Chemical and microbial ecology of the demosponge *Aplysina aerophoba*

Ecología química y microbiana de la demosponja *Aplysina aerophoba*

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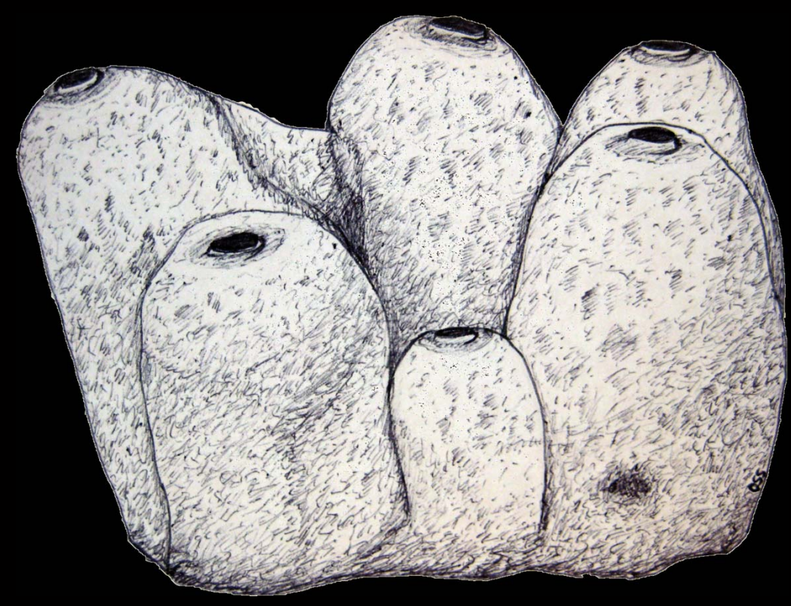

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Oriol Sacristán Soriano
June 2013

Tesis Doctoral
Universidad de Barcelona
Facultad de Biología
Programa de Doctorado de Biodiversidad

Chemical and microbial ecology of the demosponge *Aplysina aerophoba*

Memoria presentada por Oriol Sacristán Soriano
para optar al Título de Doctor
por la Universidad de Barcelona

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Barcelona, Junio de 2013

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Dra. Cruz Palacín Cabañas
Profesora UB

Als meus avis, a mis yayos
i al Manel

Agradecimientos

Como colofón final a esta tesis toca dar las gracias a todas aquellas personas que han hecho posible que este proyecto saliera adelante. Digo “toca” porqué yo no es que me caracterice por el don de la palabra y aunque a algunos ya se lo he agradecido en persona a muchos otros no. Así que me dispongo a escribir unas líneas multilingües para que los destinatarios de esta sección reciban su más que merecido reconocimiento.

El primer agradecimiento como es natural va dirigido a Mikel Becerro, mi director en el CEAB y en la distancia en los últimos dos años. Por lo que me has enseñado, por las informales clases de estadística, por tus consejos, y por ver el vaso medio lleno cuando yo lo veía medio vacío.

A la Creu Palacín, la tutora d’aquesta tesi, li vull agrair la seva disponibilitat i voluntat eterna per assumir diversos rols. Primer va començar formant part del tribunal del màster experimental que vaig realitzar a la UB, després va assumir la meva tutoria en el màster en Biodiversitat, i finalment ha acabat tutoritzant aquesta tesi doctoral.

Je veux remercier beaucoup à Bernard Banaigs pour toute l’aide dans l’analyse chimique des échantillons, sans laquelle la moitié de la thèse n'aurait pas été possible. Gràcies Susanna López per algun que altre consell. Li agraeixo també a l’Emilio O. Casamayor els coneixements que m’ha transmès sobre biologia molecular i el *savoir faire* en el laboratori.

Aunque no he incluido nada de mis estancias en Lisboa y Wageningen en la tesis, quiero agradecer su trabajo a las personas que me acogieron. Muito obrigado Madalena Humanes por me ter recebido na Universidade de Lisboa. Obrigado também a Helena Gaspar por me ter levado a mergulhar. A Alberto Reis pelos

conselhos no cultivo de bactérias d'esponjas. Estou também muito agradecido a Sandra, a pessoa responsável da minha estadia no ICAT. Thanks to Detmer Sipkema, my supervisor at Wageningen University, for his commitment and guidance during my short stay. Also thanks to all Microbiology lab staff and Detmer's team for helping me with some lab's issues. Agradecer también a Juanan Tamayo quien me acogió en su casa y a la comunidad de *Spanish* por la organización de eventos variopintos. Nunca pensé que podría asistir a tantas barbacoas en tres meses, incluso lloviendo!

Agradecimiento a las dos ex integrantes del grupo Charlotte y Villamor, en especial a Adri por sacarme de muestreo y aunque a veces al ofrecerte la mano me cogiste el brazo, una pierna, un trozo de cerebro […] (Villamor, 2010) fue un placer ayudarte y que me ayudaras otras veces.

Agrair a la Marta Ribes, a l'Eroteida Jiménez i a la Marta Jové la seva ajuda en l'organització, muntatge i seguiment d'experiments a la ZAE (CMIMA), com també a les persones responsables de la gestió d'aquesta zona d'experimentació, al Maxi i a l'Elvira.

Molts són els que m'han donat consells durant les etapes de laboratori. Gemma, JC, Andrea, Xevi, Sònia, Lucía, and specially Patrick who always had an answer that solved all my worries. Altres m'han assistit en la recol·lecció de mostres com l'Ana Pazó, la Mª João, l'Edu Serrano però, especialment, vull agrair la seva ajuda incondicional al João Gil que mensualment m'acompanyava per estar escassos 20 minuts a l'aigua. Raffaele a ti te agradezco la ayuda que me prestaste en el análisis de imágenes por satélite.

Moltes gràcies al CEAB, amb distinció especial per la Carmela i la Gemma que són dos grans pilars pel bon funcionament del centre. També agrair al Ramón el seu suport informàtic. I acabar aquesta part més formal amb l'agraïment al Xavier i a la Iosune, els meus padrins després de la marxa del Mikel al IPNA.

He conegut a molta gent durant el meu pas pel CEAB amb els quals he compartit mil històries que han fet que la tesi fos més amena, a vegades tant que oblidaves el motiu pel qual et trobaves

allà. En una primera fornada ens trobem amb personatges com la Paula risas, Romero, Deivid, Michela, Tina, Mireia, les Lauras Núñez i Serrano, els bessons Oscar i Edu, Ulla, Helena, Ari, Virgis, Mari Carmen, Marc, Valentín, Alis, Hierbas, Jenny, Peckor. Amb ells he compartit birres, congressos, immersions, i amb algun d'ells fins i tot pis. Destacar los consejos de dos doctoras como la copa de un pino, Riesgo y Roci. En una segona fornada podríem destacar al *grupo salsero blandense* (Steffi, Roser, Edu, Miquel, Laura, Aitana, Dani, Gemma, Susanna, Carmen) amb el qual grans tardes i nits hem compartit. Steffi i Roser gràcies pel vostre suport y por esas magníficas charlas como por esas cervecitas previas para coger fuerzas... como las posteriores para recuperarlas donde se incorporaban Maria Elena, Clara y Lili a veces. Carmeta, mi compañera de piso por excelencia, gracias por el apoyo y por los buenos ratos compartidos, a veces entre algún que otro grito. Willy a ti te agradezco esas tardes de tenis en este final de tesis que hemos compartido. Donatella *grazie mille* por tus consejos y los buenos momentos que hemos pasado. Dr. Barberán, gracias por las salidas a buscar setas por los alrededores del CEAB, por ese *look sexy lady* que tanto nos favorece, y por dejarme la tesis para usarla de modelo. Muchas otras personas han participado en este largo camino, en el café, durante las comidas, en eventos varios. Xavi, Pinedo, Guillem, Alessandro, Jordi, Simo, Antoni, Kathrin, Peipoch, Sandra, Juan Pablo, Mariani, Letzy, Alba, Tati, Sara, Marta, Francesco, Chiara, Mirco, Fede, Miguel, Claudia, Carlos, Marias, Celias, Cristina, Tomàs, Boris, Danilo, y alguno más que seguro me dejo (sorry).

Fora la vida del CEAB també he trobat persones interessades en el que feia. Durant la universitat vam començar a créixer com a biòlegs encara que al final les sortides per les que ha optat cada un són ben diverses. La Mire, les Laures, la Gemma, el Keko, la Núria, l'Aina, l'Albert. Tots ells algun cop han escoltat les misèries i les riqueses de la ciència. Aquí també vaig conèixer a la Lídia, fuiste mi mayor apoyo durante los primeros años de la tesis y por eso te estoy agradecido. Dels amics de tota la vida el que he rebut són, principalment, qüestions entusiastes sobre el que feia. Gràcies Marc

i Gemma, Miquel i Erola. Menció especial també pels amics de playa Carles, Rubén, Rocky i Albert.

Agraeixo a la família, sobretot als meus pares, l'interès que han demostrat per intentar esbrinar en el que estava treballant i la paciència, quasi infinita diria, que han tingut amb mi a l'hora de trobar aquelles respostes a les seves preguntes que sovint quedaven a l'aire.

Per acabar, m'has acompanyat de ben a prop els dos darrers anys i escaig d'aquest camí, amb un petit (afortunadament) parèntesi entremig, i t'haig d'agrair una infinitat de coses. El teu suport, els ànims i els teus consells han estat fonamentals per afrontar aquest darrer tram. Magda, moltíssimes gràcies.

Junio de 2013

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Resumen

1.1 Introducción general

El filo Porifera (Grant, 1836) está compuesto por metazoos sésiles con un sistema acuífero diferenciado del que forman parte los coanocitos que generan un flujo de agua unidireccional (Bergquist, 1978). A pesar de no presentar auténticos tejidos, las esponjas pueden llegar a desarrollar dos regiones bien definidas, el ectosoma (capa externa) y el coanosoma (región interna) (Boury-Esnault and Rützler, 1997). Estos organismos representan un grupo ecológicamente importante del bentos marino con gran capacidad de adaptación y dispersión en muchos hábitats, contribuyendo así en la organización y funcionamiento a nivel de comunidad y ecosistema (Van Soest et al., 2012).

Una de las claves del éxito evolutivo de las esponjas yace en la relación estrecha que presentan desde el Precámbrico con microorganismos (Wilkinson, 1984; Jackson et al., 2010; Uriz et al., 2012) a través de la cual desarrollaron una protección química. Muchas esponjas habrían utilizado los metabolitos secundarios de origen microbiano como defensa química. A día de hoy se han desarrollado dos áreas de investigación, la ecología química y la microbiana de esponjas, de las que realmente desconocemos su

origen o su impacto. Por esta razón, queríamos cuantificar el impacto de estas dos disciplinas y compararlas de una manera rigurosa.

Para evaluar la ecología química y microbiana en esponjas utilizamos una base de datos, *Science Citation Index Expanded® database*, de *Thomson Reuters Web of Knowledge℠* (http://webofknowledge.com) con el mismo criterio de búsqueda descrito por Becerro (2008), (*sponge OR porifera*) *AND chemi* AND ecolog** and (*sponge OR porifera*) *AND microb* AND ecolog**, respectivamente. Se recopilaron todas aquellas publicaciones desde 1900 hasta finales de 2012. Intentamos reducir los falso negativos ampliando la búsqueda en el límite temporal inferior (desde 1900 a 1990) con un criterio más amplio, *(sponge OR porifera) AND ecolog**. Se utilizaron varios parámetros para comparar la literatura química y microbiana como el número total de publicaciones, número total de citas recibidas, promedio de citas por publicación, número total de artículos citados, y el índice *h* (Becerro, 2008). Con la base de datos *Science Citation Index Expanded®* los resultados pueden ser clasificados en función de los autores, de categorías o disciplinas, lo que permite un análisis cuantitativo de los resultados.

Después de realizar la búsqueda con los criterios anteriormente mencionados, fueron seleccionadas 201 publicaciones de ecología química y 101 de ecología microbiana. Ambas disciplinas presentaron un aumento significativo a finales de los 90 con un incremento tanto del número de publicaciones como de las citas que recibían dichos artículos. Comparativamente, la ecología química de esponjas ha sido más productiva hasta la fecha. Abarca el doble de citas recibidas con respecto a la ecología microbiana aunque el número de citas recibidas promedio por publicación es similar y prácticamente cita dos veces más artículos. Si observamos el impacto en la comunidad científica, éste es mucho mayor en las publicaciones relacionadas con la ecología química. El porcentaje de revisiones publicadas en ambas disciplinas es similar (20%) aunque éstas presentan un impacto menor con respecto a los

artículos convencionales. El porcentaje de autores con múltiples contribuciones también es similar (10-20%) entre ambas ecologías. Mientras que un 40% de las publicaciones en ecología química pertenecen a la categoría *Marine & Freshwater Biology* seguido de un 30% englobadas en la categoría *Ecology*, un 30% de las publicaciones de ecología microbiana pertenecen a la categoría *Microbiology*, casi un 30% se engloban en *Marine & Freshwater Biology* y un 20% en la categoría de *Ecology*. Sin embargo, los artículos que citan dichas publicaciones en ecología química y microbiana se engloban en mayor proporción en categorías relacionadas con ciencias aplicadas.

Una manera de ver el estado en que se encuentra una área de investigación es evaluar el impacto de sus trabajos en la comunidad científica. Esto nos permite observar su evolución y nos ofrece la oportunidad de mejorar en el futuro. El echo de que la demanda de información en ecología química y microbiana provenga básicamente de disciplinas aplicadas yace en el potencial que presentan los simbiontes de esponjas a la hora de producir compuestos bioactivos. Esto convierte a estas asociaciones esponja-simbionte en un recurso biotecnológico importantísimo. Por otra parte, la falta de interés en trabajos relacionados con la ecología química y microbiana de esponjas por parte de la ecología, en el sentido más amplio, puede recaer en el objeto de estudio de estas publicaciones que centran su atención a nivel de especie tal y como sugiere Becerro (2008). Así que tenemos la oportunidad de continuar manteniendo el interés de farmacólogos y biotecnólogos e incrementar la atención por parte de la comunidad de ecólogos explotando la característica que hace a las esponjas particularmente relevantes, la simbiosis.

Para abordar los aspectos químicos y microbianos en esta tesis seleccionamos dos técnicas analíticas ampliamente usadas en ambas disciplinas e idóneas para el estudio de los metabolitos secundarios y de la comunidad de microorganismos asociados. Utilizamos una cromatografía líquida de alta resolución (HPLC, con sus siglas en inglés) para cuantificar los compuestos bioactivos y una

electroforesis en gel con gradiente desnaturalizante (DGGE, con sus siglas en inglés). La HPLC es un método popular de análisis no dependiente de la volatilidad o estabilidad de los compuestos a analizar que presenta varias aplicaciones incluyendo separación, identificación, purificación y cuantificación de compuestos (Lindsay and Kealey, 1987). La DGGE es una técnica basada en la amplificación de ADN por reacción en cadena de la polimerasa (PCR, con sus siglas en inglés) con una posterior separación de los fragmentos de ADN. De esta manera obtenemos un perfil de diversidad genética de la comunidad microbiana (Muyzer et al., 1993; Muyzer and Smalla, 1998).

1.2 Estructura, objetivos y resultados

Esta tesis fue concebida para estudiar la ecología química y microbiana de la esponja *Aplysina aerophoba* (Nardo, 1833), cuya química y comunidad bacteriana asociada han sido objeto de muchos estudios previos. El principal objetivo fue explorar la fuentes de variación tanto de los productos naturales adscritos a *Aplysina* como de su estructura microbiana e inferir una posible relación entre comunidad de microorganismos y la producción de metabolitos secundarios.

Los primeros 3 capítulos de esta tesis constan, primero, de un resumen completo; segundo, de una introducción general; y tercero, de los objetivos principales. A partir del cuarto capítulo nos encontramos con los distintos artículos desarrollados.

1.2.1 **Primera parte:** Variabilidad natural de los metabolitos secundarios y de la comunidad bacteriana asociada a la esponja

Conocer a que escala hay cambios tanto en la abundancia de productos naturales como en la comunidad bacteriana nos permite inferir qué factores subyacen los patrones de variabilidad

observados. Está sección incluye tres capítulos que exploran la variabilidad natural de los perfiles químicos y microbianos a distintas escales espaciales y temporales.

Capítulo 4: Explorando las conexiones entre productos naturales y comunidad bacteriana en la esponja *Aplysina aerophoba*

Aplysina aerophoba presenta una gran diversidad de alcaloides bromados y alberga una compleja comunidad microbiana. A pesar de que dichos compuestos han sido localizados en el interior de células de la esponja, las enzimas que se encargan de incorporar los elementos halógenos a los compuestos orgánicos se han descrito exclusivamente en algas, hongos, y bacterias. La comunidad bacteriana asociada a *Aplysina* podría entonces estar involucrada en la síntesis de estos compuestos. Con este estudio investigamos si los cambios observados tanto en la concentración de los compuestos bromados como en la comunidad microbiana asociada estaban correlacionados. Para acometer dicho objetivo, se cuantificaron los productos naturales mayoritarios mediante cromatografía líquida de alta resolución y se analizaron las poblaciones bacterianas mediante electroforesis en gel con gradiente desnaturalizante empleando el gen ribosómico 16S rRNA como marcador.

Identificamos asociaciones múltiples entre bacterias y productos naturales, principalmente entre una bacteria del grupo *Chloroflexi* y el compuesto aplysinamisin-1 y entre una bacteria no identificada y los compuestos aerophobin-2 e isofistularin-3. Estos resultados sugieren que dichas bacterias podrían estar implicadas en la producción de los alcaloides bromados, o bien, afectadas por ellos. Este estudio es uno de los primeros en documentar una correlación significativa entre productos naturales y poblaciones bacterianas en un organismo bentónico. Profundizar en el estudio de estas asociaciones sería beneficioso para entender la organización y funcionamiento de estos sistemas huésped-simbionte como *Aplysina aerophoba*.

Capítulo 5: Escalas espaciales relevantes de la variación química en *Aplysina aerophoba*

Conocer la escala en la cual los productos naturales presentan una variabilidad mayor es crucial porque permite conocer el tipo de factores que regulan su producción. La esponja *Aplysina aerophoba* es común en el Mediterráneo donde ocupa ambientes someros y en el área de influencia del océano Atlántico. Esta especie contiene concentraciones importantes de compuestos bromados que desempeñan varias funciones ecológicas. Este estudio investiga la variación ecológica de los metabolitos secundarios mayoritarios de *A. aerophoba* en una escala espacial comprendida entre centenares de metros hasta miles de kilómetros. Para ello, utilizamos un diseño jerarquizado de muestreo en el que se recolectaron muestras de dos regiones geográficamente distantes (Islas Canarias y Mediterráneo Occidental, separadas por 2500 km), de dos zonas dentro de cada región (separadas por unos 50 km), de dos localidades dentro de cada zona (separadas por unos 5 km), y de dos puntos dentro de cada localidad (separados por unos 500 m). Utilizamos la técnica de cromatografía líquida de alta resolución para cuantificar los compuestos bromados y con un espectrofotómetro cuantificamos la clorofila *a*.

Los resultados muestran una importante variación tanto en los productos naturales como en el contenido de clorofila *a*. Ambos patrones de variación se daban tanto en la escala geográfica más grande como en la más pequeña, aunque dependiendo del compuesto analizado prevalecía una escala u otra. Encontramos una correlación negativa entre la clorofila *a* y el compuesto isofistularin-3, lo que sugiere un impacto en la concentración de estos compuestos por parte de la comunidad simbionte. Estos resultados acentúan el complejo control de la producción de metabolitos secundarios y sugieren que factores actuando a pequeña y gran escala espacial que regularían la producción de compuestos naturales.

Capítulo 6: Tendencias temporales en la producción de metabolitos secundarios de la esponja *Aplysina aerophoba*

Se desconocen en gran medida los cambios temporales en la producción de metabolitos secundarios. Con este estudio cuantificamos la concentración de compuestos bromados tanto en el ectosoma como en el coanosoma de *Aplysina aerophoba* durante un periodo de dos años, y examinamos los patrones temporales de dichos productos naturales. Se cuantificó la concentración de aerophobin-2, aplysinamisin-1 e isofistularin-3, tres de los cuatro compuestos mayoritarios, mediante cromatografía líquida de alta resolución basándose en curvas de estándares.

Encontramos diferencias significativas en la abundancia de compuestos entre la capa externa y el interior de la esponja. Mientras que el ectosoma mostraba abundancias más altas de compuestos bromados durante los meses de verano, el coanosoma no seguía un claro patrón de variación. Además, encontramos que los compuestos aerophobin-2 e isofistularin-3 de la región del ectosoma estaban correlacionados con la temperatura del agua. Este estudio es uno de los primeros en documentar y cuantificar cambios estacionales en compuestos individuales registrados durante varios años. Estudios posteriores ayudaran a clarificar el papel de factores ambientales, biológicos y fisiológicos en determinar los patrones estacionales de los compuestos bromados.

1.2.2 **Segunda parte:** Efecto de factores ambientales en los metabolitos secundarios y comunidad bacteriana asociada a la esponja

En esta sección, pasamos a una aproximación experimental mediante la cual evaluamos el efecto de factores ambientales (como la luz) en la producción de compuestos bioactivos y en la estructuración de la comunidad bacteriana.

Capítulo 7: ¿distintas condiciones lumínicas pueden causar variación de los perfiles bacterianos y de productos naturales de la esponja *Aplysina aerophoba*?

La posible implicación de la comunidad microbiana en la química de la esponja es muy conocida a día de hoy. Sin embargo, se desconoce en gran medida cómo los factores ambientales afectarían tanto los perfiles químicos como microbianos y la implicación de estos cambios en el conjunto de la esponja. Con este estudio intentamos averiguar experimentalmente el efecto de la luz en la abundancia de metabolitos secundarios y en la comunidad bacteriana asociada a *Aplysina aerophoba*. Para ello, cuantificamos los compuestos bromados mayoritarios mediante cromatografía líquida de alta resolución y analizamos las poblaciones bacterianas mediante electroforesis en gel con gradiente desnaturalizante empleando el gen 16S rRNA como marcador.

Encontramos que los perfiles químicos y bacterianos eran distintos entre el ectosoma y el coanosoma de la esponja. A pesar que tanto los patrones de compuestos bromados como los bacterianos parecían mantener una estabilidad temporal, observamos que ciertos filotipos bacterianos prevalecían en condiciones lumínicas altas y otros en condiciones de escasa luminosidad, mientras que la mayoría de metabolitos incrementaron con independencia del régimen lumínico. Conseguimos identificar varias asociaciones entre una bacteria no identificada (OTU 84) y tres compuestos bromados (aerophobin-1, aplysinamisin-1 e isofistularin-3), y entre otra bacteria no identificada (OTU 75) y el compuesto aerophobin-2. Estos resultados sugieren que estas bacterias podrían determinar la abundancia de los compuestos bromados o a la inversa. Las asociaciones que hemos encontrado servirán para establecer nuevas hipótesis que aporten nuevo conocimiento de la organización y funcionamiento de estas complejas interacciones huésped-simbionte.

1.3 Discusión general

A finales de los años 90 tanto la ecología química como la microbiana empezaron a crecer de forma significativa en términos de productividad e impacto, aunque la primera ha recibido mayor atención por parte de la comunidad científica (**Introducción general**). Estas dos subdisciplinas de la ecología de esponjas centran su atención, principalmente, en el papel de los productos naturales y en una caracterización comparativa de los microbiomas asociados. Secundariamente, nos encontramos con algunos ejemplos que tratan sobre la variación natural y ambiental de la química de metabolitos secundarios y de simbiontes microbianos (Thompson et al., 1987; Becerro et al., 1995; Friedrich et al., 2001; Hentschel et al., 2002; Webster et al., 2004; Page et al., 2005; Abdo et al., 2007; Erwin et al., 2012b), aunque existen pocos estudios que evalúan la comunidad bacteriana de esponjas como fuente de compuestos bioactivos (Thompson et al., 1987; Becerro et al., 1995; Friedrich et al., 2001; Hentschel et al., 2002; Webster et al., 2004; Page et al., 2005; Abdo et al., 2007; Erwin et al., 2012b). Dado este vacío de publicaciones, tenemos la oportunidad de integrar estos dos campos de la ecología de esponjas investigando la relación entre productos naturales y microorganismos simbiontes, con el fin de entender la funcionalidad de la comunidad bacteriana en el huésped.

La aproximación que utilizamos consistía en explorar la variabilidad natural de los compuestos bioactivos y de la estructura microbiana de la esponja *Aplysina aerophoba* a distintas escalas espaciales (desde pocos centímetros hasta miles de kilómetros) y temporales (meses, años) (**Capítulos 4, 5, 6, y 7**), y evaluar el efecto de factores ambientales, como la luz, en los perfiles químicos y microbianos (**Capítulo 7**). Posteriormente, correlacionar ambos patrones de variación e inferir alguna asociación potencial entre productos naturales y simbiontes microbianos (**Capítulos 4, 5, y 7**). Una aproximación a escala múltiple podría aclararnos los factores que hay detrás de la variación natural (Levin, 1992).

Empezamos por preguntarnos si había diferencias a nivel intraindividual en la abundancia de metabolitos secundarios y en la microbiota asociada o si prevalecía una homogeneidad en el conjunto de *Aplysina aerophoba* (**Capítulos 4, 6, y 7**). Los patrones de variación encontrados reflejan que la esponja presenta dos entidades distintas en términos de concentración de productos naturales y composición microbiana, la capa externa (ectosoma) y el interior de la esponja (coanosoma). Este patrón de distribución también se ha observado en otras especies de esponjas (Kubanek et al., 2002; Furrow et al., 2003; Thiel et al., 2007b; Meyer and Kuever, 2008; Freeman and Gleason, 2010; Sipkema and Blanch, 2010). La compartimentalización de compuestos bioactivos podría ser explicada a través de la teoría de defensa óptima (McKey, 1974, 1979; Rhoades, 1979), con lo que las zonas más expuestas a la depredación o más críticas para la supervivencia de la especie presentarían mayor concentración de compuestos, asumiendo una limitación de recursos para la producción de defensas químicas. Debido al papel anti-depredación de los compuestos bromados de *Aplysina* (Thoms et al., 2004), la región interna enriquecida en defensas actuaría como una barrera química final para proteger funciones como la reproducción o la circulación de agua. La máxima de *todo está en todas partes pero el ambiente selecciona* (Baas Becking, 1934) sería la base para entender la distribución de las poblaciones de microbios. En el caso de *A. aerophoba*, la distribución de las cianobacterias restringidas al córtex responde a la naturaleza fototrofa de estos microorganismos que requieren de luz directa.

Conocer la escala geográfica en la cual aparecen cambios en los perfiles químicos y microbianos nos puede ayudar a desenmascarar los factores que subyacen a la variación natural (**Capítulo 5**). Los cambios en la concentración de compuestos bromados de *Aplysina aerophoba* se produjeron a pequeña (centenares de metros) y gran (miles de kilómetros) escala, variación espacial que ya había sido documentada a una escala menor (Teeyapant et al., 1993a). Diversos factores tanto bióticos

(depredación, *fouling*), como abióticos (luz, profundidad) con influencia en distintas escalas espaciales podrían ser responsables de la variación encontrada (Thompson et al., 1987; Becerro et al., 1995; Swearingen and Pawlik, 1998; Page et al., 2005; Abdo et al., 2007; Noyer et al., 2011). Los patrones de cambio de la clorofila *a* (estimación de la comunidad de microbios fototrofos) eran similares a los de los metabolitos secundarios con variaciones locales y entre las dos zonas geográficamente distantes. Cambios en las condiciones lumínicas alterarían la comunidad fotosintética (Becerro et al., 2003; Thacker, 2005; Erwin and Thacker, 2008), aunque otros factores ecológicos actuando en múltiples escalas podrían reflejar dicha variabilidad (Wilkinson, 1987; Wulff, 2006; Taylor et al., 2007b).

Es importante conocer también la variabilidad temporal en la abundancia de los productos naturales y en la microbiota para poder entender qué factores son los responsables de dicha variación (**Capítulo 6**). *Aplysina aerophoba* mostraba patrones temporales de variación de los compuestos bromados mayoritarios con periodicidad anual. Mientras que en el coanosoma los compuestos presentaban una variabilidad mensual, en el ectosoma existían cambios mensuales y estacionales en función del compuesto analizado. El patrón estacional en el córtex de la esponja con concentraciones mayores durante los meses de verano es similar al descrito en otras especies (Page et al., 2005; Abdo et al., 2007). Factores abióticos como la temperatura del agua podrían explicar las diferencias temporales en la química de *Aplysina*, aunque otros factores correlacionados con la temperatura (*fouling*, competencia) (Thompson et al., 1987; Becerro et al., 1995; Turon et al., 1996; Page et al., 2005; Ferretti et al., 2009), es decir, con marcada estacionalidad (Turon et al., 1996; Duckworth and Battershill, 2001), podrían también explicar estos patrones de las defensas químicas. La variabilidad temporal podría responder a *trade-offs* entre defensas químicas, crecimiento y reproducción (Leong and Pawlik, 2010), aunque se requieren datos de crecimiento y reproducción en *A. aerophoba* para apoyar esta hipótesis. La

variabilidad temporal de la comunidad bacteriana en esta especie debe ser testada a pesar de que en otras especies parece ser bastante estable en el tiempo (Erwin et al., 2012b).

Dado el potencial de los factores abióticos en determinar los cambios espaciales y temporales en los productos naturales y comunidad bacteriana, testamos el efecto de la luz en ambos parámetros (**Capítulo 7**). Los resultados mostraban una estabilidad general de la química y de la microbiota con un efecto de la luz restringido a un compuesto en la parte superficial de la esponja y a una pequeña fracción de la comunidad bacteriana. Si descartamos el efecto directo que tiene la luz en las defensas químicas, otros factores asociados a ésta podrían determinar la abundancia de los metabolitos secundarios (Becerro et al., 1995). El efecto positivo de la luz en poblaciones de cianobacterias ya había sido documentado (Thacker, 2005; Erwin and Thacker, 2007; Erwin and Thacker, 2008), aunque la respuesta de otros grupos bacterianos se desconoce por completo.

Otro factor a tener en cuenta que podría explicar la variación en la abundancia de los compuestos bioactivos yace en la propia microbiota que podría estar involucrada en la producción de dichos compuestos a algún nivel. Los estudios que documentan relaciones cuantitativas son escasos (**Capítulos 4, 5, y 7**). En esta tesis mostramos múltiples relaciones positivas entre varios filotipos bacterianos (entre ellos uno perteneciente a *Chloroflexi*) y diversos compuestos bromados, como una relación negativa entre la clorofila *a* y el compuesto isofistularin-3. Estas posibles asociaciones podrían ser explicadas bajo varios escenarios. Primero, la abundancia de una determinada bacteria llevaría a una mayor concentración de un compuesto. Se daría este caso si las bacterias estuviesen implicadas en la producción de los productos naturales directa o indirectamente aportando enzimas necesarias para su síntesis (Karpushova et al., 2005; Siegl and Hentschel, 2009). Segundo, la abundancia de determinados compuestos llevaría a una mayor abundancia de bacterias. En este caso las bacterias son seleccionadas por unas determinadas condiciones del ambiente,

como por ejemplo la capacidad de metabolizar los compuestos bromados de *Aplysina* (Ahn et al., 2003; Ahn et al., 2009). Tercero, las asociaciones encontradas podrían ser espurias aunque es poco probable que se den tantas exclusivamente por azar. En este caso una interacción de exclusión competitiva entre dos bacterias podría llevar a una relación indirecta entre la bacteria excluida y un compuesto (asociación clorofila *a* e isofistularin-3).

Resumiendo, la producción de metabolitos secundarios en esponjas y la composición filogenética de la microbiota asociada depende esencialmente de la especie de esponja, de los factores ecológicos con variación espacial y temporal (Becerro and Paul, 2004; Taylor et al., 2004; Webster et al., 2004; Page et al., 2005; Thiel et al., 2007b; Schöttner et al., 2013), como también del estado del huésped (si presenta o no estrés) (Webster et al., 2011). La combinación entre factores abióticos y bióticos determinaran en última instancia la abundancia de defensas químicas y la diversidad microbiana debido a una autorregulación entre ambos tipos de factores (Wulff, 2012).

1.3.1 Conclusiones

Las conclusiones principales de esta tesis son las siguientes:

- La ecología química y la microbiana de esponjas han recibido una atención importante por parte de la comunidad científica a partir de finales de los años 90, principalmente de ciencias aplicadas (**Introducción general**).
- Carencia de especialistas con un importante número de contribuciones en ambas disciplinas (**Introducción general**).
- Diferencias en la abundancia de compuestos bromados y en la composición de la comunidad microbiana entre el ectosoma y el coanosoma (**Capítulos 4, 6, y 7**).

- Los perfiles de los compuestos bromados y clorofila *a* variaban a pequeña (cientos de metros) y gran (miles de kilómetros) escala espacial (**Capítulo 5**).
- Los perfiles de los compuestos bromados mostraban cambios temporales. En el coanosoma los compuestos presentaban diferencias mensuales, mientras que el ectosoma las diferencias eran tanto mensuales como estacionales (con abundancias mayores en verano) (**Capítulo 6**).
- El efecto de la luz en los metabolitos secundarios y en la comunidad bacteriana era ínfimo. El compuesto aerophobin-1 del ectosoma y una pequeña fracción bacteriana se vieron afectados por las condiciones lumínicas (**Capítulo 7**).
- Documentamos múltiples relaciones positivas entre varios filotipos bacterianos (entre ellos uno perteneciente a *Chloroflexi*) y diversos compuestos bromados, como una relación negativa entre la clorofila *a* y el compuesto isofistularin-3 (**Capítulos 4, 5, y 7**).
- Los productos naturales y la comunidad microbiana asociada dependen esencialmente de la especie de esponja, de su estado, y de los factores ecológicos con variación espacial y temporal (**Discusión general**).

Informe del director

El Dr. Mikel A. Becerro García, Científico Titular del Instituto de Productos Naturales y Agrobiología (IPNA-CSIC) y previamente del Centro de Estudios Avanzados de Blanes (CEAB-CSIC), en calidad de Director de la tesis doctoral *Chemical and microbial ecology of the demosponge Aplysina aerophoba* presentada por Oriol Sacristán Soriano para optar al título de Doctor dentro del programa de doctorado en Biodiversidad de la Universidad de Barcelona, hace constar que la participación del aspirante a doctor en cada uno de los artículos presentados en esta memoria ha sido extensa en todos y cada uno de los aspectos necesarios para la publicación de un artículo internacional en revistas de primera línea. En todos y cada uno de los artículos Oriol ha discutido y contribuido al diseño y aproximación de la investigación, realizado la toma y manipulación de las muestras, obtención de datos de campo y laboratorio, análisis de resultados y obtención de conclusiones. A la hora de la redacción de los artículos científicos, Oriol ha mostrado una evolución espectacular hasta el punto de ser totalmente autónomo en la recta final de su tesis doctoral. Oriol ha presentado primeras versiones muy elaboradas y de una alta calidad para cumplir con los estándares de las revistas donde se han publicado sus investigaciones. Asimismo, constato que en los cuatro artículos científicos generados con esta tesis doctoral, el candidato es primer firmante de los trabajos y en ningún caso el resto de coautores ha utilizado, implícita o explícitamente, datos o resultados de estos trabajos para la elaboración de ninguna otra tesis doctoral. Tres de los cuatro artículos se encuentran ya publicados y lo han sido en revistas internacionales de referencia de la especialidad pertenecientes al primer cuartil (Q1), de la categoría *Microbiology*

como de la de *Chemistry, Medicinal* según la base de datos *Science Citation Index* (SCI).

- **Artículo I**
  Sacristán-Soriano O, Banaigs B, Casamayor EO, Becerro MA (2011) Exploring the links between natural products and bacterial assemblages n the sponge *Aplysina aerophoba*. *Applied and Environmental Microbiology* 77:862-870.
  Indicadores bibliométricos. Impact Factor (2011): 3.829; Cuartil: Q1; Categoría: *MICROBIOLOGY* (posición 28 de 114); Citas SCI acumuladas: 9.

- **Artículo II**
  Sacristán-Soriano O, Banaigs B, Becerro MA (2011) Relevant spatial scales of chemical variation in *Aplysina aerophoba*. *Marine Drugs* 9:2499-2513.
  Indicadores bibliométricos. Impact Factor (2011): 3.854; Cuartil: Q1; Categoría: *CHEMISTRY, MEDICINAL* (posición 7 de 59); Citas SCI acumuladas: 4.

- **Artículo III**
  Sacristán-Soriano O, Banaigs B, Becerro MA (2012) Temporal trends in the secondary metabolite production of the sponge *Aplysina aerophoba*. *Marine Drugs* 10:677-693.
  Indicadores bibliométricos. Impact Factor (2011): 3.854; Cuartil: Q1; Categoría: *CHEMISTRY, MEDICINAL* (posición 7 de 59); Citas SCI acumuladas: 1.

- **Artículo IV**
  Sacristán-Soriano O, Banaigs B, Becerro MA (submitted) Can different light regimes cause variations in natural product and bacterial profiles of the sponge *Aplysina aerophoba*? *Microbial Ecology*

Indicadores bibliométricos. Impact Factor (2011): 2.912; Cuartil: Q1; Categoría: *MARINE & FRESHWATER BIOLOGY* (posición 9 de 97).

El director de la tesis
Dr. Mikel A. Becerro García
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2
General introduction

Phylum Porifera (Grant, 1836) are sessile metazoans with a differentiated inhalant and exhalant aquiferous system with external pores. Unidirectional water current is generated through the body by flagellated cells (choanocytes) usually contained within chambers to accomplish physiological functions (Bergquist, 1978; Hooper and van Soest, 2002). Sponges possess different cell types with mobile and totipotent capabilities that confer this phylum a huge plasticity (Hooper and van Soest, 2002). Lacking a tissue grade of construction, sponges can reach two well-differentiated regions, the ectosome (external layer free of choanocytes) and the choanosome (internal region with choanocytes) (Boury-Esnault and Rützler, 1997). As the most likely primitive metazoans (Schutze et al., 1999; Halanych, 2004; Giribet et al., 2007), their challenging structural organization, physiology for biocalcification and trophic requirements allowed sponges to rapidly colonize distinct environments and built important sponge reefs during the Paleozoic and Mesozoic eras, making them an ecologically relevant group principally in marine benthic communities (Carrera and Botting, 2008; Jackson et al., 2010). To date, sponges are still ecologically important among benthic fauna although the role as reef builders in modern coral reefs has changed in favor of scleractinian corals

(Hooper and van Soest, 2002; Jackson et al., 2010). Nonetheless, sponges have demonstrated a huge capacity to adapt and spread in many habitats (Van Soest et al., 2012) contributing to organization and functioning at both community and ecosystem levels. These sessile metazoans act as structure builders and interact with other organisms through a variety of trophic interactions (e.g., competition, deterrence, symbiosis) (Wulff, 2006; Rützler, 2012; Wulff, 2012), and also play a role in large-scale processes such as nutrient cycling, primary production, calcification or bioerosion (Diaz and Rützler, 2001; Schlappy et al., 2010; Ribes et al., 2012; Rützler, 2012; Uriz et al., 2012).

2.1 Sponge chemical and microbial ecology

One of the keys of the evolutionary success of this group lies in the close association between sponges and microbes that dates back to the Precambrian era (Wilkinson, 1984; Jackson et al., 2010; Uriz et al., 2012). The need to be defended may have arisen from the lack of motility of sponges and several mechanisms emerged to fulfill their demand including a chemical protection (Taylor et al., 2007b; Siegl et al., 2008). Chemical defenses have been well studied in the phylum Porifera (Proksch, 1994; Müller et al., 2004; Sipkema et al., 2005a; Proksch et al., 2010), however, the origin of many compounds still remains controversial (König et al., 2006; Wang, 2006; Hentschel et al., 2012). Many sponges would have taken advantage of associated microbes to use their metabolites as a defense barrier against predators, competitors or foulers (Pawlik et al., 1995; Becerro et al., 1997a; Newbold et al., 1999; Amsler et al., 2000; Waddell and Pawlik, 2000a, b). This particular symbiosis has consequently become a key factor in biotic interactions within the sponge research (Taylor et al., 2007b). To date, chemical ecology and microbial ecology are two independent areas of the sponge research with ecological implications that occasionally converge at the same point (i.e., the chemical ecology of sponge-associated microbes). But, what do we know about these sponge research

areas? Which one appeared first? Do they have the same impact on the scientific community?

We want to analyze the evolution of the sponge chemical and microbial ecology from the very beginning, to quantify their impact on the scientific community, and to compare both research areas. There are some book chapters and reviews published in the literature in the last 2-3 years that exhaustively compile and analyze publications in both sponge ecology areas, in chemistry (Proksch et al., 2010; Paul et al., 2011; Genta-Jouve and Thomas, 2012) and microbiology (Hentschel et al., 2012; Schippers et al., 2012; Thacker and Freeman, 2012). However, we want to provide a quantitative point of view for those sponge disciplines and compare them for the first time without losing rigorousness and analytical strength.

2.2 Reference sampling and analysis

Reviewing all chemical and microbial ecology literature on sponges can be pretentious and biased from the point of view of who are performing this search. Since the appearance of Thomson Reuters Web of Knowledge℠ (http://webofknowledge.com), our idea has become more feasible and objective. Thanks to Garfield (1964, 1970) the Web of Knowledge platform includes multidisciplinary resources to search, track and measure literature in the sciences and generate statistical reports. Web of Science® is one of the available tools that provides access to the world's leading citation databases. We used Science Citation Index Expanded® database, which comprises over 8500 major journals across 150 disciplines and covers publications from 1900 up to the present, to search for sponge chemical and microbial ecology. We also used the "Analyze" tool within this database to group published papers, analyze them, and identify research trends.

For an evaluation of the chemical and microbial ecology of sponge research we followed the same criteria described by Becerro (2008) and searched for (*sponge OR porifera*) *AND chemi* AND*

*ecolog** and (*sponge OR porifera*) *AND microb** *AND ecolog**, respectively. The timespan used for these searches was from 1900 to 2012. The use of the asterisk allowed searching for variants such as 'chemical or chemistry, microbial or microbe, and ecology, ecological or ecologically'. If any of the combinations of these variants appeared in the title, abstract, or keywords, the target publications on sponge chemical and microbial ecology were likely to be selected. However, this search strategy as other analyses presents both false positive and false negative results. We reduced the false positives double checking all the publications that resulted in the analysis. We could not do so with the false negatives so we probably leaved out some publications related to sponge chemical or microbial ecology because they did not include in the title, abstract, or keywords the terms (or any variants) used in our search. Yet, we tried to reduce the false negatives in the lower time limit of the search. Thus, we did a broad search on sponge ecology using the same terms used by Becerro (2008) *(sponge OR porifera) AND ecolog** with an arbitrary timespan from 1900 to 1990 that allowed us to check all the publications found. We finally recovered only one publication related to sponge chemical ecology that was incorporated in the analysis. The criteria used for our search are simple, easy to reproduce, comparable with other searches, and unbiased.

We used several parameters to compare chemical and microbial literature. We recorded the total number of publications, total number of citations, average number of citations per publication, total number of citing articles, and the *h* index (Becerro, 2008). The total number of publications reflects the productivity of the research area but shows no information on the impact of the studies. The total number of citations provides an idea of the relevance of the research but may be biased by a few articles with an unusually high number of citations that inflate the impact of the remaining published works. The number of citations per publication is a measure of the relative importance of the research area but is biased by productivity. The total number of citing

articles is the number of previous research studies related to the research area searched. The *h* index, defined by Hirsch (2005) as the number of papers with citation number ≥*h*, is a useful and unbiased index to estimate the importance of scientific outputs. Similar *h* indexes imply similar relevance of two research fields, while higher *h* values indicate greater importance regardless of the total number of publications or citations of the two scientific areas (Hirsch, 2005).

The Science Citation Index Expanded® database provides results as a function of Authors and Subject Categories, which allows quantitative examination of the research output by contributing researchers and disciplines. Subject categories are non-exclusive, so a single article may be included in several disciplines. Consequently, adding up the number of publications or percentages for each category we obtain a number larger than the actual number of records found or percentages larger than 100%. For details on what is comprised in each subject category visit the webpage (Scope Notes, Science Citation Index Expanded®).

2.3 Trends on sponge chemical and microbial ecology

A total of 263 publications initially met the sponge chemical ecology requirements, while 148 publications were found with the microbial ecology search. After checking all published works, 63 papers of the chemical search and 47 papers of the microbial output were completely unrelated to our goal and were discarded. We found an additional publication (Green, 1977) with a broad search that also met the chemical ecology requirements and was subsequently added to the sponge chemical output (see Reference sampling above). Thus, a total of 201 and 101 publications of chemical and microbial ecology, respectively, were analyzed. The chemical output accounted for 5469 citations while microbial papers totaled 2647 citations, which showed an increasing trend over the years but with a marked rise in the early 1990s in both

cases when the number of publications started growing (Fig. 2.1). In the chemical ecology area, the number of publications showed a low increase in the early 1990s with about 4 publications per year, then almost doubled in the late 1990s and early 2000s, and has tripled in the last 6 years with 19 papers in 2012 (Fig. 2.1). In the microbial ecology field, the number of publications exhibited an irregular increase with several 'blank' periods of no publication records and isolated published works, then the rate increased at almost 3 publications per year in the late 1990s and early 2000s, and has quadrupled in the last 6 years with and average of 10 papers per year (Fig. 2.1).

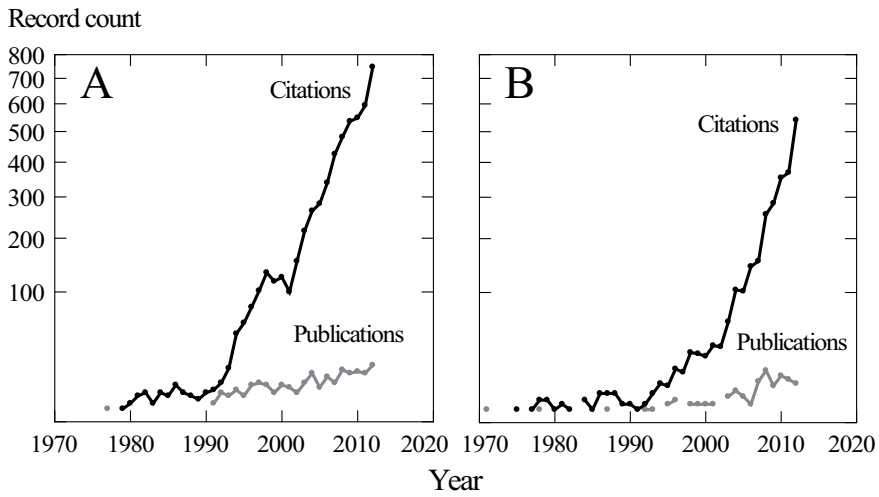


Figure 2.1. Evolution of the number of publications (in gray) and citations (in black) on sponge chemical (A) and microbial (B) ecology found in the Science Citation Index Expanded® database from 1900 to 2012. Search terms: (*sponge OR porifera*) *AND chemi* AND ecolog** and (*sponge OR porifera*) *AND microb* AND ecolog**, respectively.

Comparatively, sponge chemical ecology ranked first in terms of productivity (i.e., number of publications) doubling the number of articles published in microbial ecology (Table 2.1). Chemical ecology also ranked first for the number of citations received, showed the highest average number of citations per publication, and

cited more related references than microbial ecology (Table 2.1). The highest *h* index obtained by chemical ecology reflected a greater impact on the scientific community than those of the microbial ecology (Table 2.1). Of the 201 publications on the chemical ecology field, 40 were reviews (19.9%). Almost the same percentage of reviews occurred in sponge microbial ecology (18.8%). These reviews accounted for over 37% of the citations and around 50% of citing articles in both research areas (Table 2.1). Although these reviews exhibited over a twofold increase in the average number of citations per item compared with regular articles, the greatest relevance of the research lay in the latter that showed high *h* indexes (Table 2.1).

Table 2.1. Total number of publications (n), total number of citations (cites), average number of citations per publication (avg), total number of citing articles (art) and *h* index for both sponge research areas searched in the Science Citation Index Expanded® database from 1900 to 2012. Research output showed by research area and within each area categorized by publication type (articles and reviews).

Sponge research area	n	cites	avg	art	*h*
Chemical ecology	201	5469	27.21	3307	38
Articles	159	3305	20.79	1896	33
Reviews	40	2141	53.53	1860	21
Microbial ecology	101	2647	26.21	1795	27
Articles	81	1627	20.09	1144	24
Reviews	19	1001	52.68	887	12

The 201 publications on chemical ecology were contributed by 510 authors. Over 20% of the researchers (110) had multiple contributions (two or more publications). The number of researchers with five or more papers was 17 and just 7 had ten or more publications. The 101 publications on sponge microbial ecology were contributed by 350 researchers. Over 10% of the

authors (40) had multiple contributions and just 4 had five or more publications but below ten.

Publications on sponge chemical and microbial ecology covered over 20 subject categories in the Thomson Reuters Web of Knowledge^{SM} (27 and 26, respectively; Table 2.2). On chemical ecology, about 40% of the 201 publications belonged to the Marine & Freshwater Biology category followed by almost 30% of the records that belonged to the Ecology subject category. On microbial ecology, about 30% of the 101 papers belonged to the Microbiology category and almost 30% belonged to the Marine & Freshwater Biology. The Ecology subject category was represented by almost 20% of the publications (Table 2.2).

Table 2.2. Number of publications (n), percentage of total publications on sponge chemical and microbial ecology (%) for each subject category. Ranking for each subject category within brackets.

Subject category	**Chemical ecology**		**Microbial ecology**	
	n	%	n	%
Marine & Freshwater Biology	82	40.8 (1)	29	28.7 (2)
Ecology	59	29.4 (2)	18	17.8 (3)
Paleontology	-	-	12	11.9 (4)
Geology	-	-	9	8.9 (5)
Biochemistry & Molecular Biology	33	16.4 (3)	2	2.0 (15)
Chemistry, Medicinal	23	11.4 (4)	5	5.0 (10)
Oceanography	23	11.4 (5)	8	7.9 (6)
Microbiology	13	6.5 (6)	31	30.7 (1)
Pharmacology & Pharmacy	13	6.5 (7)	3	3.0 (13)
Biotechnology & Applied Microbiology	12	6.0 (8)	7	6.9 (7)
Multidisciplinary Sciences	12	6.0 (9)	4	4.0 (11)
Chemistry, Organic	11	5.5 (10)	-	-
Plant Sciences	11	5.5 (11)	3	3.0 (14)
Zoology	10	5.0 (12)	-	-
Environmental Sciences	9	4.5 (13)	6	5.9 (8)

Table 2.2. Continued.

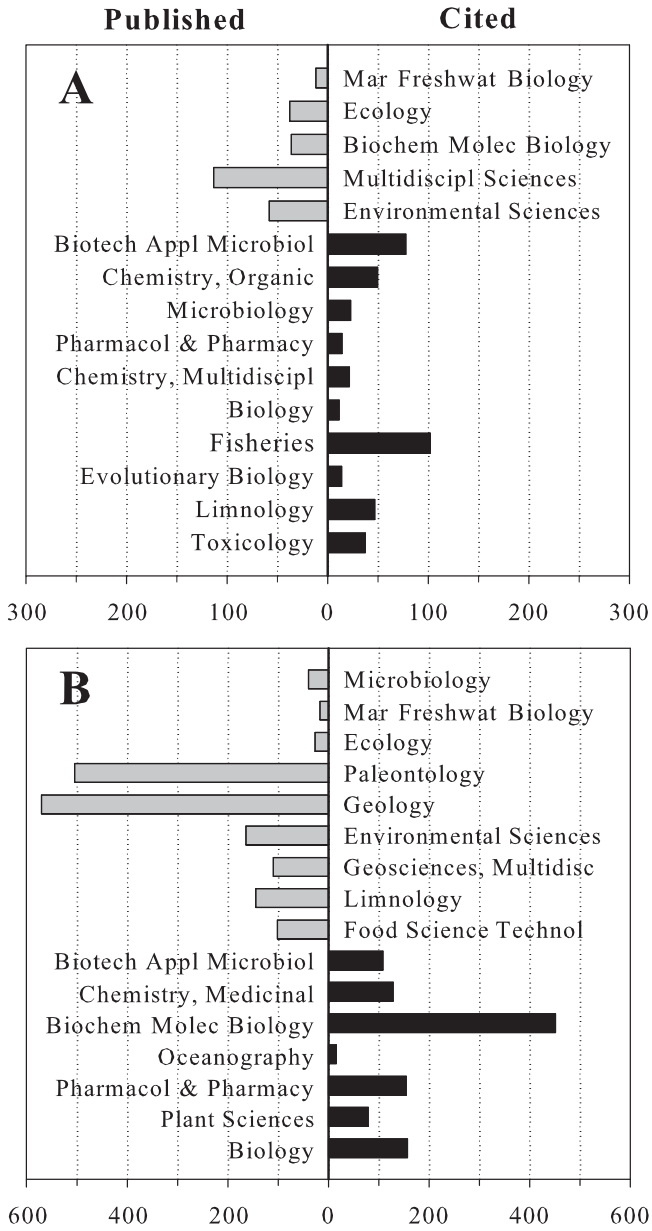
Chemistry, Multidisciplinary	7	3.5 (14)	-	-
Biology	6	3.0 (15)	1	1.0 (19)
Evolutionary Biology	5	2.5 (16)	-	-
Geosciences, Multidisciplinary	5	2.5 (17)	6	5.9 (9)
Geography, Physical	4	2.0 (18)	2	2.0 (18)
Fisheries	3	1.5 (19)	2	2.0 (16)
Limnology	2	1.0 (20)	3	3.0 (12)
Mycology	2	1.0 (21)	1	1.0 (24)
Toxicology	2	1.0 (22)	-	-
Biodiversity & Conservation	1	0.5 (23)	-	-
Economics	1	0.5 (24)	1	1.0 (21)
Environmental Studies	1	0.5 (25)	1	1.0 (22)
Genetics & Heredity	1	0.5 (26)	-	-
Physiology	1	0.5 (27)	-	-
Food Science & Technology	-	-	2	2.0 (17)
Critical Care & Medicine	-	-	1	1.0 (20)
Integrative & Complementary Medicine	-	-	1	1.0 (23)
Nursing	-	-	1	1.0 (25)
Veterinary Sciences	-	-	1	1.0 (26)

-, no records found.

More than 3200 papers representing over 100 subject categories cited the 201 publications on sponge chemical ecology, whereas the 101 records published on microbial ecology were cited by more than 1700 publications that represented over 90 categories. This large number of subject categories compared with the relatively low number of categories that represented the chemical and microbial research output (i.e., around 30) implies a great interest in sponge chemical and microbial ecology not only by the scientific community from the same research area but by other scientific disciplines. Whereas a goodness of fit (Sokal and Rohlf, 1995) showed no significant differences in the proportion of articles in specific subject categories between the 201 publications on chemical ecology and the publications that cited them ($G = 7.075$, $df = 14$, $P = 0.932$), significant differences were detected between

the 101 articles published on microbial ecology and those that cited them (G = 37.021, df = 15, $P = 0.001$). Although the proportion of articles in most of the categories was similar on sponge chemical ecology, the proportion of articles published was slightly greater in Multidisciplinary Sciences category while the articles cited showed a greater percentage in the categories Biotechnology & Applied Microbiology and Fisheries (Fig. 2.2). Contrastingly on sponge microbial ecology, the proportion of articles published exhibited a twofold increase compared with those cited in subject categories such as Environmental Sciences, Multidisciplinary Geosciences, Limnology, or Food Science & Technology and a sixfold increase in Paleontology and Geology categories (Fig. 2.2). The proportion of articles cited approximately doubled the proportion of articles published in subject categories such as Biotechnology & Applied Microbiology, Medicinal Chemistry, Pharmacology & Pharmacy, Plant Sciences, or Biology and exceeded by almost 500% in Biochemistry & Molecular Biology category (Fig. 2.2).

Figure 2.2. (next page) Increasing rate (%) of articles on chemical (A) and microbial (B) ecology published ([published-cited]/cited; in gray) or cited ([cited-published]/published; in black) within several subject categories found in the Science Citation Index Expanded® database. Subject categories were ordered within published or cited articles as a function of the percentage of the total publications represented by each category in descending order. The remaining categories were excluded either because the proportions of articles published or cited were equivalent (i.e., the relative value did not deviate from 1) or because the percentage of the total publications in a subject category was below 1%. Gray bars show higher proportion of chemical and microbial ecology papers published than cited, whereas the opposite is true for black bars. Mar Freshwat Biology: Marine & Freshwater Biology; Biochem Molec Biology: Biochemistry & Molecular Biology; Multidiscipl Sciences: Multidisciplinary Sciences; Biotech Appl Microbiol: Biotechnology & Applied Microbiology; Pharmacol & Pharmacy: Pharmacology & Pharmacy; Chemistry, Multidiscipl: Chemistry, Multidisciplinary; Geosciences, Multidisc: Geosciences, Multidisciplinary; Food Science Technol: Food Science & Technology.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Pharmacol & Pharmacy
Plant Sciences
Biology
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2.4 Sponge chemical and microbial ecology: Past, Present and Future

One of the simplest ways to evaluate the "healthy" status of a research discipline is looking at the impact of its investigations on the scientific community. With that purpose, we quantitatively analyzed references published on sponge chemical and microbial ecology gathered within the Science Citation Index Expanded® database. This quantitative analysis provides a historical view of these two disciplines over the years, a current diagnose of their status, and opportunities to improve future research on these topics.

According to our search history, literature on sponge chemical and microbial ecology appeared during the seventies. Despite the simultaneous appearance of both sponge areas, chemical ecology seems to be more productive and its impact on scientific community, as evaluated by the *h* index, is greater than those of microbial ecology. The low productivity of sponge microbial ecology may be a consequence of the low number of contributors to this particular research field. 90% of the researchers had only one publication and about 1% contributed with more than 5 papers. However, percentages were not much better in chemical ecology area where 80% of the researchers contributed with a single publication, 2% of the contributors had between 5 and 9 papers and just 1% contributed with 10 or more publications. This difference suggests that sponge chemical and microbial ecologies lack specialist researchers with a clear background in these disciplines as occurred in the broad field of sponge ecology (Becerro, 2008). So, there is a chance to growth for chemical and microbial ecology areas within the taxonomic group of Porifera.

Reviews are crucial and useful tools to collect information and update the status of a particular research field. Although reviews showed the highest average number of citations per publication on chemical and microbial ecology research, the impact of this publication type was minor compared with regular articles that showed higher *h* values. Almost 20% of the publications on

sponge chemical and microbial ecology were reviews doubling the percentage of reviews on sponge ecology (updated to 2012 from Becerro, 2008). This may suggest an overproduction of reviews in both areas within sponge ecology field that deal with topics highly related with less relevance than it would be expected.

A huge impact on the scientific community is derived from the large number of subject categories that cited publications on sponge chemical and microbial ecology. Chemical ecology research on sponges is highly cited by Biotechnology and Applied Microbiology, and Fisheries categories compared with the percentage of articles on chemical ecology published in the same categories. Sponge microbial ecology, however, is extensively cited by the categories Biotechnology and Applied Microbiology, Medicinal Chemistry, Biochemistry & Molecular Biology, Pharmacology & Pharmacy, and Biology. In both cases, the demand for information on chemical and microbial ecology basically comes from applied disciplines. Since many microbial symbionts were found to produce bioactive compounds that can be used for their biotechnological potential, microbe-sponge interactions have attracted increasing attention from applied research (Wang, 2006; Dunlap et al., 2007; Egan et al., 2008; Hentschel et al., 2012), even the ecological perspectives of these associations have become essential to gain knowledge on how those bioactive metabolites are produced (Taylor et al., 2007b). Hence, applied microbiology (i.e., organism manipulation to make products or solve problems to meet human needs) fuels part of its research by citing publications on sponge chemical and microbial ecology and applied chemistry (i.e., drug discovery and pharmacognosy) does so by citing research on sponge microbial ecology.

On the other hand, there is a slightly lack of citations from the subject categories Marine & Freshwater Biology, Ecology, and Microbiology, which accounted for the major number of publications on chemical and microbial ecology, in contrast to what we expected. This suggests that the ecological perspectives of sponge chemistry and microbiology may fall outside the interests of

researchers that published their works in those same categories and not meet the standards of ecology. The reason may fall on many sponge ecology studies that focus their attention on the species level and, consequently, their impact on the ecologist community decrease as suggested by Becerro (2008).

Overall, sponge chemical and microbial ecology are close disciplines that have been proved useful for applied sciences and will continue contributing with significant works to seek the putative roles of natural products isolated from sponge-symbiont associations and to better understand these complex interactions. Both research areas have the chance to keep appealing to pharmacologists and biotechnologists and finally meet the standards of broader ecological issues by exploiting the trait that makes sponges particularly relevant, symbiosis, perhaps the most ancient one within metazoans (Taylor et al., 2007a).

2.5 Chemical and microbial methodologies

To address chemical and microbial issues in my PhD dissertation, two analytical techniques have been used for their long background in both research fields and for the adequacy to the study of sponge chemistry and associated microbial assemblages. We used high-performance liquid chromatography (HPLC) to study sponge-derived secondary metabolites and denaturing gradient gel electrophoresis (DGGE) to analyze the sponge microbial consortium. For those who are unfamiliar with these techniques, I expose the basic principles behind these two analytical methods.

HPLC is a popular method of analysis not dependent of the volatility or stability of the sample compound. It has many applications including separation, identification, purification, and quantification of various compounds (Lindsay and Kealey, 1987).

Chemical separation. Given a particular column and mobile phase, chemical compounds differ in migration rates, which allow compound separation. Thus, the extent or degree of separation is mostly determined by the choice of a stationary phase and mobile

phase. The former refers to the solid support contained within the column over which the mobile phase continuously flows. The latter refers to the solvent being continuously injected to the column, or stationary phase. The mobile phase acts as a carrier for a sample solution. As the sample solution flows with the mobile phase through the stationary phase, the components of that solution will migrate according to the non-covalent interactions of the compounds with the stationary phase (Lindsay and Kealey, 1987).

Purification. Refers to the process of separating or extracting the target compound from other compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic conditions (Lindsay and Kealey, 1987).

Identification. To identify any compound a detector (i.e., the component that emits a response due to the eluting sample compound and, subsequently, signals a peak on the chromatogram) must first be selected. After that, a separation assay must be developed. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks. Identifying a compound by HPLC can be accomplished comparing retention times with the literature and further nuclear magnetic resonance analysis (Lindsay and Kealey, 1987).

Quantification. It is the process of determining the unknown concentration of a compound in a solution. A calibration curve is obtained injecting a series of known concentrations of the pure compound, resulting in a series of peaks that correlate to the concentration of the compound injected. Then, the chromatographer calculates the area under each peak and plots the relation between peak area and the concentration of the pure compound (calibration curve). Hence, you have the equation of the calibration curve to calculate the concentration of the compound in the unknown sample (Lindsay and Kealey, 1987).

DGGE is a genetic fingerprinting technique of polymerase chain reaction (PCR)-amplified ribosomal DNA fragments that provides a pattern or profile of genetic diversity in a microbial community (Muyzer et al., 1993; Muyzer and Smalla, 1998). This

molecular technique is based on the separation of DNA fragments of the same length but with different sequences. Separation occurs due to the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide). The melting of DNA fragments proceeds in discrete melting domains (i.e., stretches of base-pairs with an identical melting temperature). Once a domain with the lowest melting temperature reaches its melting temperature at a particular position in the denaturing gel, a transition of a helical to a partially melted molecule occurs and migration of the molecule will practically stop. Sequence variation within such domains causes the melting temperatures to differ. Molecules with different sequences will therefore stop migrating at different positions in the gel (Muyzer et al., 1993; Muyzer and Smalla, 1998).

In order to increase the percentage of sequence variant detection, a guanine-cytosine-rich sequence (so-called GC-clamp) is added to the 5'-end of one of the PCR primers. Thus, GC-clamp is introduced into the amplified DNA fragments and acts as a high melting domain preventing the two DNA strands from complete dissociation into single strands (Muyzer and Smalla, 1998).

After loading samples in different lanes and running the gel, DGGE gels are stained using DNA staining probes. The result is a pattern of bands for which the number of bands of each lane corresponds to the number of predominant microbial members in the corresponding sample and bands in the same position in the gel correspond, normally, to one bacterium's type gene. Afterwards, sequencing of the bands allows identification of the bacterial type sequence identity (Muyzer and Smalla, 1998).

DGGE of PCR-amplified 16S rDNA is widely used in many studies such as assessment of community complexity and community changes, monitoring the enrichment and isolation of bacteria, or detection of microheterogeneity in rRNA encoding genes (Muyzer et al., 1993; Brinkhoff and Muyzer, 1997;

Casamayor et al., 2000; Casamayor et al., 2002; Webster et al., 2008b).

3
Objectives

This PhD dissertation has been conceived to study the chemical and microbial ecology of sponges using the demosponge *Aplysina aerophoba* (Nardo, 1833) as a model species because its secondary chemistry and its associated microbial community are well studied and it is quite abundant in our study area (Northwestern Mediterranean and Canary Islands). Beyond the knowledge achieved about the major chemistry and bacterial assemblages in *A. aerophoba*, we have been able to explore the variation sources of the natural products and the sponge microbial consortium. Additionally, we have assessed the putative relationships between the host bacterial community and the production of secondary metabolites in this species.

This PhD dissertation is divided in two sections. The first part includes an observational approach to assess the natural variability of sponge chemistry and bacterial assemblages while the second part corresponds to an experimental approach to test the effect of environmental factors on both parameters. The detailed objectives are given below:

Part I: Natural variability of secondary metabolites and sponge-associated bacterial community

This section includes three chapters with a defined scale each one that share the specific objective of seeking at which scale natural products and microbial consortium vary the most to investigate the factors behind the observed patterns of variability.

- **Chapter 4** explores changes in the abundance of secondary metabolites and the relative composition of bacterial assemblages in *Aplysina aerophoba* at multiple intraindividual scales (between chimney-like structures, within chimneys, and between tissue layers). This study also investigates the relationship between natural products and microbial community structure by assessing whether both parameters covary at an intraspecimen scale (Sacristán-Soriano et al., 2011b).

- **Chapter 5** compares the concentration of natural products and chlorophyll *a* (used as a proxy of phototrophic bacteria) in *A. aerophoba* at multiple spatial scales from neighboring bays to distant biogeographic regions over 2500 km apart (Northwestern Mediterranean and Canary Islands). This design allows us to investigate which is the magnitude of the variance attributable to the distinct spatial scales and the most relevant scale at which the concentration of secondary metabolites and chlorophyll *a* vary. This study also evaluates the relationship between the abundance of bioactive compounds and concentrations of chlorophyll *a* (Sacristán-Soriano et al., 2011a).

- **Chapter 6** compares the abundance of secondary metabolites in *A. aerophoba* at multiple temporal scales from months to seasons, and years, and between the outer sponge layer (i.e., ectosome) and the inner core (i.e.,

choanosome). As in **Chapter 5**, the approach used allows us to investigate which is the magnitude of the variance that can be ascribed to the distinct temporal scales and the most relevant scale at which the abundance of natural products varies (Sacristán-Soriano et al., 2012).

Part II: Effects of environmental factors on secondary metabolites and sponge-associated bacterial community

In this section, we moved from an observational to an experimental approach to test for the effect of environmental factors such as light on the production of natural products and on the structure of the bacterial community. This part includes a single chapter that deals with this specific objective.

- **Chapter 7** experimentally addresses whether different light exposures can modify chemical and microbial profiles in the external and internal regions of *Aplysina aerophoba*. This study also investigates if changes in secondary metabolites and associated bacteria are correlated each other (Sacristán-Soriano et al submitted).

Part I: Natural variability of secondary metabolites and sponge-associated bacterial community

4

Exploring the links between natural products and bacterial assemblages in the sponge *Aplysina aerophoba*

Resumen

Aplysina aerophoba presenta una gran diversidad de alcaloides bromados y alberga una compleja comunidad microbiana. A pesar de que dichos compuestos han sido localizados en el interior de células de la esponja, las enzimas que se encargan de incorporar los elementos halógenos a los compuestos orgánicos se han descrito exclusivamente en algas, hongos, y bacterias. La comunidad bacteriana asociada a *Aplysina* podría entonces estar involucrada en la síntesis de estos compuestos. Con este estudio investigamos si los cambios observados tanto en la concentración de los compuestos bromados como en la comunidad microbiana asociada estaban correlacionados. Para acometer dicho objetivo, se cuantificaron los productos naturales mayoritarios mediante cromatografía líquida de

alta resolución y se analizaron las poblaciones bacterianas mediante electroforesis en gel con gradiente desnaturalizante empleando el gen ribosómico 16S rRNA como marcador. Identificamos asociaciones múltiples entre bacterias y productos naturales, principalmente entre una bacteria del grupo *Chloroflexi* y el compuesto aplysinamisin-1 y entre una bacteria no identificada y los compuestos aerophobin-2 e isofistularin-3. Estos resultados sugieren que dichas bacterias podrían estar implicadas en la producción de los alcaloides bromados, o bien, afectadas por ellos. Este estudio es uno de los primeros en documentar una correlación significativa entre productos naturales y poblaciones bacterianas en un organismo bentónico. Profundizar en el estudio de estas asociaciones sería beneficioso para entender la organización y funcionamiento de estos sistemas huésped-simbionte como *Aplysina aerophoba*.

Abstract

The sponge *Aplysina aerophoba* produces a large diversity of brominated alkaloids (BAs) and hosts a complex microbial assemblage. Although BAs are located within sponge cells, the enzymes that bind halogen elements to organic compounds have been exclusively described in algae, fungi, and bacteria. Bacterial communities within *A. aerophoba* could therefore be involved in the biosynthesis of these compounds. This study investigates whether changes in both the concentration of BAs and bacterial assemblages are correlated in *A. aerophoba*. To do so, we quantified major natural products using high-performance liquid chromatography and analyzed bacterial assemblages using denaturing gradient gel electrophoresis on the 16S rRNA gene. We identified multiple associations between bacteria and natural products, including a strong relationship between a *Chloroflexi* phylotype and aplysinamisin-1, and between an unidentified bacterium and aerophobin-2 and isofistularin-3. Our results suggest that these bacteria could either be involved in the production of BAs

or be directly affected by them. To our knowledge, this is one of the first reports that find a significant correlation between natural products and bacterial populations in any benthic organism. Further investigating these associations will shed light on the organization and functioning of host-endobiont systems such as *Aplysina aerophoba*.

4.1 Introduction

Many sponges are known to be associated with large amounts of bacteria (Moore, 2006) that can amount up to 40% of the sponge biomass (Unson et al., 1994; Friedrich et al., 2001). These types of sponges have been referred to as high-microbial-abundance sponges (Friedrich et al., 1999; Webster et al., 2001; Hentschel et al., 2002; Webster et al., 2004; Hentschel et al., 2006). While a fraction of the bacterial assemblage is used for sponge nutrition (Ruppert and Barnes, 2004), another fraction can be permanently associated with sponges (Friedrich et al., 2001). This fraction is highly diverse and phylogenetically complex, with representatives from several phyla (e.g., *Proteobacteria, Chloroflexi, Cyanobacteria, Actinobacteria, Acidobacteria*, and *Bacteroidetes* (Friedrich et al., 1999; Webster et al., 2001; Hentschel et al., 2002; Webster et al., 2004; Hentschel et al., 2006; Taylor et al., 2007b)). Variability in sponge-associated microbial communities has been assessed at several levels, including within and between specimens of the same host species (Althoff et al., 1998; Hentschel et al., 2002; Taylor et al., 2004; Webster et al., 2004; Taylor et al., 2005; Thiel et al., 2007b), between sponge species (Hentschel et al., 2002; Taylor et al., 2004), and temporal or spatial distribution within sponges (Friedrich et al., 2001; Webster and Hill, 2001; Fieseler et al., 2004; Taylor et al., 2004; Usher et al., 2004; Webster et al., 2004; Hoffmann et al., 2006).

Sponges are also known as a rich source of natural products or secondary metabolites (Faulkner et al., 1994; Weiss et al., 1996; Faulkner et al., 2000; Moore, 2006), many of which have the

potential to be used in pharmaceutical and biotechnological applications (Osinga et al., 1998; Sipkema et al., 2005a; Sipkema et al., 2005b; Taylor et al., 2007b). Understanding the true origin of sponge-derived compounds may help obtain the required amount of specific potential drugs to set up clinical trials. However, the actual biosynthetic pathways and true producers of most sponge natural products are uncertain and remain to be investigated. Some natural products seem to have a sponge origin since they are located within sponge cells (Uriz et al., 1996; Turon et al., 2000). Other compounds are associated with microbial symbionts, suggesting that microbes are the true producers (Faulkner et al., 1994; Unson et al., 1994; Althoff et al., 1998; Faulkner et al., 2000). However, location does not necessarily imply production. Microbes are known to actively excrete their natural products into the surrounding medium (Moore, 2006), and the synthesis of natural products could be accomplished in several of the multiple cell components present in sponges (Kreuter et al., 1992).

Sponges of the *Aplysinidae* family are a rich source of microorganisms (Vacelet, 1975; Friedrich et al., 2001; Thoms et al., 2003) and also contain high concentrations of brominated alkaloids (BAs) (Friedrich et al., 1999; Friedrich et al., 2001; Hentschel et al., 2001). These BAs seem to be located within sponge cells, suggesting a biosynthesis by the sponge (Turon et al., 2000). However, bromoperoxidase enzymes (responsible for the halogenation reaction that incorporates the bromine into the compound) have only been reported for bacteria, algae, fungi, and plants (Baden and Corbett, 1980; van Pée, 1990, 1996; Verdel et al., 2000; van Pée et al., 2006). Ebel et al. (1997) suggest that bacteria may produce the enzymes necessary to transform some of the secondary metabolites in *Aplysina aerophoba*. The possibility that sponge-associated bacteria, algae, or fungi might be taking part in the biosynthesis of these compounds cannot be ruled out, making it possible that the production of these BAs is a joint effort of multiple cell components in the sponge (Kreuter et al., 1992). To date, the majority of studies report the presence of compounds in specific cell

compartments or show qualitative associations between secondary metabolites and bacteria (Unson and Faulkner, 1993; Unson et al., 1994; Flowers et al., 1998; Turon et al., 2000). Quantitative data supporting a relationship between bacterial abundance and concentration of natural products is lacking, let alone experimental evidence for this hypothesis, which would benefit from a better understanding of the links between bacterial strains and concentration of natural products.

In the present study, we assess the relationship between microbial community structure and natural products in the sponge *Aplysina aerophoba* (Nardo, 1833). BAs are known to vary within this sponge (Kreuter et al., 1992; Turon et al., 2000), and we sought to detect whether the bacterial community structure covaried with the concentration of natural products at an intraspecimen scale. To achieve our goal, we used high-performance liquid chromatography (HPLC) and denaturing gradient gel electrophoresis (DGGE) to investigate the relative abundance of major secondary metabolites and the complex bacterial assemblages found in multiple tissues of *Aplysina aerophoba*. The resulting chemical and bacterial matrices were analyzed with a variety of uni- and multivariate statistical methods that can prove useful in the field of microbial ecology.

4.2 Materials and methods

4.2.1 Sampling

In spring 2006, the sponge *Aplysina aerophoba* (formerly known as *Verongia aerophoba* [Nardo, 1833]) was collected by scuba diving at four locations along the Costa Brava, northwestern Mediterranean, between 3 and 14 m in depth. We collected several specimens from each location to obtain enough material for the bulk chemical extraction necessary to set up the chemical methods. We also collected one specimen from each location to assess intraspecimen variation in both natural products and the bacterial community.

To investigate intraspecimen variability, we randomly chose two chimney-like structures for each sponge. We cut the chimneys underwater and placed them in plastic bags with seawater. Then, we placed the sealed plastic bags in a cooler with ice to transfer them to the laboratory (2 to 3 h). Each chimney was divided into apical and basal zones, and we took samples from the ectosome and choanosome of the sponge from both zones with a sterilized scalpel under seawater (see Fig. S4.1). Apical and basal zones refer to the first top and bottom centimeter of a chimney-like structure. Typically, a distance of 2 to 3 cm separates the apical and basal zones. Ectosome refers to the 2 to 3 mm outer layer of sponge, with a greener or more purple color due to the presence of *Cyanobacteria* (Turon et al., 2000; Becerro et al., 2003), while choanosome refers to the cyanobacterium-free inner layer of the sponge. Sponge samples were always under water to prevent the compound degradation that this sponge experiences in contact with air (as the species name "*aerophoba*" indicates). For each individual sponge, we had a total of eight samples (2 chimneys × 2 zones × 2 tissues) for natural product quantification and bacterial analyses.

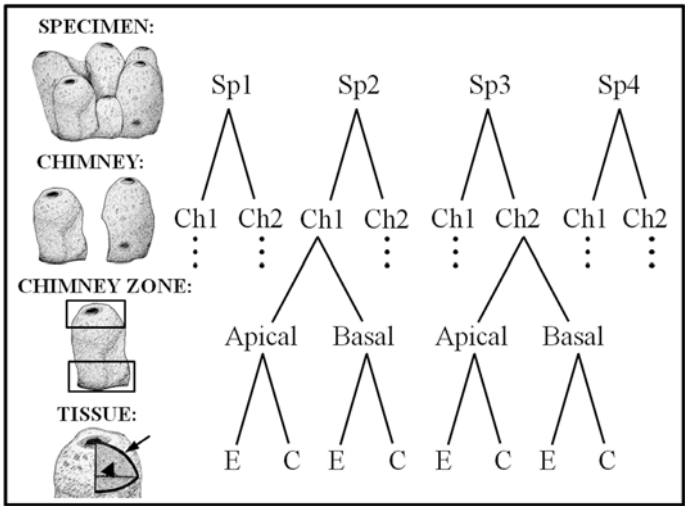


Figure S4.1. (previous page) Sampling design of Aplysina aerophoba. We sampled four Aplysina specimens (Sp1 to Sp4) and we randomly chose two chimney-like structures (Ch1 and Ch2) from each specimen. We divided each chimney (we only show two chimneys, the others follow the same pattern) into apical and basal zones (rectangular boxes) and we took one sample from the ectosome (E; arrow) and one from the choanosome (C; arrowhead).

4.2.2 Secondary metabolite isolation and identification

Sponge tissues were frozen at −20°C, freeze-dried, and extracted three times (1 h, 1 h, and overnight) with methanol (MeOH; 20 ml of MeOH per 1 g of sponge). The combined extracts were concentrated by vacuum rotary evaporation, leaving a powdery organic residue. To isolate the major compounds observed in preliminary HPLC analyses, the organic extract was first fractionated by flash chromatography using VWR LaFlash equipment on an Analogix Sepra C18 (SF25-55g) cartridge eluted with increasing amount of methanol in water, resulting in four fractions.

Fraction 1 (eluted with 30% MeOH) and fraction 3 (eluted with 100% MeOH), contained the major compounds and were further purified by semi-preparative reversed-phase HPLC (Waters 1525 binary HPLC pump and Waters 2487 dual λ absorbance detector) on a Phenomenex Gemini RP-18 (250 by 10 mm, 5 μm) column. The elution conditions consisted of 30% MeOH in wáter (peaks 1 to 5) and 75% MeOH in water (peak 6) and a flow rate of 2.5 ml min^{-1} with UV detection at 245 nm. These conditions led to purification of the four known compounds aerophobin-1 (5 mg), aerophobin-2 (5 mg), aplysinamisin-1 (7 mg), and isofistularin-3 (12 mg). These four compounds were characterized by proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR; JEOL EX 400 spectrometer), liquid chromatography-mass spectrometry (LC-MS; Thermo Scientific LCQ Fleet), UV spectrometry (Hewlett-Packard diode array spectrophotometer), and comparison of spectroscopy data with published values from the literature.

4.2.3 HPLC analysis and compound quantification

HPLC analyses were performed with a system from Waters, including the Alliance separations module 2695, the column heater, and the 2998 photodiode array detector. The equipment was controlled and the data were handled by the Empower Chromatography Data software (Waters). The HPLC conditions consisted of two eluents (eluent A [0.1% aqueous trifluoroacetic acid] and eluent B [acetonitrile]) and an elution profile based on a linear gradient from 30% eluent B to 80% eluent B within 18 min and then to 100% eluent B for an additional 10 min. Flow rate was kept constant at 0.4 ml min^{-1}. We used a Phenomenex Synergi Max-RP (80 Å, 250 by 3.0 mm, 4 µm) analytical column with a fixed temperature of 30°C.

For the quantification of the natural products, 30 mg of freeze-dried sponge tissue from each ectosomal and choanosomal samples were extracted three times with 1.5 ml of MeOH in an ultrasonic tank for 15 min each time. The crude extract was filtered through a 20-µm-pore-size polytetrafluoriethylene filter (PTFE) and added in a 5-ml beaker. The final volume was adjusted to 5 ml of crude extract solution, and an aliquot of 1.5 ml was passed through a 13-mm, 0.2-µm-pore-size PTFE syringe-filter before HPLC injection. Then, 10 µl of this filtered solution was injected into the HPLC system described above. The brominated compounds were detected at 245 nm from the data collected across the 210- to 800-nm wavelength range. Peaks were integrated by applying the detector response based on peak areas to calibration curves obtained using the previously purified and characterized compounds as external standards. The final amount of natural compounds was calculated by averaging three replicate injections. Concentrations of brominated compounds were expressed as mg per g (dry mass) of sponge tissue.

4.2.4 DNA extraction and PCR amplification

DNA was extracted from ~1 mm3 of ethanol-preserved (100% final concentration) sponge tissue (2 to 3 mg [wet mass]) using a DNeasy tissue kit (Qiagen), the effectiveness of which has been tested for environmental samples (Simonelli et al., 2009), according to the manufacturer's instructions with the following modifications recommended in the troubleshooting guide of the Qiagen kit: (i) lower amount of sample processed (2 to 5 mg), (ii) higher proteinase K digestion time (3 to 5 h, until the tissue looked well digested), and (iii) a final elution step with 40 µl of elution buffer (10 mM Tris-Cl, 0.5 mM EDTA [pH 9.0]) and incubation at room temperature for 10 min. DNA extracts were run in an agarose gel to check integrity and concentration using a standard mass ladder (DNA Smart Ladder; Eurogentec, Belgium). Small differences in yield extraction were visualized among samples. However, such differences were not expected to produce qualitative changes in the DNA mixtures, and the results were normalized using relative abundances in the fingerprinting analysis for an accurate intersample comparison.

PCR amplification of bacterial 16S rRNA gene suitable for subsequent genetic fingerprinting analysis was carried out using the universal bacterial primer combination BAC358F (5'-CCT ACG GGA GGC AGC AG-3') with a 40-nucleotide GC-rich sequence attached to the 5' end (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3') and BAC907RM (5'-CCG TCA ATT CMT TTG AGT TT-3'), which amplify a fragment approximately 568 bp long as described elsewhere (Casamayor et al., 2000). 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 of sterilized MilliQ water, 5 µl of 10× reaction buffer, 1.5 µl of $MgCl_2$ (50 mM), 1 µl of

deoxyribonucleoside triphosphates (10 mM each), 2.5 µl of each primer (10 µM), 2.25 µl of bovine serum albumin (6 mg/ml), 0.25 µl of EcoTaq polymerase (Ecogen; 5 U/µl), and 1 µl of DNA template. The amounts of DNA template ranged between 10 and 100 ng of DNA for the different samples. Within such range we did not observe any remarkably loss/gain of DGGE bands in the fingerprinting analysis. PCR products were run in an agarose gel using a standard mass ladder (DNA Smart Ladder; Eurogentec, Belgium) to quantify the PCR product obtained in each case (Casamayor et al., 2000; Demergasso et al., 2008).

4.2.5 DGGE analysis of PCR products and sequencing

DGGE was performed by using a Bio-Rad DCode universal mutation detection system (Bio-Rad) on a 6% polyacrylamide gel in 1× TAE (40 mM Tris base, 20 mM sodium acetate trihydrate, 1 mM EDTA). A comparable amplicon mass for each sample (c. 600 ng of PCR product) was added on the DGGE, and the gels were run for 4 h at a constant voltage of 200 V and 60°C in a 45 to 70% vertical denaturant gradient (100% denaturant agent is 7 M urea and 40% deionized formamide) (Casamayor et al., 2000). After electrophoresis, gels were stained for 45 min with SYBR Gold nucleic acid stain (Molecular Probes) and photographed with the UV GelDoc system (Uvitec). Image files were processed with the NIH Image software (National Institutes of Health, Bethesda, MD), and the relative band intensities were measured (Demergasso et al., 2008). Although not free of limitations, the signal intensity of the DGGE bands has been shown to be a useful tool for calculating the relative percentages of the different groups (Casamayor et al., 2000), and analysis of absence/presence data offered the same conclusions here (see Results). Obviously, absolute quantitative data on the abundance of specific bacteria requires the use of other techniques ((Casamayor et al., 2000) and references therein).

Prominent bands were excised from the gel, resuspended in 25 µl of MilliQ water, and stored at 4°C overnight. An aliquot (2 to

5 µl) of the supernatant was used for PCR reamplification with the original primer set, and the PCR product was sequenced by using external sequencing facilities (Macrogen). Sequences were sent to BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) to get a first indication of what sequences were retrieved. Sequences with >97% sequence identity to a cultured nearest phylogenetic neighbor in 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.

4.2.6 Data analysis

We used several statistical methods available in PRIMER 6 software (Clarke and Warwick, 2001) to analyze data on secondary metabolites and bacteria of *Aplysina aerophoba* as a function of specimen (four specimens), chimney (two chimneys for each specimen), chimney zone (top and bottom), and tissue type (ectosome and choanosome). Standardized and square root-transformed data were used to calculate Bray-Curtis similarity, and permutational multivariate analysis of variance (PERMANOVA) was used to test for differences in secondary metabolite and bacterial data together across the four factors. We also performed two independent PERMANOVAs on chemical and bacterial data using exclusively factors that proved significant in the previous analysis. In addition, we tested the effect that the DGGE gel might impose in the analysis of the bacterial community with a PERMANOVA with DGGE gel as a factor. Taking into account the PCR bias and the limitations of the DGGE as a quantitative technique (Muyzer and Smalla, 1998), we also used presence/absence data to validate the method.

We used ANOVA and the nonparametric Mann-Whitney U test from Systat 12 software (SPSS, 1999a) to analyze separately each secondary metabolite and each OTU across significant factors. We also performed a Mantel test (based on similarity matrices) to investigate whether there was a relationship between secondary

metabolites and bacteria. We constructed two independent matrices from secondary metabolites and bacterial data and calculated a measure of similarity between each point and all of the others. The Mantel test correlates the two n × n similarity matrices (Fortin and Gurevitch, 2001) and detects any association between the components of both matrices (secondary metabolites and bacteria in our study). Mantel tests cannot point to any specific subset of data (i.e., any specific secondary metabolites and bacteria) responsible for an overall association, so further analyses are required to identify specific relationships.

Because of the large number of secondary metabolites and bacteria analyzed in the present study, we used factor analysis to look for coherent groups of chemical and bacterial variables that were correlated with one another within groups but largely independent between groups (Tabachnick and Fidell, 2001). These groups of correlated variables or factors help interpret the underlying mechanisms that have created the relationship between variables. 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 since it minimizes the number of variables that load highly on a factor and maximizes the loading variance across factors. The resulting independent factors were used as variables in a canonical correlation analysis to test whether any chemical and bacterial factors were correlated. We then used simple correlation analysis from Systat 12 software (SPSS, 1999a) to establish the quantitative relationship behind the actual chemical and bacterial variables in the correlated factors.

4.2.7 Nucleotide sequence accession numbers

The 16S rRNA gene sequences were deposited in GenBank under accession numbers AM905024 to AM905030.

4.3 Results

4.3.1 Natural product and bacterial profiles of *Aplysina aerophoba*

We quantified a total of 32 samples (4 individual sponges x 2 chimneys for each sponge x 2 zones for each chimney x 2 tissues for each zone) to characterize the chemical profile. We found six major peaks in the crude extract of *A. aerophoba*. We labeled the peaks according to their retention time: peak 1, peak 2, peak 3, peak 4, peak 5, and peak 6 (Fig. S4.2). We identified four out of the six major peaks: aerophobin-1 (Aero1; peak 3), aerophobin-2 (Aero2; peak 4), aplysinamisin-1 (Aply1; peak 5) and isofistularin-3 (Iso3; peak 6) according to their retention times and their UV profiles in comparison with purified and characterized compounds. We used two DGGE gels to characterize the bacterial profiles of the same 32 samples previously analyzed for the chemical profile. We identified a total of 24 different band positions and we assigned each position to an OTU (Fig. S4.3). Of these 24 OTUs, 15 were shared by all specimens except for OTUs 4, 11, and 19, which were restricted to three specimens. The remaining 9 OTUs were uncommon and were restricted to a particular sample or gel.

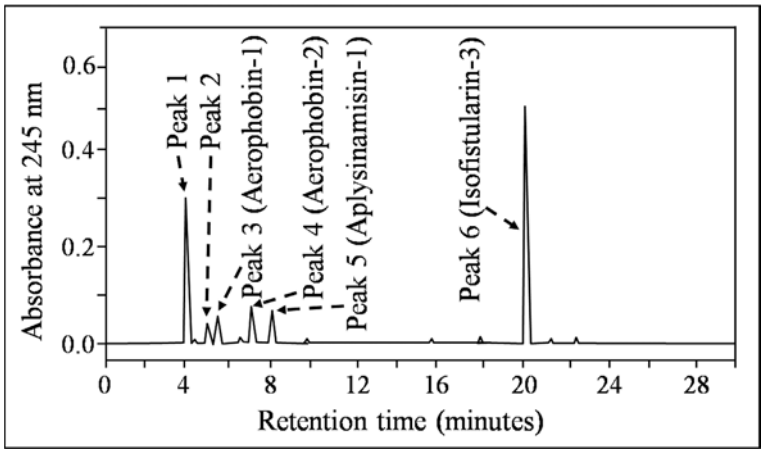


Figure S4.2. Example of HPLC chromatogram for Aplysina aerophoba. This chromatogram was obtained from a choanosome tissue from l'Escala specimen and has been edited to remove the solvent peak. The six major compounds we found are peak 1, peak 2, peak 3 (aerophobin-1), peak 4 (aerophobin-2), peak 5 (aplysinamisin-1) and peak 6 (isofistularin-3). See text for HPLC conditions.

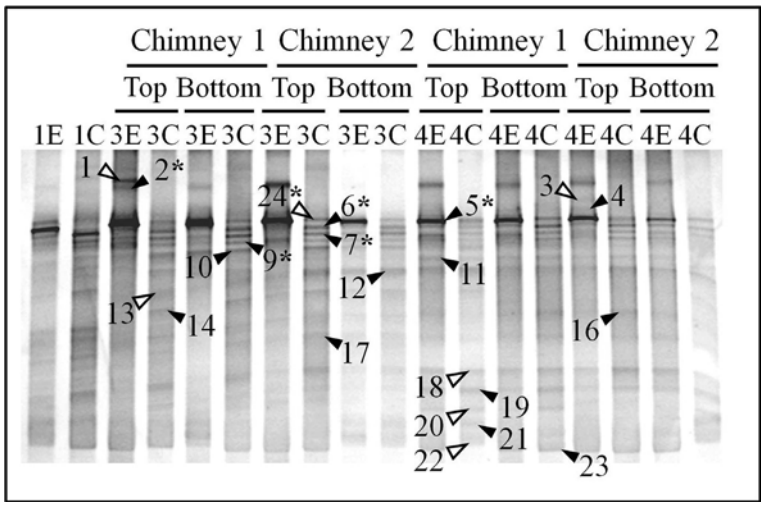


Figure S4.3. (previous page) Example of a 16S rDNA-DGGE gel with samples of Aplysina aerophoba from three localities: l'Escala (1) Tamariu (3) and Sant Feliu (4). The two samples from l'Escala were used to compare DGGE bands. We had eight samples from each specimen (2 chimneys × 2 top/bottom zones × 2 ectosome (E) and choanosome (C) tissues). Numbers (1-24) show the OTUs that were identified. Asterisks (*) show the OTUs that were sequenced. Filled arrowheads show OTUs shared by all the specimens and opened arrowheads show sample-specific OTUs.

Using both chemical and bacterial data, we found significant differences between specimens (PERMANOVA, $P = 0.001$) and between ectosome and choanosome tissues (PERMANOVA, $P = 0.018$) (Fig. 4.1). We found no differences between chimneys or between the chimney top and bottom zones (PERMANOVA, $P = 0.118$ and $P = 0.155$, respectively). We therefore ran subsequent independent PERMANOVAs analyses on chemical and bacterial data using the significant factors "specimens" and "tissue type".

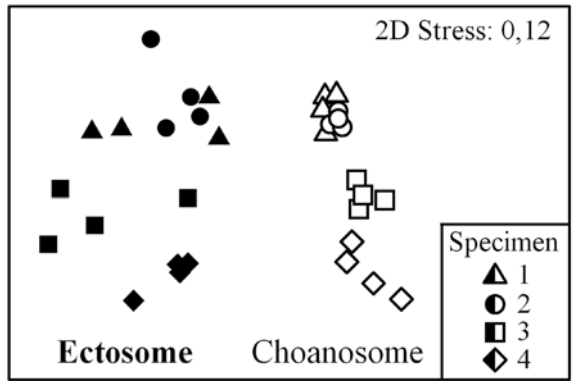


Figure 4.1. Nonmetric multidimensional scaling based on Bray-Curtis similarity matrices from standardized and square root-transformed abundances of chemical and bacterial data. Significant differences between tissues and specimens (obtained by PERMANOVA) are shown. See the text for details on PERMANOVA.

4.3.2 Natural product variation in *Aplysina aerophoba*

Ectosome and choanosome tissues significantly differed in their secondary chemistry (PERMANOVA, $P = 0.002$). Aero2, Aply1, and Iso3 were more abundant in choanosome tissues than in ectosome tissues (ANOVAs, $P = 0.013$, $P = 0.004$, and $P = 0.001$, respectively), while Aero1 showed no differences between tissues (Mann-Whitney U test, $P = 0.678$) (Fig. 4.2). We found nonsignificant differences in the secondary chemistry between specimens (PERMANOVA, $P = 0.371$).

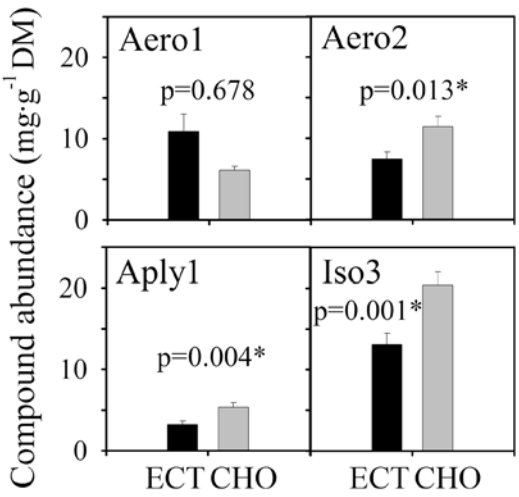


Figure 4.2. Secondary metabolite concentrations (mg g [dry mass] of sponge tissue^{-1} ± 1 standard error) of the sponge *A. aerophoba* between ectosome (ECT) and choanosome (CHO) layers. Asterisks (*) indicate significant differences between tissues ($P \leq 0.05$). Aero1, aerophobin-1; Aero2, aerophobin-2; Aply1, aplysinamisin-1; Iso3, isofistularin-3.

4.3.3 Bacterial community variation in *Aplysina aerophoba*

We found highly significant differences in the bacterial assemblage between ectosome and choanosome tissues (PERMANOVA, P = 0.001) but differences between specimens were not significant (PERMANOVA, P = 0.542).

We found a gel effect when we compared the bacterial community between DGGE gels (PERMANOVA, P = 0.004), so we analyzed both gels separately. In both gels, we found significant differences between ectosome and choanosome tissues (PERMANOVA, P = 0.001 for both gels) and nonsignificant differences between specimens (PERMANOVA, Gel 1, P = 0.695; Gel 2, P = 0.638). Analysis of presence/absence data showed the same results, i.e., significant differences between ectosome and choanosome tissues (PERMANOVA, Gel 1, P = 0.018; Gel 2, P = 0.008) and no differences between specimens (PERMANOVA, Gel 1, P = 0.437; Gel 2, P = 0.470), so the bacterial differences between tissues and the homogeneity between specimens were quantitatively well supported despite the gel effect. Of 24 bacterial types, 7 were responsible for the differences found between the two sponge tissues (Fig. 4.3). OTUs 2 and 5 were exclusively found in the ectosome, while OTUs 6 and 24 were restricted to the choanosome (Fig. 4.3). OTUs 7, 10, and 16 were distributed over both tissues but were more abundant in the choanosome of the sponge (Mann-Whitney U tests, P = 0.001, P = 0.003, and P = 0.014, respectively) (Fig. 4.3).

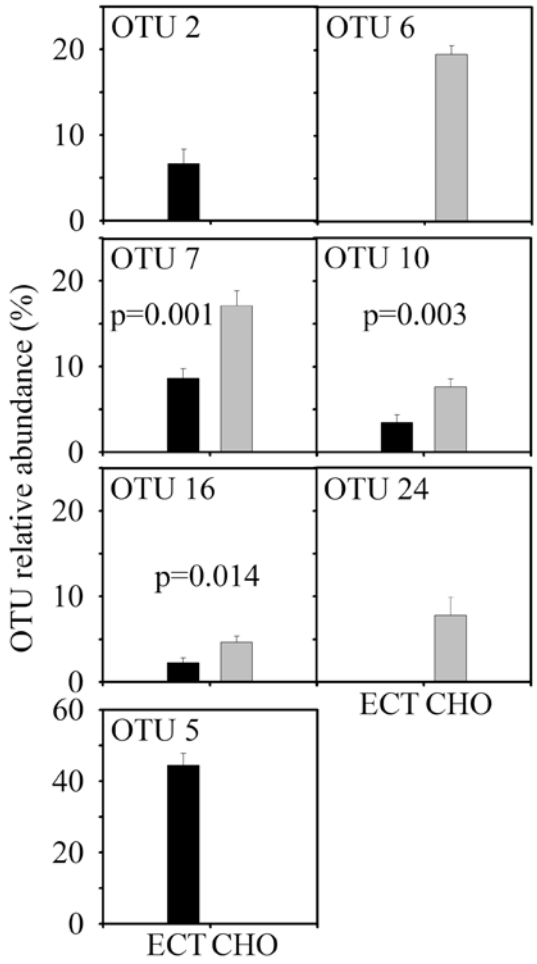


Figure 4.3. OTU relative abundances of the sponge *A. aerophoba* between ectosome (ECT) and choanosome (CHO) tissue layers. Only OTUs with significant differences between tissues ($P \leq 0.05$) are shown.

4.3.4 Relationship between natural products and bacterial community

We detected a significant correlation between the chemical and bacterial profiles regardless of the gel analyzed or their combination (Mantel test; bacterial data from both gels, $R = 0.209$, $P = 0.001$; gel 1: $R = 0.249$, $P = 0.025$; gel 2, $R = 0.315$, $P = 0.005$).

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 between groups. Factor analysis resulted in two chemical and eight bacterial factors (Tables 4.1 and 4.2) that explained 85.31 and 79.99% of the total variance, respectively. Canonical correlation analysis on the factor analysis scores resulted in four significant correlations that included three bacterial factors (BF1, BF2, and BF7) and the two chemical factors (Table 4.3). Individual correlation analysis of the secondary metabolites and bacterial types included in the correlated factors resulted in 15 uncorrected significant correlations (out of 37 possible correlations [Table 4.4]), which is an extremely unlikely event to be explained by chance (Bernoulli equation, $P = 9.25E^{-11}$). Significant correlations after Bonferroni corrections were drastically reduced and restricted to the positive relationships between OTU 19 and the compounds Aero2 and Iso3 ($R = 0.606$, $P = 0.007$; $R = 0.569$, $P = 0.020$, respectively) and between OTU 7 (*Chloroflexi*; see below) and Aply1 ($R = 0.593$, $P = 0.010$) (Fig. 4.4; Table 4.4).

Table 4.1. Bacterial factors obtained from the factor analysis of the bacterial data[a].

OTU	Loading value obtained with bacterial factor:							
	BF1	BF2	BF3	BF4	BF5	BF6	BF7	BF8
OTU5	−0.86							
OTU6	0.84							
OTU7	0.81							
OTU2	−0.74							
OTU18		−0.86						
OTU19		−0.84						
OTU24		−0.83						
OTU12		−0.69						
OTU9		0.64						
OTU20		−0.62						
OTU22			−0.86					
OTU11				0.82				
OTU4				0.75				
OTU16				0.67				
OTU13					0.82			
OTU23						−0.81		
OTU15						−0.79		
OTU8							−0.93	
OTU3								−0.80

[a] To facilitate interpretation, we exclusively show OTU loadings with absolute values greater than 0.60. The closer the absolute loading values to 1.0, the stronger the association between the variables (OTUs or compounds) and the factors. The sign of the values represents the positive or negative nature of their association. BF, bacterial factor.

Table 4.2. Chemical factors obtained from the factor analysis of the chemical data[a].

Compound	Loading value obtained with chemical factor:	
	CF1	CF2
Iso3	0.97	
Aero2	0.94	
Aply1	0.77	
Aero1		0.99

[a]The compounds aerophobin-1 (Aero1), aerophobin-2 (Aero2), aplysinamisin-1 (Aply1), and isofistularin-3 (Iso3) were evaluated. CF, chemical factor.

Table 4.3. Canonical correlation analysis between chemical and bacterial factors.

Bacterial factor	*R* (*P*)[a]	
	CF1	CF2
BF1	0.516 (0.002)	NS
BF2	−0.332 (0.032)	0.359 (0.036)
BF3	NS	NS
BF4	NS	NS
BF5	NS	NS
BF6	NS	NS
BF7	NS	−0.440 (0.012)
BF8	NS	NS

[a] The correlation coefficient (*R*) and *P* value of significant correlations ($P \leq 0.05$) are given. NS, nonsignificant correlations. BF, bacterial factor; CF, chemical factor.

Table 4.4. Correlation analyses of specific brominated alkaloids and bacterial types (OTU) included in the correlated chemical and bacterial factors.

Bacterial factor	OTU	Bacterial group	*R* (*P*)[a]			
			CF1			CF2 (Aero1)
			Aero2	Iso3	Aply1	
BF1	2	*Bacteroidetes*	NS	NS	NS	–
	5	*Cyanobacteria*	−0.432 (0.013)	−0.519 (0.002)	−0.507 (0.003)	–
	6	*Chloroflexi*	0.386 (0.029)	0.499 (0.004)	0.511 (0.003)	–
	7	*Chloroflexi*	NS	NS	0.593 (<0.001)*	–
BF2	9	*Chloroflexi*	NS	NS	NS	NS
	12	UI[b]	NS	NS	NS	NS
	18	UI	NS	0.378 (0.033)	NS	−0.365 (0.040)
	19	UI	0.606 (<0.001)*	0.569 (0.001)*	NS	NS
	20	UI	0.374 (0.035)	NS	NS	NS
	24	*Gammaproteobacteria*	0.363 (0.041)	0.386 (0.029)	NS	NS
BF7	8	*Actinobacteria*	–	–	–	0.442 (0.011)

[a] The correlation coefficients (R) and uncorrected P values of all significant correlations are shown. Asterisks indicate significant correlations after Bonferroni correction. NS, nonsignificant correlations; –, correlations not tested; BF, bacterial factor; CF, chemical factor.

[b] UI, unidentified OTU

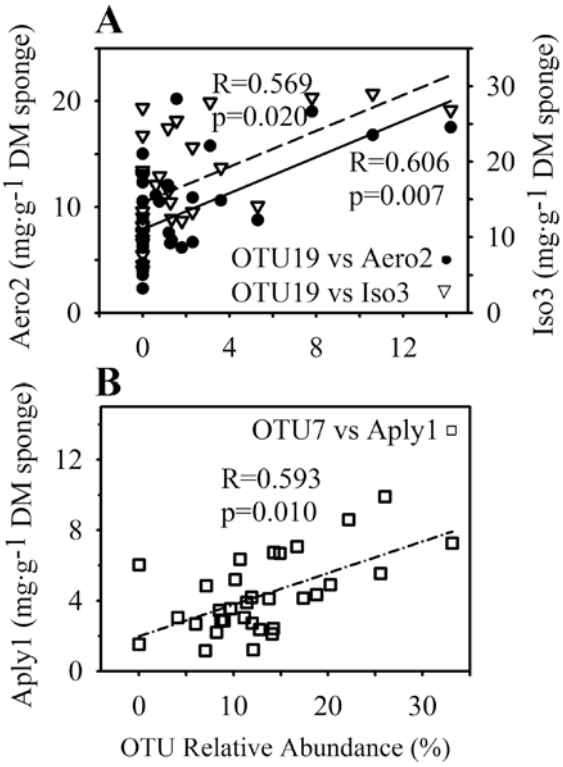


Figure 4.4. Relationship between (A) the concentrations of aerophobin-2 (Aero2) and isofistularin-3 (Iso3) and the relative abundance of the unidentified OTU19 and (B) the concentration of aplysinamisin-1 (Aply1) and the relative abundance of a Chloroflexi clade (OTU7). Concentrations of compounds in mg g (dry mass) of sponge tissue^{-1} ± 1 standard error. *P* values are significant after Bonferroni corrections (see the text for details).

4.3.5 Phylogenetic analysis of excised 16S rDNA-DGGE bands

To identify the potential bacterial populations that were related to secondary metabolites of *Aplysina aerophoba*, we excised from the gels and successfully sequenced a total of 24 16S ribosomal DNA (rDNA)-DGGE bands. These bands belonged to seven different OTUs (see Fig. S4.3), five of which were very abundant (>44% of the total bacterial abundance) and were shared by both gels. Comparison of the 16S rRNA gene sequences with GenBank database showed a large range of bacterial taxa present in *Aplysina*. The bacterial community included representatives of the *Chloroflexi* (OTU6, accession no. AM905025; OTU7, accession no. AM905026; and OTU9, accession no. AM905027), *Cyanobacteria* (OTU5, accession no. AM905024) and *Bacteroidetes* (OTU2, accession no. AM905030), as well as members of *Gammaproteobacteria* (OTU24, accession no. AM905029) and *Actinobacteria* (OTU8, accession no. AM905028) (Table 4.5). Six sequences were previously reported from marine sponges (OTU5 from *Aplysina aerophoba*, OTU6 from *Spongia agaricina*, OTU7 from *Xestospongia testudinaria*, OTU8 and OTU9 from *Ancorina alata* and OTU24 from *Ircinia felix*) and closely related to those reported in another *Aplysina* species (Table 4.5). The remaining sequence was closer to sequences from plankton (OTU2 from seawater).

Table 4.5. 16S rDNA sequence identities of representative bands excised from DGGE gels[a].

DGGE band	OTU	Division	Closest relative in database (isolation source; AN)	% Identity	Closest *Aplysina* host species (% identity; AN)
19	2	*Bacteroidetes*	Uncultured *Flavobacterium* (seawater; FN433428)	92.9	Not found
1	5	*Cyanobacteria*	*Synechococcus spongiarum* (*Aplysina aerophoba*; EF656449)	99.6	*Aplysina aerophoba* (99.6%; EF656449)
6	6	*Chloroflexi*	Uncultured clone DGGE band bd29 (*Spongia agaricina*; AM849595)	100	*Aplysina fulva* (98.7%; GU982064)
7	7	*Chloroflexi*	*Chloroflexus* sp. (*Xestospongia testudinaria*; FJ481354)	99.8	*Aplysina fulva* (99.6%; GU982078)
13	8	*Actinobacteria*	Uncultured clone AncD18 (*Ancorina alata*; FJ900572)	98.1	*Aplysina fulva* (97.3%; FM160879)
12	9	*Chloroflexi*	Uncultured clone AncB18 (*Ancorina alata*; FJ900579)	97.2	*Aplysina fulva* (96.8%; GU982098)
18	24	*Gammaproteobacteria*	Uncultured clone DGGE band IF6-3 (*Ircinia felix*; DQ661847)	97.4	*Aplysina fulva* (96.4%; FM160910)

[a] AN, accession number.

4.4 Discussion

Over the last decades, many sponges have been investigated in an attempt to independently describe both the large diversity of natural products (Blunt et al., 2005, 2006; Moore, 2006; Blunt et al., 2007, 2008) and potential symbiotic microorganisms (Hentschel et al., 2003; Hentschel et al., 2006; Taylor et al., 2007b). Sponge microbial ecology is receiving intense and increasing attention (Becerro, 2008), and one area of particular interest is to assign the true origin of these natural products, whether of sponge or microbial origin (Unson and Faulkner, 1993; Unson et al., 1994; Uriz et al., 1996; Flowers et al., 1998; Turon et al., 2000; Müller et al., 2004; Moore, 2005; 2006). The question is not trivial because it has potential applications in biotechnology and pharmacognosy, but it remains to be rigorously addressed. The traditional approach consists in locating secondary metabolites in cell components using a variety of methods including X-ray microanalysis, density gradients of dissociated cells, and others (Uriz et al., 1996; Turon et al., 2000; Müller et al., 2004). Although these methods can locate secondary metabolites (or a characteristic element of the metabolite, e.g., a bromine atom), they reveal only limited information on the true production of these compounds. This is particularly true if several parts of the compound are assembled in multiple cell compartments. This possibility cannot be ruled out and would provide additional support for the true collaboration between the sponge and its bacterial assemblage. In the present study we explored the association between bacteria and secondary chemistry by investigating relationships between the concentration of secondary metabolites and significant changes in the relative abundance of bacterial populations. Our study provides one of the first reports of a significant correlation between specific secondary metabolites and bacterial types (Flatt et al., 2005). Although we cannot infer the true functional nature of these associations, they provide more explicit and simplified paths to explore the links

between secondary metabolites and sponge-associated bacteria in *A. aerophoba*.

The characterization of chemical and bacterial profiles in *A. aerophoba* has already been reported by numerous authors (Weiss et al., 1996; Ciminiello et al., 1997; Ebel et al., 1999; Hentschel et al., 2001; Hentschel et al., 2002; Thoms et al., 2006). The relative composition of brominated alkaloids present in *A. aerophoba* seems to be highly conserved in both spatial and temporal scales. *Aplysina* individuals sampled thousands of kilometers apart showed indistinguishable secondary metabolite profiles (Ebel et al., 1999). At a small spatial scale, bromoisoxazoline patterns of two *Aplysina* species proved to be remarkably stable and unaffected either by changing the light conditions or depth (Putz et al., 2009). Similarly, the alkaloid content remained unchanged after cultivation of *Aplysina* specimens in starvation conditions and antibiotics exposure in aquaria (Friedrich et al., 2001). Nevertheless, other studies with emphasis on natural products quantification show considerable variability in the concentration of secondary metabolites within *Aplysina* species (Puyana et al., 2003) and between specimens of the same *Aplysina* species (Thoms et al., 2006). The relative composition of the main brominated compounds under *ex situ* and *in situ* cultivation of *A. aerophoba* remained stable, although the total alkaloid content increased twice as much *ex situ* than *in situ* (Klöppel et al., 2008). There is a similar concern about the variation of bacterial assemblages in sponges. The microbial community seems to be uniform over time in *A. aerophoba* (Friedrich et al., 2001) and in other sponge species (Taylor et al., 2004; Hoffmann et al., 2006).

Our results show evidence for a significant variation in the concentration of secondary metabolites in *A. aerophoba* both within and between specimens, which is consistent with the pronounced variability of secondary metabolite concentrations found not only in *Aplysina* spp. but also in other sponge species (Becerro et al., 1995; Betancourt-Lozano et al., 1998; Schupp et al., 1999; Page et al., 2005) and other sessile organisms such as bryozoans (Peters et al.,

2004). Our results also show evidence for changes in the relative abundance of bacterial populations in *A. aerophoba*. Signal intensity of DGGE bands is a useful tool to estimate the relative abundance of predominant bacterial groups (i.e., those >0.1 to 1% of total abundance) and to have a rough picture of microbial changes but does not provide detailed quantitative estimations (Casamayor et al., 2000). We were cautious in the number of PCR cycles run to avoid the "plateau" phase and in using the same amount of template in each reaction. The samples that we compared were run under the same PCR and DGGE conditions, and we carefully estimate the error among replicates. If there was any PCR bias it should be the same in all lanes, and therefore comparison among samples is still valid. The use of normalized relative abundances in the analyses also allowed us for accurate intersample comparison.

Except for aerophobin-1, we found higher concentration of every compound in the choanosome of the sponge, making a picture of a chemically rich sponge nucleus surrounded by a not-so-rich tissue layer. Traditionally, significant differences in secondary metabolites between sponge tissues are usually interpreted as evidence of their roles against predation, competition, or both (Becerro et al., 1995, 1997a; Becerro et al., 1997b; Schupp et al., 1999). The ectosome of *A. aerophoba* is packed with cyanobacteria ((Vacelet, 1970, 1975; Becerro et al., 2003); the present study) and is the preferred tissue preyed upon by the opisthobranch nudibranch *Tylodina perversa* (Becerro et al., 2003). Thoms et al. (2004) failed to detect aerophobin-1 in *A. aerophoba* but reported aerophobin-2 as one of the major constituents and the major deterrent compound against generalist predators together with isofistularin-3. Although the deterrence of aerophobin-1 is not known, chemical differences with aerophobin-2 are so minor that aerophobin-1 would be expected to be similarly deterrent against generalist predators too. If so, the high concentration of aerophobin-1 in the outer tissues of the sponge in our study could serve as a defensive barrier against generalist predators, which have never been observed feeding on *A.*

aerophoba (unpublished data). High chemical concentrations in the outer tissues could also be a defense against foulers (Becerro et al., 1994; Becerro et al., 1997b). The higher concentration of isofistularin-3 (and other compounds) in the choanosome could be acting as a final deterrent against the specialist predator *T. perversa*, which is similar to what has been described for other opisthobranch-sponge feeding interactions (Becerro et al., 1998; Becerro et al., 2006b). Other roles, such as antibacterial activities, are far less investigated but could also explain the observed variation in secondary chemistry (Becerro et al., 1994). The natural products found in *A. aerophoba* can be rapidly converted into aeroplysinin-1 and dienone, which show stronger antibiotic activity than their precursors and may protect this sponge from invasion by bacterial pathogens (Teeyapant and Proksch, 1993; Ebel et al., 1997; Thoms et al., 2004).

The predominant bacterial community in *A. aerophoba* seemed to be fairly constant between and within individuals, although some bacteria significantly varied their relative abundance between the ectosome and choanosome tissues of the sponge. These observations are in agreement with those previously reported by Thiel et al. (Thiel et al., 2007b), who found a distinct bacterial community between the outer (ectosome) and the inner (choanosome) tissues of the sponge *Tethya aurantium*. To date, the most noticeable microbial difference between ectosome and choanosome tissues of *A. aerophoba* is the presence of photosynthetic cyanobacteria in the outer tissues (Becerro et al., 2003). Our study provides evidence for a specific distribution of *Cyanobacteria* and *Bacteroidetes* in the ectosome of the sponge. Some *Chloroflexi* and *Gammaproteobacteria* were restricted to the choanosome, while other bacterial bands had dissimilar distribution between tissues. The differences between ectosome and choanosome seem to be greater than previously reported. Apart from these bacterial groups, the microbial assemblage included representatives of other *Chloroflexi* and *Actinobacteria*. Overall, the sponge-associated bacterial community investigated in our study is

as highly diverse and phylogenetically complex as described by other authors (Friedrich et al., 1999; Webster et al., 2001; Hentschel et al., 2002; Webster et al., 2004; Hentschel et al., 2006).

Our study also found significant differences in secondary chemistry between specimens of *Aplysina aerophoba* that were collected at a regional scale (< 100 km). Thoms et al. (2006) also found significant differences in the concentration of secondary metabolites between specimens of *A. aerophoba* collected in Croatia and France, supporting the idea that chemical variation seems to be common within species of the genus *Aplysina* (Puyana et al., 2003). Similarly, several bacteria differed between individuals of *A. aerophoba*, *Geodia barretti*, and *Cymbastela concentrica* (Friedrich et al., 2001; Taylor et al., 2004; Hoffmann et al., 2006). However, bacterial communities can be fairly constant between sponge species both within and between geographic regions (Hentschel et al., 2002; Webster et al., 2004). Further research is necessary to understand these contrasting differences in bacterial variability within and between sponges.

Beyond the actual chemical or bacterial variation reported in this or previous studies, our study is the first attempt to correlate changes in both secondary chemistry and bacterial assemblage. A total of 15 of the 37 correlations evaluated resulted in significant positive and negative correlations between bacterial populations and natural products. This high number of significant correlations is extremely unlikely to occur by chance alone and suggests strong interactions between bacteria and between bacteria and natural products. Some *Chloroflexi* strains were positively related to the concentration of aplysinamisin-1, while a cyanobacterial strain was negatively related to the same compound. These *Chloroflexi* and *Cyanobacteria* strains are found in the choanosome and ectosome of the sponge, respectively, and were clearly negatively associated with each other. Other bacterial types, such as the *Gammaproteobacteria* strain and the unidentified OTU 19, seemed to be positively related to the abundance of aerophobin-2 and isofistularin-3 but not to aplysinamisin-1. An *Actinobacteria* strain

was associated with the concentration of aerophobin-1. Our results show how phylogenetically distinct bacterial groups are related to multiple natural products, which is also supported by a genomic study in *A. aerophoba* that assigned secondary metabolite gene clusters to a *Chloroflexi* clade and to the candidate phylum *Poribacteria* (Siegl and Hentschel, 2009).

These correlations could be driven by multiple factors. Larger compound concentrations could lead to larger abundance of particular bacteria because of specific habitat conditions or preferences. For example, the abundance of brominated compounds in the sponge tissue would facilitate bacteria with the ability to metabolize these compounds (van Pée and Unversucht, 2003). Ahn et al. (2003) demonstrated that anaerobic bacteria harbored within the *A. aerophoba* carried out an anaerobic reductive dehalogenation of brominated aromatic compounds, suggesting that natural products would determine the presence or absence of these bacteria. Other hypotheses that could produce the same type of correlations between secondary chemistry and bacteria could include reduced competitive interactions with other bacterial species or preferential abiotic conditions. Under these hypotheses, the increasing abundance of bacteria could be either a spurious correlation or a consequence of the higher concentration of the compound. We cannot rule out that low abundant bacteria can produce metabolites that accumulate over time, although this would imply a low turnover of these compounds and the spherulous cells where they concentrate (Turon et al., 2000). However, it is well known that spherulous cells degenerate and are frequently released into the environment (Bergquist, 1978; Thompson et al., 1983; Becerro et al., 1997b; Turon et al., 2000), suggesting rapid cell turnover (De Goeij et al., 2009).

Alternatively, bacteria might be involved in the production of the natural product leading to higher concentration of compounds with increasing bacterial abundance. Various secondary metabolites of *A. aerophoba* are found in multiple sponge compartments, raising the possibility that distinct sponge compartments play a role

at specific steps of the biosynthetic pathway (Kreuter et al., 1992). Interestingly, the haloperoxidase or halogenase enzymes responsible for halogenation processes have only been reported for bacteria, algae, and fungi (Baden and Corbett, 1980; van Pée, 1990, 1996; van Pée and Unversucht, 2003; van Pée et al., 2006), which suggests that the bacterial endobionts of *A. aerophoba* could be producing these enzymes (De Goeij et al., 2009). Thus, *Aplysina* sponge cells may produce the inactive precursors of secondary metabolites, while bacteria may provide the enzymes necessary to activate them. Alternatively, the halogenation reaction could be attributed to either fungi or microalgae, which can also be associated with sponges.

Our phylogenetic analysis reveals that multiple bacterial strains were involved in these relationships. Populations of *Chloroflexi*, *Actinobacteria*, and *Cyanobacteria* are associated with the production of bioactive compounds (Jensen et al., 2005; Nett and König, 2007). The phylum *Chloroflexi* is an early branching lineage of bacteria about which little is known, especially about non free-living microbes (Bryant and Frigaard, 2006; Nett and König, 2007). We now know that prominent enzymes involved in the biosynthesis of bioactive secondary metabolites are assigned to *Chloroflexi* (non-ribosomal peptide synthetase, NRPS) and to *Poribacteria* clades (polyketide synthases, PKS) in *A. aerophoba* (Siegl and Hentschel, 2009). Mat-forming *Cyanobacteria* are a rich source of natural products (Burja et al., 2001; Moore, 2005; König et al., 2006) and symbiotic cyanobacteria seem to be involved in the production of halogenated compounds in the sponge *Dysidea herbacea* (Unson and Faulkner, 1993; Unson et al., 1994; Flowers et al., 1998). *Gammaproteobacteria*, *Deltaproteobacteria,* and *Actinobacteria* carry out halogenation or dehalogenation reactions (van Pée, 1990, 1996; van Pée and Unversucht, 2003), and the former phylum is the most likely producer of bryostatins in the bryozoan *Bugula neritina* (Davidson et al., 2001). In our specimens, we found many of these bacterial types and they were correlated with some of the brominated compounds investigated in our study.

Taken together, our data and those from the literature seem to support that sponge-associated bacteria are related to natural product synthesis.

Unraveling the true origin of natural products is challenging. The current body of evidence is too limited to make broad generalizations, but it suggests complex chemical and biological interactions that are far from being resolved. The diversity of secondary metabolites in any particular sponge species is usually large and so is the diversity of their associated bacterial communities. These multiple levels of complexity result in a demanding field, where solutions must include tools from multiple areas and disciplines. Further experimentation and new molecular techniques are needed to advance in this research area (Schirmer et al., 2005; Siegl and Hentschel, 2009). Our study presented evidence for a positive correlation between natural products and certain bacterial strains, which provides a simplified version of the sponge-microbe interactions and could be used as a starting point for hypothesis testing. Experimentally modifying the concentration of secondary metabolites or the abundance of endobiotic bacteria would be critical to elucidate their true association and to infer functional relationships, and such research is under way in our laboratory. A further step would be to use quantitative reverse transcriptase PCR (Ding and Cantor, 2004) to study the expression of genes involved in the production of secondary metabolites and the enzymes responsible for the biotransformation of natural products. Applying modern chemical, molecular, and ecological techniques will substantially improve our understanding of the organization and functioning of these truly complex host-endobiont ecosystems.

Acknowledgments

This research was supported by CSIC postgraduate and predoctoral grants to O.S.-S. and funded by the Spanish Ministry of Science and Innovation (grants CTM2007-66635 to M.A.B. and CGL2009-

13318 to E.O.C.) and by the Agence Nationale de la Recherche (France; ECIMAR, ANR-06-BDIV-001-04).

We thank A. Riesgo for her comments on previous versions of the manuscript. We also thank R. Thacker and anonymous reviewers for suggestions that improved the final version of the manuscript.

5

Relevant spatial scales of chemical variation in *Aplysina aerophoba*

Resumen

Conocer la escala en la cual los productos naturales presentan una variabilidad mayor es crucial porque permite conocer el tipo de factores que regulan su producción. La esponja *Aplysina aerophoba* es común en el Mediterráneo donde ocupa ambientes someros y en el área de influencia del océano Atlántico. Esta especie contiene concentraciones importantes de compuestos bromados que desempeñan varias funciones ecológicas. Este estudio investiga la variación ecológica de los metabolitos secundarios mayoritarios de *A. aerophoba* en una escala espacial comprendida entre centenares de metros hasta miles de kilómetros. Para ello, utilizamos un diseño jerarquizado de muestreo en el que se recolectaron muestras de dos regiones geográficamente distantes (Islas Canarias y Mediterráneo Occidental, separadas por 2500 km), de dos zonas dentro de cada región (separadas por unos 50 km), de dos localidades dentro de

cada zona (separadas por unos 5 km), y de dos puntos dentro de cada localidad (separados por unos 500 m). Utilizamos la técnica de cromatografía líquida de alta resolución para cuantificar los compuestos bromados y con un espectrofotómetro cuantificamos la clorofila *a*. Los resultados muestran una importante variación tanto en los productos naturales como en el contenido de clorofila *a*. Ambos patrones de variación se daban tanto en la escala geográfica más grande como en la más pequeña, aunque dependiendo del compuesto analizado prevalecía una escala u otra. Encontramos una correlación negativa entre la clorofila *a* y el compuesto isofistularin-3, lo que sugiere un impacto en la concentración de estos compuestos por parte de la comunidad simbionte. Estos resultados acentúan el complejo control de la producción de metabolitos secundarios y sugieren que factores actuando a pequeña y gran escala espacial que regularían la producción de compuestos naturales.

Abstract

Understanding the scale at which natural products vary the most is critical because it sheds light on the type of factors that regulate their production. The sponge *Aplysina aerophoba* is a common Mediterranean sponge inhabiting shallow waters in the Mediterranean and its area of influence in Atlantic Ocean. This species contains large concentrations of brominated alkaloids (BAs) that play a number of ecological roles in nature. Our research investigates the ecological variation in BAs of *A. aerophoba* from a scale of hundred of meters to thousand kilometers. We used a nested design to sample sponges from two geographically distinct regions (Canary Islands and Mediterranean, over 2500 km), with two zones within each region (less than 50 km), two locations within each zone (less than 5 km), and two sites within each location (less than 500 m). We used high-performance liquid chromatography to quantify multiple BAs and a spectrophotometer to quantify chlorophyll *a* (Chl *a*). Our results show a striking degree

of variation in both natural products and Chl *a* content. Significant variation in Chl *a* content occurred at the largest and smallest geographic scales. The variation patterns of BAs also occurred at the largest and smallest scales, but varied depending on which BA was analyzed. Concentrations of Chl *a* and isofistularin-3 were negatively correlated, suggesting that symbionts may impact the concentration of some of these compounds. Our results underline the complex control of the production of secondary metabolites, with factors acting at both small and large geographic scales affecting the production of multiple secondary metabolites.

5.1 Introduction

Conducting research at multiple spatial or temporal scales can substantially increase our understanding of numerous ecological processes, making scale a central problem in ecology (Levin, 1992). Ecological processes and the mechanisms behind them show large spatial and temporal variation, which results in a high degree of heterogeneity across wide ranges of space, time, and biological organization (e.g., from ecosystems, to species, to species traits). Moreover, a multiple scale approach could shed light on the factors behind natural variation, improve our understanding of the ecological role of specific traits, and show evidence of the relevance or universality of ecological mechanisms and processes (Levin, 1992). Besides, there is no single natural scale at which ecological studies should be studied (Levin, 1992). Yet, ecological studies over large spatial and temporal scales are often hard to carry out and are uncommon on numerous ecological areas.

The production of marine natural products is an area that could benefit tremendously from a multi scale approach. We know that marine natural products play multiple roles in nature (Paul and Vanalstyne, 1992; Schmitt et al., 1995; Becerro et al., 1997a) and vary remarkably as a function of space and time (Matlock et al., 1999; Wright et al., 2000; Pennings et al., 2001). The factors behind such variation are far from being fully understood and this research

area is in chronic need of empirical data (Hay, 1996; Paul et al., 2006). In fact, the poor understanding of the processes that control chemical diversity and variation is hindering the development of marine chemical ecology (Paul et al., 2006). It is therefore critical to investigate chemical variation because it will shed light on the factors that regulate the production of chemical defenses, building up the field of marine chemical ecology.

The production of natural products is widespread in the benthic realm (Blunt et al., 2011). Sponges are consistently the richest source of marine natural products (Blunt et al., 2006; Blunt et al., 2011) and have also received considerable attention from a chemical ecology perspective (Paul et al., 2006; Becerro, 2008). Sponge secondary chemistry is known to vary significantly within species as a function of time (Turon et al., 1996; Duckworth and Battershill, 2001; Page et al., 2005; Abdo et al., 2007), geographic region (Abdo et al., 2007; Noyer et al., 2011), habitat/community (Thompson, 1985; Becerro et al., 1995; Becerro and Paul, 2004), specimens (Puyana et al., 2003; Ettinger-Epstein et al., 2008), tissues (Unson et al., 1994; Becerro et al., 1998; Sacristán-Soriano et al., 2011b), and cells (Bewley et al., 1996; Turon et al., 2000). In particular, there is abundant information on secondary chemistry and chemical ecology of the genus *Aplysina*. There are multiple bromotyrosine alkaloids (BAs) described for *Aplysina* spp. (Tymiak and Rinehart, 1981; Bergquist and Wells, 1983; Ciminiello et al., 1994; Ciminiello et al., 1996; Ciminiello et al., 1997), which can represent up to 13% of the sponge dry mass (Teeyapant et al., 1993a). These compounds have a variety of biological activities (Teeyapant et al., 1993b; Koulman et al., 1996; Weiss et al., 1996; Encarnacion-Dimayuga et al., 2003) and play multiple ecological roles in nature (Kelly et al., 2003; Thoms et al., 2004; Thoms et al., 2006). Changes in the chemical structure of these compounds alter their biological activity (Thoms et al., 2004; Thoms et al., 2006), so the exact composition and concentration of compounds in the sponge tissues could translate into differential ecological roles.

BAs may vary remarkably within the same *Aplysina* species (Nunez et al., 2008), which could have important ecological implications. The BAs of *Aplysina aerophoba* vary between cells (Turon et al., 2000), tissues (Kreuter et al., 1992; Becerro et al., 2003; Sacristán-Soriano et al., 2011b), specimens (Kreuter et al., 1992; Putz et al., 2009), and geographic location (Teeyapant et al., 1993a). Moreover, this species is distributed in the Mediterranean and Canary islands, where it can be locally abundant (Teeyapant et al., 1993a; Becerro et al., 2003). These characteristics make *Aplysina aerophoba* a perfect organism to investigate natural product variation across multiple spatial scales ranging from a few meters to thousands of kilometers apart, and thus provide a great opportunity to assess the spatial scale at which chemical variation varies the most.

It is known that photosynthetic symbionts can contribute to the production of secondary metabolites (Unson and Faulkner, 1993; Unson et al., 1994; Piel, 2004) and might be involved somehow in the secondary chemistry of *A. aerophoba* (Siegl and Hentschel, 2009; Sacristán-Soriano et al., 2011b). Since concentration of Chlorophyll *a* (Chl *a*) can be used as a traditional proxy of the abundance of photosynthetic symbionts in sponges (Becerro et al., 1998; Becerro et al., 2003; Erwin and Thacker, 2007; Freeman and Thacker, 2011), we assessed whether the concentration of Chl *a* varied at the same spatial scale as BAs and further investigated whether both Chl *a* and secondary metabolites were related.

Is there a spatial pattern of variation in BAs? Do BAs vary the most between, near, medium, or far away locations? Do individual BAs follow the same pattern of variation? Do these patterns match the pattern of variation in Chl *a*? In this study we tested these hypotheses by looking at the spatial variation in BAs and Chl *a* of the sponge *Aplysina aerophoba* in neighboring bays to locations over 2500 km apart. We followed a nested design with two distant geographic regions, two zones within each region, two locations within each zone, and two sites within each location. This design

calculates the magnitude of the variance attributable to the four spatial scales and will suggest the most relevant scale at which chemical variation should be addressed in this species. We found a spatial scale where BAs and Chl *a* varied the most and shed light on the complex mechanisms behind the production of natural products within a single species. This is the first report to take a broad spatial approach in marine chemical ecology, but further research will clarify whether this complex trend in the production of natural products is common among benthic organisms.

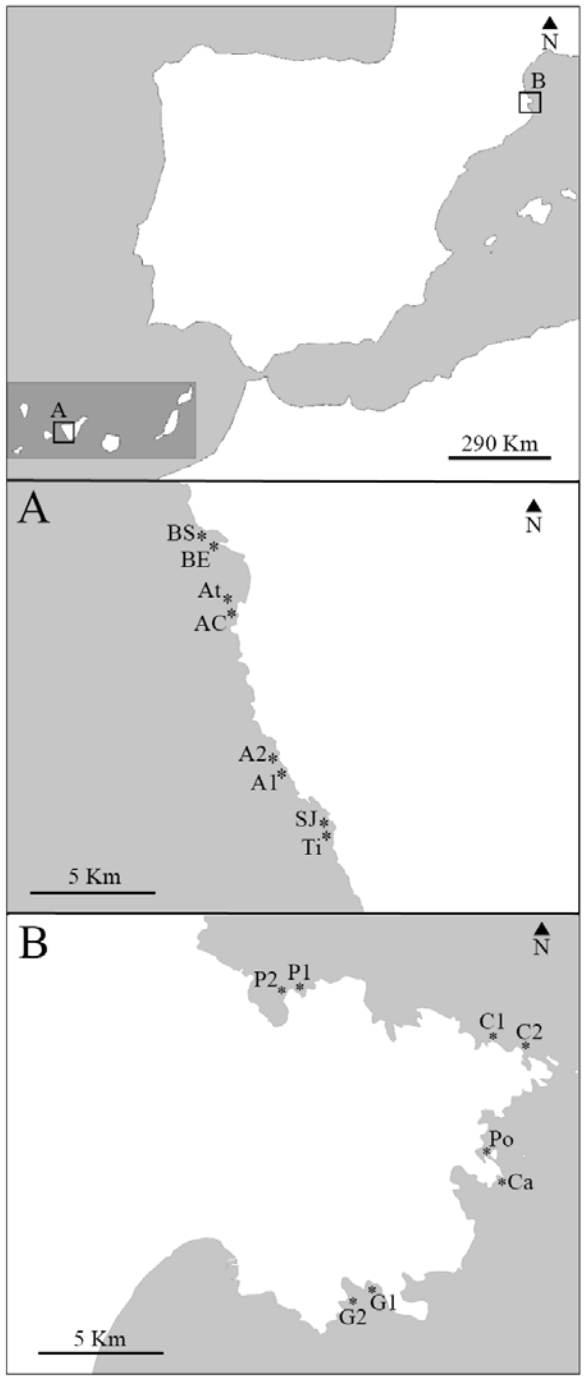
5.2 Materials and methods

In March 2003, the sponge *Aplysina aerophoba* (Nardo, 1833) was collected by scuba diving at different sampling sites of Tenerife (Canary Islands) and Cap de Creus (Northwestern Mediterranean) (Fig. 5.1). We collected several specimens from each location to obtain enough material for the bulk chemical extraction necessary to set up the chemical methods. To assess a geographic variation in natural products, we used a nested design covering a range of biogeographic scales to collect the whole chimney-like structures representative of this species, which are known to have the same secondary chemistry (Sacristán-Soriano et al., 2011b), of about 10 specimens in each site. We used a sharp knife to cut chimneys off by the base and placed them in independent plastic bags. Immediately after collection, samples were placed in coolers with ice to prevent changes in the secondary chemistry and all samples were frozen at −20 °C within 4 h of collection. Once in the laboratory, samples were freeze-dried under dark and a small portion of the top half of the chimney away from the cutting surface was selected for the quantification of the BAs. There are no differences in secondary chemistry between the top and bottom zones of the chimneys (Sacristán-Soriano et al., 2011b).

For BA isolation, 50 g of freeze-dried sponge were extracted three times (1 h, 1 h, and overnight) with methanol (MeOH, 20 mL MeOH per 1 g sponge). The crude extract (CE) was first

fractionated by flash-chromatography. High-performance liquid chromatography (HPLC) was performed on a Waters HPLC with an Alliance separation module 2695, column heater, and 2998 photodiode array detector. Separation was achieved using a hydrophobic column (Phenomenex Gemini C18, 110 Å, 250 × 10.0 mm, 5 μm). Mobile phase consisted of 70% of MeOH, 30% of MilliQ water; the flow rate was 3 $mL \cdot min^{-1}$ and the injection volume was 100 μL. Peaks were detected at 320 nm and each BA compound detected was identified and carefully isolated. Dry pure BAs were recovered using rotatory evaporation for use as standards. Then, series of dilution on pure compounds coupled to peak area calculation in HPLC (at 245 nm) allowed tracing calibration-curves. The major compounds observed in the HPLC chromatograms were characterized by classic spectrometric techniques (liquid chromatography/mass spectrometry, nuclear magnetic resonance, UV profile) and by their retention times.

Figure 5.1. (next page) Study area that comprises two biogeographic regions; Tenerife (**A**; Canary Islands) and Cap de Creus (**B**; Northwestern Mediterranean). (**A**) Sampling sites of Tenerife. Punta Tixera (Ti), San Juan (SJ), Alcalá 1 (A1), Alcalá 2 (A2), Atlántida Coast (AC), Atlántida (At), Barranco del Eco (BE), Barranco Seco (BS); (**B**) Sampling sites of Cap de Creus. Gat 1 (G1), Gat 2 (G2), Caials (Ca), Portlligat (Po), Club Med 1 (C1), Club Med 2 (C2), Port de la Selva 1 (P1), Port de la Selva 2 (P2).


N
B
A
290 Km
A
N
BS
BE
At
AC
A2
A1
SJ
Ti
5 Km
B
N
P2
P1
C1
C2
Po
Ca
G2
G1
5 Km

For BA quantification, approximately 50 mg of freeze-dried sponge tissue was extracted three times with 1.5 mL of MeOH in an ultrasonic tank for 15 min each time. The CE was filtered through a 20 µm polytetrafluoroethylene filter (PTFE) and added in a 5 mL beaker. The final volume was adjusted to 5 mL with MeOH and an aliquot of 1.5 mL was passed through a 13 mm, 0.2 µm PTFE syringe-filter before HPLC injection. Separation was achieved using a hydrophobic column (Phenomenex Synergi Max-RP, 80 Å, 250 × 3.0 mm, 4 µm) with a mobile phase of buffered (0.1% trifluoroacetic acid) water and acetonitrile. We used a linear gradient from 30% to 80% acetonitrile over 18 min, with an additional 10 min at 100% acetonitrile at the end of the run, with a flow rate of 0.4 $mL \cdot min^{-1}$. Samples were injected in 10 µL volumes and column temperature was maintained at 30 °C. Peaks were identified at 245 nm and integrated by applying the detector response based on peak areas to calibration curves. Concentrations of brominated compounds were expressed as $mg \cdot g^{-1}$ of dry mass of sponge tissue.

We used the concentration of chlorophyll *a* (Chl *a*) as an indication on the density of autotrophic symbionts within *A. aerophoba*. Approximately 100 mg of freeze-dried sponge tissue was extracted with 5 mL of 90% acetone for 12 h in the dark at 1 °C. 2 mL of the extract solution were passed through a 0.2 µm PTFE syringe-filter. The 8452A diode array spectrophotometer (Hewlett Packard) was used to perform the absorbance measurements. We measured absorbance at four wavelength (630, 647, 664, and 750 nm) according to the trichromatic equation of Jeffrey and Humphrey (Jeffrey and Humphrey, 1975) to calculate the Chl *a* concentration (1). Chl *a* was expressed as $ng \cdot mg^{-1}$ of dry mass of sponge tissue.

$$\begin{aligned}\text{Chl } a \left(\text{mg L}^{-1}\right) &= 11.85\left(\text{Abs664} - \text{Abs750}\right) - 1.54\left(\text{Abs647} - \text{Abs750}\right) \\ &-0.08\left(\text{Abs630} - \text{Abs750}\right)\end{aligned} \quad (1)$$

We used several statistical methods from SYSTAT 12 software (SPSS, 1999a, b) to analyze secondary metabolite abundances and Chl *a* content. Nested analyses of covariance (ANCOVAs) were performed on ranked compound abundances and Chl *a* concentrations with region (2 regions), zone (4 zones), location (8 locations), and sampling site (16 sites) as factors (Fig. 5.1) and depth as a covariate. We used *a posteriori* pairwise comparisons with Bonferroni test to identify the groups responsible for the main significant factors. We also used simple correlation analysis to establish the quantitative relationships between secondary metabolites, Chl *a* and depth.

5.3 Results and discussion

5.3.1 Natural product and chlorophyll *a* quantification

We quantified a total of 126 samples (2 regions × 2 zones per region × 2 locations per zone × 2 sampling sites per location × 6–10 individual sponges per sampling site; Fig. 1) to characterize the chemical profile and determine the chlorophyll *a* concentration (Chl *a*) of *Aplysina aerophoba*. We identified and quantified the four major brominated alkaloids in our samples: aerophobin-1 (Aero1; peak 3), aerophobin-2 (Aero2; peak 4), aplysinamisin-1 (Aply1; peak 5), and isofistularin-3 (Iso3; peak 6), by comparing their retention times and UV profiles to those of purified, characterized standard compounds.

The secondary metabolites identified were in agreement with the abundant literature available for this species (Ciminiello et al., 1997; Thoms et al., 2004; Thoms et al., 2006; Putz et al., 2009). To minimize compound degradation and the enzymatic transformation of the high molecular weight (HMW) BAs quantified in our study into the low molecular weight (LMW) natural products reported elsewhere (Teeyapant and Proksch, 1993; Teeyapant et al., 1993b; Ebel et al., 1997; Thoms et al., 2006), we used a sampling protocol that minimized manipulation of live tissues, froze our samples

rapidly after collection, and used methanol alone to obtain crude extracts. Since these LMW natural products result from bioconversion of precursors (but see (Puyana et al., 2003)), their presence in our chromatograms could cast doubt on the actual concentration of the precursors quantified in our samples (Thoms et al., 2006). We failed to observe these bioconverted natural products in our chromatograms or they were at such low concentrations that cannot explain the large variation in BAs observed in our samples (see below). The concentrations of the four compounds we quantified were positively correlated, except for the negative correlation between aerophobin-2 and aplysinamisin-1 (Table 5.1). Although this negative correlation could be indicative of bioconversion between these two compounds (concentration of a precursor decreases as concentration of the resulting compound increases), the degradation of aerophobin-2 and isofistularin-3 results in the LMW compounds (Weiss et al., 1996), which were undetected in our extracts.

Table 5.1. Correlation coefficients (below diagonal) and uncorrected *P*-values (above diagonal) between depth and the concentrations of chlorophyll *a* (Chl *a*), aerophobin-1 (Aero1), aerophobin-2 (Aero2), aplysinamisin-1 (Aply1), isofistularin-3 (Iso3), and the four BAs combined (All).

	Depth	Chl *a*	All	Aero1	Aero2	Aply1	Iso3
Depth		<0.001*	0.001*	0.008	0.276	0.494	<0.001*
Chl *a*	−0.391*		0.001*	0.059	0.459	0.796	<0.001*
All	0.294*	−0.283*		---	---	---	---
Aero1	0.236	−0.168	---		<0.001*	<0.001*	<0.001*
Aero2	0.098	−0.067	---	0.403*		<0.001*	0.001*
Aply1	0.062	−0.023	---	0.543*	−0.466*		0.508
Iso3	0.349*	−0.430*	---	0.385*	0.294*	0.060	

* show significant correlation coefficients and *P*-values after Bonferroni correction; --- correlation between the four individual compounds and their combined concentration are meaningless and not reported.

5.3.2 Secondary metabolite variation

The total concentration of BAs varied between sampling sites but not between regions, zones, or locations (nested analysis of covariance, ANCOVA, $P < 0.001$; $P = 0.722$; $P = 0.086$; and $P = 0.650$, respectively, Table 5.2). Because we found that depth could explain 8.6% of the variability in the total concentration of BAs ($R^2 = 0.086$, Table 5.1) we used depth as a covariate in our analyses. Total concentration of BAs did not vary with depth (Table 5.2), but 28.41% of the total variance in BAs occurred between sites that are less than 500 m apart (Table 5.2).

Table 5.2. *P*-values and percentage of the total variance explained by depth, region, zone, location, and site. Values obtained from nested ANCOVAs used to test the effect of spatial scale on the concentration of chlorophyll *a* (Chl *a*), aerophobin-1 (Aero1), aerophobin-2 (Aero2), aplysinamisin-1 (Aply1), isofistularin-3 (Iso3), and total concentration of BAs (All) with Depth as covariate. We also show the percent variance unexplained by the factors (Error). Geographic scale included Region (over 2500 km), Zone (less than 50 km), Location (less than 5 km), and Site (less than 500 m).

Compound	Depth	Region	Zone	Location	Site	Error
Chl *a*	0.069	<0.001/31.76%	0.905	0.236	0.038/9.46%	59.33%
All	0.275	0.722	0.086	0.650	<0.001/28.41%	68.58%
Aero1	0.382	0.762	0.054	0.694	<0.001/21.95%	53.40%
Aero2	0.064	0.945	0.060	0.736	0.001/28.47%	72.27%
Aply1	0.595	<0.001/18.34%	0.955	0.102	0.273	76.26%
Iso-3	0.937	0.012/46.27%	0.555	0.388	0.110	49.20%

Separate nested ANCOVAs on the concentrations of each of the four compounds showed the relevance of spatial scale in the production of these compounds. The abundance of aerophobin-1 and aerophobin-2 differed significantly at the lowest scale (sampling site; $P < 0.001$ for both compounds; Fig. 5.2 and Table 5.2) while the concentrations of aplysinamisin-1 and isofistularin-3

did not vary at this scale (Aply1, $P = 0.273$; Iso3, $P = 0.110$, Table 5.2). This pattern reversed at the largest geographic scale investigated in our study (Region, over 2500 km apart). The compounds that did not vary between sampling sites, varied significantly across regions (over 2500 km apart) and *vice versa* (region; Aply1, $P < 0.001$; Iso3, $P = 0.012$; Aero1, $P = 0.762$; Aero2, $P = 0.945$; Fig. 5.3 and Table 5.2). Intermediate scales were nonsignificant for all compounds, although zone was almost significant for aerophobin-1 and aerophobin-2 (Table 5.2). Thus, Region explained 18.34% and 46.27% of the total variance in aplysinamisin-1 and isofistularin-3 while Sampling Site explained 21.95% and 28.47% of the total variance of aerophobin-1 and aerophobin-2 (Table 5.2). Depth failed to explain the concentration of any of the four BAs investigated (Table 5.2).

An earlier study on the concentration of BAs in *Aplysina aerophoba* around the Canary Islands showed that the concentration of BAs varied spatially, despite the striking chemical similarity in the secondary chemistry of this species in locations over 500 km apart (Teeyapant et al., 1993a). Our study suggests that the secondary chemistry of *A. aerophoba* varies over small and large spatial scales and our nested design allowed us to quantify the geographic scale that explained the largest variation in the concentration of BAs (Sokal and Rohlf, 1995). We found that the largest variation in the total concentration of BAs occurs at the lowest geographic scale investigated, i.e., specimens less than 500 m apart showed the largest differences in the total concentration of BAs. This pattern was also observed in the compounds aerophobin-1 and aerophobin-2, which varied the most at low spatial scales, while aplysinamisin-1 and isofistularin-3 varied the most across broad spatial scales.

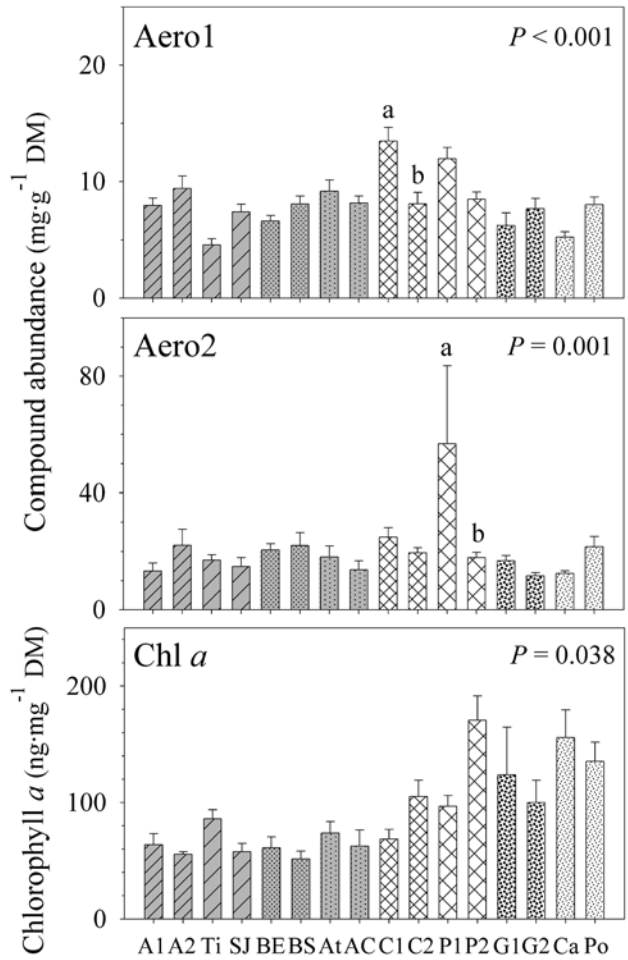


Figure 5.2. Significant differences in aerophobin-1, aerophobin-2 ($mg \cdot g^{-1}$ dry mass sponge tissue ± 1 SE), and chlorophyll *a* concentrations ($ng \cdot mg^{-1}$ dry mass sponge tissue ± 1 SE) of *Aplysina aerophoba* between sampling sites. A1 = Alcalá 1 ($N = 6$); A2 = Alcalá 2 ($N = 7$); Ti = Punta Tixera ($N = 7$); SJ = San Juan ($N = 9$); BE = Barranco del Eco ($N = 8$); BS = Barranco Seco ($N = 7$); At = Atlántida ($N = 6$); AC = Atlántida Coast ($N = 7$); C1 = Club Med 1 ($N = 10$); C2 = Club Med 2 ($N = 10$); P1 = Port de la Selva 1 ($N = 10$); P2 = Port de la Selva 2 ($N = 10$); G1 = Gat 1 ($N = 6$); G2 = Gat 2 ($N = 8$); Ca = Caials ($N = 7$); Po = Portlligat ($N = 8$). Aero1 = aerophobin-1; Aero2 = aerophobin-2; Chl *a* = chlorophyll *a*. Letters indicate significant differences ($P \leq 0.05$) of pairwise comparisons between sampling sites of the same location (showed as the same pattern design).

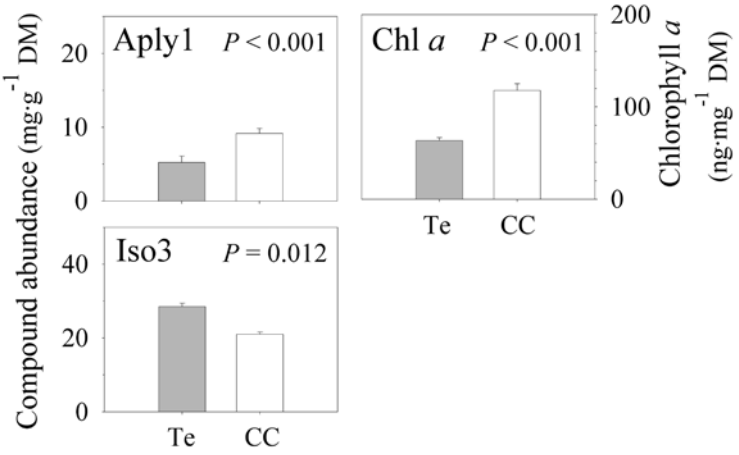


Figure 5.3. Significant differences in aplysinamisin-1, isofistularin-3 ($mg \cdot g^{-1}$ dry mass sponge tissue ± 1 SE), and chlorophyll *a* concentrations ($ng \cdot mg^{-1}$ dry mass sponge tissue ± 1 SE) of *Aplysina aerophoba* between Tenerife (Te, N = 57) and Cap de Creus (CC, N = 69). Aply1 = aplysinamisin-1; Iso3 = isofistularin-3; Chl *a* = chlorophyll *a*.

Our current knowledge of the production of BAs is incomplete, but it seems reasonable to argue that multiple factors acting at multiple spatial scales determine the concentration of these compounds. Studies from multiple disciplines often report increasing diversities with distance (Coykendall et al., 2011; Poulin et al., 2011; Noyer and Becerro, 2012) but changes in sponge secondary chemistry occur at wide range of distances (Swearingen and Pawlik, 1998; Puyana et al., 2003; Abdo et al., 2007). A study on the chemical diversity of the Atlanto-Mediterranean *Spongia lamella* showed two types of compounds (Noyer et al., 2011). One group remained constant among 9 populations scattered along the western Mediterranean and Atlantic coast of Portugal (half the distance of our study) while the other group varied significantly

across this scale (Noyer et al., 2011). The two aerophobin compounds seemed to be affected by factors acting at small spatial scale, whereas aplysinamisin-1 and isofistularin-3 varied significantly across large spatial scales. Isofistularin-3 and aerophobin-2 provide effective chemical defense against predators (Thoms et al., 2004) and other BAs present in *A. aerophoba* show strong cytotoxic, algicide, and antibacterial activities (Koulman et al., 1996; Weiss et al., 1996). Geographic differences in predation, fouling, competition, or symbionts (see below) among other factors could account for the contrasting patterns of variation of BAs in *A. aerophoba* (Becerro et al., 1995; Swearingen and Pawlik, 1998; Page et al., 2005; Abdo et al., 2007; Noyer et al., 2011).

Alternatively, our data could be interpreted as an intriguing evidence of the chaotic nature of chemical variability. This chaotic vision could be sustained by true variation in BAs, or could be the result of multiple factors (e.g., sample manipulation, compound quantification, data analyses). Experimental errors, however, would either amplify or reduce our perception of the existing natural variation. Since our methods minimized compound alteration in this species and were consistent across all samples, we believe that our study accurately describes the natural variation in the production of BAs in *A. aerophoba* from local to regional geographic scale. Although a significant percentage of the variation in BAs in *A. aerophoba* was explained (up to 47%), between 49% and 77% remained unexplained (Table 5.2); thus it is clear that other, currently unknown, factors account for such variability. Further analyses and understanding of the production of natural products will shed light on this area.

5.3.3 Chlorophyll *a* variation

Chl *a* slightly decreased with increasing depth, which explained over 15.29% of the variation in Chl *a* in our samples (Table 5.1). However, the effect of spatial scale was larger than depth (Table 5.2). Our results showed that 31.76% of the variance in Chl *a*

occurred between the two distant geographic regions and 9.46% between sites less than 500 m apart (Table 5.2). The pattern of Chl *a* variation mirrored the pattern of BAs variation. Chl *a*, aplysinamisin-1, and isofistularin-3 varied the most between the two biogeographic regions 2500 km apart. Total concentration of BAs, aerophobin-1, and aerophobin-2 varied the most between sites less than 500 m apart, which was the second source of variation in Chl *a*. Because concentration of Chl *a* can be used as a proxy of the abundance of photosynthetic symbionts in sponges (Becerro et al., 1998; Becerro et al., 2003; Erwin and Thacker, 2007) and photosynthetic symbionts can contribute to the production of secondary metabolites (Unson and Faulkner, 1993; Unson et al., 1994; Piel, 2004) (including *Chloroflexi* photosynthetic bacteria in *A. aerophoba* (Siegl and Hentschel, 2009; Sacristán-Soriano et al., 2011b)), we further investigated whether the concentration of Chl *a* and secondary metabolites was related.

5.3.4 Relationship between natural products and chlorophyll *a*

Individual correlation analysis resulted in one significant negative correlation between the abundance of isofistularin-3 and the concentration of Chl *a* ($R = -0.430$, $P < 0.001$; Fig. 5.4, Table 5.1). We detected no further relationships between Chl *a* and remaining compounds (Table 5.1).

Chl *a* in *Aplysina aerophoba* may come from multiple symbiotic sources including photosynthetic cyanobacteria and *Chloroflexi* (Sacristán-Soriano et al., 2011b). Our results suggested that the higher the abundance of photosynthetic symbionts in the sponge, the lower the concentration of isofistularin-3. Whether this negative association represents a direct relationship between this compound and any of the photosynthetic symbionts in *A. aerophoba* is unclear, but isofistularin-3 has been shown to be positively associated with the abundance of a symbiotic bacterium (Sacristán-Soriano et al., 2011b). Whether this bacterium decreases its

abundance with increasing photosymbiont concentrations remains unknown, but these correlations may reflect a chemically mediated biotic interaction between the sponge host and potential symbionts or between two members of the symbiotic microbial community within *A. aerophoba*.

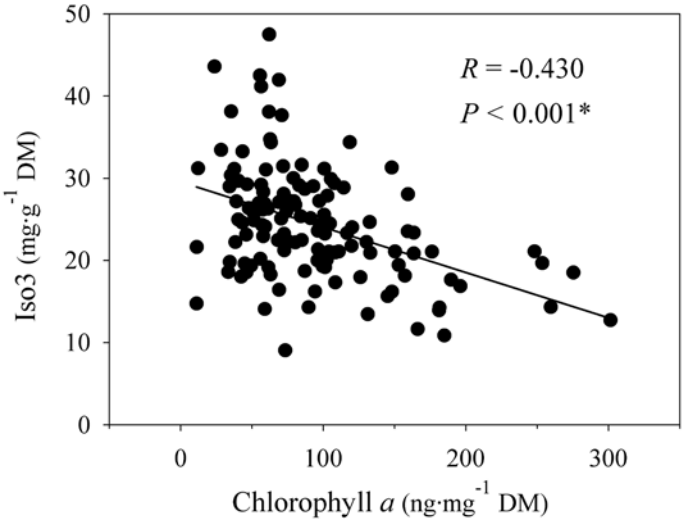


Figure 5.4. Relationship between the abundance of isofistularin-3 (Iso3; $mg \cdot g^{-1}$ dry mass sponge tissue ± 1 SE) and the concentration of chlorophyll *a* (Chl *a*; $ng \cdot mg^{-1}$ dry mass sponge tissue ± 1 SE). * Significant *P*-value after Bonferroni correction.

The diversity of symbiotic communities and natural products found in marine invertebrates is a challenge to assess the role of the symbionts in the production of the compounds typically assigned to the hosts. This is an area that clearly needs further research. *Aplysina aerophoba* has been studied extensively, but we still know very little about factors shaping the production of BAs in this species. Although the BAs seem to be stored in sponge cells (Turon et al., 2000), multiple cell components might be involved in their production and the role of symbionts is largely unknown (Kreuter et al., 1992; Ebel et al., 1997; Siegl and Hentschel, 2009; Sacristán-

Soriano et al., 2011b). This hypothesis was launched over a decade ago but it remains to be experimentally tested. The advent of new biotechnological methods and approaches will surely shed light on the role of symbionts in the production of natural products (Ding and Cantor, 2004; Donia and Schmidt, 2011; Sacristán-Soriano et al., 2011b).

5.4 Conclusions

The spatial scale explaining the largest variation in the concentration of natural products within a single species depends on the actual compound investigated. Our study showed that the rich secondary chemistry of *Aplysina aerophoba* varied quantitatively from sites less than 500 m apart to geographic regions over 2500 km apart. Aerophobin-1 and aerophobin-2, and the combined concentration of all BA compounds varied the most between sampling sites less than 500 m apart, while aplysinamisin-1 and isofistularin-3 varied across larger spatial scales.

Depth played a trivial role, if any, regulating the concentration of BAs in *A. aerophoba*. Individual correlation analyses with all our data showed that depth could explain a small percentage (8–12%) of the variation in BAs. This minor effect was however overshadowed by the larger influence of spatial scale in the concentrations of BAs.

The putative role of symbionts on the production of BAs in *A. aerophoba* remains an open question. There is indirect evidence that supports the role of symbionts, including the correlation between isofistularin-3 and chlorophyll *a* detected in this study. The circumstantial evidence available provides a number of testable hypotheses that, with the use of new molecular techniques, should contribute to the development of this particularly elusive research area.

Acknowledgments

We thank Sandra Duran, Andrea Blanquer, and Natalia Ortega for their invaluable help in the Canarian sampling trip. We also thank the anonymous reviewers for suggestions that improved the final version of the manuscript.

This research is a part of OSS PhD Thesis, who was supported by a CSIC predoctoral grant. Research partially funded by the Spanish Ministry of Science and Innovation (SOLID: CTM2010-17755 and BENTHOMICS: CTM2010-22218-C02-01) and by the Agence Nationale de la Recherche (France; ECIMAR project, ANR-06-BDIV-001-04). This research is a contribution of the Consolidated Research Group "Grupo de Ecología Bentónica" (SGR2009-655) of the Catalan Goverment.

6

Temporal trends in the secondary metabolite production of the sponge *Aplysina aerophoba*

Resumen

Se desconocen en gran medida los cambios temporales en la producción de metabolitos secundarios. Con este estudio cuantificamos la concentración de compuestos bromados tanto en el ectosoma como en el coanosoma de *Aplysina aerophoba* durante un periodo de dos años, y examinamos los patrones temporales de dichos productos naturales. Se cuantificó la concentración de aerophobin-2, aplysinamisin-1 e isofistularin-3, tres de los cuatro compuestos mayoritarios, mediante cromatografía líquida de alta resolución basándose en curvas de estándares. Encontramos diferencias significativas en la abundancia de compuestos entre la capa externa y el interior de la esponja. Mientras que el ectosoma mostraba abundancias más altas de compuestos bromados durante

los meses de verano, el coanosoma no seguía un claro patrón de variación. Además, encontramos que los compuestos aerophobin-2 e isofistularin-3 de la región del ectosoma estaban correlacionados con la temperatura del agua. Este estudio es uno de los primeros en documentar y cuantificar cambios estacionales en compuestos individuales registrados durante varios años. Estudios posteriores ayudaran a clarificar el papel de factores ambientales, biológicos y fisiológicos en determinar los patrones estacionales de los compuestos bromados.

Abstract

Temporal changes in the production of secondary metabolites are far from being fully understood. Our study quantified, over a two-year period, the concentrations of brominated alkaloids in the ectosome and the choanosome of *Aplysina aerophoba*, and examined the temporal patterns of these natural products. Based on standard curves, we quantified the concentrations of aerophobin-2, aplysinamisin-1, and isofistularin-3: three of the four major peaks obtained through chemical profiling with high-performance liquid chromatography. Our results showed a striking variation in compound abundance between the outer and inner layers of the sponge. The ectosome showed high concentrations of bromocompounds during the summer months, while the choanosome followed no pattern. Additionally, we found that, from the outer layer of the sponge, aerophobin-2 and isofistularin-3 were significantly correlated with water temperature. The present study is one of the first to document quantitative seasonal variations in individual compounds over multiple years. Further studies will clarify the role of environmental, biological, and physiological factors in determining the seasonal patterns in the concentration of brominated alkaloids.

6.1 Introduction

Marine invertebrates are involved in a great variety of interactions, many of which are chemically mediated (Paul et al., 2006; Egan et al., 2008). Not surprisingly, marine invertebrates are a potential source of natural, bioactive products that act against external threats (Sipkema et al., 2005a; Paul et al., 2006). These compounds often play multiple ecological roles, primarily protection against predators (Pawlik et al., 1995; Waddell and Pawlik, 2000a, b; Burns et al., 2003; Ruzicka and Gleason, 2009), competitors for space (Engel and Pawlik, 2000; Luter and Duckworth, 2010), biofoulers (Uriz et al., 1992; Becerro et al., 1994), or opportunistic pathogenic microorganisms (Newbold et al., 1999; Kelman et al., 2001).

Natural products have generated pharmaceutical and biotechnological interest due to their potential roles in human diseases (Newman and Cragg, 2007). However, novel drug development requires large amounts of the candidate species in screening for potentially bioactive compounds (Munro et al., 1999; Newman and Cragg, 2004). To that end, biological and ecological studies can provide guidelines for applied and biotechnological research. To collect sufficient amounts of bioactive compounds for testing, we need to know where the natural products are produced and stored (e.g., specific tissue or cells), what organisms are involved in their production (i.e., the marine invertebrate, an associated microorganism, or multiple organisms), and what factors affect their production (e.g., biotic, abiotic factors). An important point to understand natural product production is whether or not secondary metabolites vary over time, behavior which is unknown for most species. Continuous quantitative data over multiple years will contribute significantly to understand their natural variability, providing additional information to contrast with proposed mechanisms of production and natural function.

Sponges are among the best-known producers of bioactive compounds (Faulkner, 2000; Paul et al., 2006; Erwin et al., 2010). Sponges are distributed in multiple, diverse habitats (Zenkevitch

and Birstein, 1960; Reiswig, 1973; Wilkinson and Cheshire, 1989; Uriz et al., 1992; Corriero et al., 2000; Voultsiadou, 2005); this property has generated an interest in sponges in the field of chemical ecology and in the pharmacognosy industry (Becerro, 2008). To date, research has focused mostly on novel drug discovery with highlights on the origin of natural products. In comparison, little is known about the temporal variability of the vast number of known bioactive compounds. Secondary metabolite production may vary as predicted by the optimal defense theory (ODT), outlined by McKey (1974, 1979) and Rhoades (1979), in plants, and tested by several authors (Skogsmyr and Fagerstrom, 1992; Zangerl and Rutledge, 1996). The ODT assumes that the metabolic cost of secondary metabolite production for chemical defenses must meet the organisms' needs and be balanced against the other terms in the energy budget (i.e., reproduction and growth). This theoretical framework of terrestrial plant defenses is particularly relevant to sponge chemical ecology (Becerro et al., 1995; Pawlik et al., 2008). Variations in the abundance of natural products may respond to physical constraints such as hydrodynamism (Page et al., 2005), depth (Thompson et al., 1987; Page et al., 2005; Ferretti et al., 2009), or water temperature (Abdo et al., 2007). Habitat (Becerro et al., 1995), sponge size (Becerro et al., 1995), competition for space (Turon et al., 1996), or against fouling (Duckworth and Battershill, 2001) may also cause changes in sponge secondary chemistry. Many of these biotic or abiotic factors vary between months, seasons, and years (Turon et al., 1996; Becerro et al., 1997b; Duckworth and Battershill, 2001) so the production of secondary metabolites may have strong temporal patterns.

The aim of the present study was to quantitatively assess the temporal variability of bioactive compounds in the demosponge *Aplysina aerophoba* (Nardo 1833). This verongid is a rich source of brominated alkaloids (BAs), which are chemically well characterized (Weiss et al., 1996; Ciminiello et al., 1997; Ebel et al., 1997; Thoms et al., 2006). BAs in this sponge are known to vary

within the same specimen and at multiple geographic scales (Kreuter et al., 1992; Turon et al., 2000; Sacristán-Soriano et al., 2011a; 2011b), but the temporal patterns of variation remain undescribed. Here, we aimed to determine whether the concentration of natural products varied at multiple temporal scales, from months, to seasons, and years. Our results showed that temporal changes in the concentration of BAs occurred in the outer layer (i.e., ectosome) of the sponge while we did not detect any variation in the internal region (i.e., choanosome).

6.2 Materials and methods

From May 2008 to April 2010, five different healthy specimens of the sponge *Aplysina aerophoba* were sampled monthly by scuba diving in Portbou (Northwestern Mediterranean) to depths between 7 and 10 m. Sampling was always conducted on fresh individuals with similar sizes, which were separated from each other between 2 and 100 m. Underwater, we cut off the upper part of one chimney per specimen and placed them in plastic bags with seawater. Immediately after collection, samples were placed in coolers with ice to prevent changes in the secondary chemistry. Once in the laboratory, a small portion of the top half of the chimney away from the cutting surface (i.e., injured tissue) was selected for the quantification of the BAs, minimizing a potential effect of manipulation and the biotransformation of high molecular weight metabolites into low molecular weight compounds (Ebel et al., 1997). We used a sterilized scalpel to excise samples from the ectosome (1 mm-thickness) and choanosome (2 mm-thickness) of the sponge under seawater. Samples were then frozen at −20 °C within 3 hours of collection until processing.

The major brominated alkaloids (BAs) of *Aplysina aerophoba* were extracted, isolated and identified as described by Sacristán-Soriano et al. (2011b). Briefly, we reduced sponge manipulation, avoided air exposure of fresh samples, froze sponges immediately after sample preparation (less than 4 hours after collection), freeze-

dried material and keep them at −20 °C, and used methanol only for extraction. With this methodology, we have never detected the biotransformation process that converts the high molecular weight (HMW) BAs into low molecular weight (LMW) BAs described for this species. LMW BAs were either absent in our chromatograms or at concentrations below our detection threshold. We purified the four major and known compounds aerophobin-1, aerophobin-2, aplysinamisin-1, and isofistularin-3 as described by Sacristán-Soriano et al. (2011b). We characterized these compounds with proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR; JEOL EX 400 spectrometer), liquid chromatography-mass spectrometry (LC-MS; Thermo Scientific LCQ Fleet), UV spectrometry (Hewlett Packard diode array spectrophotometer), and comparison of spectroscopy data with published values from the literature (Cimino et al., 1983; Ciminiello et al., 1997). Full details on the chemical methods can be found in Sacristán-Soriano et al. (2011b).

HPLC analyses were performed with a system from Waters that included the Alliance separations module 2695, the column heater, and the 2998 photodiode array detector. The equipment was controlled and the data were handled by the Empower Chromatography Data software (Waters). The HPLC conditions consisted of two eluents (A: 0.1% aqueous trifluoroacetic acid, B: acetonitrile) and an elution profile based on a linear gradient from 30% B to 80% B within 18 min and then to 100% B in an additional 10 min. The flow rate was constant at 0.4 $mL \cdot min^{-1}$. We used a Phenomenex Synergi Max-RP (80 Å, 250 × 3.0 mm, 4 µm) analytical column with a fixed temperature of 30 °C.

For quantification of the natural products, 30 mg of freeze-dried sponge tissue from ectosomal and choanosomal samples were prepared by extracting three times with 1.5 mL of methanol (MeOH) in an ultrasonic tank for 15 min (Sacristán-Soriano et al., 2011b). The crude extract was filtered through a 20-µm polytetrafluoroethylene filter (PTFE) into a 5 mL beaker. The final volume was adjusted to 5 mL of crude extract. Aliquots of 1.5 mL

were passed through a 13 mm, 0.2-µm PTFE syringe-filter before injecting 10 µL into the HPLC system. The brominated compounds 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 previously purified and characterized external standard compounds. The final amounts of the natural compounds were calculated by averaging three replicate injections. Concentrations of BAs were expressed in $mg \cdot g^{-1}$ of dry mass of sponge tissue.

We obtained sea surface temperatures (SSTs) at the study site throughout the 2-year study (May 2008 to April 2010) by accessing moderate-resolution imaging spectrometer measurements (Aqua MODIS satellite) made by the NASA Goddard Space Flight Center [Available online: http://oceancolor.gsfc.nasa.gov/ (accessed on 1 March 2011)]. "Ocean Level-2" HDF data were read and processed with MathLab R2009a software. High-quality readings were obtained with flag values of 0 or 1 and we discarded unreliable data (flag values of 2 or 3). We used a flag value of 1 because insufficient readings were obtained with a flag of 0. Suitable SST readings (N = 293) corresponded to daily means in a 9-km^2 area centered at the following coordinates: 42°25'33.6"N–3°12'40.32"E (Portbou). We also measured seawater temperatures with *in situ* data loggers (Onset Computer Corporation, model HOBO Pendant UA-002-08) placed at a depth of 7 m for one year (June 2009 to April 2010) for comparisons with satellite-derived SST data. Monthly average temperatures were calculated for both satellite-derived and logger-derived data.

We used PRIMER 6 software (Clarke and Warwick, 2001) to analyze data on secondary metabolites of *Aplysina aerophoba* as a function of tissue type (ectosome or choanosome). Square root-transformed data were used to calculate Bray-Curtis similarities. We used permutational multivariate analysis of variance (PERMANOVA) to test for differences in secondary metabolites among different tissue layers. Results were plotted with non-metric multidimensional scaling (MDS).

We used several statistical methods from SYSTAT 12 software (SPSS, 1999a, b) to analyze each secondary metabolite separately. We used a nested design of ranked compound abundances with year (first and second), season (spring, summer, autumn, and winter), and month (12 months) as factors. Seasons were defined by natural periods (not by temperature) so they contained the same months in both years. Nested designs require random subordinate groups. We treated month and season as random factors because their actual conditions vary tremendously between years, i.e., despite sharing the same name, months and seasons could be confined to their specific years. In nested analyses, lower variables are always tested before upper variables. To test for the significance of an upper level, the model considers the variance associated with the lower level, so there may be differences in an upper level regardless the significance of the levels below. With this hierarchical approach, we can therefore partition the variance into components associated with each temporal scale (year, season within years, and months within seasons) to assess the temporal scale at which concentration of BAs vary the most. This approach can provide hints on the mechanisms behind the temporal pattern. We then used Tukey HSD pairwise comparisons to test for differences between the levels of each significant temporal scale. We used simple correlation analysis to establish the quantitative relationships between secondary metabolites. We also performed trend analyses of compound data to assess for tendencies and seasonal variations over time in the abundances of secondary metabolites.

Monthly logger and satellite-derived temperature data from the same period were compared with paired t-tests to identify differences between methodologies. Pearson's correlation was used to examine associations between ranked compound abundances and satellite-derived SSTs.

6.3 Results and discussion

6.3.1 Chemical profile of *Aplysina aerophoba*

We quantified a total of 240 samples (2 sampling years × 12 months per year × 5 individual sponges per month × 2 tissues per specimen) to characterize the chemical profile. We identified four out of six major peaks: aerophobin-1 (Aero1; peak 3), aerophobin-2 (Aero2; peak 4), aplysinamisin-1 (Aply1; peak 5), and isofistularin-3 (Iso3; peak 6), by comparing their retention times and UV profiles to those of purified, characterized standard compounds. Of these, we finally quantified the three major BAs (Aero2, Aply1, and Iso3), because Aero1 was often under the detection limit in the HPLC analyses.

6.3.2 Natural product variations

The three compounds quantified showed significant differences in abundance in the ectosomal (outer) and choanosomal (inner) layers of the sponge (PERMANOVA, $P = 0.001$; Fig. 6.1). Therefore, we subsequently performed separate analyses of the chemical data for these two layers.

We found striking differences in the concentrations of secondary metabolites between the ectosome and the choanosome of *Aplysina aerophoba*. These differences were not only caused by changes in the abundance of the secondary metabolites but also in their dynamics. As reported for other sponges (Turon et al., 1996; Becerro et al., 1998; Schupp et al., 1999), *A. aerophoba* produces a heterogeneous mix of natural products. This heterogeneous mix of compounds is not uniformly distributed within the sponge. Based on X-ray energy dispersive microanalysis to locate bromine atoms (Turon et al., 2000), BAs in *A. aerophoba* were found in two sponge structures: the spherulous cells and the sponge fibers. Kreuter et al. (1992) also detected intraspecimen differences in the amount of the low molecular weight (LMW) compounds, aeroplysinin-1 and dienone, between the external (i.e., outer layer,

oscular region) and the internal regions of *A. aerophoba*. These results were in agreement with a recent study (Sacristán-Soriano et al., 2011b) that found significant differences in the accumulation of high molecular weight (HMW) BAs between the choanosome (i.e., inner region) and the ectosome (i.e., outer layer) of the same species. The concentrations of the three compounds we quantified were positively correlated in both layers: ectosomal (Aero2-Aply1, $R = 0.560$; Aero2-Iso3, $R = 0.658$; Aply1-Iso3, $R = 0.916$; $P < 0.001$ for all comparisons) and choanosomal (Aero2-Aply1, $R = 0.363$; Aero2-Iso3, $R = 0.571$; Aply1-Iso3, $R = 0.849$; $P < 0.001$ for all comparisons). This could indicate that all compounds might be responding in the same way and at the same scale to the regulating factor(s).

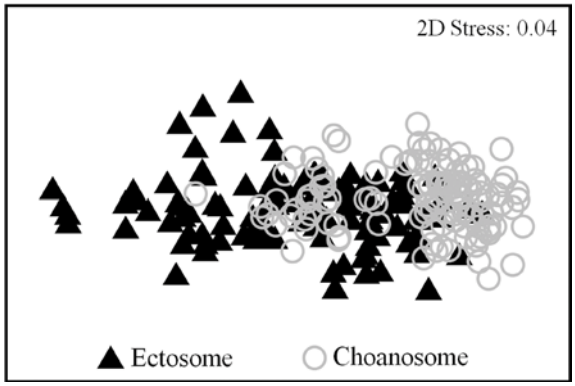


Figure 6.1. Metabolite abundances in the ectosome and choanosome of *Aplysina aerophoba*. Non-metric multidimensional scaling (MDS) was based on Bray-Curtis similarity matrices from standardized and square root-transformed abundances of chemical data. Significant differences were found between both layers.

The HMW BAs have been described as undergoing biotransformation when the tissue has been damaged, resulting in the LMW BAs aeroplysinin-1 and dienone, a process that is supposed to be enzyme-mediated (Weiss et al., 1996; Ebel et al., 1997). The presence of these LMW BAs in our chromatograms

could cast doubt on the actual concentration of the precursors quantified in our samples (Thoms et al., 2006). Yet, we never detected these bioconverted natural products in our chromatograms or they were at such low concentrations that cannot explain the large variation in BAs observed in our samples. This is expected, because our methods were designed to minimize the probability of biotransformation, keeping tissue manipulation to a minimum and avoiding the use of water. Moreover, Puyana et al. (2003) found no evidence for biotransformation in two *Aplysina* species from the Caribbean. There is an unresolved controversy as to whether or not biotransformation occurs in *Aplysina* species and our study can neither support nor refute this biotransformation hypothesis. Our study was designed to investigate the temporal variation of the unaltered secondary chemistry of *Aplysina aerophoba*. If biotransformation does occur, our methods proved ideal to avoid biotransformation because we only found HMW BAs in our chromatograms, so biotransformation can neither affect nor explain the intraindividual variation observed in our study.

At the ectosomal level, there was no change in the abundances of the three compounds between years (nested ANOVA, $P > 0.05$ for the three BAs; Table 6.1). We found significant seasonal differences in the concentration of Aply1 and Iso3, with greater abundances in summer (nested ANOVA, $P < 0.01$ for both BAs; Fig. 6.2, Table 6.1). However, there was no seasonal change in the abundance of Aero2 (nested ANOVA, $P > 0.05$; Table 6.1). Significant monthly differences were detected in the concentration of Aero2; the abundance increased in August and decreased in February (nested ANOVA, $P < 0.05$; Fig. 6.3, Table 6.1). Nevertheless, the abundance of Aply1 and Iso3 did not vary between months (nested ANOVA, $P > 0.05$ for both; Table 6.1). Our results showed that "year" failed to explain the concentration of any of the three BAs investigated in the external layer of the sponge. However, "season" explained 25.82% and 24.44% of the total variance in Aply1 and Iso3 while "month" explained 14.85% of the total variance in Aero2 (Table 6.1).

Table 6.1. *P*-values and percentage of the total variance explained by year, season, and month in the ectosome and the choanosome of the sponge. Values obtained from nested ANOVAs used to test the effect of timescale on the concentration of aerophobin-2 (Aero2), aplysinamisin-1 (Aply1), and isofistularin-3 (Iso3). We also show the percent variance unexplained by the factors (Error).

	Ectosome				Choanosome			
Compound	Year	Season	Month	Error	Year	Season	Month	Error
Aero2	0.869	0.190	0.003/24.44%	78.13%	0.059	0.491	0.030/13.67%	76.29%
Aply1	0.368	0.001/25.82%	0.003/24.44%	74.18%	0.463	0.438	0.025/15.80%	83.80%
Iso3	0.260	0.003/24.44%	0.322	74.18%	0.551	0.201	0.031/14.03%	79.60%

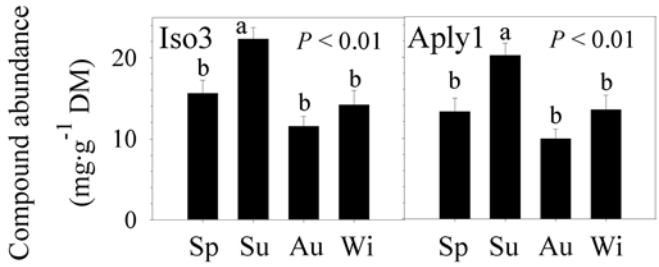


Figure 6.2. Seasonal changes in BA abundances. Secondary metabolite concentrations (mg·g^{-1} dry mass sponge tissue) ± 1 standard error of the mean (SE) observed in different seasons in the ectosome of *Aplysina aerophoba*. Sp = spring (N = 30); Su = summer (N = 30); Au = autumn (N = 30); Wi = winter (N = 30). Aply1 = aplysinamisin-1; Iso3 = isofistularin-3. Means (i.e., seasons) with different letters are significantly different from each other ($P \leq 0.05$; pairwise comparisons).

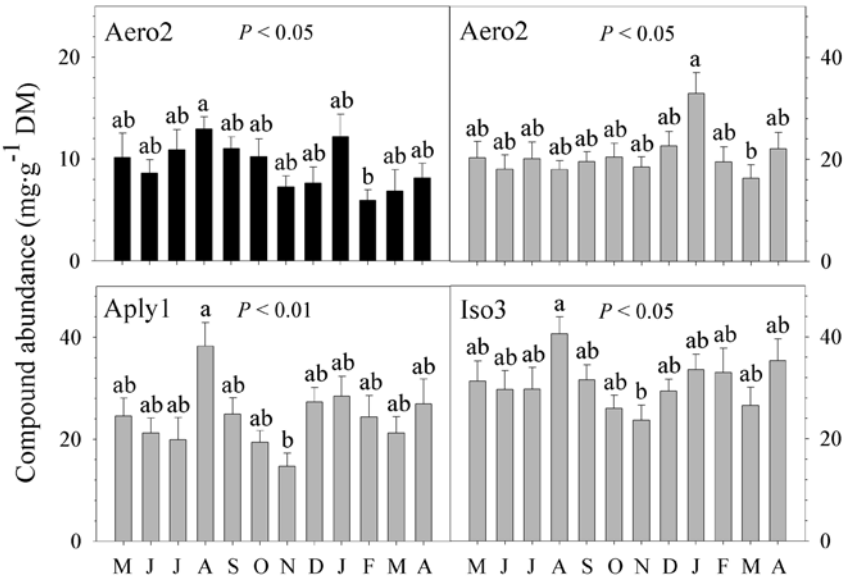


Figure 6.3. Monthly changes in metabolite concentrations. Secondary metabolite abundances (mg·g^{-1} dry mass sponge tissue ± 1 SE) in the ectosome (black) and in the choanosome (grey) of *Aplysina aerophoba* from May to April (N = 10 per month). We only show compounds in which there were significant monthly changes in metabolite concentrations. Aero2 = aerophobin-2; Aply1 = aplysinamisin-1; Iso3 = isofistularin-3. Means (i.e., months) with different letters are significantly different from each other ($P \leq 0.05$; pairwise comparisons).

At the choanosomal level, we found no annual differences in the abundances of the three compounds (nested ANOVA, $P > 0.05$ for the three BAs; Table 6.1). The concentration of the three BAs did not vary either between seasons (nested ANOVA, $P > 0.05$ for the three BAs; Table 6.1). However, we found significant monthly changes in the abundances of the three compounds (nested ANOVA, $P < 0.05$ for the three BAs; Figure 6.3, Table 6.1).

Whereas the abundance of Aply1 and Iso3 increased in August and decreased in November, the concentration of Aero2 was higher in January and lower in March (Fig. 6.3). Neither "year" nor "season" explained the concentration of the three BAs in the internal part of the sponge. But, "month" explained 13.67%, 15.80%, and 14.03% of the total variance in aerophobin-2, aplysinamisin-1, and isofistularin-3 (Table 6.1).

6.3.3 Natural product trends

Looking at the complete 2-year-long temporal series, we found in the external layer of the sponge significant changes in the concentration of Aply1 and Iso3 over time but not in Aero2 (P = 0.035, P = 0.038, and P = 0.711, respectively; Fig. 6.4). Contrastingly, we found no significant trend or seasonality in the abundance of BAs in the choanosome of the sponge (Aero2, P = 0.083; Aply1, P = 0.509; and Iso3, P = 0.566; Fig. 6.5).

Natural variations in sponge secondary chemistry have received little attention. An environmental influence on bioactive compound production was first documented by Thompson et al. (1987) in the sponge *Rhopaloeides odorabile*. Seasonal patterns were reported later in several sponge species, where bioactivity was used to measure metabolite biosynthesis. Turon et al. (1996) found *Crambe crambe* most biologically active in late summer and autumn. *Latrunculia* sp. nov. was most active in spring (Duckworth and Battershill, 2001). Swearingen & Pawlik (1998) documented that *Chondrilla nucula* was most toxic in summer. In contrast, other sponge species, *Agelas oroides* and *Petrosia ficiformis*, appeared to be most bioactive during winter (Ferretti et al., 2009). But, relatively few studies have described long-term, quantitative, temporal changes in individual biologically active compounds.

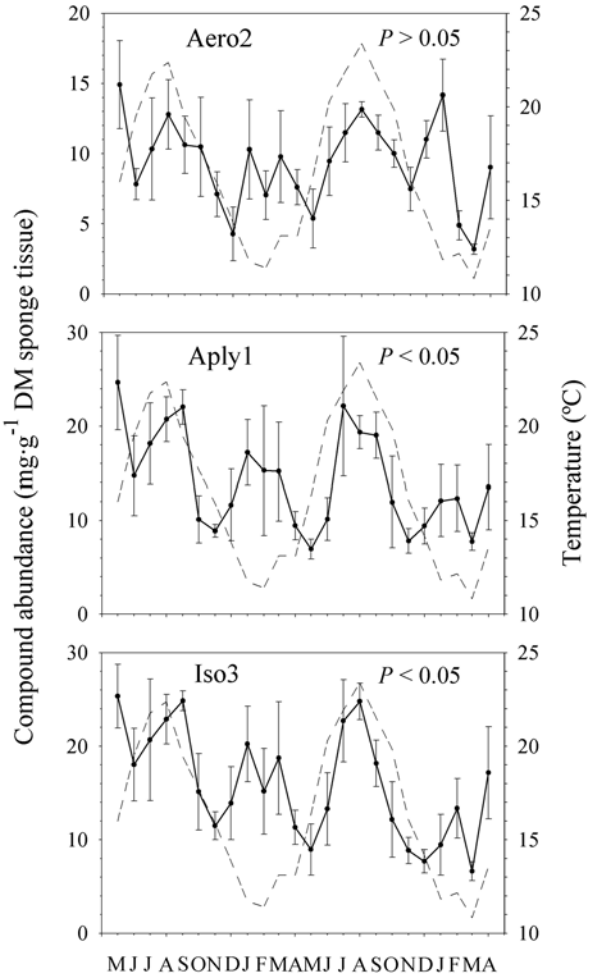


Figure 6.4. Temporal trends in metabolite abundances in the ectosome. Compound abundances (mg·g^{-1} dry mass sponge tissue ± 1 SE) observed in the ectosome of *Aplysina aerophoba* over a two-year survey (N = 5 per month). Aero2 = aerophobin-2; Aply1 = aplysinamisin-1; Iso3 = isofistularin-3. Significant differences $P \leq 0.05$. The sea surface temperatures (°C) are also shown for the whole period (discontinuous line).

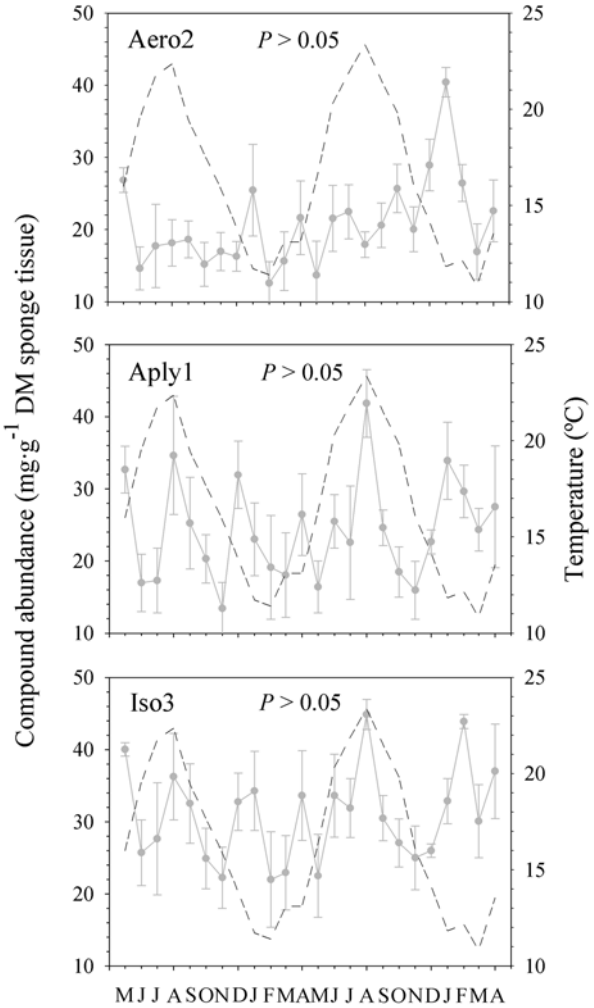


Figure 6.5. Temporal trends in metabolite abundances in the choanosome. Compound abundances (mg·g^{-1} dry mass sponge tissue ± 1 SE) observed in the choanosome of *Aplysina aerophoba* over a two-year survey (N = 5 per month). Aero2 = aerophobin-2; Aply1 = aplysinamisin-1; Iso3 = isofistularin-3. Significant differences $P \leq 0.05$. The sea surface temperatures (°C) are also shown for the whole period (discontinuous line).

We detected changes within but not between years in the BAs of *Aplysina aerophoba*. Our data showed that temporal variations in BAs were tissue-specific. The internal region (i.e., choanosome) did not appear to follow a clear pattern of production, but changes in sponge secondary chemistry occurred at low temporal scale (i.e., months). By contrasting, there seemed to be a clear trend in the external layer (i.e., ectosome) with the highest production period in summer and a lower peak in winter. Whereas aerophobin-2 seemed to be more prone to variation between months, aplysinamisin-1 and isofistularin-3 varied significantly between seasons. In fact, we also showed evidence for an annual periodicity with greater abundances of these two compounds in the warmer season. Page et al. (2005) reported a similar seasonal pattern in the concentration of two bioactive compounds of *Mycale hentscheli*, although they did not present evidence of an annual periodicity in secondary metabolite production. Similarly, Abdo et al. (2007) documented that the concentration of one bioactive compound of *Haliclona* sp. was significantly higher in summer than in winter.

Alternatively, our data could be the result of the chaotic nature of chemical variability. Our results could be supported by true variation in BAs, or could be the consequence of laboratory errors (as a result of our extraction methods and analyses). Experimental errors, however, could either amplify or reduce the true natural variation. Since our methods minimized compound alteration in this species and were consistent across all samples, we believe that our study accurately describes the natural temporal variation in the production of BAs in *A. aerophoba*. Although a notable percentage of the variation in BAs in *A. aerophoba* was explained by the temporal scales investigated in our study (up to 26%), most variance remained unexplained (Table 6.1). It is clear that other unknown factors account for such variability. Further analyses and understanding of the production of natural products will shed light on this area.

Variations in defense patterns among tissues can be explained by environmental or physiological constraints. We found a

chemically rich sponge core surrounded by a less chemically rich tissue layer (Sacristán-Soriano et al., 2011b). Traditionally, intraindividual differences in bioactive natural products were typically interpreted as evidence of the distinct roles of different tissues against predation, competition, or both (Becerro et al., 1995, 1997a; 1997b; Schupp et al., 1999). In *Aplysina*, the BAs aerophobin-2, aplysinamisin-1, and isofistularin-3 seem to be anti-predatory deterrents (Teeyapant et al., 1993b; Weiss et al., 1996).

From the perspective of the ODT, one would expect a more chemically enriched external layer due to its exposure to the environment. However, the higher concentration of BAs in the choanosome could suggest a sufficiently protected sponge in the external layer or low biological pressure from other marine organisms. Indeed, the nudibranch *Tylodina perversa* is the only known predator of *A. aerophoba* and preys preferentially on the ectosome of the sponge (Becerro et al., 2003). Thus, the higher internal abundance of BAs might minimize the effect of this specialist predator in the inner core of the sponge, similar to what has been described for other opisthobranch-sponge feeding interactions (Becerro et al., 1998; 2006a). Concentrations of chemical defenses—up to 10% of sponge dry mass in the ectosome—might be sufficient to protect the sponge against generalist fish predators, as tested by Thoms et al. (2004). In our study, we found that surface concentrations of BAs varied between 0.3 and 2.6%, which may not be high enough to deter predators, as opposed to the chemically enriched inner core that might also act to effectively deter generalist predators (Thoms et al., 2004). However, the deterrent activity of BAs at such low concentrations should be tested. Fluctuations in the abundance of *T. perversa* could explain part of the variation found in BA production, but most of the ecological and biological data of this gastropod is unknown (e.g., abundance, annual cycle).

Other roles (i.e., anti-fouling, competition, antibacterial) could also explain variations in secondary chemistry (Uriz et al., 1992; Becerro et al., 1994). The higher bioactivity during the warmer

period may have been a response to increased fouling on the sponge surface (Duckworth and Battershill, 2001) or to an increase in competitors (Turon et al., 1996). However, it seems unlikely that BA production was constrained by variations in the abundance of competitors or foulers, based on the anti-predatory role of the HMW BAs studied (Teeyapant et al., 1993b; Weiss et al., 1996). Indeed, only the LMW BA aeroplysinin-1 and dienone show strong antibiotic activity and may protect this sponge from invasion of bacterial pathogens (Teeyapant and Proksch, 1993; Ebel et al., 1997; Thoms et al., 2004) but these two compounds were absent or below threshold detection in our samples. These LMW BAs could however be readily available after biotransformation of the HMW BA precursors quantified in our study (Weiss et al., 1996; Ebel et al., 1997; Thoms et al., 2006). The possibility cannot be entirely ruled out that these biological constraints (i.e., foulers, competitors, pathogens) vary over the year, causing seasonal changes in the biosynthesis of bioactive compounds.

Seasonal changes in secondary chemistry may be also attributed to physiological factors related to differential investment in growth or reproduction. Leong and Pawlik (2010) described a resource allocation trade-off between chemical defenses and growth in two *Aplysina* species, with maximum growth in summer months. The reproductive biology of verongid sponges is little known. *A. aerophoba* is thought to be oviparous and dioecious, common traits in the order Verongida (Gallissian and Vacelet, 1976; Tsurumi and Reiswig, 1997; Bergquist and Cook, 2002). This order displays a restricted gamete production period between May and July (Scalera-Liaci et al., 1971; Gallissian and Vacelet, 1976). However, rope-form sponges (e.g., *Aplysina* species) appear to be adapted to undertake asexual reproduction by fragmentation; sexual reproduction is considered as a functional alternative with small investment in gamete production (Tsurumi and Reiswig, 1997). Data on growth and reproduction of *A. aerophoba* is scarce but it is likely to be similar to that of other verongid sponges. Taking into account data on other verongid species, maximum growth could

coincide with maximum metabolite production in summer just after gamete production, suggesting that these sponges were not limited in energetic resources during the warmer season. However, there is no evidence to support this hypothesis and formal data on growth and reproduction would be required.

6.3.4 Seawater temperature and concentration of BAs

We found no differences between the *in situ* and satellite-derived temperature data (paired *t*-test; $t = 0.884$, $df = 10$, $P = 0.397$) and the measurements were highly significantly correlated ($R = 0.990$, $P < 0.001$). Therefore, we used satellite-derived sea surface temperatures (SSTs) as a proxy for water temperature.

At the ectosomal level, the abundances of Aero2 and Iso3 were positively correlated with SST ($R = 0.500$, $P = 0.013$; and $R = 0.465$, $P = 0.022$, respectively; Fig. 6.6). If any, Aply1 was marginally correlated with the satellite-derived SST ($R = 0.395$, $P = 0.056$). Contrastingly, the abundances of Aero2, Aply1, and Iso3 in the choanosome were not correlated with SST ($R = -0.079$, $P = 0.713$; $R = -0.009$, $P = 0.968$; and $R = 0.086$, $P = 0.689$, respectively).

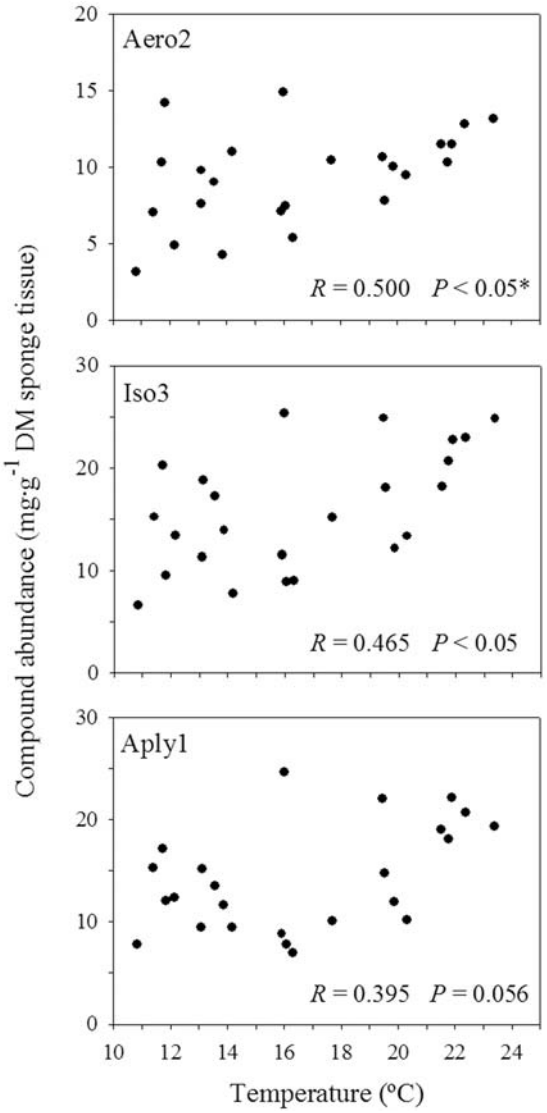


Figure 6.6. Correlations between compound abundances and satellite-derived sea surface temperatures. Changes in compound abundances ($mg \cdot g^{-1}$ dry mass sponge tissue) measured in the ectosome of *Aplysina aerophoba* were related to changes in sea surface temperature (°C). Aero2 = aerophobin-2; Aply1 = aplysinamisin-1; Iso3 = isofistularin-3. Significant differences $P \leq 0.05$. * Significant correlation after Bonferroni correction.

Variations in chemical defenses may be also attributed to environmental factors, such as water temperature. We showed that satellite-derived SST data could be accurately used as a proxy for seawater temperature pattern, consistent with other reports (Smale and Wernberg, 2009; Maldonado et al., 2010). Some BAs were significantly correlated with seawater temperature in the external part of *Aplysina aerophoba*. Water temperature could be directly affecting the secondary metabolite production as reported in *Haliclona* sp. (Abdo et al., 2007) or other factors (i.e., biological or physiological) correlated with seawater temperature might be causing such variation (Thompson et al., 1987; Becerro et al., 1995; Turon et al., 1996; Duckworth and Battershill, 2001; Page et al., 2005; Ferretti et al., 2009). In our study there appeared to be other factors that regulate the production of BAs. We based this hypothesis on the low correlation strength (R value ≤ 0.5) and the partial mismatch between temperature and ectosome patterns over time (Fig. 6.4). The relationship between BAs and temperature could imply no causation. In our opinion, water temperature does not cause changes in secondary chemistry, since those changes should then occur both in the ectosome and choanosome of the sponge. Other environmental factors (e.g., light exposure) could differentially affect the surface layer but not the inner region of the sponge (Thompson et al., 1987; Page et al., 2005) and could be driving these trends. Biotic factors that also preferentially affect the ectosome of the sponge and are associated with temperature could be behind the increased compound concentration of the ectosome in summer (Becerro et al., 1997b). It is known that photosynthetic symbionts can contribute to the production of secondary metabolites (Unson and Faulkner, 1993; Unson et al., 1994; Piel, 2004). Since most photosymbionts are found in the outer layer of the sponge, these associated bacteria could be driving somehow the secondary chemistry of *A. aerophoba* (Siegl and Hentschel, 2009; Sacristán-Soriano et al., 2011b). Although the BAs seem to be stored in sponge cells (Turon et al., 2000), multiple cell components might be involved in their production (Kreuter et al., 1992; Ebel et al., 1997;

Siegl and Hentschel, 2009; Sacristán-Soriano et al., 2011b). The actual role of symbionts in the production of secondary metabolites, however, is largely unknown.

6.4 Conclusions

To date, the present study was one of the first to document long-term (two years) quantitative seasonal variations in bioactive secondary metabolites in sponges. We provided evidence for high compound accumulation during summer months in the verongid *Aplysina aerophoba*, as previously described for other sponges. This extended our understanding of the underlying patterns of production of biologically active metabolites. Beyond the biological and ecological consequences, being aware of such variation can contribute to a sustainable wild harvest or aquaculture supply for biotechnological research (Munro et al., 1999), minimizing the extraction of sponge biomass. For example, our data could cause a two-fold increase in the supply efficiency of aplysinamisin-1 or isofistularin-3, should they be targets of biotechnological research or commercial applications. So, just by harvesting sponges at times of peak metabolite concentration we could reduce by up to 50% our impact in natural populations and our expenses to extract the target natural product.

Acknowledgments

We thank J. Gil and A. Villamor for their assistance with sample collection and R. Bernardello for assistance in processing HDF temperature data. We also thank anonymous reviewers for suggestions that improved the final version of the manuscript. This research is a part of OSS PhD Thesis, who was supported by a CSIC predoctoral grant. Research partially funded by the Spanish Ministry of Science and Innovation (SOLID: CTM2010-17755 and BENTHOMICS: CTM2010-22218) and by the Agence Nationale de la Recherche (France; ECIMAR project, ANR-06-BDIV-001-

04). This research is a contribution of the Consolidated Research Group “Grupo de Ecología Bentónica” (SGR2009-655) of the Catalan Government.

Part II: Effects of environmental factors on secondary metabolites and sponge-associated bacterial community

7

Can different light regimes cause variations in natural product and bacterial profiles of the sponge *Aplysina aerophoba*?

Resumen

La posible implicación de la comunidad microbiana en la química de la esponja es muy conocida a día de hoy. Sin embargo, se desconoce en gran medida cómo los factores ambientales afectarían tanto los perfiles químicos como microbianos y la implicación de estos cambios en el conjunto de la esponja. Con este estudio intentamos averiguar experimentalmente el efecto de la luz en la abundancia de metabolitos secundarios y en la comunidad bacteriana asociada a *Aplysina aerophoba*. Para ello, cuantificamos los compuestos bromados mayoritarios mediante cromatografía líquida de alta resolución y analizamos las poblaciones bacterianas

mediante electroforesis en gel con gradiente desnaturalizante empleando el gen 16S rRNA como marcador. Encontramos que los perfiles químicos y bacterianos eran distintos entre el ectosoma y el coanosoma de la esponja. A pesar que tanto los patrones de compuestos bromados como los bacterianos parecían mantener una estabilidad temporal, observamos que ciertos filotipos bacterianos prevalecían en condiciones lumínicas altas y otros en condiciones de escasa luminosidad, mientras que la mayoría de metabolitos incrementaron con independencia del régimen lumínico. Conseguimos identificar varias asociaciones entre una bacteria no identificada (OTU 84) y tres compuestos bromados (aerophobin-1, aplysinamisin-1 e isofistularin-3), y entre otra bacteria no identificada (OTU75) y el compuesto aerophobin-2. Estos resultados sugieren que estas bacterias podrían determinar la abundancia de los compuestos bromados o a la inversa. Las asociaciones que hemos encontrado servirán para establecer nuevas hipótesis que aporten nuevo conocimiento de la organización y funcionamiento de estas complejas interacciones huésped-simbionte.

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 high-performance liquid chromatography and analyzed sponge-associated bacteria using denaturing gradient gel electrophoresis on 16S rRNA gene amplicons. We identified distinct chemical and bacterial profiles between the ectosome and the choanosome of *A. aerophoba*. Although the chemical and microbial patterns seem to be stable over time, we observed some

phylotypes that preferred high light regimes and others preferred low illumination conditions while most of the secondary metabolite abundances increased regardless the illumination regime. We identified multiple associations between bacteria and natural products, including a strong relationship between OTU 84 and three brominated compounds (i.e., aerophobin-1, aplysinamisin-1, and isofistularin-3) and between OTU 75 and the compound aerophobin-2. Our results suggest that these bacteria could determine the abundance of BAs or *viceversa*. The associations found would serve to outline further hypotheses that improve our understanding of the organization and functioning of these complex host-symbiont interactions.

7.1 Introduction

Sponges are benthic invertebrates with a 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 and Thacker, 2008; Ribes et al., 2012), considering this association as a sponge holobiont. The role of those microbial symbionts has been well investigated in the last few years and has led the development of the sponge microbiology research (Grozdanov and Hentschel, 2007; Taylor et al., 2007b; 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 continues focusing its main effort on chemical defenses against predators. Studies of secondary metabolites with activities against competitors or fouling

came after (Paul et al., 2011). Comparatively, fewer studies concentrate their attention on sponge-associated microbial diversity as a source of potential bioactive compounds. Although sponge-cyanobacteria symbioses are quite well studied (Thacker, 2005; Erwin and Thacker, 2007; Erwin and Thacker, 2008), for most microbial symbionts their persistence in the host and their function is completely unknown. Understanding the processes that control the natural variation of chemical and bacterial diversity in sponges may help shed light on the symbionts' roles. A poor comprehension of those processes is hindering the development of marine chemical ecology, particularly in sponges (Paul et al., 2006).

A considerable effort has been done to elucidate potential biotic and abiotic factors that cause variation in the concentration of natural products. Among the biotic factors, changes in secondary chemistry have been observed due to sponge size, competition for space, predation, or fouling (Becerro et al., 1995; Turon et al., 1996; Duckworth and Battershill, 2001). Among the abiotic factors, depth, illumination, hydrodynamism, or water temperature appear to constrain 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., intraindividual, geographic, temporal variability) must be also considered to distinguish between natural variation and external modulation of the production of secondary metabolites (Kreuter et al., 1992; Turon et al., 1996; Duckworth and Battershill, 2001; Page et al., 2005; Abdo et al., 2007; Ferretti et al., 2009; Freeman and Gleason, 2010; Noyer et al., 2011; Sacristán-Soriano et al., 2011a; Sacristán-Soriano et al., 2011b; Sacristán-Soriano et al., 2012).

The variability of sponge-associated microbes has received comparable efforts. To date, the majority of studies have been restricted to evaluate intraspecific (i.e., within and between specimens of the same host (Althoff et al., 1998; Taylor et al., 2004; Taylor et al., 2005; Thiel et al., 2007b) over a number of spatial and temporal scales (Friedrich et al., 2001; Webster and Hill, 2001; Webster et al., 2004; Hoffmann et al., 2006; Erwin et al., 2012b))

and interspecific (i.e., between host species (Hentschel et al., 2002; Taylor et al., 2004; Usher et al., 2004; Noyer et al., 2010)) variation. 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; Webster et al., 2008b; Erwin et al., 2012b; Simister et al., 2012).

In the present study, we tested whether light can cause fluctuations of natural products and symbionts in the sponge *Aplysina aerophoba* (Nardo, 1833). Are chemical and microbial profiles affected by different light exposures? Are changes in secondary metabolites and associated bacteria correlated? Quantitative data supporting a relationship between bacteria and natural products is limited (Sacristán-Soriano et al., 2011b) and would benefit tremendously our understanding of the links between sponge-associated bacteria and the production of natural products. The sponge *A. aerophoba* was chosen to experimentally address these concerns. 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*.

7.2 Materials and methods

7.2.1 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 Ziploc® bags underwater, and, once at the surface,

immediately transferred to two 60-l barrels. Sponges were then transported (2 to 3 h) to the Marine and Environmental Research Mediterranean Centre in Barcelona (CMIMA, CSIC). The experiment was carried out in two tanks with running seawater. We put nine specimens in each tank and created a light gradient by combining two floodlights and a black mesh (Fig. 7.1). We measured light intensity in the field with *in situ* data loggers (Onset Computer Corporation, model HOBO Pendant UA-002-08) placed from 14:00 to 15:00 hrs at 0 m, 1 m and 3 m depth in Blanes (NW Mediterranean) to get a rough idea of the maximum light conditions that sponges could meet. Experimental light conditions mimicked the maximum light intensity encountered by sponges in our study area at a depth of 1 m to total dark, but following a natural light cycle (see Fig. 7.1 for details in 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 one week before the beginning of the experiment. The experiment lasted four months (March-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 compound degradation 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 after the end of the experiment. Samples consisted of small pieces (less than 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 ºC until processed. These samples were used to characterize the chemical and bacterial profiles of *Aplysina aerophoba* as described below.

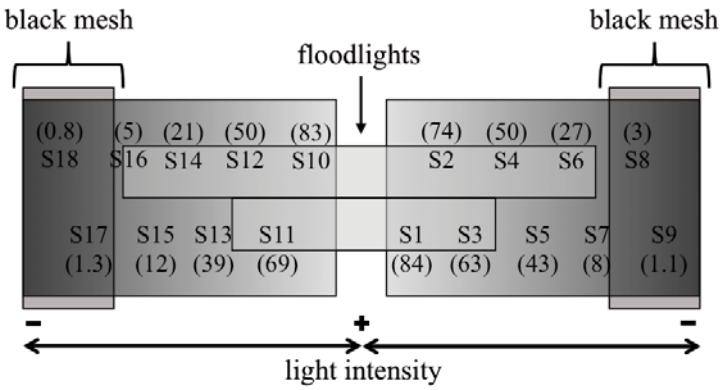


Figure 7.1. Experimental design. We put eighteen specimens of *Aplysina aerophoba* in two tanks with running seawater (S1 to S18). We created a light gradient with high intensities in bright zones (+) and low intensities in dark zones (-). We show light measurements, expressed in microeinsteins ($\mu E \cdot m^{-2} \cdot s^{-1}$), of each individual in brackets (see Materials and methods for details).

7.2.2 HPLC analysis and compound quantification

The major brominated alkaloids (BAs) of *Aplysina aerophoba* were quantified following the methodology described in Sacristán-Soriano et al. (2011a; 2011b; 2012). With this methodology, we have never detected the biotransformation process that converts the High Molecular Weight (HMW) BAs into Low Molecular Weight (LMW) BAs described for this species. 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 them at −20 °C. With our methods, LMW BAs were either absent in our chromatograms or at concentrations below our detection threshold.

HPLC analyses were performed as described in Sacristán-Soriano et al. (2011a; 2011b; 2012). The four major compounds (aerophobin-1, aerophobin-2, aplysinamisin-1, and isofistularin-3)

observed in the HPLC chromatograms had been previously isolated and characterized by classic spectrometric techniques (^{1}H and ^{13}C nuclear magnetic resonance; liquid chromatography-mass spectrometry; UV spectrometry) 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 were extracted with methanol (MeOH) following the methodology described in Sacristan-Soriano et al. (2011a; 2011b; 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 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.

7.2.3 DNA extraction and PCR amplification

We took a small piece of each frozen sample and preserved them with ethanol (EtOH; 100% final concentration) until processed. DNA was extracted from approximately 2 mg of EtOH-preserved wet mass sponge tissue using the DNeasy Tissue kit (Qiagen) 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, Belgium). Small differences in yield extraction were

visualized among samples. However, such differences were not expected to produce qualitative changes in the DNA mixtures, and the results were normalized using relative abundances in the fingerprinting analysis for an accurate intersample comparison.

PCR amplification of bacterial 16S rRNA gene suitable for subsequent genetic fingerprinting analysis was carried out using the universal bacterial primer combination BAC358F with a GC-clamp 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 of sterilized MilliQ water, 5 µl of 10X reaction buffer, 1.5 µl of $MgCl_2$ (50 mM), 1 µl of deoxyribonucleoside triphosphates (10 mM each), 2.5 µl of each primer (10 µM), 2.25 µl of bovine serum albumin (6 $mg \cdot ml^{-1}$), 0.25 µl of EcoTaq polymerase (ECOGEN, 5$U \cdot \mu l^{-1}$) and 1 µl of 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 such range of DNA concentrations. PCR products were run in an agarose gel using a standard mass ladder (DNA Smart Ladder; Eurogentec, Belgium) to quantify the PCR product obtained in each case (Casamayor et al., 2000; Demergasso et al., 2008).

7.2.4 DGGE analysis of PCR products and sequencing

Denaturing gradient gel electrophoresis (DGGE) was performed as described in Sacristán-Soriano et al. (Sacristán-Soriano et al., 2011b). A comparable amplicon mass for each sample (c. 600 ng of PCR product) was added on the DGGE, and gels were run for 4 h at a constant voltage of 200 V and 60ºC in a 40 to 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 the NIH Image software (National Institutes of Health, Bethesda, MD) and the relative band intensities were measured (Demergasso et al., 2008; Sacristán-Soriano et al., 2011b). The signal intensity of the DGGE bands has been shown to be a useful tool for calculating the relative percentages of the different groups (Casamayor et al., 2000; Sacristán-Soriano et al., 2011b). Because of the limitations of the technique, we transformed the relative percentages into absence/presence data, which resulted in the same results and conclusions (see Results). Obviously, absolute quantitative data on the abundance of specific bacteria requires the use of other techniques (Casamayor et al., 2000), and references therein]. Although we do not expect differences in relative signal intensity 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, resuspended in 25 µl of MilliQ water, and stored at 4°C overnight. An aliquot (2 to 5 µl) of the supernatant was used for PCR reamplification with the original primer set, and the PCR product was sequenced using external sequencing facilities (Macrogen). Sequences were aligned and edited with SeaView 4 software (Gouy et al., 2010). Sequences were then sent to BLAST search (November 2012; 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 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.

7.2.5 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 the same host species (Hentschel et al., 2002; Pabel et al., 2003; Erwin and Thacker, 2007; Bayer et al., 2008; Erwin and Thacker, 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), tops matching sequences 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 resampled 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 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).

7.2.6 Data analysis

We used multivariate statistical methods available in PRIMER 6 software (Clarke and Warwick, 2001) to analyze 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 the 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 the DGGE as a quantitative technique (Muyzer and Smalla, 1998), we also used presence/absence bacterial data as a more conservative approach to test whether observed patterns were caused by qualitative vs. quantitative changes in bacteria. Individual analyses of variance (ANOVA) were used to analyze each compound and each OTU across tissue type. We used square-root or rank transformation when raw data failed to meet parametric assumptions.

We used repeated measures analysis of covariance (ANCOVA) from Systat 12 software (SPSS, 1999a, b) to analyze separately each secondary metabolite and each OTU from both tissue layers. We used ANCOVA on chemical and bacterial data to test for the effect of light in the course of the experiment (time as a factor and light as a covariate). 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 vs. quantitative changes in bacteria.

Because of the large number of variables (i.e., secondary metabolites and OTUs) analyzed 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 between groups (Tabachnick and Fidell, 2001; Sacristán-Soriano et al., 2011b). We specifically 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 since it minimizes the number of variables that load highly on a factor and maximizes the loading variance across factors. The independent factors we obtained (i.e., factors' scores) were used as variables in a canonical correlation analysis to test for any correlation between chemical and bacterial factors. We then

used simple correlation analysis (Pearson correlation) to point out the numerical relationship behind the actual secondary metabolites and OTUs within the correlated factors.

7.2.7 Nucleotide sequence accession numbers

The 16S rRNA gene sequences were deposited in GenBank under accession numbers KC143239 to KC143286.

7.3 Results

7.3.1 Natural product variation

We analyzed a total of 72 samples (18 individual sponges × 2 tissue layers × 2 sampling times) to characterize the chemical profile. We quantified the four major brominated alkaloids (BAs) of the sponge *Aplysina aerophoba*, 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 at the beginning of the experiment were analyzed. We found that both 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 ectosomal and choanosomal layers (one-way ANOVA, $P = 0.051$). We therefore treated both sponge tissue layers separately in the analysis of the effect of light over time.

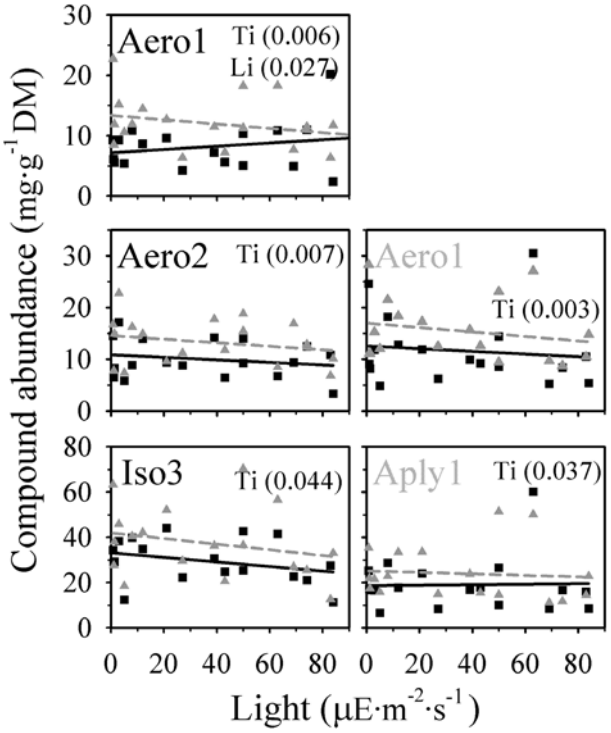


Figure 7.2. Repeated measures analysis of covariance on secondary metabolite concentrations ($mg \cdot g^{-1}$ [dry mass] of sponge tissue ± 1 standard error). Black squares represent measures at the beginning of the experiment and dark gray triangles represent measures at the end. Compounds in black symbolize the ectosome and compounds in gray symbolize the choanosome. Significant factors are shown with *P*-values in brackets ($P \leq 0.05$). Ti, time; Li, light; Aero1, aerophobin-1; Aero2, aerophobin-2; Aply1, aplysinamisin-1; Iso3, isofistularin-3.

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 four months. In the ectosome, the concentration of Aero1, Aero2, and Iso3 was greater after the course of the experiment (ANCOVA, $P = 0.006$, $P = 0.007$, $P = 0.044$, respectively; Fig. 7.2), 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. 7.2), while the amount of Aero2 and Iso3 remained statistically invariable (ANCOVA, $P = 0.308$, $P = 0.070$, respectively). We failed to detect a direct effect of light in BA abundances in both the ectosomal and the choanosomal layers (ANCOVA, $P > 0.05$ in all cases). Only the concentration of Aero1 in the outer part of the sponge was significantly affected by light (ANCOVA, $P = 0.027$; Fig. 7.2). We also failed to detect any effect of different light exposures over the course of the experiment in compound concentrations showed by the nonsignificant interaction between light and time (ANCOVA, $P > 0.05$ in all cases).

7.3.2 Bacterial community variation

We analyzed different specimens for direct comparison on a single DGGE gel. To compare the relative abundance of bacteria between the ectosome and the choanosome, we analyzed 18 samples from 9 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. 7.3). We found highly significant differences in the bacterial assemblage between the ectosome and choanosome of the sponge (one-way PERMANOVA, $P = 0.001$). The analysis of presence/absence data showed the same results (one-way PERMANOVA, $P = 0.001$), so bacterial differences between tissue layers were qualitatively and quantitatively well supported. Of those 18 different OTUs, 11 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. OTUs 7 and 10 were distributed over both tissue layers but were more abundant in the external part of the sponge (one-way ANOVA, $P = 0.002$, $P = 0.044$, respectively). The remaining 7 OTUs either had a homogeneous distribution between layers (OTUs 8, 9, and 11; one-way ANOVA, $P = 0.109$, $P = 0.551$, $P = 0.323$, respectively) or

were uncommon and restricted to a particular sample (OTUs 2, 12, 13, and 14).

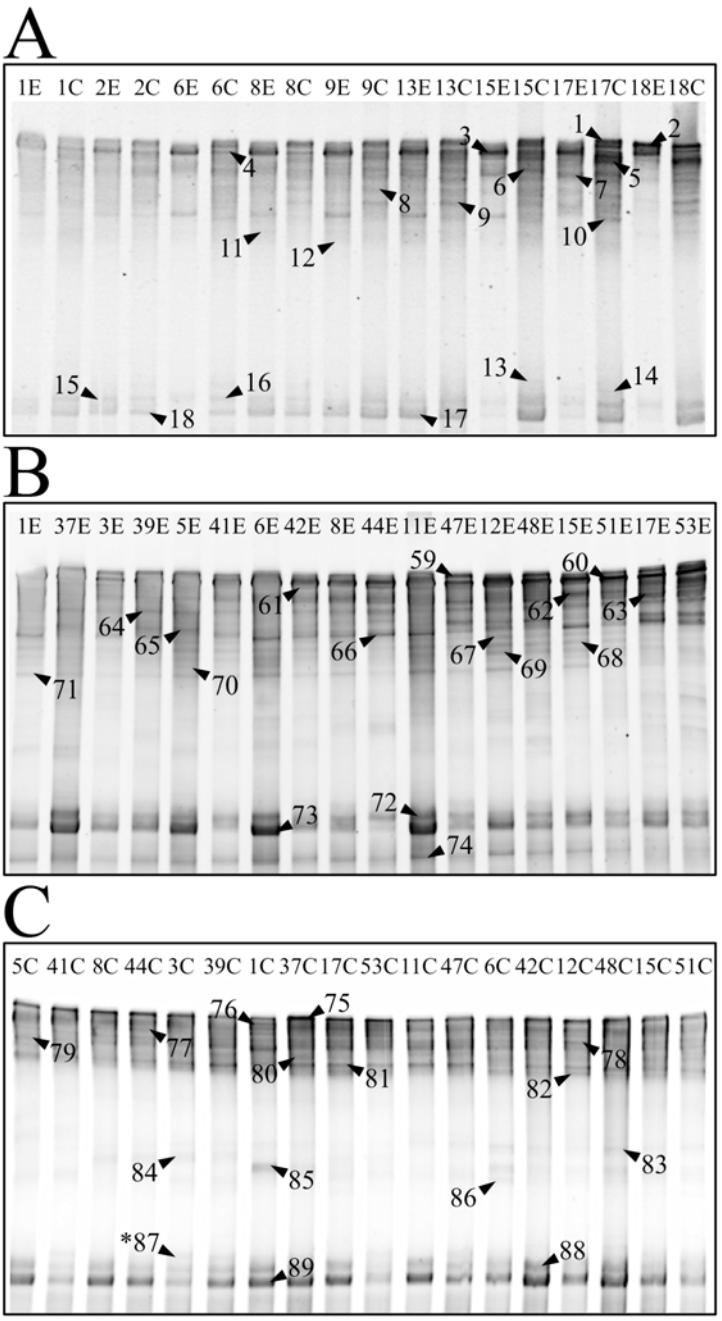


Figure 7.3. (previous page) 16S rDNA-DGGE gels of *Aplysina aerophoba* samples. (**A**) We compared the bacterial community between the ectosome (E) and the choanosome (C) of 9 specimens (1, 2, 6, 8, 9, 13, 15, 17, and 18) at the beginning of the experiment. We tested the effect of light across time 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 9 individuals covering the whole light gradient. Numbers (1-18; 59-89) show the OTUs that were identified and sequenced. Asterisk (*) shows an unclassified OTU.

To assess the effect of light over time, we analyzed 9 specimens at the beginning and at the end of the experiment and we treated both tissue layers separately (i.e., a total of 18 samples in each gel; Fig. 7.3). In the ectosome, we identified a total of 16 OTUs (from OTU 59 to 74; Fig. 7.3). Although we found nonsignificant differences in the relative abundance of all the OTUs over the time course of the experiment (ANCOVA, $P > 0.05$ in all cases), we detected a significant effect of light in four of them, OTUs 62, 64, 68, and 73 (ANCOVA, $P = 0.006$, $P = 0.045$, $P = 0.046$, $P = 0.032$, respectively; Fig. 7.4). The remaining OTUs were not affected by light (ANCOVA, $P > 0.05$ in all cases). In the choanosome, though, we identified 15 OTUs (from OTU 75 to 89; Fig. 7.3), most of which showed nonsignificant differences in their relative abundances over time (ANCOVA, $P > 0.05$ in all cases). However, we found that OTU 77 and 81 significantly differed their percentages after four months of the experiment (ANCOVA, $P = 0.031$, $P = 0.047$, respectively; Fig. 7.4). We also found a significant effect of light in the same OTUs 77 and 81 at the end of the experiment (ANCOVA interactions, $P = 0.013$, $P = 0.021$, respectively; Fig. 7.4). From the analysis of presence/absence OTU data, we found almost the same results. OTU 62 showed a significant effect of light in the ectosome (ANCOVA, $P = 0.035$). In the choanosome, OTU 77 showed significant differences over time (ANCOVA, $P = 0.039$) and a significant effect of light after four

months of the experiment (ANCOVA interaction, $P = 0.017$). The other significant effects we found from the quantitative analyses could not be tested due to a lack of variation between OTU presence/absence data at the beginning and at the end of the experiment.

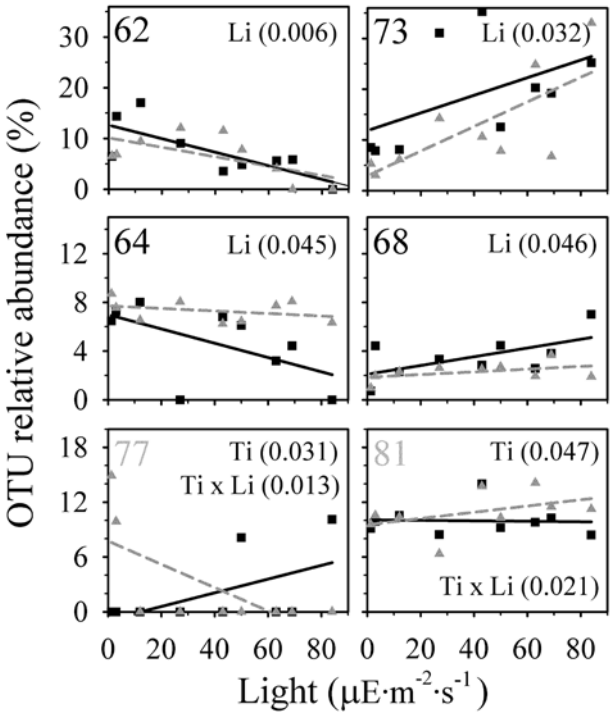


Figure 7.4. Repeated measures analysis of covariance on OTU relative abundances (%). Black squares represent measures at the beginning of the experiment and dark gray triangles represent measures at the end. OTUs in black symbolize the ectosome and OTUs in gray symbolize the choanosome. Significant factors are shown with *P*-values in brackets ($P \leq 0.05$). Ti, time; Li, light; Ti x Li; interaction between time and light.

7.3.3 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 between groups. Both tissue layers were analyzed separately. In the ectosome, factor analysis resulted in one chemical (CF) and five bacterial factors (BF) that explained 73.98 and 86.56% of the total variance, respectively (Tables 7.1 and 7.2). In the choanosome, however, factor analysis resulted in two CFs and six BFs that explained 98.80 and 87.80% of the total variance, respectively (Tables 7.1 and 7.2). Canonical correlation analysis (CCA) on the factor analysis scores resulted in one significant correlation between CF1 and BF1 in the ectosome, whereas CCA in the choanosome resulted in three significant correlations that included the two chemical and three bacterial factors (BF3, BF4, and BF5; Table 7.3). Individual correlation analysis of compounds and bacterial types included in the correlated factors resulted in 9 uncorrected significant correlations in the ectosome (of 16 possible correlations; Table 7.4) and 5 in the choanosome (of 17 possible correlations; Table 7.4), which is an unlikely event to be explained by chance (Binomial distribution, $P = 1.56E^{-8}$, $P = 1.04E^{-3}$, respectively). Significant correlations after Bonferroni corrections were drastically reduced and restricted to the internal part of the sponge between OTU 84 and the compounds Aero1, Aply1, and Iso3 (R = 0.699, $P = 0.019$, R = 0.683, $P = 0.027$, R = 0.797, $P = 0.001$, respectively) and between OTU 75 and Aero2 (R = 0.676, $P = 0.004$) (Fig. 7.5; Table 7.4).

Table 7.1. Loading values obtained with chemical factors (CF) from the factor analysis of chemical data. We show the factors from the ectosome (CF1) and the choanosome (CF2 and CF3) of *Aplysina aerophoba*[a].

	Ectosome	Choanosome	
Compound	CF1	CF2	CF3
Aero1	0.98	0.99	-
Iso3	0.99	0.99	-
Aply1	0.88	0.98	-
Aero2	0.50	-	0.99

[a] To facilitate interpretation, we exclusively 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 (compounds) and the factors. The compounds aerophobin-1 (Aero1), aerophobin-2 (Aero2), aplysinamisin-1 (Aply1), and isofistularin-3 (Iso3) were evaluated.

Table 7.2. Loading values obtained with bacterial factors (BF) from the factor analysis of bacterial data. We show the factors from the ectosome and (BF1-BF5) the choanosome (BF6-BF11) of *Aplysina aerophoba*[a].

	Ectosome						Choanosome					
OTU	BF1	BF2	BF3	BF4	BF5	OTU	BF6	BF7	BF8	BF9	BF10	BF11
68	0.88	-	-	-	-	89	−0.96	-	-	-	-	-
71	0.80	-	-	-	-	88	−0.91	-	-	-	-	-
74	0.77	-	-	-	-	87	0.70	-	-	-	-	-
64	−0.59	-	-	-	-	78	-	−0.91	-	-	-	-
72	-	−0.88	-	-	-	76	-	0.88	-	-	-	-
63	-	0.87	-	-	-	86	-	-	0.87	-	-	-
73	-	−0.69	-	-	-	82	-	-	0.70	-	-	-
66	-	-	−0.85	-	-	75	-	-	-	−0.93	-	-
59	-	-	0.81	-	-	79	-	-	-	0.72	-	-
62	-	-	−0.72	-	-	85	-	-	-	-	−0.78	-
65	-	-	0.69	-	-	83	-	-	-	-	−0.76	-
60	-	-	−0.62	-	-	84	-	-	-	-	0.70	-
67	-	-	-	0.81	-	80	-	-	-	-	-	−0.93
61	-	-	-	−0.67	-	81	-	-	-	-	-	−0.73
70	-	-	-	-	−0.92	77	-	-	-	-	-	0.53
69	-	-	-	-	−0.73							

[a] To facilitate interpretation, we exclusively 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 7.3. Canonical correlation analyses between chemical (CF) and bacterial (BF) factors from the ectosome and choanosome of *Aplysina aerophoba*.

Ectosome	R (P)[a]	Choanosome	R (P)[a]	
	CF1		CF2	CF3
BF1	−0.529 (0.027)	BF6	NS	NS
BF2	NS	BF7	NS	NS
BF3	NS	BF8	0.421 (0.042)	NS
BF4	NS	BF9	NS	−0.619 (0.006)
BF5	NS	BF10	0.553 (0.012)	NS
		BF11	NS	NS

[a] The correlation coefficient (R) and P value of significant correlations ($P \leq 0.05$) are given. NS, nonsignificant correlations.

7.3.4 Phylogenetic analysis of excised 16S rRNA-DGGE bands

We excised from the gels and sequenced 82 16S ribosomal RNA-DGGE bands that belonged to 48 different OTUs (Fig. 7.3). This meant an average of almost two sequenced bands per OTU with identical sequence, although we selected only a sequence representing each OTU (Table 7.5). Comparison of the 16S rRNA gene sequences with GenBank database showed a large range of bacterial taxa present in *Aplysina*. After assessing the taxonomic affiliation, we discarded the sequences with poor taxonomic resolution and with a low identity percentage in the BLAST search (Table 7.5) for subsequent analyses and interpretation of results. 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. 7.6). OTUs 62, 66, 73, and 82, however, were related to sequences derived from a non-sponge source (Fig. 7.6). Phylogenetic analysis was also used to qualitatively compare the bacterial community

recovered from both tissue layers. The ectosome included representatives of *Cyanobacteria* (*N* = 12) as exclusive group, representatives of *Alpha-* (*N* = 5) *Delta-* (*N* = 1), and *Gammaproteobacteria* (*N* = 1), and one sequence affiliated to *Actinobacteria* (*N* = 1). In the choanosome, however, sequences recovered belonged to *Gammaproteobacteria* (*N* = 3), *Alphaproteobacteria* (*N* = 2), and *Deltaproteobacteria* (*N* = 2).

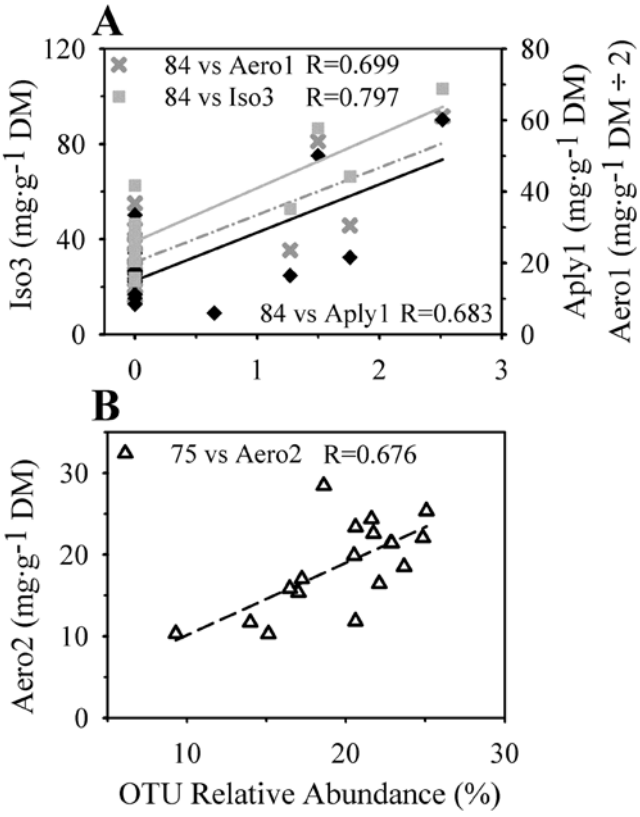


Figure 7.5. Relationship between (**A**) the concentrations of aerophobin-1 (Aero1), aplysinamisin-1 (Aply1) and isofistularin-3 (Iso3) and the relative abundance of OTU 84 and (**B**) the concentration of aerophobin-2 (Aero2) and the relative abundance of OTU 75. Concentrations of compounds in $mg \cdot g^{-1}$ [dry mass] of sponge tissue ± 1 standard error. All relationships shown are significant ($P \leq 0.05$) after Bonferroni corrections (see also Table 7.4).

Table 7.4. Correlation analyses of the specific brominated alkaloids and OTUs included in the correlated chemical (CF) and bacterial (BF) factors from the ectosome and choanosome of *Aplysina aerophoba.*

Bacterial Factor	OTU	Bacterial Group	R (*P*)[a]			
Ectosome			CF1			
			Aero1	Aero2	Aply1	Iso3
BF1	64	*δ-Proteobacteria*	NS	−0.546 (0.019)	NS	0.490 (0.039)
	68	*Cyanobacteria*	−0.546 (0.019)	NS	−0.502 (0.034)	−0.573 (0.013)
	71	*Cyanobacteria*	−0.546 (0.019)	NS	−0.502 (0.034)	−0.573 (0.013)
	74	*Cyanobacteria*	NS	−0.502 (0.034)	NS	NS
Choanosome			CF2			CF3 (Aero2)
			Aero1	Aply1	Iso3	
BF8	82	*γ-Proteobacteria*	NS	NS	NS	-
	86	*δ-Proteobacteria*[b]	NS	0.490 (0.039)	NS	-
BF9	75	*Firmicutes*[b]	-	-	-	0.676 (0.002)*
	79	*δ-Proteobacteria*	-	-	-	NS
BF10	83	*Actinobacteria*[b]	NS	NS	NS	-
	84	*Chloroflexi*[b]	0.699 (0.001)*	0.683 (0.002)*	0.797 (<0.001)*	-
	85	*Firmicutes*[b]	NS	NS	NS	-

[a] The correlation coefficients (R) and uncorrected *P* values of all significant correlations are shown. Asterisks indicate significant correlations after Bonferroni correction. NS, nonsignificant correlations; -, correlations not tested.

[b] Affiliation group suspicious

Table 7.5. 16S rDNA sequence identities of sequenced bands excised from DGGE gels.

OTU	Putative Division	Closest relative in database (isolation source; AN[a])	% Identity
1	*Firmicutes*	*Thermanaeromonas toyohensis* strain ToBE (subterranean water; 219857149)	83.5
2	*Cyanobacteria*	*Prochlorococcus marinus* subsp. *pastoris* str. PCC 9511 (seawater; 265678459)	96.0
3	*Cyanobacteria*	*Prochlorococcus marinus* subsp. *pastoris* str. 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* str. PCC 9511 (seawater; 265678459)	96.0
12	*Cyanobacteria*	*Prochlorococcus marinus* subsp. *pastoris* str. PCC 9511 (seawater; 265678459)	96.0
13	*Nitrospira*	*Thermodesulfovibrio hydrogeniphilus* strain Hbr5 (terrestrial hot spring; 343205695)	80.3

Table 7.5. Continued.

14	*Firmicutes*	*Thermacetogenium phaeum* strain PB (thermal wastewater; 219856869)	81.4
15	*Cyanobacteria*	*Prochlorococcus marinus* subsp. *pastoris* str. PCC 9511 (seawater; 265678459)	96.0
16	*Alphaproteobacteria*	*Oceanibaculum indicum* strain P24 (deep seawater; 343206001)	85.5
17	*Cyanobacteria*	*Prochlorococcus marinus* subsp. *pastoris* str. 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* str. 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* str. PCC 9511 (seawater; 265678459)	95.8

Table 7.5. Continued.

68	*Cyanobacteria*	*Prochlorococcus marinus* subsp. *pastoris* str. PCC 9511 (seawater; 265678459)	96.1
69	*Cyanobacteria*	*Prochlorococcus marinus* subsp. *pastoris* str. PCC 9511 (seawater; 265678459)	96.1
70	*Firmicutes*	*Thermacetogenium phaeum* strain PB (thermal wastewater; 219856869)	85.9
71	*Cyanobacteria*	*Prochlorococcus marinus* subsp. *pastoris* str. 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* str. 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

Table 7.5. Continued.

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] AN, NCBI gi accession number.

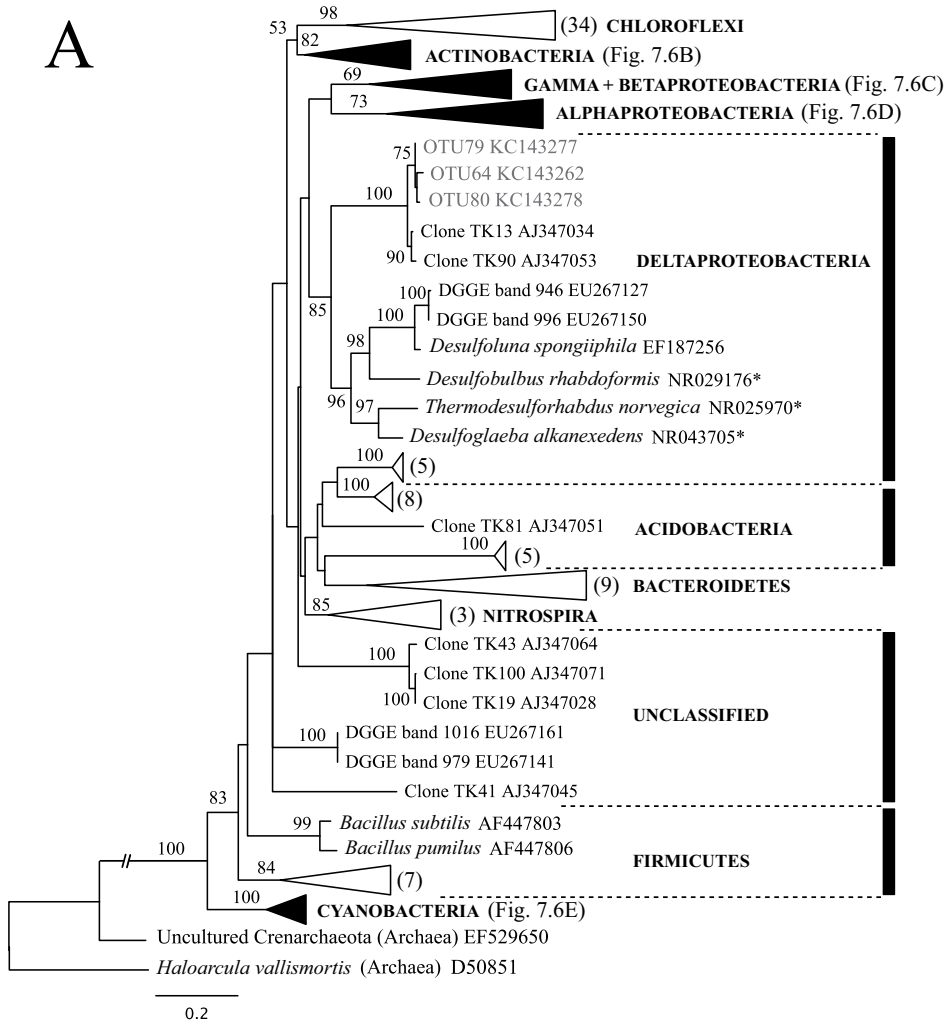


Figure 7.6. (**A**) Phylogeny of bacterial 16S rRNA gene sequences from *Aplysina aerophoba*. Tree topology was constructed using ML criteria, and numbers on nodes represent bootstrap support (< 50% not shown). Terminal nodes denote the sequence source and GenBank accession nos., and gray values correspond 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 sections **B**, **C**, **D**, and **E** for condensed black clades.

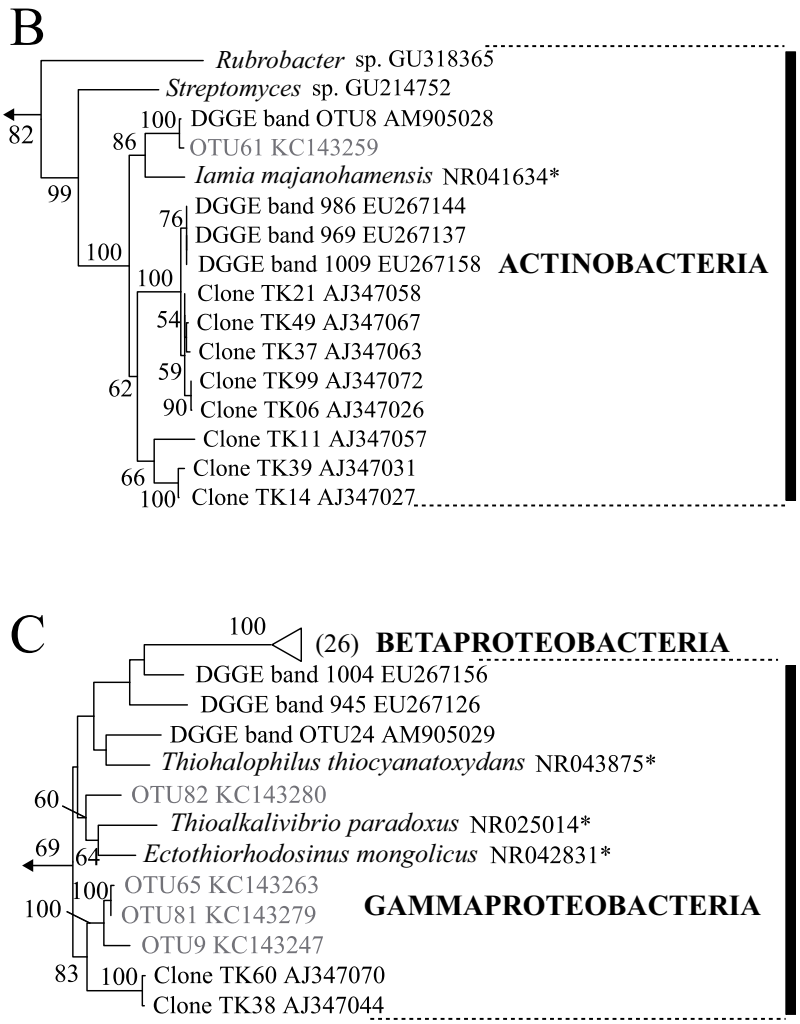


Figure 7.6. Continued.

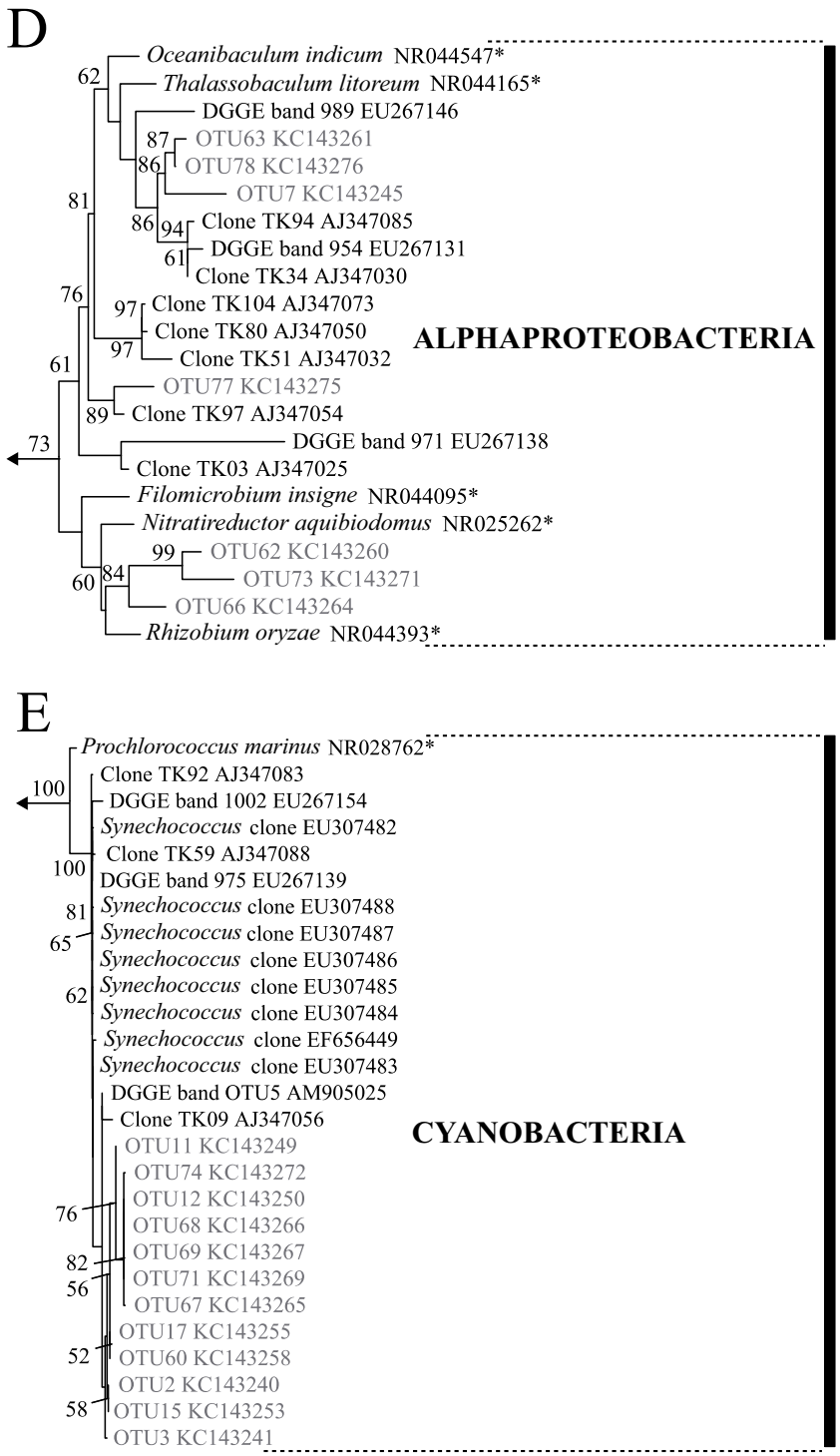


Figure 7.6. Continued.

7.4 Discussion

7.4.1 Natural products: intraindividual and *ex situ* variability

A preliminary comparison of external and internal tissue layers of *Aplysina aerophoba* showed an enriched core in brominated alkaloids (BAs), which possess a markedly deterrent activity (Thoms et al., 2004). These differences support those reported previously in other studies of the same species (Sacristán-Soriano et al., 2011b; Sacristán-Soriano et al., 2012). *Ircinia* spp. showed the same distribution pattern with higher concentrations of secondary compounds in inner as opposed to outer tissue regions (Freeman and Gleason, 2010). However, other studies could not detect differences in chemical defense between sponge surface and inner tissue (Swearingen and Pawlik, 1998; Burns et al., 2003; Rohde and Schupp, 2011). A third pattern has been also 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 and Gleason, 2010). All patterns could be explained by the optimal defense theory (ODT), from which areas more prone to predatory attack or more critical for species survival would allocate more defensive metabolites under the assumption that resources used in the production of chemical defenses are limited (McKey, 1974, 1979; Rhoades, 1979). Predatory feeding types (i.e., predators making deep or superficial bites) may be driving the distribution of secondary compounds (Freeman and Gleason, 2010). The *Aplysina* predator *Tylodina perversa* is known to feed preferentially on the outer ectosome layer (Becerro et al., 2003). The predator could be deterred by the larger concentration of chemical defenses in the inner choanosome, where basic sponge physiological processes such as reproduction and water pumping occur, and could be left to feed on a tissue more prone to regenerate after predation. In fact, artificial wounds emulating bite marks left

by a predator may regenerate within days or weeks (Wulff, 2010) but these hypotheses remain to be tested in *A. aerophoba*.

Ex situ experiment testing the effect of light on *Aplysina* individuals under the given cultivation conditions revealed that the relative composition of the four main BAs (aerophobin-1, aerophobin-2, aplysinamisin-1, and isofistularin-3) remained almost invariable. However, most of the BAs fairly increased its abundance after four months of *ex situ* cultivation in both sponge layers. Both Klöppel et al. (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 aerophobin-1 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 reported that neither transplanting sponges to different depth regimes nor cultivating them under different light conditions affected the alkaloid patterns (Thoms et al., 2003; Putz et al., 2009). In contrast, several sponge species have been found more toxic in shaded than in well-illuminated habitats, accumulating more bioactive compounds (Becerro et al., 1995; Ferretti et al., 2009). Differently, 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 and Paul, 2004). The diterpene content of the sponge *Rhopaloeides odorabile* may also experience a light-induced production (Thompson et al., 1987). Alternatively to a direct light effect, other factors associated with light (e.g., competition, predation) may be determining the abundance of bioactive compounds.

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 might explain the increase in the amount of sponge secondary metabolites after *ex situ*

cultivation. The ectosome of *Aplysina aerophoba* is known to seasonally increase the concentration of brominated metabolites during the months where the experiment was carried out (Sacristán-Soriano et al., 2012). Our experiment used running seawater from the ocean at the natural occurring temperature, 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 explanation would be a potential loss of sponge tissue under maintenance conditions (Klöppel et al., 2008; Gerçe et al., 2009) that may possibly lead 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.

7.4.2 Associated bacteria: intraindividual and *ex situ* variability

Denaturing gradient gel electrophoresis (DGGE) has been found useful to assess changes in microbial consortium inhabiting sponge body at different spatio-temporal scales and compare those communities between sponge species (Friedrich et al., 2001; Taylor et al., 2004; Webster et al., 2004; Taylor et al., 2005; Thiel et al., 2007b; Meyer and Kuever, 2008; Mohamed et al., 2008; Hardoim et al., 2012), even to estimate the relative abundance of predominant bacterial groups (i.e., those >0.1 to 1% of total abundance) and get a rough picture of microbial variability (Sacristán-Soriano et al., 2011b). Despite that 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 reduce precise phylogenetic analyses, but still allow a broad phylogenetic affiliation (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 persists through time, and (ii) a given band is the same in different sample types (i.e. different tissue layers, sampling times). Other intrinsic biases associated with PCR-DGGE are well documented (Suzuki and Giovannoni, 1996; von Wintzingerode et al., 1997; Muyzer and Smalla, 1998) and, as for any PCR-based approach, can lead to a misinterpretation of results. However, we proceed in a way to minimize technique biases and validate intersample comparison, as described in Sacristán-Soriano et al. (2011b).

Comparing the ectosome and the choanosome of *Aplysina aerophoba*, we observed striking differences in bacteria inhabiting both tissue layers. Distinct phylotypes, represented by DGGE bands, were affiliated with different regions of the sponge. This local distribution of associated microbial populations was also observed in other sponge species (Thiel et al., 2007b; Meyer and Kuever, 2008; Sipkema and Blanch, 2010). On the phylum-level, changes were basically due to *Cyanobacteria* that have 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 four months revealed slight shifts in a small fraction (19.4%) of the sponge microbiota, while most microbes (80.6%) remained stable. Erwin et al. (2012b) also documented a remarkable stability of bacterial symbionts of *Ircina* spp. *in situ* over 1.5 years, where changes were restricted to some bacteria, 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 in 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 show 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., 2007b; Lee et al., 2011; Thacker and Freeman, 2012; White et al., 2012; Schöttner et al., 2013).

Among the variable bacterial fraction, there seemed to be two different patterns between both tissue layers. In the sponge cortex, four bacterial strains were directly affected by different light regimes. A *Cyanobacteria* phylotype (OTU 68) and an *Alphaproteobacteria* (OTU 73) phylotype were benefited by high light conditions, while two *Alpha-* (OTU 62) and *Deltaproteobacteria*-derived (OTU 64) sequences were advantaged by low illumination regimes. *Cyanobacteria*-host symbioses are quite well studied (Thacker, 2005; Erwin and Thacker, 2007; Erwin and Thacker, 2008) and respond to light in the same way as we found. The other potential associations are completely unknown. In the inner sponge, two *Alpha-* (OTU 77) and *Gammaproteobacteria*-affiliated (OTU 81) phylotypes varied over time depending on the luminosity received. However, we do not know whether these phylotypes belong to the permanent (i.e., symbiotic) or the transient fraction of *Aplysina* 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., 2007b; Webster et al., 2010; Erwin et al., 2011; Lee et al., 2011; Hardoim et al., 2012; Thacker and Freeman, 2012; White et al., 2012; Schöttner et al., 2013), the little variability observed here may be ascribed to transient components of the sponge microbiota, as microbes recovered from sponge tissue may come from food source (Pile et al., 1996), invasion (Webster et al., 2002), or simply from the surrounding environment.

Briefly, the abundance, phylogenetic composition, and diversity of the host-associated microbial community mainly depends to a large extent on the sponge-species and host state (i.e., sponge health) as demonstrated by earlier studies (Taylor et al., 2004; Webster et al., 2004; Taylor et al., 2005; Hill et al., 2006; Li et al., 2007; Thiel et al., 2007a; Thiel et al., 2007b; Meyer and

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 sponge stress may be the main driver of the symbiotic shifts causing a breakdown in the sponge health and making unstable the symbiont communities (Webster et al., 2011). Yet, what and how environmental stressors such as light and temperature alter the sponge health need to be further investigated.

7.4.3 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 on sponge secondary chemistry is well known and widely documented in the literature (Dunlap et al., 2007; Grozdanov and Hentschel, 2007; Taylor et al., 2007b; Egan et al., 2008; Siegl et al., 2008; Hentschel et al., 2012). However, studies with quantitative relationships between secondary metabolites and individual microbes are scarce. Here, we attempt to correlate variations in both natural products and bacterial assemblage. A total of 14 of the 33 correlations evaluated resulted in significant positive and negative correlations between bacterial populations and secondary metabolites, which is extremely unlikely to occur by chance alone. It suggests strong interactions between bacteria and between bacteria and brominated compounds. Our results showed that two phylotypes were positively related to the concentration of the four alkaloids analyzed. OTU 84 (unidentified phylotype) was related to aerophobin-1, aplysinamisin-1, and isofistularin-3, while OTU 75 (unidentified phylotype) was related with aerophobin-2. In both cases, the relationship occurred in the inner part of the sponge. In a previous study, an association between aplysinamisin-1 and a *Chloroflexi*-affiliated phylotype was pointed out (Sacristán-Soriano et al., 2011b). Further, a *Chloroflexi*

bacterium isolated from *Aplysina aerophoba* was pointed as the likely producer of a novel non-ribosomal peptide synthetase (Siegl and Hentschel, 2009). Thus, *Chloroflexi* might 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* that were described to encode polyketide synthase gene clusters (Zhang et al., 2009). Also isolates from *A. aerophoba* that were tested for antimicrobial and antifungal activities (Hentschel et al., 2001; Pabel et al., 2003).

These correlations could be explained by multiple factors. If bacteria were involved in the production of natural products, microbes could be implicated either directly by producing themselves the bioactive compounds or indirectly by synthesizing enzymes that would be crucial for the biogenesis of secondary metabolites. Accordingly, bacteria would determine the abundance of natural products. Several studies have been described the true origin of natural products by the sponge symbionts (Unson and 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). Yet, little is known about the cooperation between sponges and their microbial consortia 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) essential for the synthesis of halogenated compounds in the marine environment.

Alternatively, the microenvironment created by the sponge would select for the presence of a particular microbe. This hypothesis may be the case 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 posterior 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* could also undergo anaerobic reductive dehalogenation (Field and Sierra-Alvarez, 2008). So, it is likely that sponge-associated bacteria belonging to those phyla (Hentschel et al., 2001; Hentschel et al., 2002; Noyer et al., 2010; Erwin et al., 2011; Erwin et al., 2012a) may be capable of metabolizing sponge-derived halogenated compounds. Although sponge-associated bacteria seem to be related to natural product synthesis and our results are very unlikely to be explained by chance alone, we cannot completely exclude that the associations we found were spurious correlations as a result of complex trophic interactions with concomitant organisms of the sponge microenvironment that we do not know yet.

Overall, we detected a differential distribution of natural products and sponge-associated bacteria between tissue layers. Although a stress reaction of the sponges to captivity could not be discarded, the stability in chemical and microbial profiles prevailed against a restricted effect of light. So, the stress would have been minor. We also showed a relationship between natural products and two bacterial phylotypes that could be used to outline further hypotheses in the future. Our data and those from the literature suggest complex ecological interactions that are far from being understood. Advances in metagenomics 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.

Acknowledgments

We thank Marta Ribes and Eroteida Jiménez for their assistance with experimental set up and data collection.

This research is a part of OSS PhD Thesis, who was supported by a CSIC predoctoral grant. Research partially funded

by the Spanish Ministry of Science and Innovation (SOLID: CTM2010-17755 and BENTHOMICS: CTM2010-22218) and by the Agence Nationale de la Recherche (France; ECIMAR project, ANR-06-BDIV-001-04). This research is a contribution of the Consolidated Research Group "Grupo de Ecología Bentónica" (SGR2009-655) of the Catalan Government.

General discussion

8
Concluding remarks

Since the late 90s sponge chemical and microbial studies have steadily increased their contribution to the field of Ecology, but the former have received greater importance from the scientific community, as shown in the **General introduction** section. Both areas lack specialist researchers with a clear background in these disciplines and with high number of contributions. However, their works are highly cited by applied sciences due to the biotechnological potential derived from multiple of their results. These disciplines mainly focus their attention on natural product roles and a comparative characterization of sponge microbiomes. Although away from the main focus, there are a few examples in the literature of works that deal with natural and environmental variation in secondary chemistry and microbial endobionts (Thompson et al., 1987; Becerro et al., 1995; Friedrich et al., 2001; Hentschel et al., 2002; Webster et al., 2004; Page et al., 2005; Abdo et al., 2007; Erwin et al., 2012b). However, fewer studies concentrate their effort on sponge microbiome as a source of potential bioactive compounds (Grozdanov and Hentschel, 2007; Taylor et al., 2007b; Siegl et al., 2008; Hentschel et al., 2012). So, in this thesis, we have had the opportunity to integrate these two fields of ecology by studying the relationship between secondary

metabolites and microbial symbionts, which promotes understanding of the function that microbial consortia may play in sponge hosts.

The approach used consisted in assessing the natural variability of bioactive compounds and sponge-associated bacteria in the demosponge *Aplysina aerophoba* at multiple spatial (i.e., from few centimeters to thousand kilometers) and temporal (i.e., months and years) scales (**Chapters 4, 5, 6 and 7**) and evaluating how environmental factors (i.e., light) affected both profiles (**Chapter 7**). We then correlated those changes to infer any potential association between natural products and microbial symbionts (**Chapters 4, 5, and 7**). Shifts on sponge microbes at different temporal scales remain to be tested in *A. aerophoba* although microbial consortia seem to be quite stable over time (Erwin et al., 2012b).

A multiple scale approach could shed light on the factors behind natural variation (Levin, 1992). Accordingly, the morphology of *Aplysina aerophoba* forming chimney-like structures led to a question about whether there were intraindividual differences in secondary metabolites and associated microbes or homogeneity prevailed within the sponge body (**Chapters 4, 6, and 7**). Our data showed two distinct entities in terms of natural product abundances and microbial community diversity of the sponge, the skinny outer layer (i.e., the ectosome) and the inner core of the sponge (i.e., the choanosome). These particular allocation patterns of chemicals and microbes have also been observed in other sponge species (Kubanek et al., 2002; Furrow et al., 2003; Thiel et al., 2007b; Meyer and Kuever, 2008; Freeman and Gleason, 2010; Sipkema and Blanch, 2010). The enriched core in bioactive compounds and the surface layer packed with *Cyanobacteria* may act as two functional units *a priori* with different degrees of bioactivity and diverse microbial physiologies, but this hypothesis should be tested.

At such small spatial scale, the relevance and universality of some ecological processes can be tested. The local distribution of

bioactive compounds within the sponge body may respond to the optimal defense theory (ODT), outlined by McKey (1974, 1979) and Rhoades (1979) in plants but particularly relevant to sponge chemical ecology (Pawlik et al., 2008). The ODT postulates that areas more prone to predatory attack or more critical for species survival would allocate more defensive metabolites, assuming limited resources for the production of chemical defenses. The major brominated compounds described in *A. aerophoba* provide effective chemical defense against predators (Thoms et al., 2004), so the enriched internal region may act as a final deterrent barrier trying to protect basic sponge physiological processes that occur herein such as reproduction and water pumping. Contrastingly, the only known predator of *Aplysina*, *Tylodina perversa*, may be left to feed on the external layer or simply feed on the *Cyanobacteria*-rich tissue without decreasing the species fitness (Becerro et al., 2003).

Other ecological principles may serve to explain the distinct distribution of microbial populations within this complex microenvironment described as holobiont (i.e., assemblage of sponge cells and microbes in close association). The principle postulated by Baas Becking (1934), *everything is everywhere, but the environment selects*, provides the basis for understanding microbial dispersal and complexity of symbiotic communities. The local distribution of *Cyanobacteria* within the *Aplysina* cortex respond to the phototrophic nature of these microbes that may need direct light exposure. However, it has been found phototrophic microorganisms to be evenly distributed over the outer and the inner regions of *Tethya* spp. (Thiel et al., 2007b; Sipkema and Blanch, 2010). The unusual association of *Cyanobacteria* with the sponge endosome, which does not receive direct light exposure, would be facilitated by light transmission via bundles of radiate spicules (Thiel et al., 2007b). The particular traits of the *Aplysina* microenvironment (i.e., lack of spicules) confine *Cyanobacteria* to the sponge surface (including the surface within the oscula).

Unraveling the type of factors that modulate chemical and microbial patterns is not an easy task. Natural product and microbial

community profiles are highly diverse, the microbe-host associations are extremely complex, and their nature largely unknown. Understanding the geographic scale at which changes in secondary metabolites and associated bacteria occur may help untangle the factors behind natural variation (**Chapter 5**). The variation pattern of brominated alkaloids (BAs) in *A. aerophoba* mirrored that of chlorophyll *a* (Chl *a*), used as a proxy of phototrophic bacteria (Becerro et al., 2003; Thacker, 2005; Erwin and Thacker, 2008) that may play multiple key roles for the ecological success of sponges (Erwin and Thacker, 2008). Considering a geographic scale comprised from hundred meters to thousand kilometers, the variation patterns occurred at the smallest and largest geographic scales.

Despite the striking similarity of chemical profiles of *Aplysina aerophoba* collected from such distant regions, the heterogeneity in compound concentrations was remarkable with some compounds that varied at the smallest scale (i.e., local) and others at the largest scale (i.e., regional). An earlier study on the concentration of brominated compounds in this species around the Canary Islands also showed spatial variation in chemical compound abundances (Teeyapant et al., 1993a). Shifts in secondary chemistry occur at wide range distances within the phylum Porifera (Swearingen and Pawlik, 1998; Puyana et al., 2003; Abdo et al., 2007; Noyer et al., 2011). Interpreting clearly the factors that act at such spatial scales with our current knowledge of the production of BAs would be inappropriate, despite the remarkable variation (up to 46%) explained by those spatial scales. But, it seems reasonable to discuss that multiple factors acting at multiple spatial scales may modulate the concentration of these bioactive compounds. BAs play an anti-predatory role but other compounds show cytotoxic and antibacterial activities (Koulman et al., 1996; Weiss et al., 1996), so geographic differences in predation, fouling, or competition could account for the contrasting patterns of natural products in *A. aerophoba* (Becerro et al., 1995; Swearingen and Pawlik, 1998; Page et al., 2005; Abdo et al., 2007; Noyer et al., 2011).

Additionally, abiotic factors (e.g., light, depth) that differ spatially could also be responsible for part of the variation reported (Thompson et al., 1987; Page et al., 2005).

The variation pattern of Chl *a* mimicked that of *Aplysina* secondary metabolites. The concentration of Chl *a* varied the most between the two distant geographic regions but changes also occurred locally between sites less than 500 m apart. Changes in light conditions between compared regions alter Chl *a* concentrations, and thus the sponge photosynthetic community (Becerro et al., 2003; Thacker, 2005; Erwin and Thacker, 2008). However, it seems unlikely that light may affect differentially the Chl *a* abundance in samples located at such close sites. Although an important percentage of variation (up to 32%) was explained by the spatial scales investigated here, most variance remained unexplained. So, multiple ecological processes may act at multiple scales to cause such differences within this complex habitat, where microbial populations compete for the space and resources under a host control (Wilkinson, 1987; Wulff, 2006; Taylor et al., 2007a; 2007b).

Ecological processes and the mechanisms behind them vary not only spatially but also temporally, which render a high degree of heterogeneity to specific traits such as secondary chemistry or associated microbial consortium. Understanding the temporal scale at which changes in natural products and bacterial community occur may also provide knowledge of the factors underlying the natural variation (**Chapter 6**). Although ecological studies over a large temporal scale are often hard to carry out and, for that reason, uncommon, they are much more appropriate to detect global patterns of variation. Given a two-year period, we could assess the variation in secondary metabolite abundances and microbial community in *Aplysina aerophoba* within years and check the reproducibility of the observed patterns over the years.

Long-term studies documenting quantitative, temporal changes in individual biologically active compounds are scarce and we contribute to fulfill this demand. *Aplysina* showed temporal

patterns of variation in BA concentrations within years with an annual periodicity. However, those changes were tissue-specific. While BAs varied the most at low (i.e., months) or large (i.e., seasons) temporal scales in the ectosome depending on the compound analyzed, BAs varied the most at low temporal scale in the choanosome of the sponge. This result supports the hypothesis of two distinct functional entities in *A. aerophoba*, the external layer and the core of the sponge, as I commented above. Whereas aerophobin-2 showed a chaotic pattern, aplysinamisin-1 and isofistularin-3 exhibited a clear pattern with monthly differences in the choanosome between August (maximum) and November (minimum) and seasonal differences in the ectosome, where higher abundances were detected during summer. This variation pattern of greater abundances in the warmer season is shared with other sponge species (Page et al., 2005; Abdo et al., 2007).

Factors acting at such temporal scales (e.g., water temperature) may be modulating compound patterns as suggested by the positive correlation between some BAs and seawater temperature. Indeed, a notable percentage of the variation (up to 26%) was explained by the temporal scales explored in our study. Water temperature has a consistent seasonal pattern but factors do not vary alone, so other factors (i.e., abiotic or biotic) that correlate well with seawater temperature may be responsible for such variation (Thompson et al., 1987; Becerro et al., 1995; Turon et al., 1996; Page et al., 2005; Ferretti et al., 2009). Seasonal changes in the known predator *T. perversa* could explain part of the variation found in BAs, but most of the ecological and biological data of this gastropod is unknown (e.g., abundance, annual cycle). Other biotic interactions (i.e., fouling, competition) with marked seasonality (Turon et al., 1996; Duckworth and Battershill, 2001) could also explain BA patterns in *A. aerophoba* since the products of the biotransformation (aeroplysinin-1 and dienone) of the precursors studied in our study show strong antibiotic and cytotoxic activities and may protect the sponge from invasion of pathogens or foulers (Teeyapant et al., 1993b; Ebel et al., 1997; Thoms et al., 2004).

Contrastingly, seasonal changes could respond to resource allocation trade-offs between chemical defenses, growth, and reproduction (Leong and Pawlik, 2010). Yet, data on growth and reproduction of *A. aerophoba* would be required to support this hypothesis.

Long-term studies documenting temporal changes in the sponge microbiome are also uncommon. Although the microbial community in *Ircinia* spp. seems to be quite stable over time (Erwin et al., 2012b), this long-term stability remains to be tested in *A. aerophoba*. Despite such a strong symbiont-sponge association documented in several species (Lemoine et al., 2007; Webster et al., 2008a; Erwin et al., 2011; Erwin et al., 2012b), how the microbiota may be affected by environmental factors is largely unknown.

Given the potential effect of abiotic factors (e.g., light exposure) in determining spatial and temporal changes in secondary chemistry and microbial assemblages, the effect of distinct illumination regimes has been experimentally tested in *Aplysina aerophoba* (**Chapter 7**). An overall stability of chemical and microbial profiles prevailed against a restricted effect of light. Although the relative composition of BAs remained almost invariable, most BAs increased its abundance over the time course of the experiment regardless the illumination regime with the exception of aerophobin-1 that seemed to be affected by light in the outer layer of the sponge. The bacterial community also remained unchanged except for a small fraction that seemed to be affected by different light conditions.

The factors behind the increase in BA concentration have not yet been determined but probably are related to a seasonal increase (see above) rather than a direct light modulation of secondary chemistry. Similarly, other studies on *Aplysina* spp. documented no light effect on the alkaloid patterns after *in situ* and *ex situ* cultivation under different illumination regimes (Thoms et al., 2003; Putz et al., 2009). Nevertheless, there are some examples in the literature reporting a possible light effect on sponge natural products either promoting (Thompson et al., 1987; Becerro and

Paul, 2004) or hindering (Becerro et al., 1995; Ferretti et al., 2009) its production. As the effect of light has not been really tested in those studies, it seems that other factors associated with light may be determining the abundance of bioactive compounds. Shifts in illumination produce changes in benthic community diversity altering biotic interactions, and thus sponge chemistry (Becerro et al., 1995).

Contrastingly to the chemical profile, it was reported a slight effect of light on some phylotypes although most bacteria remained invariable. In the outer layer, whereas a *Cyanobacteria* phylotype and an *Alphaproteobacteria* phylotype prevailed in high light conditions, an *Alpha*- and a *Deltaproteobacteria* phylotypes predominated in low illumination regimes. In the inner core, however, an *Alpha*- and a *Gammaproteobacteria* phylotypes varied depending on the light received. The positive effect of light on *Cyanobacteria* has already been documented (Thacker, 2005; Erwin and Thacker, 2007; 2008). The other possible associations, on the other hand, are completely unknown. As the sponge microbiome of a particular species is highly stable over space and time (Webster et al., 2010; Erwin et al., 2011; 2012b; White et al., 2012; Schöttner et al., 2013), this variable fraction could be ascribed to transient bacteria rather than true symbionts.

Microbial symbionts, nevertheless, play multiple roles in their hosts. The last factor to be considered that could account for the variation of natural products at multiple scales in *Aplysina aerophoba* may lie in the potential contribution of associated microbes in secondary metabolite production, which is widely documented (Dunlap et al., 2007; Taylor et al., 2007b; Egan et al., 2008; Siegl et al., 2008; Hentschel et al., 2012). Yet, studies reporting quantitative relationships are uncommon (**Chapters 4, 5, and 7**). Here, we pointed out multiple positive relationships between a *Chloroflexi* phylotype and aplysinamisin-1 and between three unidentified phylotypes and several BAs, and we also described a negative association between Chl *a* (i.e., photosynthetic bacteria) and isofistularin-3.

Larger abundance of particular bacteria could lead to larger compound concentrations if those bacteria were directly involved in the production of natural products. Siegl and Hentschel (2009) have found prominent enzyme classes responsible for the synthesis of bioactive compounds in various *Aplysina* microbes, including members of the *Chloroflexi* phylum. However, BAs of *A. aerophoba* seem to be located within sponge cells (Turon et al., 2000), suggesting a sponge origin. The location of secondary metabolites in multiple sponge compartments (i.e., the ectosome and choanosome) raises the possibility of cooperation between the sponge and its microbiota in compound production, but this hypothesis has received little attention. Bacteria of the genus *Bacillus* (phylum *Firmicutes*) isolated from *A. aerophoba* have been described to be a potential source for enzymes involved in the halogenation process (i.e., incorporation of Bromine atoms into brominated compounds) (Karpushova et al., 2005). Thus, the host may produce the inactive precursors of secondary metabolites, while microbial symbionts may provide the enzymes to activate them.

Alternatively, higher concentrations of compounds could lead to higher bacterial abundance because of specific habitat conditions or preferences. The abundance of brominated compounds may facilitate bacteria capable of metabolizing them (van Pée and Unversucht, 2003). Two studies on *A. aerophoba* microbiology have revealed that bacteria of the genus *Desulfoluna* (*Deltaproteobacteria*) are able to metabolize brominated compounds (Ahn et al., 2003; Ahn et al., 2009). But, this metabolic process is not exclusive of this group. *Chloroflexi* bacteria could also undergo dehalogenation of sponge-derived aromatic compounds (Field and Sierra-Alvarez, 2008).

Although these multiple associations are unlikely to be explained by chance alone, we cannot completely exclude a spurious correlation. Brominated compounds and sponge microbes might be related indirectly. A potential positive relationship between natural products and particular bacteria or simply the

abiotic conditions predominant in a specific sponge region may determine the presence of these bacteria, leading to a competitive exclusion of other microbes. This could be the case of the negative correlation between isofistularin-3 and phototrophic bacteria (measured as Chl *a*).

In summary, the production of natural products in sponges and the abundance and phylogenetic composition of the host-associated microbial community mainly depend to a large extent on the sponge-species and the ecological factors with spatial and temporal variations (e.g., light, predation, competition, fouling) (Becerro and Paul, 2004; Taylor et al., 2004; Webster et al., 2004; Page et al., 2005; Thiel et al., 2007a; 2007b; Schöttner et al., 2013). The host state (i.e., stress) is also a key factor that may be the main driver of symbiotic shifts causing a breakdown in the sponge health and making the symbiont communities unstable (Webster et al., 2011) and likely the sponge chemical defense. The combination between abiotic and biotic factors may finally determine the concentration of bioactive compounds and associated microbial diversity, as the abiotic environmental context can control the outcomes of biotic interactions, and biotic interactions often moderate the effect of abiotic factors (Wulff, 2012). For that reason, it is not an easy task to actually figure out the factors that limit or enhance chemical and microbial variability. Further experiments and time-series observations are needed to reveal the underlying processes hidden.

9

Conclusions

The main conclusions of this PhD dissertation are:

- Sponge chemical and microbial ecologies have received a greater impact from the scientific community since the late 90s, but mainly from applied sciences (**General introduction**).
- There is a lack of specialist researchers with a clear background in these disciplines and with a significant number of contributions (**General introduction**).
- *Aplysina aerophoba* showed intraindividual differences in the abundance of brominated compounds and microbial community composition between the outer layer (i.e., the ectosome) and the inner core of the sponge (i.e., the choanosome) (**Chapters 4, 6, and 7**).
- The largest scales of variation in the concentration of brominated alkaloid profiles and chlorophyll *a* (used as a proxy of phototrophic bacteria) were at hundred meters and thousand of kilometers (**Chapter 5**).
- *Aplysina aerophoba* showed temporal changes in the abundance of secondary metabolites. While compound

concentrations varied between months in the choanosome, the abundance of natural products in the ectosome differed between months or seasons depending on the compound analyzed. Those compounds with seasonal changes (aplysinamisin-1 and isofistularin-3) showed greater abundances during summer (**Chapter 6**).

- The effect of light on secondary chemistry and associated bacterial community was almost negligible. Only aerophobin-1 in the outer sponge layer and a small bacterial fraction were affected by different illumination regimes (**Chapter 7**).
- We showed multiple positive relationships between a *Chloroflexi* phylotype and aplysinamisin-1 and between three unidentified phylotypes and several brominated compounds. We also described a negative association between Chl *a* (i.e., photosynthetic bacteria) and isofistularin-3 (**Chapters 4, 5, and 7**).
- The sponge secondary chemistry and the associated microbial community mainly depend on the sponge-species, the host state, and the ecological factors with spatial and temporal variations (**Concluding remarks**).

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Appendices

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2011, p. 862–870 Vol. 77, No. 3
0099-2240/11/$12.00 doi:10.1128/AEM.00100-10

Exploring the Links between Natural Products and Bacterial Assemblages in the Sponge *Aplysina aerophoba*▽†

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Received 14 January 2010/Accepted 18 November 2010

The sponge *Aplysina aerophoba* produces a large diversity of brominated alkaloids (BAs) and hosts a complex microbial assemblage. Although BAs are located within sponge cells, the enzymes that bind halogen elements to organic compounds have been exclusively described in algae, fungi, and bacteria. Bacterial communities within *A. aerophoba* could therefore be involved in the biosynthesis of these compounds. This study investigates whether changes in both the concentration of BAs and the bacterial assemblages are correlated in *A. aerophoba*. To do so, we quantified major natural products using high-performance liquid chromatography and analyzed bacterial assemblages using denaturing gradient gel electrophoresis on the 16S rRNA gene. We identified multiple associations between bacteria and natural products, including a strong relationship between a *Chloroflexi* phylotype and aplysinamisin-1 and between an unidentified bacterium and aerophobin-2 and isofistularin-3. Our results suggest that these bacteria could either be involved in the production of BAs or be directly affected by them. To our knowledge, this is one of the first reports that find a significant correlation between natural products and bacterial populations in any benthic organism. Further investigating these associations will shed light on the organization and functioning of host-endobiont systems such as *Aplysina aerophoba*.

Many sponges are known to be associated with large amounts of bacteria (46) that can amount up to 40% of the sponge biomass (35, 76). These types of sponges have been referred to as high-microbial-abundance sponges (36, 38, 40, 87, 88). While a fraction of the bacterial assemblage is used for sponge nutrition (56), another fraction can be permanently associated with sponges (35). This fraction is highly diverse and phylogenetically complex, with representatives from several phyla (e.g., *Proteobacteria*, *Chloroflexi*, *Cyanobacteria*, *Actinobacteria*, *Acidobacteria*, and *Bacteroidetes* [36, 38, 40, 65, 87, 88]). Variability in sponge-associated microbial communities has been assessed at several levels, including within and between specimens of the same host species (2, 38, 66, 67, 69, 87), between sponge species (38, 66), and temporal or spatial distribution within sponges (31, 35, 41, 66, 78, 86, 87).

Sponges are also known as a rich source of natural products or secondary metabolites (29, 30, 46, 89), many of which have the potential to be used in pharmaceutical and biotechnological applications (51, 61, 62, 65). Understanding the true origin of sponge-derived compounds may help obtain the required amount of specific potential drugs to set up clinical trials. However, the actual biosynthetic pathways and true producers of most sponge natural products are uncertain and remain to be investigated. Some natural products seem to have a sponge origin since they are located within sponge cells (74, 77). Other compounds are associated with microbial symbionts, suggesting that microbes are the true producers (2, 29, 30, 32, 76). However, location does not necessarily imply production. Microbes are known to actively excrete their natural products into the surrounding medium (46), and the synthesis of natural products could be accomplished in several of the multiple cell components present in sponges (45).

Sponges of the *Aplysinidae* family are a rich source of microorganisms (35, 72, 80) and also contain high concentrations of brominated alkaloids (BAs) (35, 36, 39). These BAs seem to be located within sponge cells, suggesting a biosynthesis by the sponge (74). However, bromoperoxidase enzymes (responsible for the halogenation reaction that incorporates the bromine into the compound) have only been reported for bacteria, algae, fungi, and plants (3, 81–83, 85). Ebel et al. (27) suggest that bacteria may produce the enzymes necessary to transform some of the secondary metabolites in *Aplysina aerophoba*. The possibility that sponge-associated bacteria, algae, or fungi might be taking part in the biosynthesis of these compounds cannot be ruled out, making it possible that the production of these BAs is a joint effort of multiple cell components in the sponge (45). To date, the majority of studies report the presence of compounds in specific cell compartments or show qualitative associations between secondary metabolites and bacteria (33, 74–76). Quantitative data supporting a relationship between bacterial abundance and concentration of natural products is lacking, let alone experimental evidence for this hypothesis, which would benefit from a better understanding of the links between bacterial strains and concentration of natural products.

In the present study, we assess the relationship between microbial community structure and natural products in the

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 29 November 2010.

sponge *Aplysina aerophoba* (Nardo, 1843). BAs are known to vary within this sponge (45, 74), and we sought to detect whether the bacterial community structure covaried with the concentration of natural products at an intraspecimen scale. To achieve our goal, we used high-performance liquid chromatography (HPLC) and denaturing gradient gel electrophoresis (DGGE) to investigate the relative abundance of major secondary metabolites and the complex bacterial assemblages found in multiple tissues of *Aplysina aerophoba*. The resulting chemical and bacterial matrices were analyzed with a variety of uni- and multivariate statistical methods that can prove useful in the field of microbial ecology.

MATERIALS AND METHODS

Sampling. In spring 2006, the sponge *Aplysina aerophoba* (formerly known as *Verongia aerophoba* [Nardo, 1843]) was collected by scuba diving at four locations along the Costa Brava, northwestern Mediterranean, between 3 and 14 m in depth. We collected several specimens from each location to obtain enough material for the bulk chemical extraction necessary to set up the chemical methods. We also collected one specimen from each location to assess intraspecimen variation in both natural products and the bacterial community.

To investigate intraspecimen variability, we randomly chose two chimney-like structures for each sponge. We cut the chimneys underwater and placed them in plastic bags with seawater. Then, we placed the sealed plastic bags in a cooler with ice to transfer them to the laboratory (2 to 3 h). Each chimney was divided into apical and basal zones, and we took samples from the ectosome and choanosome of the sponge from both zones with a sterilized scalpel under seawater (see Fig. S1 in the supplemental material). Apical and basal zones refer to the first top and bottom centimeter of a chimney-like structure. Typically, a distance of 2 to 3 cm separates the apical and basal zones. Ectosome refers to the 2 to 3 mm outer layer of sponge, with a greener or more purple color due to the presence of cyanobacteria (10, 74), while choanosome refers to the cyanobacterium-free inner layer of the sponge. Sponge samples were always under water to prevent the compound degradation that this sponge experiences in contact with air (as the species name "*aerophoba*" indicates). For each individual sponge, we had a total of eight samples (2 chimneys × 2 zones × 2 tissues) for natural product quantification and bacterial analyses.

Secondary metabolite isolation and identification. Sponge tissues were frozen at −20°C, freeze-dried, and extracted three times (1 h, 1 h, and overnight) with methanol (MeOH; 20 ml of MeOH per 1 g of sponge). The combined extracts were concentrated by vacuum rotary evaporation, leaving a powdery organic residue. To isolate the major compounds observed in preliminary HPLC analyses, the organic extract was first fractionated by flash chromatography using VWR LaFlash equipment on an Analogix Sepra C18 (SF25-55g) cartridge eluted with increasing amount of methanol in water, resulting in four fractions.

Fraction 1 (eluted with 30% MeOH) and fraction 3 (eluted with 100% MeOH), contained the major compounds and were further purified by semipreparative reversed-phase HPLC (Waters 1525 binary HPLC pump and Waters 2487 dual λ absorbance detector) on a Phenomenex Gemini RP-18 (250 by 10 mm, 5 μm) column. The elution conditions consisted of 30% MeOH in water (peaks 1 to 5) and 75% MeOH in water (peak 6) and a flow rate of 2.5 ml min^{-1} with UV detection at 245 nm. These conditions led to purification of the four known compounds aerophobin-1 (5 mg), aerophobin-2 (5 mg), aplysinamisin-1 (7 mg), and isofistularin-3 (12 mg). These four compounds were characterized by proton and carbon nuclear magnetic resonance (^1H and ^{13}C NMR; JEOL EX 400 spectrometer), liquid chromatography-mass spectrometry (LC-MS; Thermo Scientific LCQ Fleet), UV spectrometry (Hewlett-Packard diode array spectrophotometer), and comparison of spectroscopy data with published values from the literature.

HPLC analysis and compound quantification. HPLC analyses were performed with a system from Waters, including the Alliance separations module 2695, the column heater, and the 2998 photodiode array detector. The equipment was controlled and the data were handled by the Empower Chromatography Data software (Waters). The HPLC conditions consisted of two eluents (eluent A [0.1% aqueous trifluoroacetic acid] and eluent B [acetonitrile]) and an elution profile based on a linear gradient from 30% eluent B to 80% eluent B within 18 min and then to 100% eluent B for an additional 10 min. Flow rate was kept constant at 0.4 ml min^{-1}. We used a Phenomenex Synergi Max-RP (80 Å, 250 by 3.0 mm, 4 μm) analytical column with a fixed temperature of 30°C.

For the quantification of the natural products, 30 mg of freeze-dried sponge tissue from each ectosomal and choanosomal samples were extracted three times with 1.5 ml of MeOH in an ultrasonic tank for 15 min each time. The crude extract was filtered through a 20-μm-pore-size polytetrafluoriethylene filter (PTFE) and added in a 5-ml beaker. The final volume was adjusted to 5 ml of crude extract solution, and an aliquot of 1.5 ml was passed through a 13-mm, 0.2-μm-pore-size PTFE syringe-filter before HPLC injection. Then, 10 μl of this filtered solution was injected into the HPLC system described above. The brominated compounds were detected at 245 nm from the data collected across the 210- to 800-nm wavelength range. Peaks were integrated by applying the detector response based on peak areas to calibration curves obtained using the previously purified and characterized compounds as external standards. The final amount of natural compounds was calculated by averaging three replicate injections. Concentrations of brominated compounds were expressed as mg per g (dry mass) of sponge tissue.

DNA extraction and PCR amplification. DNA was extracted from ~1 mm^3 of ethanol-preserved (100% final concentration) sponge tissue (2 to 3 mg [wet mass]) using a DNeasy tissue kit (Qiagen), the effectiveness of which has been tested for environmental samples (60), according to the manufacturer's instructions with the following modifications recommended in the troubleshooting guide of the Qiagen kit: (i) lower amount of sample processed (2 to 5 mg), (ii) higher proteinase K digestion time (3 to 5 h, until the tissue looked well digested), and (iii) a final elution step with 40 μl of elution buffer (10 mM Tris-Cl, 0.5 mM EDTA [pH 9.0]) and incubation at room temperature for 10 min. DNA extracts were run in an agarose gel to check integrity and concentration using a standard mass ladder (DNA Smart Ladder; Eurogentec, Belgium). Small differences in yield extraction were visualized among samples. However, such differences were not expected to produce qualitative changes in the DNA mixtures, and the results were normalized using relative abundances in the fingerprinting analysis for an accurate intersample comparison.

PCR amplification of bacterial 16S rRNA gene suitable for subsequent genetic fingerprinting analysis was carried out using the universal bacterial primer combination BAC358F (5′-CCT ACG GGA GGC AGC AG-3′) with a 40-nucleotide GC-rich sequence attached to the 5′ end (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′) and BAC907RM (5′-CCG TCA ATT CMT TTG AGT TT-3′), which amplify a fragment approximately 568 bp long as described elsewhere (20). 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 of sterilized MilliQ water, 5 μl of 10× reaction buffer, 1.5 μl of $MgCl_2$ (50 mM), 1 μl of deoxyribonucleoside triphosphates (10 mM each), 2.5 μl of each primer (10 μM), 2.25 μl of bovine serum albumin (6 mg/ml), 0.25 μl of EcoTaq polymerase (Ecogen; 5 U/μl), and 1 μl of DNA template. The amounts of DNA template ranged between 10 and 100 ng of DNA for the different samples. Within such range we did not observe any remarkably loss/gain of DGGE bands in the fingerprinting analysis. PCR products were run in an agarose gel using a standard mass ladder (DNA Smart Ladder; Eurogentec, Belgium) to quantify the PCR product obtained in each case (20, 25).

DGGE analysis of PCR products and sequencing. DGGE was performed by using a Bio-Rad DCode universal mutation detection system (Bio-Rad) on a 6% polyacrylamide gel in 1× TAE (40 mM Tris base, 20 mM sodium acetate trihydrate, 1 mM EDTA). A comparable amplicon mass for each sample (c. 600 ng of PCR product) was added on the DGGE, and the gels were run for 4 h at a constant voltage of 200 V and 60°C in a 45 to 70% vertical denaturant gradient (100% denaturant agent is 7 M urea and 40% deionized formamide) (20). After electrophoresis, gels were stained for 45 min with SYBR Gold nucleic acid stain (Molecular Probes) and photographed with the UV GelDoc system (Uvitec). Image files were processed with the NIH Image software (National Institutes of Health, Bethesda, MD), and the relative band intensities were measured (25). Although not free of limitations, the signal intensity of the DGGE bands has been shown to be a useful tool for calculating the relative percentages of the different groups (20), and analysis of absence/presence data offered the same conclusions here (see Results). Obviously, absolute quantitative data on the abundance of specific bacteria requires the use of other techniques (see reference 20 and references therein).

Prominent bands were excised from the gel, resuspended in 25 μl of MilliQ water, and stored at 4°C overnight. An aliquot (2 to 5 μl) of the supernatant was used for PCR reamplification with the original primer set, and the PCR product was sequenced by using external sequencing facilities (Macrogen). Sequences were sent to BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) to get a first indication of what sequences were retrieved. Sequences with >97% sequence

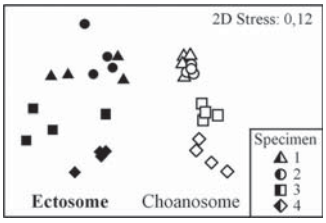

FIG. 1. Nonmetric multidimensional scaling based on Bray-Curtis similarity matrices from standardized and square root-transformed abundances of chemical and bacterial data. Significant differences between tissues and specimens (obtained by PERMANOVA) are shown. See the text for details on PERMANOVA.

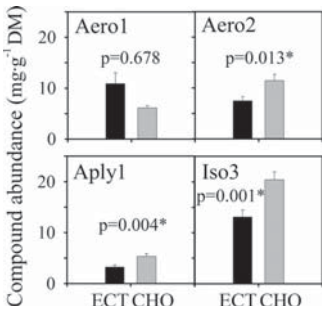

FIG. 2. Secondary metabolite concentrations (mg g [dry mass] of sponge tissue^{-1} ± 1 standard error) of the sponge *A. aerophoba* between ectosome (ECT) and choanosome (CHO) layers. Asterisks (*) indicate significant differences between tissues ($P \leq 0.05$). Aero1, aerophobin-1; Aero2, aerophobin-2; Aply1, aplysinamisin-1; Iso3, isofistularin-3.

identity to a cultured nearest phylogenetic neighbor in 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.

Data analysis. We used several statistical methods available in PRIMER 6 software (22) to analyze data on secondary metabolites and bacteria of *Aplysina aerophoba* as a function of specimen (four specimens), chimney (two chimneys for each specimen), chimney zone (top and bottom), and tissue type (ectosome and choanosome). Standardized and square root-transformed data were used to calculate Bray-Curtis similarity, and permutational multivariate analysis of variance (PERMANOVA) was used to test for differences in secondary metabolite and bacterial data together across the four factors. We also performed two independent PERMANOVAs on chemical and bacterial data using exclusively factors that proved significant in the previous analysis. In addition, we tested the effect that the DGGE gel might impose in the analysis of the bacterial community with a PERMANOVA with DGGE gel as a factor. Taking into account the PCR bias and the limitations of the DGGE as a quantitative technique (49), we also used presence/absence data to validate the method.

We used ANOVA and the nonparametric Mann-Whitney U test from Systat 12 software (63, 64) to analyze separately each secondary metabolite and each OTU across significant factors. We also performed a Mantel test (based on similarity matrices) to investigate whether there was a relationship between secondary metabolites and bacteria. We constructed two independent matrices from secondary metabolites and bacterial data and calculated a measure of similarity between each point and all of the others. The Mantel test correlates the two $n \times n$ similarity matrices (34) and detects any association between the components of both matrices (secondary metabolites and bacteria in our study). Mantel tests cannot point to any specific subset of data (i.e., any specific secondary metabolites and bacteria) responsible for an overall association, so further analyses are required to identify specific relationships.

Because of the large number of secondary metabolites and bacteria analyzed in the present study, we used factor analysis to look for coherent groups of chemical and bacterial variables that were correlated with one another within groups but largely independent between groups (64). These groups of correlated variables or factors help interpret the underlying mechanisms that have created the relationship between variables. 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 since it minimizes the number of variables that load highly on a factor and maximizes the loading variance across factors. The resulting independent factors were used as variables in a canonical correlation analysis to test whether any chemical and bacterial factors were correlated. We then used simple correlation analysis from Systat 12 software (63, 64) to establish the quantitative relationship behind the actual chemical and bacterial variables in the correlated factors.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences were deposited in GenBank under accession numbers AM905024 to AM905030.

RESULTS

Natural product and bacterial profiles of *A. aerophoba*. We quantified a total of 32 samples (4 individual sponges × 2 chimneys for each sponge × 2 zones for each chimney × 2 tissues for each zone) to characterize the chemical profile. We found six major peaks in the crude extract of *A. aerophoba*. We labeled the peaks according to their retention times: peak 1, peak 2, peak 3, peak 4, peak 5, and peak 6 (see Fig. S2 in the supplemental material). We identified four of the six major peaks: aerophobin-1 (Aero1; peak 3), aerophobin-2 (Aero2; peak 4), aplysinamisin-1 (Aply1; peak 5), and isofistularin-3 (Iso3; peak 6) according to their retention times and their UV profiles in comparison with purified and characterized compounds. We used two DGGE gels to characterize the bacterial profiles of the same 32 samples previously analyzed for the chemical profile. We identified a total of 24 different band positions, and we assigned each position to an OTU (see Fig. S3 in the supplemental material). Of these 24 OTUs, 15 were shared by all specimens except for OTUs 4, 11, and 19, which were restricted to three specimens. The remaining 9 OTUs were uncommon and were restricted to a particular sample or gel.

Using both chemical and bacterial data, we found significant differences between specimens (PERMANOVA, $P = 0.001$) and between ectosome and choanosome tissues (PERMANOVA, $P = 0.018$) (Fig. 1). We found no differences between chimneys or between the chimney top and bottom zones (PERMANOVA, $P = 0.118$ and $P = 0.155$, respectively). We therefore ran subsequent independent PERMANOVAs on chemical and bacterial data using the significant factors "specimens" and "tissue type."

Natural product variation in *A. aerophoba*. Ectosome and choanosome tissues significantly differed in their secondary chemistry (PERMANOVA, $P = 0.002$). Aero2, Aply1, and Iso3 were more abundant in choanosome tissues than in ectosome tissues (ANOVA, $P = 0.013$, $P = 0.004$, and $P = 0.001$, respectively), while Aero1 showed no differences between tissues (Mann-Whitney U test, $P = 0.678$) (Fig. 2). We found nonsignificant differences in the secondary chemistry between specimens (PERMANOVA, $P = 0.371$).

Bacterial community variation in *A. aerophoba*. We found highly significant differences in the bacterial assemblage between ectosome and choanosome tissues (PERMANOVA, $P = 0.001$) but differences between specimens were not significant (PERMANOVA, $P = 0.542$).

We found a gel effect when we compared the bacterial com-

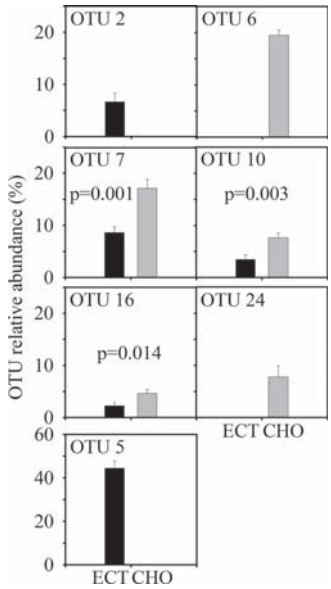

FIG. 3. OTU relative abundances of the sponge *A. aerophoba* between ectosome (ECT) and choanosome (CHO) tissue layers. Only OTUs with significant differences between tissues ($P \leq 0.05$) are shown.

munity between DGGE gels (PERMANOVA, $P = 0.004$), so we analyzed both gels separately. In both gels, we found significant differences between ectosome and choanosome tissues (PERMANOVA, $P = 0.001$ for both gels) and nonsignificant differences between specimens (PERMANOVA; gel 1, $P = 0.695$; gel 2, $P = 0.638$). Analysis of presence/absence data showed the same results, i.e., significant differences between ectosome and choanosome tissues (PERMANOVA; gel 1, $P = 0.018$; gel 2, $P = 0.008$) and no differences between specimens (PERMANOVA; gel 1, $P = 0.437$; gel 2, $P = 0.470$), so the bacterial differences between tissues and the homogeneity between specimens were quantitatively well supported despite the gel effect. Of 24 bacterial types, 7 were responsible for the differences found between the two sponge tissues (Fig. 3). OTUs 2 and 5 were exclusively found in the ectosome, while OTUs 6 and 24 were restricted to the choanosome (Fig. 3). OTUs 7, 10, and 16 were distributed over both tissues but were more abundant in the choanosome of the sponge (Mann-Whitney U tests; $P = 0.001$, $P = 0.003$, and $P = 0.014$, respectively) (Fig. 3).

Relationship between natural products and bacterial community. We detected a significant correlation between the chemical and bacterial profiles regardless of the gel analyzed or their combination (Mantel test; bacterial data from both gels, $R = 0.209$, $P = 0.001$; gel 1, $R = 0.249$, $P = 0.025$; and gel 2, $R = 0.315$, $P = 0.005$).

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 between groups. Factor analysis resulted in two chemical and eight bacterial factors (Tables 1 and 2) that explained 85.31 and 79.99% of the total variance, respectively. Canonical correlation analysis on the factor analysis scores resulted in four significant correlations that included three bacterial factors (BF1, BF2, and BF7) and the two chemical factors (Table 3). Individual correlation analysis of the secondary metabolites and bacterial types included in the correlated factors resulted in 15 uncorrected significant correlations (of 37 possible correlations [Table 4]), which is an extremely unlikely event to be explained by chance (Bernoulli equation, $P = 9.25E^{-11}$). Significant correlations after Bonferroni corrections were drastically reduced and restricted to the positive relationships between OTU19 and the compounds Aero2 and Iso3 ($R = 0.606$, $P = 0.007$, and $R = 0.569$, $P = 0.020$, respectively) and between OTU 7 (*Chloroflexi*; see below) and Aply1 ($R = 0.593$, $P = 0.010$) (Fig. 4; Table 4).

TABLE 1. Bacterial factors obtained from the factor analysis of the bacterial data[a]

OTU	Loading value obtained with bacterial factor:							
	BF1	BF2	BF3	BF4	BF5	BF6	BF7	BF8
OTU5	–0.86							
OTU6	0.84							
OTU7	0.81							
OTU2	–0.74							
OTU18		–0.86						
OTU19		–0.84						
OTU24		–0.83						
OTU12		–0.69						
OTU9		0.64						
OTU20		–0.62						
OTU22			–0.86					
OTU11				0.82				
OTU4				0.75				
OTU16				0.67				
OTU13					0.82			
OTU23						–0.81		
OTU15						–0.79		
OTU8							–0.93	
OTU3								–0.80

[a] To facilitate interpretation, we exclusively show OTU loadings with absolute values greater than 0.60. The closer the absolute loading values to 1.0, the stronger the association between the variables (OTUs or compounds) and the factors. The sign of the values represents the positive or negative nature of their association. BF, bacterial factor.

Phylogenetic analysis of excised 16S rDNA-DGGE bands. To identify the potential bacterial populations that were related to secondary metabolites of *Aplysina aerophoba*, we excised from the gels and successfully sequenced 24 16S ribo-

TABLE 2. Chemical factors obtained from the factor analysis of the chemical data[a]

Compound	Loading value obtained with chemical factor:	
	CF1	CF2
Iso3	0.97	
Aero2	0.94	
Aply1	0.77	
Aero1		0.99

[a] The compounds aerophobin-1 (Aero1), aerophobin-2 (Aero2), aplysinamisin-1 (Aply1), and isofistularin-3 (Iso3) were evaluated. CF, chemical factor.

TABLE 3. Canonical correlation analysis between chemical and bacterial factors

Bacterial factor	*R* (*P*)[a]	
	CF1	CF2
BF1	0.516 (0.002)	NS
BF2	–0.332 (0.032)	0.359 (0.036)
BF3	NS	NS
BF4	NS	NS
BF5	NS	NS
BF6	NS	NS
BF7	NS	–0.440 (0.012)
BF8	NS	NS

[a] The correlation coefficient (*R*) and *P* value of significant correlations ($P \leq 0.05$) are given. NS, nonsignificant correlations. BF, bacterial factor; CF, chemical factor.

somal DNA (rDNA)-DGGE bands. These bands belonged to seven different OTUs (see Fig. S3 in the supplemental material), five of which were very abundant (>44% of the total bacterial abundance) and were shared by both gels. Comparison of the 16S rRNA gene sequences with GenBank database showed a large range of bacterial taxa present in *Aplysina*. The bacterial community included representatives of the *Chloroflexi* (OTU6, accession no. AM905025; OTU7, accession no. AM905026; and OTU9, accession no. AM905027), *Cyanobacteria* (OTU5, accession no. AM905024), and *Bacteroidetes* (OTU2, accession no. AM905030), as well as members of the *Gammaproteobacteria* (OTU24, accession no. AM905029) and *Actinobacteria* (OTU8, accession no. AM905028) (Table 5). Six sequences were previously reported from marine sponges (OTU5 from *Aplysina aerophoba*, OTU6 from *Spongia agaricina*, OTU7 from *Xestospongia testudinaria*, OTU8 and OTU9 from *Ancorina alata*, and OTU 24 from *Ircinia felix*) and closely related to those reported in another *Aplysina* species (Table 5). The remaining sequence was closer to sequences from plankton (OTU2 from seawater).

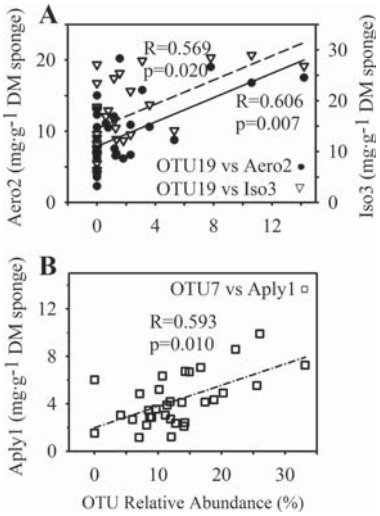

FIG. 4. Relationship between (A) the concentrations of aerophobin-2 (Aero2) and isofistularin-3 (Iso3) and the relative abundance of the unidentified OTU19 and (B) the concentration of aplysinamisin-1 (Aply1) and the relative abundance of a *Chloroflexi* clade (OTU7). Concentrations of compounds in mg g (dry mass) of sponge tissue^{-1} ± 1 standard error. *P* values are significant after Bonferroni corrections (see the text for details).

DISCUSSION

Over the last decades, many sponges have been investigated in an attempt to independently describe both the large diversity of natural products (14–17, 46) and potential symbiotic microorganisms (37, 40, 65). Sponge microbial ecology is receiving intense and increasing attention (4), and one area of particular interest is to assign the true origin of these natural products, whether of sponge or microbial origin (33, 46–48,

TABLE 4. Correlation analyses of the specific brominated alkaloids and bacterial types (OTU) included in the correlated chemical and bacterial factors

Bacterial factor	OTU	Bacterial group	*R* (*P*)[a]			
			CF1			CF2 (Aero1)
			Aero2	Iso3	Aply1	
BF1	2	*Bacteroidetes*	NS	NS	NS	–
	5	*Cyanobacteria*	–0.432 (0.013)	–0.519 (0.002)	–0.507 (0.003)	–
	6	*Chloroflexi*	0.386 (0.029)	0.499 (0.004)	0.511 (0.003)	–
	7	*Chloroflexi*	NS	NS	0.593 (<0.001)*	–
BF2	9	*Chloroflexi*	NS	NS	NS	NS
	12	UI[b]	NS	NS	NS	NS
	18	UI	NS	0.378 (0.033)	NS	–0.365 (0.040)
	19	UI	0.606 (<0.001)*	0.569 (0.001)*	NS	NS
	20	UI	0.374 (0.035)	NS	NS	NS
	24	*Gammaproteobacteria*	0.363 (0.041)	0.386 (0.029)	NS	NS
BF7	8	*Actinobacteria*	–	–	–	0.442 (0.011)

[a] The correlation coefficients (*R*) and uncorrected *P* values of all significant correlations are shown. Asterisks indicate significant correlations after Bonferroni correction. NS, nonsignificant correlations; –, correlations not tested; BF, bacterial factor; CF, chemical factor.
[b] UI, unidentified OTU.

TABLE 5. 16S rDNA sequence identities of representative bands excised from DGGE gels[a]

DGGE band	OTU	Division	Closest relative in database (isolation source; AN)	% Identity	Closest *Aplysina* host species (% identity; AN)
19	2	*Bacteroidetes*	Uncultured *Flavobacterium* (seawater; FN433428)	92.9	Not found
1	5	*Cyanobacteria*	*Synechococcus spongiarum* (*Aplysina aerophoba*; EF656449)	99.6	*Aplysina aerophoba* (99.6; EF656449)
6	6	*Chloroflexi*	Uncultured clone DGGE band bd29 (*Spongia agaricina*; AM849595)	100	*Aplysina fulva* (98.7; GU982064)
7	7	*Chloroflexi*	*Chloroflexus* sp. (*Xestospongia testudinaria*; FJ481354)	99.8	*Aplysina fulva* (99.6; GU982078)
13	8	*Actinobacteria*	Uncultured clone AncD18 (*Ancorina alata*; FJ900572)	98.1	*Aplysina fulva* (97.3; FM160879)
12	9	*Chloroflexi*	Uncultured clone AncB18 (*Ancorina alata*; FJ900579)	97.2	*Aplysina fulva* (96.8; GU982098)
18	24	*Gammaproteobacteria*	Uncultured clone DGGE band IF6-3 (*Ircinia felix*; DQ661847)	97.4	*Aplysina fulva* (96.4; FM160910)

[a] AN, accession number.

74–77). The question is not trivial because it has potential applications in biotechnology and pharmacognosy, but it remains to be rigorously addressed. The traditional approach consists in locating secondary metabolites in cell components using a variety of methods including X-ray microanalysis, density gradients of dissociated cells, and others (48, 74, 77). Although these methods can locate secondary metabolites (or a characteristic element of the metabolite, e.g., a bromine atom), they reveal only limited information on the true production of these compounds. This is particularly true if several parts of the compound are assembled in multiple cell compartments. This possibility cannot be ruled out and would provide additional support for the true collaboration between the sponge and its bacterial assemblage. In the present study we explored the association between bacteria and secondary chemistry by investigating relationships between the concentration of secondary metabolites and significant changes in the relative abundance of bacterial populations. Our study provides one of the first reports of a significant correlation between specific secondary metabolites and bacterial types (32). Although we cannot infer the true functional nature of these associations, they provide more explicit and simplified paths to explore the links between secondary metabolites and sponge-associated bacteria in *A. aerophoba*.

The characterization of chemical and bacterial profiles in *A. aerophoba* has already been reported by numerous authors (21, 28, 38, 39, 71, 89). The relative composition of BAs present in *A. aerophoba* seems to be highly conserved in both spatial and temporal scales. *Aplysina* individuals sampled thousands of kilometers apart showed indistinguishable secondary metabolite profiles (28). At a small spatial scale, bromoisoxazoline patterns of two *Aplysina* species proved to be remarkably stable and unaffected either by changing the light conditions or depth (54). Similarly, the alkaloid content remained unchanged after cultivation of *Aplysina* specimens in starvation conditions and antibiotics exposure in aquaria (35). Nevertheless, other studies with emphasis on natural products quantification show considerable variability in the concentration of secondary metabolites within *Aplysina* species (55) and between specimens of the same *Aplysina* species (62). The relative composition of the main brominated compounds under *ex situ* and *in situ* cultivation of *A. aerophoba* remained stable, although the total alkaloid content increased twice as much *ex situ* than *in situ* (43). There is a similar concern about the variation of bacterial assemblages in sponges. The microbial community seems to be uniform over time in *A. aerophoba* (35) and in other sponge species (41, 66).

Our results show evidence for a significant variation in the concentration of secondary metabolites in *A. aerophoba* both within and between specimens, which is consistent with the pronounced variability of secondary metabolite concentrations found not only in *Aplysina* spp. but also in other sponge species (9, 13, 52, 58) and other sessile organisms such as bryozoans (53). Our results also show evidence for changes in the relative abundances of bacterial populations in *A. aerophoba*. Signal intensity of DGGE bands is a useful tool to estimate the relative abundance of predominant bacterial groups (i.e., those >0.1 to 1% of total abundance) and to have a rough picture of microbial changes but does not provide detailed quantitative estimations (20). We were cautious in the number of PCR cycles run to avoid the "plateau" phase and in using the same amount of template in each reaction. The samples that we compared were run under the same PCR and DGGE conditions, and we carefully estimate the error among replicates. If there was any PCR bias it should be the same in all lanes, and therefore comparison among samples is still valid. The use of normalized relative abundances in the analyses also allowed for accurate intersample comparison.

Except for aerophobin-1, we found higher concentrations of every compound in the choanosome of the sponge, making a picture of a chemically rich sponge nucleus surrounded by a not-so-rich tissue layer. Traditionally, significant differences in secondary metabolites between sponge tissues are usually interpreted as evidence of their roles against predation, competition, or both (8, 9, 11, 58). The ectosome of *A. aerophoba* is packed with cyanobacteria (10, 79, 80; the present study) and is the preferred tissue preyed upon by the opisthobranch nudibranch *Tylodina perversa* (10). Thoms et al. (73) failed to detect aerophobin-1 in *A. aerophoba* but reported aerophobin-2 as one of the major constituents and the major deterrent compound against generalist predators together with isofistularin-3. Although the deterrence of aerophobin-1 is not known, chemical differences from aerophobin-2 are so minor that aerophobin-1 would be expected to be similarly deterrent against generalist predators too. If so, the high concentration of aerophobin-1 in the outer tissues of the sponge in the present study could serve as a defensive barrier against generalist predators, which have never been observed feeding on *A.*

aerophoba (unpublished data). High chemical concentrations in the outer tissues could also be a defense against foulers (6, 11). The higher concentration of isofistularin-3 (and other compounds) in the choanosome could be acting as a final deterrent against the specialist predator *T. perversa*, which is similar to what has been described for other opisthobranch-sponge feeding interactions (5, 7). Other roles, such as antibacterial activities, are far less investigated but could also explain the observed variation in secondary chemistry (6). The natural products found in *A. aerophoba* can be rapidly converted into aeroplysinin-1 and dienone, which show stronger antibiotic activity than their precursors and may protect this sponge from invasion by bacterial pathogens (27, 68, 73).

The predominant bacterial community in *A. aerophoba* seemed to be fairly constant between and within individuals, although some bacteria significantly varied their relative abundance between the ectosome and choanosome tissues of the sponge. These observations are in agreement with those previously reported by Thiel et al. (69), who found a distinct bacterial community between the outer (ectosome) and the inner (choanosome) tissues of the sponge *Tethya aurantium*. To date, the most noticeable microbial difference between ectosome and choanosome tissues of *A. aerophoba* is the presence of photosynthetic cyanobacteria in the outer tissues (10). Our study provides evidence for a specific distribution of *Cyanobacteria* and *Bacteroidetes* in the ectosome of the sponge. Some *Chloroflexi* and *Gammaproteobacteria* were restricted to the choanosome, while other bacterial bands had dissimilar distributions between tissues. The differences between the ectosome and the choanosome seem to be greater than previously reported. Apart from these bacterial groups, the microbial assemblage included representatives of other *Chloroflexi* and *Actinobacteria*. Overall, the sponge-associated bacterial community investigated in our study is as highly diverse and phylogenetically complex as described by other authors (36, 38, 40, 87, 88).

Our study also found significant differences in secondary chemistry between specimens of *Aplysina aerophoba* that were collected at a regional scale (<100 km). Thoms et al. (71) also found significant differences in the concentrations of secondary metabolites between specimens of *A. aerophoba* collected in Croatia and France, supporting the idea that chemical variation seems to be common within species of the genus *Aplysina* (55). Similarly, several bacteria differed between *A. aerophoba*, *Geodia barretti*, and *Cymbastela concentrica* (35, 41, 66). However, bacterial communities can be fairly constant between sponge species both within and between geographic regions (38, 87). Further research is necessary to understand these contrasting differences in bacterial variability within and between sponges.

Beyond the actual chemical or bacterial variation reported in this or previous studies, our study is the first attempt to correlate changes in both secondary chemistry and bacterial assemblage. A total of 15 of the 37 correlations evaluated resulted in significant positive and negative correlations between bacterial populations and natural products. This high number of significant correlations is extremely unlikely to occur by chance alone and suggests strong interactions between bacteria and between bacteria and natural products. Some *Chloroflexi* strains were positively related to the concentration of aplysinamisin-1, while a cyanobacterial strain was negatively related to the same compound. These *Chloroflexi* and *Cyanobacteria* strains are found in the choanosome and ectosome of the sponge, respectively, and were clearly negatively associated with each other. Other bacterial types, such as the *Gammaproteobacteria* strain and the unidentified OTU19, seemed to be positively related to the abundance of aerophobin-2 and isofistularin-3 but not to aplysinamisin-1. An *Actinobacteria* strain was associated with the concentration of aerophobin-1. Our results show how phylogenetically distinct bacterial groups are related to multiple natural products, which is also supported by a genomic study in *A. aerophoba* that assigned secondary metabolite gene clusters to a *Chloroflexi* clade and to the candidate phylum *Poribacteria* (59).

These correlations could be driven by multiple factors. Larger compound concentrations could lead to larger abundance of particular bacteria because of specific habitat conditions or preferences. For example, the abundance of brominated compounds in the sponge tissue would facilitate bacteria with the ability to metabolize these compounds (84). Ahn et al. (1) demonstrated that anaerobic bacteria harbored within the *A. aerophoba* carried out an anaerobic reductive dehalogenation of brominated aromatic compounds, suggesting that natural products would determine the presence or absence of these bacteria. Other hypotheses that could produce the same type of correlations between secondary chemistry and bacteria could include reduced competitive interactions with other bacterial species or preferential abiotic conditions. Under these hypotheses, the increasing abundance of bacteria could be either a spurious correlation or a consequence of the higher concentration of the compound. We cannot rule out that low abundant bacteria can produce metabolites that accumulate over time, although this would imply a low turnover of these compounds and the spherulous cells where they concentrate (74). However, it is well known that spherulous cells degenerate and are frequently released into the environment (11, 12, 70, 74), suggesting rapid cell turnover (24).

Alternatively, bacteria might be involved in the production of the natural product leading to higher concentration of compounds with increasing bacterial abundance. Various secondary metabolites of *A. aerophoba* are found in multiple sponge compartments, raising the possibility that distinct sponge compartments play a role at specific steps of the biosynthetic pathway (45). Interestingly, the haloperoxidase or halogenase enzymes responsible for halogenation processes have only been reported for bacteria, algae, and fungi (3, 81–84), which suggests that the bacterial endobionts of *A. aerophoba* could be producing these enzymes (24). Thus, *Aplysina* sponge cells may produce the inactive precursors of secondary metabolites, while bacteria may provide the enzymes necessary to activate them. Alternatively, the halogenation reaction could be attributed to either fungi or microalgae, which can also be associated with sponges.

Our phylogenetic analysis reveals that multiple bacterial strains were involved in these relationships. Populations of *Chloroflexi*, *Actinobacteria*, and *Cyanobacteria* are associated with the production of bioactive compounds (42, 50). The phylum *Chloroflexi* is an early branching lineage of bacteria about which little is known, especially about non-free-living microbes (18, 50). We now know that prominent enzymes

involved in the biosynthesis of bioactive secondary metabolites are assigned to *Chloroflexi* (nonribosomal peptide synthetase) and to *Poribacteria* clades (polyketide synthases) in *A. aerophoba* (59). Mat-forming *Cyanobacteria* are a rich source of natural products (19, 44, 47), and symbiotic cyanobacteria seem to be involved in the production of halogenated compounds in the sponge *Dysidea herbacea* (33, 75, 76). *Gammaproteobacteria*, *Deltaproteobacteria*, and *Actinobacteria* carry out halogenation or dehalogenation reactions (81, 82, 84), and the former phylum is the most likely producer of bryostatins in the bryozoan *Bugula neritina* (23). In our specimens, we found many of these bacterial types and they were correlated with some of the brominated compounds investigated in our study. Taken together, our data and those from the literature seem to support that sponge-associated bacteria are related to natural product synthesis.

Unraveling the true origin of natural products is challenging. The current body of evidence is too limited to make broad generalizations, but it suggests complex chemical and biological interactions that are far from being resolved. The diversity of secondary metabolites in any particular sponge species is usually large and so is the diversity of their associated bacterial communities. These multiple levels of complexity result in a demanding field, where solutions must include tools from multiple areas and disciplines. Further experimentation and new molecular techniques are needed to advance in this research area (57, 59). Our study presented evidence for a positive correlation between natural products and certain bacterial strains, which provides a simplified version of the sponge-microbe interactions and could be used as a starting point for hypothesis testing. Experimentally modifying the concentrations of secondary metabolites or the abundance of endobiotic bacteria would be critical to elucide their true association and to infer functional relationships, and such research is under way in our laboratory. A further step would be to use quantitative reverse transcriptase PCR (26) to study the expression of genes involved in the production of secondary metabolites and the enzymes responsible for the biotransformation of natural products. Applying modern chemical, molecular, and ecological techniques will substantially improve our understanding of the organization and functioning of these truly complex host-endobiont ecosystems.

ACKNOWLEDGMENTS

This research was supported by CSIC postgraduate and predoctoral grants to O.S.-S. and funded by the Spanish Ministry of Science and Innovation (grants CTM2007-66635 to M.A.B. and CGL2009-13318 to E.O.C.) and by the Agence Nationale de la Recherche (France; ECIMAR, ANR-06-BDIV-001-04).

We thank A. Riesgo for her comments on previous versions of the manuscript. We also thank R. Thacker and anonymous reviewers for suggestions that improved the final version of the manuscript.

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Mar. Drugs **2011**, *9*, 2499-2513; doi:10.3390/md9122499

OPEN ACCESS

Marine Drugs

ISSN 1660-3397

www.mdpi.com/journal/marinedrugs

Article

Relevant Spatial Scales of Chemical Variation in *Aplysina aerophoba*

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Received: 11 October 2011; in revised form: 14 November 2011 / Accepted: 16 November 2011 / Published: 28 November 2011

Abstract: Understanding the scale at which natural products vary the most is critical because it sheds light on the type of factors that regulate their production. The sponge *Aplysina aerophoba* is a common Mediterranean sponge inhabiting shallow waters in the Mediterranean and its area of influence in Atlantic Ocean. This species contains large concentrations of brominated alkaloids (BAs) that play a number of ecological roles in nature. Our research investigates the ecological variation in BAs of *A. aerophoba* from a scale of hundred of meters to thousand kilometers. We used a nested design to sample sponges from two geographically distinct regions (Canary Islands and Mediterranean, over 2500 km), with two zones within each region (less than 50 km), two locations within each zone (less than 5 km), and two sites within each location (less than 500 m). We used high-performance liquid chromatography to quantify multiple BAs and a spectrophotometer to quantify chlorophyll *a* (Chl *a*). Our results show a striking degree of variation in both natural products and Chl *a* content. Significant variation in Chl *a* content occurred at the largest and smallest geographic scales. The variation patterns of BAs also occurred at the largest and smallest scales, but varied depending on which BA was analyzed. Concentrations of Chl *a* and isofistularin-3 were negatively correlated, suggesting that symbionts may impact the concentration of some of these compounds. Our results underline the complex control of the production of secondary metabolites, with factors acting at both small and large geographic scales affecting the production of multiple secondary metabolites.

Keywords: brominated alkaloids; geographic variation; natural products; Porifera; sponges; secondary metabolites

1. Introduction

Conducting research at multiple spatial or temporal scales can substantially increase our understanding of numerous ecological processes, making scale a central problem in ecology [1]. Ecological processes and the mechanisms behind them show large spatial and temporal variation, which results in a high degree of heterogeneity across wide ranges of space, time, and biological organization (e.g., from ecosystems, to species, to species traits). Moreover, a multiple scale approach could shed light on the factors behind natural variation, improve our understanding of the ecological role of specific traits, and show evidence of the relevance or universality of ecological mechanisms and processes [1]. Besides, there is no single natural scale at which ecological studies should be studied [1]. Yet, ecological studies over large spatial and temporal scales are often hard to carry out and are uncommon on numerous ecological areas.

The production of marine natural products is an area that could benefit tremendously from a multi scale approach. We know that marine natural products play multiple roles in nature [2–4] and vary remarkably as a function of space and time [5–7]. The factors behind such variation are far from being fully understood and this research area is in chronic need of empirical data [8,9]. In fact, the poor understanding of the processes that control chemical diversity and variation is hindering the development of marine chemical ecology [9]. It is therefore critical to investigate chemical variation because it will shed light on the factors that regulate the production of chemical defenses, building up the field of marine chemical ecology.

The production of natural products is widespread in the benthic realm [10]. Sponges are consistently the richest source of marine natural products [10,11] and have also received considerable attention from a chemical ecology perspective [9,12]. Sponge secondary chemistry is known to vary significantly within species as a function of time [13–16], geographic region [13,17], habitat/community [18–20], specimens [21,22], tissues [23–25], and cells [26,27]. In particular, there is abundant information on secondary chemistry and chemical ecology of the genus *Aplysina*. There are multiple bromotyrosine alkaloids (BAs) described for *Aplysina* spp. [28–32], which can represent up to 13% of the sponge dry mass [33]. These compounds have a variety of biological activities [34–37] and play multiple ecological roles in nature [38–40]. Changes in the chemical structure of these compounds alter their biological activity [39,40], so the exact composition and concentration of compounds in the sponge tissues could translate into differential ecological roles.

BAs may vary remarkably within the same *Aplysina* species [41], which could have important ecological implications. The BAs of *Aplysina aerophoba* vary between cells [27], tissues [24,42,43], specimens [43,44], and geographic location [33]. Moreover, this species is distributed in the Mediterranean and Canary islands, where it can be locally abundant [33,42]. These characteristics make *Aplysina aerophoba* a perfect organism to investigate natural product variation across multiple

spatial scales ranging from a few meters to thousands of kilometers apart, and thus provide a great opportunity to assess the spatial scale at which chemical variation varies the most.

It is known that photosynthetic symbionts can contribute to the production of secondary metabolites [25,45,46] and might be involved somehow in the secondary chemistry of *A. aerophoba* [24,47]. Since concentration of Chlorophyll *a* (Chl *a*) can be used as a traditional proxy of the abundance of photosynthetic symbionts in sponges [23,42,48,49], we assessed whether the concentration of Chl *a* varied at the same spatial scale as BAs and further investigated whether both Chl *a* and secondary metabolites were related.

Is there a spatial pattern of variation in BAs? Do BAs vary the most between, near, medium, or far away locations? Do individual BAs follow the same pattern of variation? Do these patterns match the pattern of variation in Chl *a*? In this study we tested these hypotheses by looking at the spatial variation in BAs and Chl *a* of the sponge *Aplysina aerophoba* in neighboring bays to locations over 2500 km apart. We followed a nested design with two distant geographic regions, two zones within each region, two locations within each zone, and two sites within each location. This design calculates the magnitude of the variance attributable to the four spatial scales and will suggest the most relevant scale at which chemical variation should be addressed in this species. We found a spatial scale where BAs and Chl *a* varied the most and shed light on the complex mechanisms behind the production of natural products within a single species. This is the first report to take a broad spatial approach in marine chemical ecology, but further research will clarify whether this complex trend in the production of natural products is common among benthic organisms.

2. Results and Discussion

2.1. Natural Product and Chlorophyll a *Quantification*

We quantified a total of 126 samples (2 regions × 2 zones per region × 2 locations per zone × 2 sampling sites per location × 6–10 individual sponges per sampling site; Figure 1) to characterize the chemical profile and determine the Chlorophyll *a* concentration (Chl *a*) of *Aplysina aerophoba*. We identified and quantified the four major brominated alkaloids in our samples: aerophobin-1 (Aero1; peak 3), aerophobin-2 (Aero2; peak 4), aplysinamisin-1 (Aply1; peak 5), and isofistularin-3 (Iso3; peak 6), by comparing their retention times and UV profiles to those of purified, characterized standard compounds.

The secondary metabolites identified were in agreement with the abundant literature available for this species [30,39,40,44]. To minimize compound degradation and the enzymatic transformation of the high molecular weight (HMW) BAs quantified in our study into the low molecular weight (LMW) natural products reported elsewhere [36,39,50,51], we used a sampling protocol that minimized manipulation of live tissues, froze our samples rapidly after collection, and used methanol alone to obtain crude extracts. Since these LMW natural products result from bioconversion of precursors (but see [22]), their presence in our chromatograms could cast doubt on the actual concentration of the precursors quantified in our samples [39]. We failed to observe these bioconverted natural products in our chromatograms or they were at such low concentrations that cannot explain the large variation in BAs observed in our samples (see below). The concentrations of the four compounds we quantified

were positively correlated, except for the negative correlation between Aerophobin-2 and Aplysinamisin-1 (Table 1). Although this negative correlation could be indicative of bioconversion between these two compounds (concentration of a precursor decreases as concentration of the resulting compound increases), the degradation of Aerophobin-2 and Isofistularin-3 results in the LMW compounds [37], which were undetected in our extracts.

Figure 1. Study area that comprises two biogeographic regions; Tenerife (**A**; Canary Islands) and Cap de Creus (**B**; Northwestern Mediterranean). (**A**) Sampling sites of Tenerife. Punta Tixera (Ti), San Juan (SJ), Alcalá 1 (A1), Alcalá 2 (A2), Atlántida Coast (AC), Atlántida (At), Barranco del Eco (BE), Barranco Seco (BS); (**B**) Sampling sites of Cap de Creus. Gat 1 (G1), Gat 2 (G2), Caials (Ca), Portlligat (Po), Club Med 1 (C1), Club Med 2 (C2), Port de la Selva 1 (P1), Port de la Selva 2 (P2).

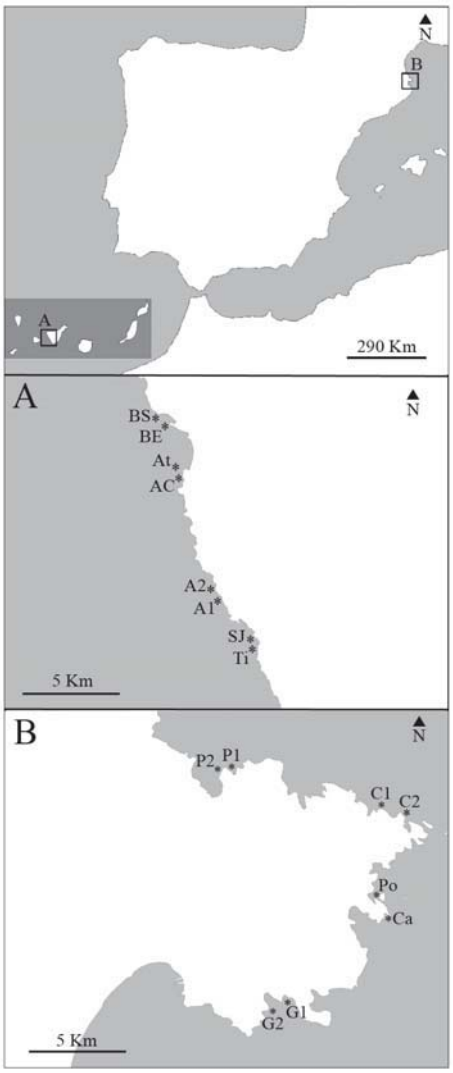


Table 1. Correlation coefficients (below diagonal) and uncorrected *P*-values (above diagonal) between depth and the concentrations of chlorophyll *a* (Chl *a*), Aerophobin-1 (Aero1), Aerophobin-2 (Aero2), Aplysinamisin-1 (Aply1), Isofistularin-3 (Iso3), and the four BAs combined (All).

	Depth	**Chl *a***	**All**	**Aero1**	**Aero2**	**Aply1**	**Iso3**
Depth		<0.001 *	0.001 *	0.008	0.276	0.494	<0.001 *
Chl *a*	−0.391 *		0.001 *	0.059	0.459	0.796	<0.001 *
All	0.294 *	−0.283 *		---	---	---	---
Aero1	0.236	−0.168	---		<0.001 *	<0.001 *	<0.001 *
Aero2	0.098	−0.067	---	0.403 *		<0.001 *	0.001 *
Aply1	0.062	−0.023	---	0.543 *	−0.466 *		0.508
Iso3	0.349 *	−0.430 *	---	0.385 *	0.294 *	0.060	

* show significant correlation coefficients and *P*-values after Bonferroni correction; --- correlation between the four individual compounds and their combined concentration are meaningless and not reported.

2.2. Secondary Metabolite Variation

The total concentration of BAs varied between sampling sites but not between regions, zones, or locations (nested analysis of covariance, ANCOVA, $P < 0.001$; $P = 0.722$; $P = 0.086$; and $P = 0.650$, respectively, Table 2). Because we found that depth could explain 8.6% of the variability in the total concentration of BAs ($R^2 = 0.086$, Table 1) we used depth as a covariate in our analyses. Total concentration of BAs did not vary with depth (Table 2), but 28.41% of the total variance in BAs occurred between sites that are less than 500 m apart (Table 2).

Table 2. *P*-values and percentage of the total variance explained by depth, region, zone, location, and site. Values obtained from nested ANCOVAs used to test the effect of spatial scale on the concentration of chlorophyll *a*, Aerophobin-1, Aerophobin-2, Aplysinamisin-1, Isofistularin-3, and total concentration of BAs with Depth as covariate. We also show the percent variance unexplained by the factors (Error). Geographic scale included Region (over 2500 km), Zone (less than 50 km), Location (less than 5 km), and Site (less than 500 m).

Compound	**Depth**	**Region**	**Zone**	**Location**	**Site**	**Error**
Chlorophyll *a*	0.069	<0.001/31.76%	0.905	0.236	0.038/9.46%	59.33%
Total BAs	0.275	0.722	0.086	0.650	<0.001/28.41%	68.58%
Aerophobin-1	0.382	0.762	0.054	0.694	<0.001/21.95%	53.40%
Aerophobin-2	0.064	0.945	0.060	0.736	0.001/28.47%	72.27%
Aplysinamisin-1	0.595	<0.001/18.34%	0.955	0.102	0.273	76.26%
Isofistularin-3	0.937	0.012/46.27%	0.555	0.388	0.110	49.20%

Separate nested ANCOVAs on the concentrations of each of the four compounds showed the relevance of spatial scale in the production of these compounds. The abundance of Aerophobin-1 and Aerophobin-2 differed significantly at the lowest scale (sampling site; $P < 0.001$ for both compounds; Figure 2 and Table 2) while the concentrations of Aplysinamisin-1 and Isofistularin-3 did not vary at this scale (Aply1, $P = 0.273$; Iso3, $P = 0.110$, Table 2). This pattern reversed at the largest geographic

scale investigated in our study (Region, over 2500 km apart). The compounds that did not vary between sampling sites, varied significantly across regions (over 2500 km apart) and *vice versa* (region; Aply1, $P < 0.001$; Iso3, $P = 0.012$; Aero1, $P = 0.762$; Aero2, $P = 0.945$; Figure 3 and Table 2). Intermediate scales were nonsignificant for all compounds, although zone was almost significant for Aerophobin-1 and Aerophobin-2 (Table 2). Thus, Region explained 18.34% and 46.27% of the total variance in Aplysinamisin-1 and Isofistularin-3 while Sampling Site explained 21.95% and 28.47% of the total variance of Aerophobin-1 and Aerophobin-2 (Table 2). Depth failed to explain the concentration of any of the four BAs investigated (Table 2).

Figure 2. Significant differences in Aerophobin-1, Aerophobin-2 ($mg \cdot g^{-1}$ dry mass sponge tissue ± 1 SE), and Chlorophyll *a* concentrations ($ng \cdot mg^{-1}$ dry mass sponge tissue ± 1 SE) of *Aplysina aerophoba* between sampling sites. A1 = Alcalá 1 ($N = 6$); A2 = Alcalá 2 ($N = 7$); Ti = Punta Tixera ($N = 7$); SJ = San Juan ($N = 9$); BE = Barranco del Eco ($N = 8$); BS = Barranco Seco ($N = 7$); At = Atlántida ($N = 6$); AC = Atlántida Coast ($N = 7$); C1 = Club Med 1 ($N = 10$); C2 = Club Med 2 ($N = 10$); P1 = Port de la Selva 1 ($N = 10$); P2 = Port de la Selva 2 ($N = 10$); G1 = Gat 1 ($N = 6$); G2 = Gat 2 ($N = 8$); Ca = Caials ($N = 7$); Po = Portlligat ($N = 8$). Aero1 = aerophobin-1; Aero2 = aerophobin-2; Chl *a* = chlorophyll *a*. Letters indicate significant differences ($P \leq 0.05$) of pairwise comparisons between sampling sites of the same location (showed as the same pattern design).

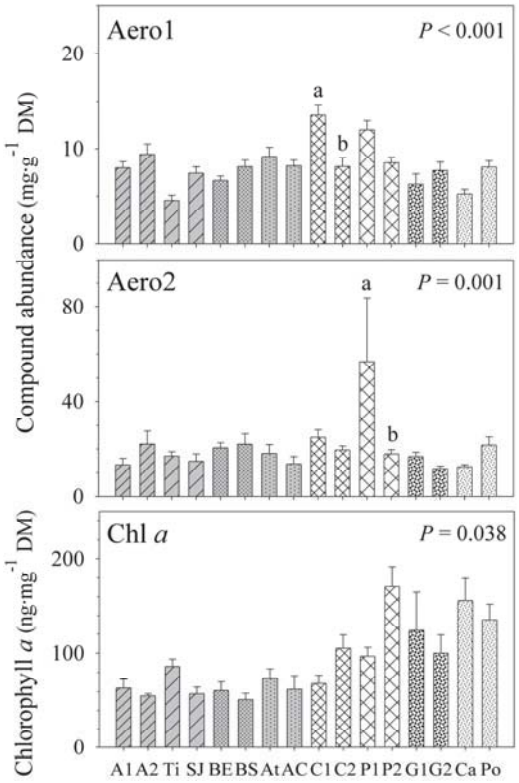
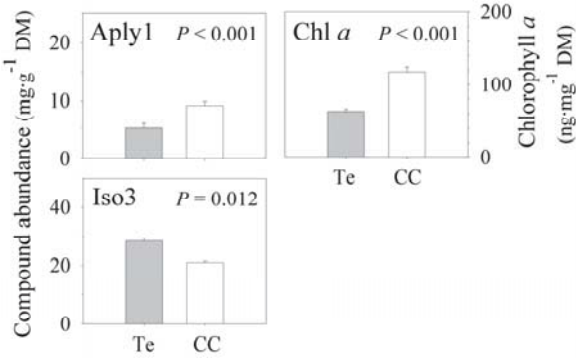


Figure 3. Significant differences in Aplysinamisin-1, Isofistularin-3 (mg·g^{-1} dry mass sponge tissue ± 1 SE), and Chlorophyll *a* concentrations (ng·mg^{-1} dry mass sponge tissue ± 1 SE) of *Aplysina aerophoba* between Tenerife (Te, N = 57) and Cap de Creus (CC, N = 69). Aply1 = aplysinamisin-1; Iso3 = isofistularin-3; Chl *a* = chlorophyll *a*.



An earlier study on the concentration of BAs in *Aplysina aerophoba* around the Canary Islands showed that the concentration of BAs varied spatially, despite the striking chemical similarity in the secondary chemistry of this species in locations over 500 km apart [33]. Our study suggests that the secondary chemistry of *A. aerophoba* varies over small and large spatial scales and our nested design allowed us to quantify the geographic scale that explained the largest variation in the concentration of BAs [52]. We found that the largest variation in the total concentration of BAs occurs at the lowest geographic scale investigated, *i.e.*, specimens less than 500 m apart showed the largest differences in the total concentration of BAs. This pattern was also observed in the compounds Aerophobin-1 and Aerophobin-2, which varied the most at low spatial scales, while Aplysinamisin-1 and Isofistularin-3 varied the most across broad spatial scales.

Our current knowledge of the production of BAs is incomplete, but it seems reasonable to argue that multiple factors acting at multiple spatial scales determine the concentration of these compounds. Studies from multiple disciplines often report increasing diversities with distance [53–55] but changes in sponge secondary chemistry occur at wide range of distances [13,22,56]. A study on the chemical diversity of the Atlanto-Mediterranean *Spongia lamella* showed two types of compounds [17]. One group remained constant among 9 populations scattered along the western Mediterranean and Atlantic coast of Portugal (half the distance of our study) while the other group varied significantly across this scale [17]. The two aerophobin compounds seemed to be affected by factors acting at small spatial scale, whereas Aplysinamisin-1 and Isofistularin-3 varied significantly across large spatial scales. Isofistularin-3 and Aerophobin-2 provide effective chemical defense against predators [40] and other BAs present in *A. aerophoba* show strong cytotoxic, algicide, and antibacterial activities [35,37]. Geographic differences in predation, fouling, competition, or symbionts (see below) among other factors could account for the contrasting patterns of variation of BAs in *A. aerophoba* [13,15,17,19,56].

Alternatively, our data could be interpreted as an intriguing evidence of the chaotic nature of chemical variability. This chaotic vision could be sustained by true variation in BAs, or could be the result of multiple factors (e.g., sample manipulation, compound quantification, data analyses). Experimental errors, however, would either amplify or reduce our perception of the existing natural

variation. Since our methods minimized compound alteration in this species and were consistent across all samples, we believe that our study accurately describes the natural variation in the production of BAs in *A. aerophoba* from local to regional geographic scale. Although a significant percentage of the variation in BAs in *A. aerophoba* was explained (up to 47%), between 49% and 77% remained unexplained (Table 2); thus it is clear that other, currently unknown, factors account for such variability. Further analyses and understanding of the production of natural products will shed light on this area.

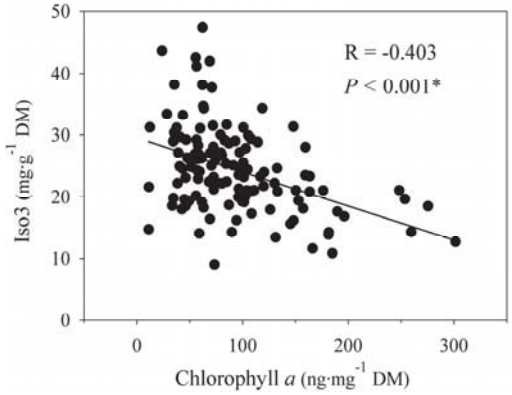
2.3. Chlorophyll a *Variation*

Chl *a* slightly decreased with increasing depth, which explained over 15.29% of the variation in Chl *a* in our samples (Table 1). However, the effect of spatial scale was larger than depth (Table 2). Our results showed that 31.76% of the variance in Chl *a* occurred between the two distant geographic regions and 9.46% between sites less than 500 m apart (Table 2). The pattern of Chl *a* variation mirrored the pattern of BAs variation. Chl *a*, Aplysinamisin-1, and Isofistularin-3 varied the most between the two biogeographic regions 2500 km apart. Total concentration of BAs, Aerophobin-1, and Aerophobin-2 varied the most between sites less than 500 m apart, which was the second source of variation in Chl *a*. Because concentration of Chl *a* can be used as a proxy of the abundance of photosynthetic symbionts in sponges [23,42,48] and photosynthetic symbionts can contribute to the production of secondary metabolites [25,45,46] (including *Chloroflexi* photosynthetic bacteria in *A. aerophoba* [24,47]), we further investigated whether the concentration of Chl *a* and secondary metabolites was related.

2.4. Relationship Between Natural Products and Chlorophyll a

Individual correlation analysis resulted in one significant negative correlation between the abundance of Isofistularin-3 and the concentration of Chl *a* ($R = -0.430$, $P < 0.001$; Figure 4, Table 1). We detected no further relationships between Chl *a* and remaining compounds (Table 1).

Figure 4. Relationship between the abundance of isofistularin-3 (Iso3; mg·g^{-1} dry mass sponge tissue ± 1 SE) and the concentration of chlorophyll *a* (Chl *a*; ng·mg^{-1} dry mass sponge tissue ± 1 SE). * Significant *P*-value after Bonferroni correction.



Chl *a* in *Aplysina aerophoba* may come from multiple symbiotic sources including photosynthetic cyanobacteria and *Chloroflexi* [24]. Our results suggested that the higher the abundance of photosynthetic symbionts in the sponge, the lower the concentration of Isofistularin-3. Whether this negative association represents a direct relationship between this compound and any of the photosynthetic symbionts in *A. aerophoba* is unclear, but Isofistularin-3 has been shown to be positively associated with the abundance of a symbiotic bacterium [24]. Whether this bacterium decreases its abundance with increasing photosymbiont concentrations remains unknown, but these correlations may reflect a chemically mediated biotic interaction between the sponge host and potential symbionts or between two members of the symbiotic microbial community within *A. aerophoba*.

The diversity of symbiotic communities and natural products found in marine invertebrates is a challenge to assess the role of the symbionts in the production of the compounds typically assigned to the hosts. This is an area that clearly needs further research. *Aplysina aerophoba* has been studied extensively, but we still know very little about factors shaping the production of BAs in this species. Although the BAs seem to be stored in sponge cells [27], multiple cell components might be involved in their production and the role of symbionts is largely unknown [24,43,47,50]. This hypothesis was launched over a decade ago but it remains to be experimentally tested. The advent of new biotechnological methods and approaches will surely shed light on the role of symbionts in the production of natural products [24,57,58].

3. Experimental Section

In March 2003, the sponge *Aplysina aerophoba* (Nardo, 1843) was collected by scuba diving at different sampling sites of Tenerife (Canary Islands) and Cap de Creus (Northwestern Mediterranean) (Figure 1). We collected several specimens from each location to obtain enough material for the bulk chemical extraction necessary to set up the chemical methods. To assess a geographic variation in natural products, we used a nested design covering a range of biogeographic scales to collect the whole chimney-like structures representative of this species, which are known to have the same secondary chemistry [24], of about 10 specimens in each site. We used a sharp knife to cut chimneys off by the base and placed them in independent plastic bags. Immediately after collection, samples were placed in coolers with ice to prevent changes in the secondary chemistry and all samples were frozen at −20 °C within 4 h of collection. Once in the laboratory, samples were freeze-dried under dark and a small portion of the top half of the chimney away from the cutting surface was selected for the quantification of the BAs. There are no differences in secondary chemistry between the top and bottom zones of the chimneys [24].

For BA isolation, 50 g of freeze-dried sponge were extracted three times (1 h, 1 h, and overnight) with methanol (MeOH, 20 mL MeOH per 1 g sponge). The crude extract (CE) was first fractionated by Flash-Chromatography. High Performance Liquid Chromatography (HPLC) was performed on a Waters HPLC with an Alliance separation module 2695, column heater, and 2998 photodiode array detector. Separation was achieved using a hydrophobic column (Phenomenex Gemini C18, 110 Å, 250 × 10.0 mm, 5 μm). Mobile phase consisted of 70% of MeOH, 30% of MilliQ water; the flow rate was 3 $mL \cdot min^{-1}$ and the injection volume was 100 μL. Peaks were detected at 320 nm and each BA compound detected was identified and carefully isolated. Dry pure BAs were recovered using rotatory

evaporation for use as standards. Then, series of dilution on pure compounds coupled to peak area calculation in HPLC (at 245 nm) allowed tracing calibration-curves. The major compounds observed in the HPLC chromatograms were characterized by classic spectrometric techniques (Liquid Chromatography/Mass Spectrometry, Nuclear Magnetic Resonance, UV profile) and by their retention times.

For BA quantification, approximately 50 mg of freeze-dried sponge tissue was extracted three times with 1.5 mL of MeOH in an ultrasonic tank for 15 min each time. The CE was filtered through a 20 µm polytetrafluoroethylene filter (PTFE) and added in a 5 mL beaker. The final volume was adjusted to 5 mL with MeOH and an aliquot of 1.5 mL was passed through a 13 mm, 0.2 µm PTFE syringe-filter before HPLC injection. Separation was achieved using a hydrophobic column (Phenomenex Synergi Max-RP, 80 Å, 250 × 3.0 mm, 4 µm) with a mobile phase of buffered (0.1% trifluoroacetic acid) water and acetonitrile. We used a linear gradient from 30% to 80% acetonitrile over 18 min, with an additional 10 min at 100% acetonitrile at the end of the run, with a flow rate of 0.4 $mL \cdot min^{-1}$. Samples were injected in 10 µL volumes and column temperature was maintained at 30 °C. Peaks were identified at 245 nm and integrated by applying the detector response based on peak areas to calibration curves. Concentrations of brominated compounds were expressed as $mg \cdot g^{-1}$ of dry mass of sponge tissue.

We used the concentration of Chlorophyll *a* (Chl *a*) as an indication on the density of autotrophic symbionts within *A. aerophoba*. Approximately 100 mg of freeze-dried sponge tissue was extracted with 5 mL of 90% acetone for 12 h in the dark at 1 °C. 2 mL of the extract solution were passed through a 0.2 µm PTFE syringe-filter. The 8452A diode array spectrophotometer (Hewlett Packard) was used to perform the absorbance measurements. We measured absorbance at four wavelength (630, 647, 664, and 750 nm) according to the trichromatic equation of Jeffrey and Humphrey [59] to calculate the Chl *a* concentration (1). Chl *a* was expressed as $ng \cdot mg^{-1}$ of dry mass of sponge tissue.

$$\text{Chl } a \left(\text{mg L}^{-1}\right) = 11.85\left(\text{Abs664} - \text{Abs750}\right) - 1.54\left(\text{Abs647} - \text{Abs750}\right) - 0.08\left(\text{Abs630} - \text{Abs750}\right) \quad (1)$$

We used several statistical methods from SYSTAT 12 software [60,61] to analyze secondary metabolite abundances and Chl *a* content. Nested analyses of covariance (ANCOVAs) were performed on ranked compound abundances and Chl *a* concentrations with region (2 regions), zone (4 zones), location (8 locations), and sampling site (16 sites) as factors (Figure 1) and depth as a covariate. We used *a posteriori* pairwise comparisons with Bonferroni test to identify the groups responsible for the main significant factors. We also used simple correlation analysis to establish the quantitative relationships between secondary metabolites, Chl *a* and depth.

4. Conclusions

The spatial scale explaining the largest variation in the concentration of natural products within a single species depends on the actual compound investigated. Our study showed that the rich secondary chemistry of *Aplysina aerophoba* varied quantitatively from sites less than 500 m apart to geographic regions over 2500 km apart. Aerophobin-1 and Aerophobin-2, and the combined concentration of all BA compounds varied the most between sampling sites less than 500 m apart, while Aplysinamisin-1 and Isofistularin-3 varied across larger spatial scales.

Depth played a trivial role, if any, regulating the concentration of BAs in *A. aerophoba*. Individual correlation analyses with all our data showed that depth could explain a small percentage (8–12%) of the variation in BAs. This minor effect was however overshadowed by the larger influence of spatial scale in the concentrations of BAs.

The putative role of symbionts on the production of BAs in *A. aerophoba* remains an open question. There is indirect evidence that supports the role of symbionts, including the correlation between Isofistularin-3 and chlorophyll *a* detected in this study. The circumstantial evidence available provides a number of testable hypotheses that, with the use of new molecular techniques, should contribute to the development of this particularly elusive research area.

Acknowledgments

We thank Sandra Duran, Andrea Blanquer, and Natalia Ortega for their invaluable help in the Canarian sampling trip. We also thank the anonymous reviewers for suggestions that improved the final version of the manuscript.

This research is a part of OSS PhD Thesis, who was supported by a CSIC predoctoral grant. Research partially funded by the Spanish Ministry of Science and Innovation (SOLID: CTM2010-17755 and BENTHOMICS: CTM2010-22218-C02-01) and by the Agence Nationale de la Recherche (France; ECIMAR project, ANR-06-BDIV-001-04). This research is a contribution of the Consolidated Research Group "Grupo de Ecología Bentónica" (SGR2009-655) of the Catalan Goverment.

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Mar. Drugs **2012**, *10*, 677-693; doi:10.3390/md10040677

OPEN ACCESS

Marine Drugs

ISSN 1660-3397

www.mdpi.com/journal/marinedrugs

Article

Temporal Trends in the Secondary Metabolite Production of the Sponge *Aplysina aerophoba*

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Received: 24 February 2012; in revised form: 12 March 2012 / Accepted: 14 March 2012 / Published: 23 March 2012

Abstract: Temporal changes in the production of secondary metabolites are far from being fully understood. Our study quantified, over a two-year period, the concentrations of brominated alkaloids in the ectosome and the choanosome of *Aplysina aerophoba*, and examined the temporal patterns of these natural products. Based on standard curves, we quantified the concentrations of aerophobin-2, aplysinamisin-1, and isofistularin-3: three of the four major peaks obtained through chemical profiling with high-performance liquid chromatography. Our results showed a striking variation in compound abundance between the outer and inner layers of the sponge. The ectosome showed high concentrations of bromocompounds during the summer months, while the choanosome followed no pattern. Additionally, we found that, from the outer layer of the sponge, aerophobin-2 and isofistularin-3 were significantly correlated with water temperature. The present study is one of the first to document quantitative seasonal variations in individual compounds over multiple years. Further studies will clarify the role of environmental, biological, and physiological factors in determining the seasonal patterns in the concentration of brominated alkaloids.

Keywords: *Aplysina*; chemical defenses; chemical ecology; natural products; Porifera; satellite-derived SST; seasonality; temporal variation

1. Introduction

Marine invertebrates are involved in a great variety of interactions, many of which are chemically mediated [1,2]. Not surprisingly, marine invertebrates are a potential source of natural, bioactive products that act against external threats [2,3]. These compounds often play multiple ecological roles, primarily protection against predators [4–8], competitors for space [9,10], biofoulers [11,12], or opportunistic pathogenic microorganisms [13,14].

Natural products have generated pharmaceutical and biotechnological interest due to their potential roles in human diseases [15]. However, novel drug development requires large amounts of the candidate species in screening for potentially bioactive compounds [16,17]. To that end, biological and ecological studies can provide guidelines for applied and biotechnological research. To collect sufficient amounts of bioactive compounds for testing, we need to know where the natural products are produced and stored (e.g., specific tissue or cells), what organisms are involved in their production (*i.e.*, the marine invertebrate, an associated microorganism, or multiple organisms), and what factors affect their production (e.g., biotic, abiotic factors). An important point to understand natural product production is whether or not secondary metabolites vary over time, behavior which is unknown for most species. Continuous quantitative data over multiple years will contribute significantly to understand their natural variability, providing additional information to contrast with proposed mechanisms of production and natural function.

Sponges are among the best-known producers of bioactive compounds [2,18,19]. Sponges are distributed in multiple, diverse habitats [12,20–24]; this property has generated an interest in sponges in the field of chemical ecology and in the pharmacognosy industry [25]. To date, research has focused mostly on novel drug discovery with highlights on the origin of natural products. In comparison, little is known about the temporal variability of the vast number of known bioactive compounds. Secondary metabolite production may vary as predicted by the optimal defense theory (ODT), outlined by McKey [26,27] and Rhoades [28], in plants, and tested by several authors [29,30]. The ODT assumes that the metabolic cost of secondary metabolite production for chemical defenses must meet the organisms' needs and be balanced against the other terms in the energy budget (*i.e.*, reproduction and growth). This theoretical framework of terrestrial plant defenses is particularly relevant to sponge chemical ecology [31,32]. Variations in the abundance of natural products may respond to physical constraints such as hydrodynamism [33], depth [33–35], or water temperature [36]. Habitat [31], sponge size [31], competition for space [37], or against fouling [38] may also cause changes in sponge secondary chemistry. Many of these biotic or abiotic factors vary between months, seasons, and years [37–39] so the production of secondary metabolites may have strong temporal patterns.

The aim of the present study was to quantitatively assess the temporal variability of bioactive compounds in the demosponge *Aplysina aerophoba* (Nardo 1833). This verongid is a rich source of brominated alkaloids (BAs), which are chemically well characterized [40–43]. BAs in this sponge are known to vary within the same specimen and at multiple geographic scales [44–47], but the temporal patterns of variation remain undescribed. Here, we aimed to determine whether the concentration of natural products varied at multiple temporal scales, from months, to seasons, and years. Our results showed that temporal changes in the concentration of BAs occurred in the outer layer (*i.e.*, ectosome) of the sponge while we did not detect any variation in the internal region (*i.e.*, choanosome).

2. Results and Discussion

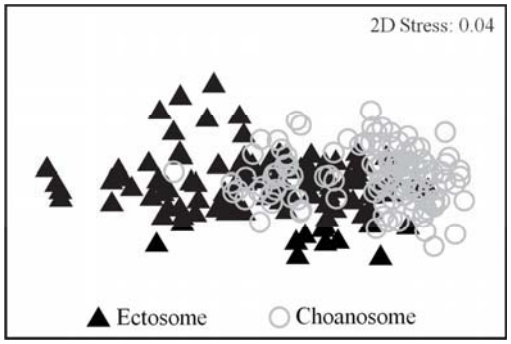
2.1. Chemical Profile of Aplysina aerophoba

We quantified a total of 240 samples (2 sampling years × 12 months per year × 5 individual sponges per month × 2 tissues per specimen) to characterize the chemical profile. We identified four out of six major peaks: aerophobin-1 (Aero1; peak 3), aerophobin-2 (Aero2; peak 4), aplysinamisin-1 (Aply1; peak 5), and isofistularin-3 (Iso3; peak 6), by comparing their retention times and UV profiles to those of purified, characterized standard compounds. Of these, we finally quantified the three major BAs (Aero2, Aply1, and Iso3), because Aero1 was often under the detection limit in the HPLC analyses.

2.2. Natural Product Variations

The three compounds quantified showed significant differences in abundance in the ectosomal (outer) and choanosomal (inner) layers of the sponge (PERMANOVA, $P = 0.001$; Figure 1). Therefore, we subsequently performed separate analyses of the chemical data for these two layers.

Figure 1. Metabolite abundances in the ectosome and choanosome of *Aplysina aerophoba*. Non-metric multidimensional scaling (MDS) was based on Bray-Curtis similarity matrices from standardized and square root-transformed abundances of chemical data. Significant differences were found between both layers.



We found striking differences in the concentrations of secondary metabolites between the ectosome and the choanosome of *Aplysina aerophoba*. These differences were not only caused by changes in the abundance of the secondary metabolites but also in their dynamics. As reported for other sponges [37,48,49], *A. aerophoba* produces a heterogeneous mix of natural products. This heterogeneous mix of compounds is not uniformly distributed within the sponge. Based on X-ray energy dispersive microanalysis to locate bromine atoms [46], BAs in *A. aerophoba* were found in two sponge structures: the spherulous cells and the sponge fibers. Kreuter *et al.* [44] also detected intraspecimen differences in the amount of the low molecular weight (LMW) compounds, aeroplysinin-1 and dienone, between the external (*i.e.*, outer layer, oscular region) and the internal regions of *A. aerophoba*. These results were in agreement with a recent study [45] that found significant differences in the accumulation of high molecular weight (HMW) BAs between the choanosome (*i.e.*, inner region) and the ectosome (*i.e.*, outer layer) of the same species. The concentrations of the three compounds we

quantified were positively correlated in both layers: ectosomal (Aero2-Aply1, $R = 0.560$; Aero2-Iso3, $R = 0.658$; Aply1-Iso3, $R = 0.916$; $P < 0.001$ for all comparisons) and choanosomal (Aero2-Aply1, $R = 0.363$; Aero2-Iso3, $R = 0.571$; Aply1-Iso3, $R = 0.849$; $P < 0.001$ for all comparisons). This could indicate that all compounds might be responding in the same way and at the same scale to the regulating factor(s).

The HMW BAs have been described as undergoing biotransformation when the tissue has been damaged, resulting in the LMW BAs aeroplysinin-1 and dienone, a process that is supposed to be enzyme-mediated [41,43]. The presence of these LMW BAs in our chromatograms could cast doubt on the actual concentration of the precursors quantified in our samples [42]. Yet, we never detected these bioconverted natural products in our chromatograms or they were at such low concentrations that cannot explain the large variation in BAs observed in our samples. This is expected, because our methods were designed to minimize the probability of biotransformation, keeping tissue manipulation to a minimum and avoiding the use of water. Moreover, Puyana *et al.* [50] found no evidence for biotransformation in two *Aplysina* species from the Caribbean. There is an unresolved controversy as to whether or not biotransformation occurs in *Aplysina* species and our study can neither support nor refute this biotransformation hypothesis. Our study was designed to investigate the temporal variation of the unaltered secondary chemistry of *Aplysina aerophoba*. If biotransformation does occur, our methods proved ideal to avoid biotransformation because we only found HMW BAs in our chromatograms, so biotransformation can neither affect nor explain the intraindividual variation observed in our study.

Table 1. *P*-values and percentage of the total variance explained by year, season, and month in the ectosome and the choanosome of the sponge. Values obtained from nested ANOVAs used to test the effect of timescale on the concentration of aerophobin-2 (Aero2), aplysinamisin-1 (Aply1), and isofistularin-3 (Iso3). We also show the percent variance unexplained by the factors (Error).

	Ectosome				Choanosome			
Compound	**Year**	**Season**	**Month**	**Error**	**Year**	**Season**	**Month**	**Error**
Aero2	0.869	0.190	0.027/14.85%	78.13%	0.059	0.491	0.030/13.67%	76.29%
Aply1	0.368	0.001/25.82%	0.539	74.18%	0.463	0.438	0.025/15.80%	83.80%
Iso3	0.260	0.003/24.44%	0.322	69.30%	0.551	0.201	0.031/14.03%	79.60%

At the ectosomal level, there was no change in the abundances of the three compounds between years (nested ANOVA, $P > 0.05$ for the three BAs; Table 1). We found significant seasonal differences in the concentration of Aply1 and Iso3, with greater abundances in summer (nested ANOVA, $P < 0.01$ for both BAs; Figure 2, Table 1). However, there was no seasonal change in the abundance of Aero2 (nested ANOVA, $P > 0.05$; Table 1). Significant monthly differences were detected in the concentration of Aero2; the abundance increased in August and decreased in February (nested ANOVA, $P < 0.05$; Figure 3, Table 1). Nevertheless, the abundance of Aply1 and Iso3 did not vary between months (nested ANOVA, $P > 0.05$ for both; Table 1). Our results showed that "year" failed to explain the concentration of any of the three BAs investigated in the external layer of the sponge. However, "season" explained 25.82% and 24.44% of the total variance in Aply1 and Iso3 while "month" explained 14.85% of the total variance in Aero2 (Table 1).

Figure 2. Seasonal changes in BA abundances. Secondary metabolite concentrations (mg·g^{-1} dry mass sponge tissue) ± 1 standard error of the mean (SE) observed in different seasons in the ectosome of *Aplysina aerophoba*. Sp = spring (N = 30); Su = summer (N = 30); Au = autumn (N = 30); Wi = winter (N = 30). Aply1 = aplysinamisin-1; Iso3 = isofistularin-3. Means (*i.e.*, seasons) with different letters are significantly different from each other ($P \leq 0.05$; pairwise comparisons).

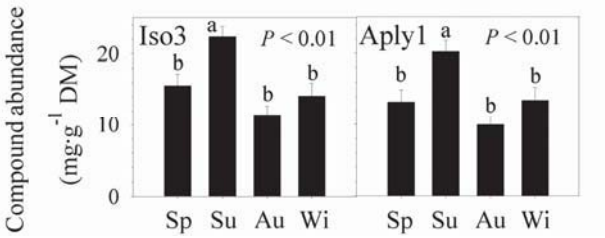
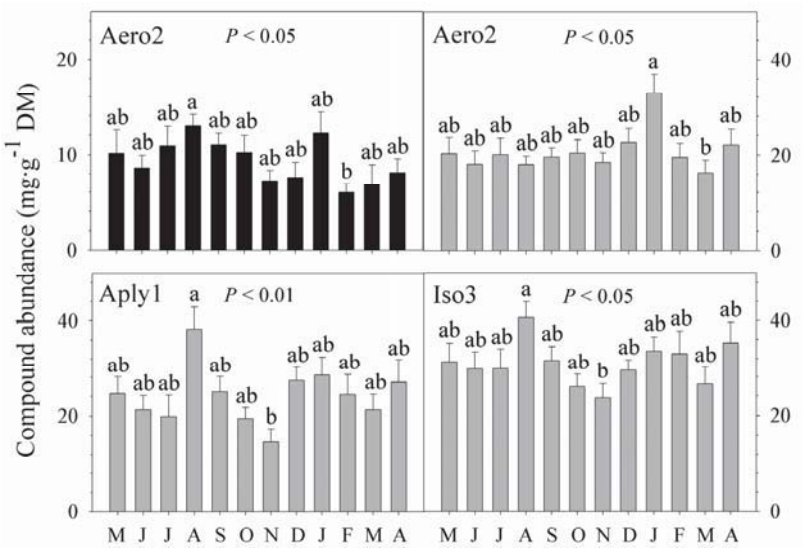


Figure 3. Monthly changes in metabolite concentrations. Secondary metabolite abundances (mg·g^{-1} dry mass sponge tissue ± 1 SE) in the ectosome (black) and in the choanosome (grey) of *Aplysina aerophoba* from May to April (N = 10 per month). We only show compounds in which there were significant monthly changes in metabolite concentrations. Aero2 = aerophobin-2; Aply1 = aplysinamisin-1; Iso3 = isofistularin-3. Means (*i.e.*, months) with different letters are significantly different from each other ($P \leq 0.05$; pairwise comparisons).



At the choanosomal level, we found no annual differences in the abundances of the three compounds (nested ANOVA, $P > 0.05$ for the three BAs; Table 1). The concentration of the three BAs did not vary either between seasons (nested ANOVA, $P > 0.05$ for the three BAs; Table 1). However, we found significant monthly changes in the abundances of the three compounds (nested ANOVA, $P < 0.05$ for the three BAs; Figure 3, Table 1). Whereas the abundance of Aply1 and Iso3 increased in

August and decreased in November, the concentration of Aero2 was higher in January and lower in March (Figure 3). Neither "year" nor "season" explained the concentration of the three BAs in the internal part of the sponge. But, "month" explained 13.67%, 15.80%, and 14.03% of the total variance in aerophobin-2, aplysinamisin-1, and isofistularin-3 (Table 1).

2.3. Natural Product Trends

Looking at the complete 2-year-long temporal series, we found in the external layer of the sponge significant changes in the concentration of Aply1 and Iso3 over time but not in Aero2 (P = 0.035, P = 0.038, and P = 0.711, respectively; Figure 4). In contrasting, we found no significant trend or seasonality in the abundance of BAs in the choanosome of the sponge (Aero2, P = 0.083; Aply1, P = 0.509; and Iso3, P = 0.566; Figure 5).

Figure 4. Temporal trends in metabolite abundances in the ectosome. Compound abundances (mg·g^{-1} dry mass sponge tissue ± 1 SE) observed in the ectosome of *Aplysina aerophoba* over a two-year survey (N = 5 per month). Aero2 = aerophobin-2; Aply1 = aplysinamisin-1; Iso3 = isofistularin-3. Significant differences $P \leq 0.05$. The sea surface temperatures (°C) are also shown for the whole period (discontinuous line).

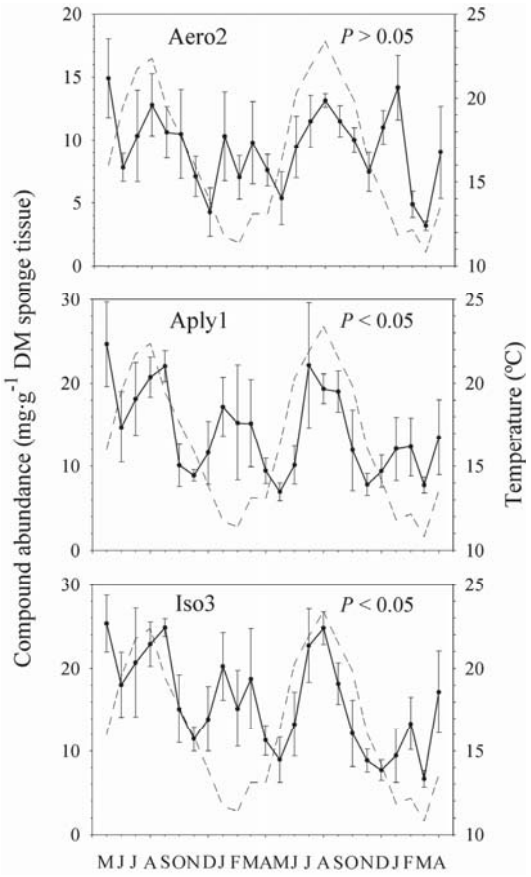
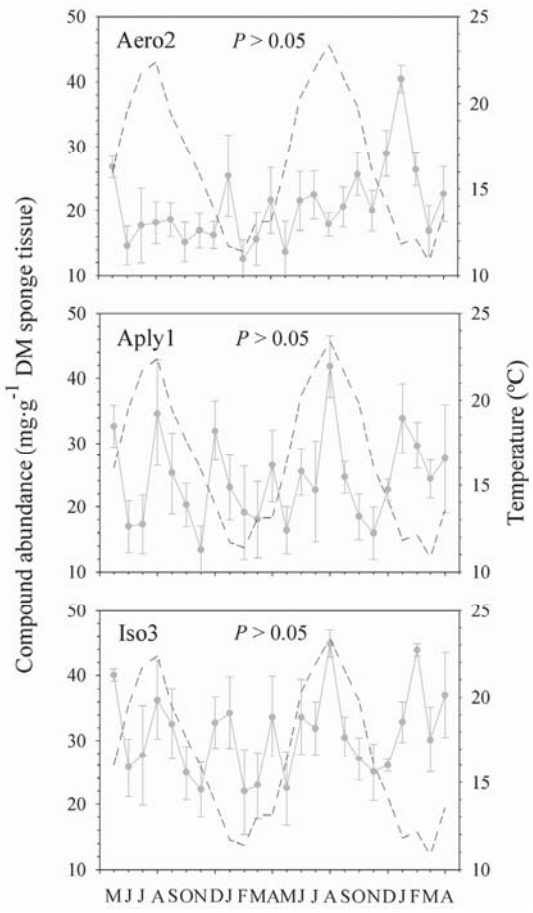


Figure 5. Temporal trends in metabolite abundances in the choanosome. Compound abundances (mg·g^{-1} dry mass sponge tissue ± 1 SE) observed in the choanosome of *Aplysina aerophoba* over a two-year survey (N = 5 per month). Aero2 = aerophobin-2; Aply1 = aplysinamisin-1; Iso3 = isofistularin-3. Significant differences $P \leq 0.05$. The sea surface temperatures (°C) are also shown for the whole period (discontinuous line).



Natural variations in sponge secondary chemistry have received little attention. An environmental influence on bioactive compound production was first documented by Thompson *et al.* [35] in the sponge *Rhopaloeides odorabile*. Seasonal patterns were reported later in several sponge species, where bioactivity was used to measure metabolite biosynthesis. Turon *et al.* [37] found *Crambe crambe* most biologically active in late summer and autumn. *Latrunculia* sp. nov. was most active in spring [38]. Swearingen & Pawlik [51] documented that *Chondrilla nucula* was most toxic in summer. In contrast, other sponge species, *Agelas oroides* and *Petrosia ficiformis*, appeared to be most bioactive during winter [34]. But, relatively few studies have described long-term, quantitative, temporal changes in individual biologically active compounds.

We detected changes within but not between years in the BAs of *Aplysina aerophoba*. Our data showed that temporal variations in BAs were tissue-specific. The internal region (*i.e.*, choanosome) did

not appear to follow a clear pattern of production, but changes in sponge secondary chemistry occurred at low temporal scale (*i.e.*, months). By contrasting, there seemed to be a clear trend in the external layer (*i.e.*, ectosome) with the highest production period in summer and a lower peak in winter. Whereas aerophobin-2 seemed to be more prone to variation between months, aplysinamisin-1 and isofistularin-3 varied significantly between seasons. In fact, we also showed evidence for an annual periodicity with greater abundances of these two compounds in the warmer season. Page *et al.* [33] reported a similar seasonal pattern in the concentration of two bioactive compounds of *Mycale hentscheli*, although they did not present evidence of an annual periodicity in secondary metabolite production. Similarly, Abdo *et al.* [36] documented that the concentration of one bioactive compound of *Haliclona* sp. was significantly higher in summer than in winter.

Alternatively, our data could be the result of the chaotic nature of chemical variability. Our results could be supported by true variation in BAs, or could be the consequence of laboratory errors (as a result of our extraction methods and analyses). Experimental errors, however, could either amplify or reduce the true natural variation. Since our methods minimized compound alteration in this species and were consistent across all samples, we believe that our study accurately describes the natural temporal variation in the production of BAs in *A. aerophoba*. Although a notable percentage of the variation in BAs in *A. aerophoba* was explained by the temporal scales investigated in our study (up to 26%), most variance remained unexplained (Table 1). It is clear that other unknown factors account for such variability. Further analyses and understanding of the production of natural products will shed light on this area.

Variations in defense patterns among tissues can be explained by environmental or physiological constraints. We found a chemically rich sponge core surrounded by a less chemically rich tissue layer [45]. Traditionally, intraindividual differences in bioactive natural products were typically interpreted as evidence of the distinct roles of different tissues against predation, competition, or both [31,39,48,52]. In *Aplysina*, the BAs aerophobin-2, aplysinamisin-1, and isofistularin-3 seem to be anti-predatory deterrents [43,53].

From the perspective of the ODT, one would expect a more chemically enriched external layer due to its exposure to the environment. However, the higher concentration of BAs in the choanosome could suggest a sufficiently protected sponge in the external layer or low biological pressure from other marine organisms. Indeed, the nudibranch *Tylodina perversa* is the only known predator of *A. aerophoba* and preys preferentially on the ectosome of the sponge [54]. Thus, the higher internal abundance of BAs might minimize the effect of this specialist predator in the inner core of the sponge, similar to what has been described for other opisthobranch-sponge feeding interactions [49,55]. Concentrations of chemical defenses—up to 10% of sponge dry mass in the ectosome—might be sufficient to protect the sponge against generalist fish predators, as tested by Thoms *et al.* [56]. In our study, we found that surface concentrations of BAs varied between 0.3 and 2.6%, which may not be high enough to deter predators, as opposed to the chemically enriched inner core that might also act to effectively deter generalist predators [56]. However, the deterrent activity of BAs at such low concentrations should be tested. Fluctuations in the abundance of *T. perversa* could explain part of the variation found in BA production, but most of the ecological and biological data of this gastropod is unknown (e.g., abundance, annual cycle).

Other roles (*i.e.*, anti-fouling, competition, antibacterial) could also explain variations in secondary chemistry [11,12]. The higher bioactivity during the warmer period may have been a response to increased fouling on the sponge surface [38] or to an increase in competitors [37]. However, it seems unlikely that BA production was constrained by variations in the abundance of competitors or foulers, based on the anti-predatory role of the HMW BAs studied [43,53]. Indeed, only the LMW BA aeroplysinin-1 and dienone show strong antibiotic activity and may protect this sponge from invasion of bacterial pathogens [41,56,57] but these two compounds were absent or below threshold detection in our samples. These LMW BAs could however be readily available after biotransformation of the HMW BA precursors quantified in our study [41–43]. The possibility cannot be entirely ruled out that these biological constraints (*i.e.*, foulers, competitors, pathogens) vary over the year, causing seasonal changes in the biosynthesis of bioactive compounds.

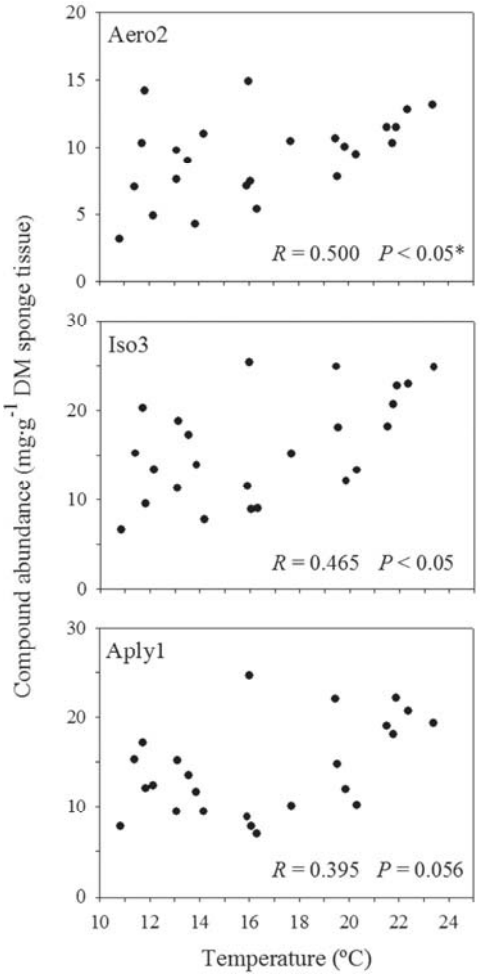
Seasonal changes in secondary chemistry may be also attributed to physiological factors related to differential investment in growth or reproduction. Leong and Pawlik [58] described a resource allocation trade-off between chemical defenses and growth in two *Aplysina* species, with maximum growth in summer months. The reproductive biology of verongid sponges is little known. *A. aerophoba* is thought to be oviparous and dioecious, common traits in the order Verongida [59–61]. This order displays a restricted gamete production period between May and July [59,62]. However, rope-form sponges (e.g., *Aplysina* species) appear to be adapted to undertake asexual reproduction by fragmentation; sexual reproduction is considered as a functional alternative with small investment in gamete production [60]. Data on growth and reproduction of *A. aerophoba* is scarce but it is likely to be similar to that of other verongid sponges. Taking into account data on other verongid species, maximum growth could coincide with maximum metabolite production in summer just after gamete production, suggesting that these sponges were not limited in energetic resources during the warmer season. However, there is no evidence to support this hypothesis and formal data on growth and reproduction would be required.

2.4. Sea Water Temperature and Concentration of BAs

We found no differences between the *in situ* and satellite-derived temperature data (paired *t*-test; $t = 0.884$, $df = 10$, $P = 0.397$) and the measurements were highly significantly correlated ($R = 0.990$, $P < 0.001$). Therefore, we used satellite-derived sea surface temperatures (SSTs) as a proxy for water temperature.

At the ectosomal level, the abundances of Aero2 and Iso3 were positively correlated with SST ($R = 0.500$, $P = 0.013$; and $R = 0.465$, $P = 0.022$, respectively; Figure 6). If any, Aply1 was marginally correlated with the satellite-derived SST ($R = 0.395$, $P = 0.056$). Contrastingly, the abundances of Aero2, Aply1, and Iso3 in the choanosome were not correlated with SST ($R = -0.079$, $P = 0.713$; $R = -0.009$, $P = 0.968$; and $R = 0.086$, $P = 0.689$, respectively).

Figure 6. Correlations between compound abundances and satellite-derived sea surface temperatures. Changes in compound abundances (mg·g^{-1} dry mass sponge tissue) measured in the ectosome of *Aplysina aerophoba* were related to changes in sea surface temperature (°C). Aero2 = aerophobin-2; Aply1 = aplysinamisin-1; Iso3 = isofistularin-3. Significant differences $P \leq 0.05$. * Significant correlation after Bonferroni correction.



Variations in chemical defenses may be also attributed to environmental factors, such as water temperature. We showed that satellite-derived SST data could be accurately used as a proxy for sea water temperature pattern, consistent with other reports [63,64]. Some BAs were significantly correlated with sea water temperature in the external part of *Aplysina aerophoba*. Water temperature could be directly affecting the secondary metabolite production as reported in *Haliclona* sp. [36] or other factors (*i.e.*, biological or physiological) correlated with sea water temperature might be causing such variation [31,33–35,37,38]. In our study there appeared to be other factors that regulate the production of BAs. We based this hypothesis on the low correlation strength (R value ≤ 0.5) and the partial mismatch between temperature and ectosome patterns over time (Figure 4). The relationship

between BAs and temperature could imply no causation. In our opinion, water temperature does not cause changes in secondary chemistry, since those changes should then occur both in the ectosome and choanosome of the sponge. Other environmental factors (e.g., light exposure) could differentially affect the surface layer but not the inner region of the sponge [33,35] and could be driving these trends. Biotic factors that also preferentially affect the ectosome of the sponge and are associated with temperature could be behind the increased compound concentration of the ectosome in summer [39]. It is known that photosynthetic symbionts can contribute to the production of secondary metabolites [65–67]. Since most photosymbionts are found in the outer layer of the sponge, these associated bacteria could be driving somehow the secondary chemistry of *A. aerophoba* [45,68]. Although the BAs seem to be stored in sponge cells [46], multiple cell components might be involved in their production [41,44,45,68]. The actual role of symbionts in the production of secondary metabolites, however, is largely unknown.

3. Experimental Section

From May 2008 to April 2010, five different healthy specimens of the sponge *Aplysina aerophoba* (Nardo 1833) were sampled monthly by scuba diving in Portbou (Northwestern Mediterranean) to depths between 7 and 10 m. Sampling was always conducted on fresh individuals with similar sizes, which were separated from each other between 2 and 100 m. Underwater, we cut off the upper part of one chimney per specimen and placed them in plastic bags with sea water. Immediately after collection, samples were placed in coolers with ice to prevent changes in the secondary chemistry. Once in the laboratory, a small portion of the top half of the chimney away from the cutting surface (*i.e.*, injured tissue) was selected for the quantification of the BAs, minimizing a potential effect of manipulation and the biotransformation of high molecular weight metabolites into low molecular weight compounds [41]. We used a sterilized scalpel to excise samples from the ectosome (1 mm-thickness) and choanosome (2 mm-thickness) of the sponge under sea water. Samples were then frozen at −20 °C within 3 hours of collection until processing.

The major brominated alkaloids (BAs) of *Aplysina aerophoba* were extracted, isolated and identified as described by Sacristán-Soriano *et al.* [45]. Briefly, we reduced sponge manipulation, avoided air exposure of fresh samples, froze sponges immediately after sample preparation (less than 4 hours after collection), freeze-dried material and keep them at −20 °C, and used methanol only for extraction. With this methodology, we have never detected the biotransformation process that converts the High Molecular Weight (HMW) BAs into Low Molecular Weight (LMW) BAs described for this species. LMW BAs were either absent in our chromatograms or at concentrations below our detection threshold. We purified the four major and known compounds aerophobin-1, aerophobin-2, aplysinamisin-1, and isofistularin-3 as described by Sacristán-Soriano *et al.* [45]. We characterized these compounds with proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR; JEOL EX 400 spectrometer), liquid chromatography-mass spectrometry (LC-MS; Thermo Scientific LCQ Fleet), UV spectrometry (Hewlett Packard diode array spectrophotometer), and comparison of spectroscopy data with published values from the literature [40,69]. Full details on the chemical methods can be found in Sacristán-Soriano *et al.* [45].

HPLC analyses were performed with a system from Waters that included the Alliance separations module 2695, the column heater, and the 2998 photodiode array detector. The equipment was controlled and the data were handled by the Empower Chromatography Data software (Waters). The HPLC conditions consisted of two eluents (A: 0.1% aqueous trifluoroacetic acid, B: acetonitrile) and an elution profile based on a linear gradient from 30% B to 80% B within 18 min and then to 100% B in an additional 10 min. The flow rate was constant at 0.4 $mL \cdot min^{-1}$. We used a Phenomenex Synergi Max-RP (80 Å, 250 × 3.0 mm, 4 µm) analytical column with a fixed temperature of 30 °C.

For quantification of the natural products, 30 mg of freeze-dried sponge tissue from ectosomal and choanosomal samples were prepared by extracting three times with 1.5 ml of methanol (MeOH) in an ultrasonic tank for 15 min [45]. The crude extract was filtered through a 20-µm polytetrafluoroethylene filter (PTFE) into a 5 mL beaker. The final volume was adjusted to 5 ml of crude extract. Aliquots of 1.5 ml were passed through a 13 mm, 0.2-µm PTFE syringe-filter before injecting 10 µL into the HPLC system. The brominated compounds 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 previously purified and characterized external standard compounds. The final amounts of the natural compounds were calculated by averaging three replicate injections. Concentrations of BAs were expressed in $mg \cdot g^{-1}$ of dry mass of sponge tissue.

We obtained sea surface temperatures (SSTs) at the study site throughout the 2-year study (May 2008 to April 2010) by accessing moderate-resolution imaging spectrometer measurements (Aqua MODIS satellite) made by the NASA Goddard Space Flight Center [70]. "Ocean Level-2" HDF data were read and processed with MathLab R2009a software. High-quality readings were obtained with flag values of 0 or 1 and we discarded unreliable data (flag values of 2 or 3). We used a flag value of 1 because insufficient readings were obtained with a flag of 0. Suitable SST readings (N = 293) corresponded to daily means in a 9-km^2 area centered at the following coordinates: 42°25'33.6"N–3°12'40.32"E (Portbou). We also measured sea water temperatures with *in situ* data loggers (Onset Computer Corporation, model HOBO Pendant UA-002-08) placed at a depth of 7 m for one year (June 2009 to April 2010) for comparisons with satellite-derived SST data. Monthly average temperatures were calculated for both satellite-derived and logger-derived data.

We used PRIMER 6 software [71] to analyze data on secondary metabolites of *Aplysina aerophoba* as a function of tissue type (ectosome or choanosome). Square root-transformed data were used to calculate Bray-Curtis similarities. We used permutational multivariate analysis of variance (PERMANOVA) to test for differences in secondary metabolites among different tissue layers. Results were plotted with non-metric multidimensional scaling (MDS).

We used several statistical methods from SYSTAT 12 software [72,73] to analyze each secondary metabolite separately. We used a nested design of ranked compound abundances with year (first and second), season (spring, summer, autumn, and winter), and month (12 months) as factors. Seasons were defined by natural periods (not by temperature) so they contained the same months in both years. Nested designs require random subordinate groups. We treated month and season as random factors because their actual conditions vary tremendously between years, *i.e.*, despite sharing the same name, months and seasons could be confined to their specific years. In nested analyses, lower variables are always tested before upper variables. To test for the significance of an upper level, the model considers the variance associated with the lower level, so there may be differences in an upper level regardless

the significance of the levels below. With this hierarchical approach, we can therefore partition the variance into components associated with each temporal scale (year, season within years, and months within seasons) to assess the temporal scale at which concentration of BAs vary the most. This approach can provide hints on the mechanisms behind the temporal pattern. We then used Tukey HSD pairwise comparisons to test for differences between the levels of each significant temporal scale. We used simple correlation analysis to establish the quantitative relationships between secondary metabolites. We also performed trend analyses of compound data to assess for tendencies and seasonal variations over time in the abundances of secondary metabolites.

Monthly logger and satellite-derived temperature data from the same period were compared with paired t-tests to identify differences between methodologies. Pearson's correlation was used to examine associations between ranked compound abundances and satellite-derived SSTs.

4. Conclusions

To date, the present study was one of the first to document long-term (two years) quantitative seasonal variations in bioactive secondary metabolites in sponges. We provided evidence for high compound accumulation during summer months in the verongid *Aplysina aerophoba*, as previously described for other sponges. This extended our understanding of the underlying patterns of production of biologically active metabolites. Beyond the biological and ecological consequences, being aware of such variation can contribute to a sustainable wild harvest or aquaculture supply for biotechnological research [16], minimizing the extraction of sponge biomass. For example, our data could cause a two-fold increase in the supply efficiency of aplysinamisin-1 or isofistularin-3, should they be targets of biotechnological research or commercial applications. So, just by harvesting sponges at times of peak metabolite concentration we could reduce by up to 50% our impact in natural populations and our expenses to extract the target natural product.

Acknowledgments

We thank J. Gil and A. Villamor for their assistance with sample collection and R. Bernardello for assistance in processing HDF temperature data. We also thank anonymous reviewers for suggestions that improved the final version of the manuscript. This research is a part of OSS PhD Thesis, who was supported by a CSIC predoctoral grant. Research partially funded by the Spanish Ministry of Science and Innovation (SOLID: CTM2010-17755 and BENTHOMICS: CTM2010-22218) and by the Agence Nationale de la Recherche (France; ECIMAR project, ANR-06-BDIV-001-04). This research is a contribution of the Consolidated Research Group "Grupo de Ecología Bentónica" (SGR2009-655) of the Catalan Government.

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Samples Availability: Available from the authors.