge bodies at different spatio-temporal scales and to compare these communities among sponge species (Friedrich *et al.* 2001; Taylor *et al.* 2004, 2005; Webster *et al.* 2004; Thiel *et al.* 2007b; Meyer & Kuever 2008; Mohamed *et al.* 2008; Hardoim *et al.* 2012). Despite this usefulness, this molecular technique has some limitations that are well reported. First, DGGE does not provide detailed quantitative estimations (Casamayor *et al.* 2000). Second, only short fragments with a length around 500 bp can be separated (Myers *et al.* 1985). Short sequences derived from DGGE gels do not allow precise phylogenetic analyses, but broad phylogenetic affiliations can still be determined (Diez *et al.* 2001). Third, the same DGGE band can represent more than one bacterial strain (Jackson *et al.* 2000). Thus, we sought to confirm for selected bands that (i) a given band persisted through time and that (ii) a given band was the same in the different sample types (*i.e.* different tissue layers, sampling times). Other intrinsic biases associated with PCR-DGGE are well documented (Suzuki & Giovannoni 1996; von Wintzingerode *et al.* 1997; Muyzer & Smalla 1998) and, as for any PCR-based approach, can lead to a misinterpretation of results. However, we used binary data and proceeded in a way that minimized technique biases and validated inter-sample comparisons, as described in Sacristán-Soriano *et al.* (2011b).

Comparing the ectosome and the choanosome of *Aplysina aerophoba*, we observed striking differences in the bacteria inhabiting the two tissue layers. Distinct phylotypes, represented by DGGE bands, were affiliated with different regions of the sponge. This local distribution of associated microbial populations has also been observed in other sponge species (Thiel *et al.* 2007b; Meyer & Kuever 2008; Sipkema & Blanch 2010). Changes were basically due to Cyanobacteria that have been found to be restricted to the cortex of the sponge as previously described in the same species (Becerro *et al.* 2003; Sacristán-Soriano *et al.* 2011b), where light energy is available for photosynthesis.

Ex situ maintenance of *A. aerophoba* over 4 months revealed slight shifts in a small fraction (6.5%) of the sponge microbiota, while most microbes (93.5%) remained stable. Erwin *et al.* (2012b) also documented a remarkable stability of bacterial symbionts of *Ircina* spp. *in situ* over 1.5 years. Changes were restricted to only some of the bacteria present, despite large fluctuations in temperature and irradiance. Further, the stability of the sponge microbiome has been assessed in different host species within their thermo-tolerance range (Lemoine *et al.* 2007; Webster *et al.* 2008a; Simister *et al.* 2012) and at different life stages of the sponge (Webster *et al.* 2011). Despite such a strong symbiont–sponge association, how the microbial structure may be affected by environmental factors is largely unknown. Here, we found little evidence

for bacterial shifts in response to different light conditions, supporting the hypothesis of a stable association between bacteria and sponges (Taylor *et al.* 2007; Lee *et al.* 2011; Thacker & Freeman 2012; White *et al.* 2012; Schöttner *et al.* 2013).

Among the variable bacterial fraction, an Alphaproteobacteria-affiliated phylotype (OTU 62) was directly affected by different light regimes in the sponge cortex, while another Alphaproteobacteria-affiliated phylotype (OTU 77) varied over time depending on the level of light reaching the inner sponge. Such potential associations are undescribed in the literature; however, we do not know whether these phylotypes belong to the permanent (*i.e.* symbiotic) or the transient fraction of the *Aplysina aerophoba* microbial community. If the sponge symbiotic microbiome is host-specific and highly stable over space and time, as suggested by several studies (Taylor *et al.* 2007; Webster *et al.* 2010; Erwin *et al.* 2011; Lee *et al.* 2011; Hardoim *et al.* 2012; Thacker & Freeman 2012; White *et al.* 2012; Schöttner *et al.* 2013), the low level of variability observed here may be ascribed to transient components of the sponge microbiota, as microbes recovered from sponge tissue may come from food sources (Pile *et al.* 1996), invasion (Webster *et al.* 2002) or simply from the surrounding environment.

Overall, the phylogenetic composition and diversity of the host-associated microbial community appear to depend to a large extent upon the sponge species and host state (*i.e.* sponge health), as demonstrated by earlier studies (Taylor *et al.* 2004, 2005; Webster *et al.* 2004; Hill *et al.* 2006; Li *et al.* 2007; Thiel *et al.* 2007a,b; Meyer & Kuever 2008; Schöttner *et al.* 2013), in contrast to the hypothesis of a uniform microbial signature of sponges across spatial and temporal scales (Hentschel *et al.* 2002). Thus, in a particular species the stress level of the sponge may be the main driver of symbiotic shifts that cause a breakdown in the health of the sponge and make the symbiont communities unstable (Webster *et al.* 2011). Yet, how environmental stressors such as light and temperature alter sponge health need to be further investigated.

Relationship between natural products and associated bacteria

The highly diverse microbial consortia inhabiting sponges are presumed to translate into metabolic diversity, resulting in the potential for new bioactive compounds to be discovered (Penesyan *et al.* 2010). The role of associated microbiota in sponge secondary chemistry is well known and widely documented in the literature (Dunlap *et al.* 2007; Grozdanov & Hentschel 2007; Taylor *et al.* 2007; Egan *et al.* 2008; Siegl *et al.* 2008; Hentschel *et al.* 2012). However, studies that link secondary metabolites and individual microbes are scarce. Here, we attempted to correlate variations in both natural products and the bacterial assemblage. A total of seven of the 18 regressions evaluated in the choanosome resulted in significant positive correlations between bacterial populations and secondary metabolites, which is unlikely to have occurred by chance alone. It suggests strong interactions, both among bacteria and between bacteria and brominated compounds. Our results show that three unidentified phylotypes (OTUs 84, 86 and 87) were positively related to the concentration of three alkaloids present (Aero1, Aply1 and Iso3) in the inner part of the sponge. An association between Aply1 and a Chloroflexi-affiliated phylotype was also found in a previous study (Sacristán-Soriano *et al.* 2011b). Further, a Chloroflexi bacterium isolated from *Aplysina aerophoba* was identified as the likely producer of a novel non-ribosomal peptide synthetase (Siegl & Hentschel 2009). Thus, Chloroflexi may play important roles in sponge nutrition and defense. Other bacteria may also play a role in sponge chemistry, such as members of the phylum Firmicutes, which have been found to encode polypeptide syntheses gene clusters (Zhang *et al.* 2009). In addition, isolates from *A. aerophoba* have been found to exhibit antimicrobial and antifungal activities (Hentschel *et al.* 2001; Pabel *et al.* 2003).

These correlations may be explained by multiple factors. If bacteria are involved in the production of natural products, microbes could be implicated either directly by producing the bioactive compounds themselves or indirectly by synthesizing enzymes that are crucial for the biogenesis of secondary metabolites. Accordingly, bacteria would determine the abundance of natural products. Several studies have described that sponge symbionts are the true producers of natural products (Unson & Faulkner 1993; Unson *et al.* 1994; Bewley *et al.* 1996; Jadulco *et al.* 2002; Mitova *et al.* 2003; Flatt *et al.* 2005; Schroder *et al.* 2006). However, in *A. aerophoba* the BAs seem to be located within sponge cells (Turon *et al.* 2000), suggesting a sponge origin. Thus, the associated microbes may co-operate with the sponge in the biosynthetic pathways of BAs, somehow either halogenating these compounds (van Pée *et al.* 2006) or activating them at the end of the process (Thoms *et al.* 2006). Far from being fully understood, the biogenesis of natural products in *Aplysina* is still controversial and little is known about the co-operation, if any, between the sponge and its microbial consortium in secondary metabolite production. Karpushova *et al.* (2005) reported that *Bacillus* spp. isolated from *A. aerophoba* may be the source of enzymes, such as esterases or peroxidases, that the host could use in the halogenation process (van Pée 1990, 1996; van Pée *et al.* 2006) essen-

tial for the synthesis of halogenated compounds in the marine environment.

Alternatively, the microenvironment created by the sponge could select for the presence of a particular microbe. This hypothesis may explain the distribution of bacteria with the ability to metabolize these compounds. Ahn *et al.* (2003) reported microbially mediated mechanisms for degradation of halogenated compounds in *A. aerophoba* by closely related Deltaproteobacteria. A later study (Ahn *et al.* 2009) revealed that these bacteria were affiliated to the genus *Desulfoluna*. Within the class of Deltaproteobacteria, *Desulfovibrio* spp. were also able to debrominate aromatic compounds (Boyle *et al.* 1999), which were predominant in *Aplysina cavernicola* (Friedrich *et al.* 1999). Members of the phylum Chloroflexi can also undergo anaerobic reductive dehalogenation (Field & Sierra-Alvarez 2008). Therefore, it is likely that sponge-associated bacteria belonging to those phyla (Hentschel *et al.* 2001, 2002; Noyer *et al.* 2010; Erwin *et al.* 2011, 2012a) may be capable of metabolizing sponge-derived halogenated compounds. Our results suggest that sponge-associated bacteria are related to natural product synthesis either by participating in their production or by responding to the internal chemical conditions within the sponge. Although our results are very unlikely to be explained by chance alone, we cannot completely exclude that the associations that we found were spurious correlations as a result of complex trophic interactions with concomitant organisms of the sponge microenvironment that we are unaware of yet. Clearly, further research on this topic will help untangle the underlying factors behind these chemical/bacterial associations.

Conclusions

Overall, we detected a differential distribution of natural products and sponge-associated bacteria between sponge tissue layers. Although a stress reaction of the sponges to captivity can not be discarded, the stability in chemical and microbial profiles prevailed with a restricted effect of light. Thus, any stress would have been minor. We also found multiple relationships between natural products and bacterial phylotypes. The probability of occurrence of those bacteria increased with increasing concentrations of correlated compounds. Our data and those from the literature suggest complex ecological interactions that are far from being understood. Although the microbial role in the sponge chemistry could not be clarified, the co-operation sponge/microbe in natural product synthesis needs further attention. The advances in metagenomic techniques applied to the sponge holobiont will significantly improve our understanding of the organization and functioning of these truly complex host–symbiont ecosystems by revealing the hidden diversity of genes involved in sponge chemistry.

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