Tapping the archives: The sterol composition of marine sponge species, as determined non-invasively from museum preserved specimens, reveals biogeographical features

Lengger, SK

http://hdl.handle.net/10026.1/5322

Geobiology

All content in PEARL is protected by copyright law. Author manuscripts are made available in accordance with publisher policies. Please cite only the published version using the details provided on the item record or document. In the absence of an open licence (e.g. Creative Commons), permissions for further reuse of content should be sought from the publisher or author.
Tapping the archives: The sterol composition of marine sponge species, as determined non-invasively from museum preserved specimens, reveals biogeographical features

Sabine K. Lengger\textsuperscript{a,*,#}, Jane Fromont\textsuperscript{b} and Kliti Grice\textsuperscript{a}

\textsuperscript{a} WA Organic and Isotope Geochemistry Centre, The Institute for Geoscience Research, Department of Chemistry, Curtin University, GPO Box U1987, Perth, WA 6845, Australia

\textsuperscript{b} Western Australian Museum, Locked Bag 49, Welshpool DC, WA 6986, Australia

\# Present address: Organic Geochemistry Unit, School of Chemistry, University of Bristol, Cantock’s Close, Bristol BS8 1TS, U.K; and Biogeochemistry Research Centre, University of Plymouth, Drake Circus, Plymouth PL4 8AA, U.K

*corresponding author. e-mail: sabine.lengger@plymouth.ac.uk, Davy Building, Room 603b University of Plymouth, Drake Circus, Plymouth PL4 8AA, U.K
tel: +44(0)1752 585966, fax: +44 (0)1752 584710

Publication in: Geobiology (2016)
Abstract

Over 8,600 species are currently recorded in the phylum Porifera (sponges). They produce a large diversity of biochemical compounds including sterols, with more than 250 different sterols identified. Some of these sterols are of great interest, due to their use for fingerprinting in ecological and biomarker (molecular fossil) studies. As a large number of identified extant species from biodiversity surveys are housed in museum collections, preserved in ethanol, these present a potentially rich source of identified specimens for comparative lipid analyses. Here, we show that, in at least one species, sterol distributions obtained from the ethanol used to preserve specimens of sponges were representative, and comparable to the sterol distribution obtained from wet frozen, and from freeze dried tissue from the same species. We employed both GC-MS as well as two-dimensional gas chromatography – time of flight mass spectrometry (GC×GC-TOFMS), with an improved signal-to-noise ratio for even minor constituents. Analysis of two additional specimens of the same species, but of different provenance, resulted in detection of marked differences in sterol composition which could be attributed to variations in geography, environmental conditions, microbial communities, diet or cryptic speciation. The possibility of using ethanol from identified, preserved museum sponges could drastically increase the number of available samples. This could enable the study of their sterol complements, and the detailed investigation of differences due to geographical and oceanographic, phylogenetic and other factors in unprecedented detail.
Introduction

The number of species in the phylum Porifera (sponges) is rapidly rising with over 8,600 currently recognized species, and suggestions that there could be more than twice as many species globally (Van Soest et al., 2012). Sponges are widespread in many shallow and deep water reef systems, and, as filter feeders, they occupy a key role in the carbon cycle of marine ecosystems (Van Soest et al., 2012). The phylum is deeply branching in the Metazoa and their phylogeny is of great interest to evolutionary biologists (Wörheide et al., 2012).

Sponges are known to produce a vast number of highly diverse natural products (Genta-Jouve & Thomas, 2012), including over 200, often unusual triterpenoids and steroids (Bergmann, 1949; D’Auria et al., 1993).

Djerassi and Silva (1991) concluded that the composition of most sponges consists of fairly common sterols, while some contain unusual sterols. This was a result of their analyses of sponge sterols (the most common types of steroids, with a hydroxyl group on C-3, Fig. 1) by mass spectrometry and nuclear magnetic resonance (NMR) in different specimens (De Rosa et al., 1973; Bergquist et al., 1980; Kerr & Baker, 1991). Unusual sterols include the cyclopropyl-side chain containing sterols found in sponges of the order Haplosclerida (Proudfoot & Djerassi, 1987; Gauvin et al., 1998; Giner et al., 1999), the unusual 19-norsterols present in some members of the genus Axinella (Minale & Sodano, 1974; Crist & Djerassi, 1983), or the multiply alkylated side chains produced by members of the order Halichondrida (Stoilov et al., 1986a, 1986b). Of particular interest to geobiologists is 24-isopropylcholesterol, which was isolated first from Pseudaxinyssa sp. (Hofheinz & Oesterhelt, 1979), now accepted as Axinyssa sp., family Halichondriidae, order Suberitida.

Sponges are the only known extant organisms where this compound is present in large amounts (McCaffrey et al., 1994; Love & Summons, 2015). This finding resulted in the interpretation of high abundances of its geologically stable derivative, 24-isopropylcholestane.
compared to 24-n-propylcholestane in the rock record as a proxy for the abundance of
demosponges (Love et al., 2009; Kelly et al., 2011). However, the validity of this biomarker
is debated (Antcliffe, 2013; Love & Summons, 2015) and should be applied with caution as
small amounts of it are also produced by marine algae. Molecular clock studies of the
biosynthetic genes though have recently shown that pelagophyte algae evolved the gene for
the synthesis of this particular sterol later than the Cryogenian, when the first massive
occurrence of this molecular fossil is observed (Gold et al., 2016). It provides a tantalizing
possibility for determining the rise of animal life.

As only a select number of sponge species and specimens has been analysed so far, the
relationship of sterol composition with phylogeny is not entirely clear: Bergquist et al. (1991)
reported a correlation, but others such as Fromont et al. (1994), concluded that sterol
composition was not necessarily related to phylogeny. Future opportunities lie in combining
DNA based phylogeny and elucidation of biosynthetic pathways, but in order to provide
comprehensive results, a representative number of species and specimens needs to be
analysed (Erpenbeck & van Soest, 2007). This is particularly important when considering that
sponges do not only employ \textit{de novo} biosynthesis of sterols, but are also capable of
modifying dietary sterols (Bergquist, 1978; Silva et al., 1991; Silva & Djerassi, 1992).

Consequently, the determining factors on the sterol composition of sponges are of high
interest to geochemists and geobiologists.

Analysis of sterols usually entails the extraction of collected or cultivated sponge tissue,
followed by purification through a gravity column or high performance liquid column
chromatography (HPLC) procedures (Popov et al., 1976). Analysis by gas chromatography-
mass spectrometry (GC-MS) is then usually carried out on the derivatized sterols, carrying
either a trimethylsilyl group or an acetyl group (Goad & Akihisa, 1997). These
derivatizations have been reported to affect the distribution of measured sterols (Mitrevski et
al., 2008). However, sponge tissue can be difficult to obtain due to the necessity of sampling permits, as many locations are marine protected areas, and due to logistical reasons for sampling in deep waters (trawls, remote operated vehicles have to be employed). Identification of these sponge samples requires a taxonomist, is very time consuming and presents one of the main bottlenecks in sponge research. Therefore, analyzing large numbers of identified sponge samples for sterols would be useful for investigating and comparing sterol distributions with respect to phylogenetic relationships, identifying unusual sterols of potential biomedical interest, and of biomarker potential as a chemotaxonomy tool e.g. in the field of paleontology (Erpenbeck & van Soest, 2007).

Hence, here we investigate the potential of using ethanol that has been used to preserve sponge specimens in museum collections (a standard procedure), for sterol analyses. Sponge tissue was stored in ethanol in glass jars for several years, causing polar extractable organic compounds to be leached into the solution. As the samples are usually stored in the dark and at a controlled temperature, chemical alteration is reduced to a minimum. Therefore, these collections present a valuable resource for the analysis of natural products, allowing non-invasive sampling of identified specimens. We employed conventional GC-MS and two dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOFMS; Liu and Phillips (1991)), the latter in order to circumvent interferences due to co-elutions of other polar compounds in the first dimension. Previous application of GC×GC coupled to flame ionization detection allowed unprecedented resolving power for sterols in environmental samples (Truong et al., 2003), and quantification of steroids in a urine sample (Mitrevski et al., 2008).

In order to determine the suitability of ethanol, used to preserve sponge specimens, for the analysis of sterols, we analyzed ethanol from a preserved specimen of Agelas sp. collected off the Western Australian coast and subsequently stored in the dark and at 18 °C and compared
it to an extract of samples of the same specimen, one of which was freeze dried and stored at
18 °C and one frozen at –20 °C. We also analyzed two other species preserved by the above
three methods, however, the specimens had been collected from various locations at different
times. Here, we resolve these sterols by GC×GC-TOFMS; demonstrate that the ethanol
collections of museum specimens can be a valuable resource for lipid and potentially other
natural products research, or for large scale studies in marine chemical ecology, and discuss
the differences between sponges of the same species but collected at different locations.

Methodology

Sampling

A specimen of the sponge *Agelas* sp. MF1 (family Agelasidae, order Agelasida) was
collected off the south-western Australian coastline during cruises and surveys as specified
(Table 1, Fig. S1). A part of the sponge was wet frozen at - 20°C, one part was preserved in
75% ethanol on board, and one part was wet frozen on board and lyophilized at the Western
Australian Museum. One specimen of *Petrosia* sp. 1 (family Petrosiidae, order Haplosclerida)
and one specimen of *Ecionemia* sp. SS1 (family Ancorinidae, order Tetractinellida) were
collected at Ningaloo (Table 1, Fig. S1) and stored in ethanol, and two specimens of each
were collected at Kalbarri (Table 1, Fig. S1) and stored wet frozen at -20°C and freeze dried,
respectively.

The ethanol preserved (6 to 9 years, analysis in 2014, see date of collection in Table 1) and
lyophilized tissue was stored in the dark at 18°C, while the frozen tissue was stored in the
dark and at -20°C. The frozen, freeze dried and ethanol preserved tissue was extracted as
outlined in section 2.2. 10 – 20 mL of the ethanol was sampled, dried under a stream of N₂,
dissolved in dichloromethane (DCM) / methanol (MeOH) 1:1 (v/v) and dried over MgSO₄,
dissolved to a concentration of 10 mg/mL and purified as detailed below.
**Tissue extraction**

Analysis steps are summarized in Fig. 2. Ethanol preserved tissue was dried under atmospheric pressure at 22°C, wet frozen tissue was lyophilized, and lyophilized tissue obtained from the museum was used without modification. The extraction protocol for the aliquots of the *Agelas* MF1 specimen are also represented in Fig. 1. Dry tissue (0.5 – 1 g) was ground with a pestle and mortar and sonicated in 10 mL DCM/MeOH 1:1 (v/v) (10 min).

After centrifugation at 3,000 rpm (5 min), the supernatant was collected. This procedure was repeated twice; the combined supernatant was dried under $\text{N}_2$ and over anhydrous MgSO$_4$, and constituted the total lipid extract (TLE).

For analysis of free sterols, 2.5 mg of TLE was subjected to gravity column chromatography, and the polar fraction was eluted from 0.8 g activated 60 mesh SiO$_2$ with 4 mL DCM/MeOH 1:1 after the apolar and aromatic compounds had been eluted with 4 mL hexane and 4 mL Hex/DCM 3:7 (v/v). The polar fraction was dried under a stream of $\text{N}_2$ and dissolved in $n$-hexane prior to analysis by GC-MS and GC×GC-TOFMS. For some of the extracts, free hydroxyl groups were converted to trimethylsilyl (TMS)-ethers by reaction with 50 µL pyridine and 50 µL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 70°C for one hour, and evaporated to dryness under a stream of $\text{N}_2$ before dissolving in hexane.

In addition, for the *Agelas* samples, the total extracted, i.e. free and bound, sterols were determined. For this, 2.5 mg of the TLE was saponified: it was dissolved in 2 mL 1N KOH in MeOH and refluxed for 2 h at 80°C. 2 mL of water and 2 mL of cyclohexane were added, shaken, and the cyclohexane containing the sterols was collected. This was repeated twice and the combined cyclohexane fractions were dried, dissolved in diethylether/ethylacetate 1:1 (v/v), eluted over SiO$_2$ and dissolved in hexane for analysis.
In order to determine the sterols bound in the biomass residue of the ethanol preserved *Agelas*, the residue of the extracted biomass was dried and refluxed in 25 mL 1N KOH in MeOH (1 h). The pH was adjusted to 6 with 2N HCl in MeOH, water was added in equal amounts to the MeOH, and the aqueous phase was extracted three times with 10 mL DCM. The combined DCM phases were dried under N$_2$ and over MgSO$_4$, dissolved in diethylether/ethylacetate 1:1 (v/v) and purified by elution from a silica (SiO$_2$) column.

*GC-MS and GC×GC-TOFMS analyses of sterols*

For GC-MS, an Agilent 5973 mass-selective detector coupled to a 6890 gas chromatograph was employed, using a 30 m x 0.25 mm ID x 0.25 µm film capillary column of type DB5-MS, with a temperature programmed from 40 to 325°C at 10°C . min$^{-1}$ and held at the final temperature for 20 min. Samples were injected in ethylacetate on a split/splitless injector in pulsed splitless mode at 320°C. The carrier gas was He at a constant flow of 1.1 mL/min.

Ionization was carried out at 70 eV, with an electron multiplier voltage of 1800 V and the source kept at 230°C. Masses scanned ranged from 50 to 750 Da. Data analysis of GC-MS data was carried out using Wsearch32 (www.wsearch.com.au).

For GC×GC-TOFMS, splitless injection at 310°C inlet temperature was employed, on an 7890 Agilent GC modified for GC×GC, coupled to a Pegasus 4D TOF-MS with linear modulation (LECO Corporation, St. Joseph, MI) employing electron ionization (EI). Primary column was a 30 m Restek CP5-Sil of 0.25 mm inner diameter and 0.25 µm film thickness and secondary column a 1.5 m 17Sil-MS (equivalent to 50 % phenyl) of dimensions 0.18 mm / 0.18 µm with helium as a carrier gas at a flow rate of 1.05 mL. min$^{-1}$. Modulation was carried out directly on the secondary column and modulation time was 5s (0.8 s hot jet, 1.70 s cold jet). The temperature was ramped from 40°C to 300°C at a rate of 3°C . min$^{-1}$, with the modulator at a 15°C and the secondary column at a 40°C offset. The Pegasus 4D was
operated at 100 Hz and at a mass range of 50-650 Daltons, with the transfer line at 290°C and
the ion source at 230°C. This configuration and program was optimized on the secondary
column separation, as using the small differences in polarity of the sterols analyzed allowed
for separation of several co-eluting compounds under these conditions. Data analysis was
conducted using ChromaTOF automatic peak detection with a signal to noise ratio and peak
width of 20 and 0.1 s, respectively, for small peaks and 300/0.4 s for larger peaks, areas of
TIC were used in order to calculate the area percentages of individual sterols of the total
sterol area.

Results and Discussion

Sterol abundances

The structures of the sterols detected are shown in Fig. 1 and their identification is described
in the supplementary material.

The ethanol from Agelas sp. MF1 contained sterols with 27, 28 and 29 carbon atoms (Fig. 3,
Fig. 4, Table 2). The sterols from the cholestane series consisted of 2, 3, 4 and 7, the sterols
from the ergostane series of 6, 10, 13, 14 and 17, and the ones from the stigmasterane series
9, 15, 16, 17, 23 and 26. The two sterols present in largest abundance were 4 and 20, other
major sterols included 2, 6, 10, 13, 23 and 26.

The ethanol from Ecionemia sp. SS1 contained a large variety of sterols, including 1 - 8, 11,
12, 15, 18, 19, and 22-25 (Fig. 3, Fig. 5, Table 3). However, the sterols of both the frozen and
the freeze dried samples consisted mainly of 22, with some other minor constituents (Fig. 5).
The sterols obtained from the ethanol of the Petrosia specimen consisted of 2, 4, 6, 11, 15,
19, 21 and 22, in a distribution largely similar to the preserved specimen. The wet frozen and
lyophilized Petrosia sp. 1 contained a slightly larger variety of sterols, with also 1, 3, 5 and 8
present in considerable proportions (Table 3). It is possible that some of these were not
detected in the ethanol due to a very large peak of 4 and 17. The ethanol of Petrosia sp. 1 also
contained a number of 3-oxosterols, which were not observed in the wet frozen and
lyophilized specimens. It is possible that these were degradation products, however, they
were not observed in any of the other ethanol preserved samples and it is thus more likely
that they were present in the sponge. No sterols of less than 27 and more than 29 carbon
atoms were detected, but it is possible that these were present in minor amounts.

Comparison of extraction methods using one specimen of Agelas preserved in three different
ways

The Agelas MF1 specimen was split into aliquots when collected in 2007, and analysis of the
wet frozen and the lyophilized samples thus allowed for a direct comparison of the sterol
composition to the ethanol preserved aliquot. The same sterols were detected, with the
exception of 3, which was only present in ethanol (Fig. 3 A). The distribution was slightly
different, with 20 being present in larger proportions in the wet frozen, and even larger
proportions in the lyophilized sample. Other differences in proportional amounts were minor.
This confirms that a representative amount of sterols is leached into the preservation fluid;
and that alteration during storage is minimal.

In order to analyze the completeness of extraction achieved by storage in ethanol, we also
extracted some of the sponge tissue that had been preserved in ethanol, and had leached the
sterols. This resulted in similar sterol compositions to those observed in the ethanol (Fig. 4B).
However, we obtained a slightly larger amount of sterol 20 (22.1 / 25.7 %), which, in
conjunction with the larger amounts present in the wet frozen and lyophilized samples,
suggests that ethanol might not completely extract 20. No Δ₅,7 sterols were detected in any of
the samples. Whilst these sterols are known to be chemically rather labile, they were not
detected in the frozen and the lyophilized samples either, therefore this is probably not an artefact of the preservation method.

When the residue of the ethanol preserved sponge after tissue extraction was subjected to saponification in order to release the more strongly bound sterols, a similar distribution to the ethanol extracted sterols was observed (Fig. 4B). However, interestingly, it was also observed that a large number of (unidentified) triterpenoids were released, as exemplified by the extracted ion current (EIC) for $m/z$ 191, a common ion observed in many triterpenoids (Fig. S4). These compounds were thus present as more strongly bound, non-extractable lipids, or potentially were associated with symbionts. Many sponges are able to source carbon and energy from a number of symbionts they harbor within their tissue (Webster & Blackall, 2009; Thacker & Freeman, 2012), many of which are known to produce bacteriohopanoids (Ourisson & Albrecht, 1992).

In addition to the analysis of the free sterols, we also determined whether significant amounts of sterol esters had been extracted by ethanol leaching or DCM/MeOH extraction, and saponified the extracts of *Agelas* sp. MF1 in order to obtain the sum of free and bound sterols (= total). Negligible changes in their distributions were observed (Table 2, Fig. 4 B), suggesting that (i) the sterol esters are present in similar proportions to the free sterols, that (ii) there are no sterol esters, or that (iii) sterol esters are not leached into the ethanol during preservation. Distributions of extracts gained by wet or lyophilized tissue extraction in DCM/MeOH similarly showed only negligible changes in distribution upon saponification (Table 2), thus suggesting reason (i) or (ii) was the cause.

Our results show that the ethanol taken from preserved museum specimens contains sterols that can be representative in type and distribution for an individual sponge. This technique could be more widely applicable and make a pool of samples accessible for larger screening.
studies for identification of new compounds for biomedical research, for geochemical research relying on biomarkers (‘unique’ compounds), or for ecological and phylogenetic studies investigating sterol distributions and their determining factors.

Enhancement of sterol analysis by GC×GC

GC×GC was first used by, and consists of the employment of two capillary columns of orthogonal selectivity, e.g. an apolar column effecting separation by volatility, followed by a polar column where retention increases with increasing polarity. The eluting compounds from the primary column are frozen for a certain period of time (the “modulation period”), usually from 2-10 seconds, and then released onto the secondary column which is shorter in length by a steep increase in temperature. This technique has, especially in the past decade, been extensively developed and applied to many fields as reviewed by e.g. Adachour et al. (2008). The advantages include an improved signal-to-noise ratio, increased separation efficiency and structured chromatograms, in which structurally similar compounds elute in roof-tile like sections, which can substantially improve compound identification, without the need for separation procedures (Eiserbeck et al., 2012; Naehler et al., 2016), and separation of structural and stereoisomers (Eiserbeck et al., 2011). Sterols are amenable to GC, show specific and varied polarities, and many potential isomers occur, which can be difficult to fully separate by one-dimensional GC without extensive pre-fractionation steps. This makes them very suitable for GC×GC-TOFMS, which allows separation not only by boiling point, but also by polarity, and hence results in a structured, two-dimensional chromatogram with grouped compound classes. Handling of the samples for identification and voucher sample preservation in ethanol instead of for lipid analysis could introduce a number of contaminants, which can unnecessarily complicate GC-chromatograms, but can easily be separated by GC×GC. It also allows for simple separation of the 3-oxo compounds from the 3-hydroxy compounds (Fig. 3 B, C), which is not possible employing one-dimensional
analysis (Fig. S2, S3) as the former exhibit a higher retention time in the second dimension (polar column; Rt). This results in additional confidence in structural identifications. Moreover, a number of different isomers were detected, such as compounds 6 / 7, which were co-eluting in one dimensional analysis (Fig. S2, S3). If some of these compounds are present in trace amounts, the signals could be difficult to deconvolute. GC×GC chromatograms also allow for sophisticated untargeted comparison of samples, thus potentially allowing untargeted cross sample comparison (Reichenbach et al., 2011; Marney et al., 2013).

Further, whilst here, analysis was conducted following simple gravity column chromatography separation, GC×GC also allows the analysis of an untreated extract, thereby removing any possibilities of bias and loss of compounds present in low concentrations during the workup. With appropriate derivatization, it could also be possible to determine a range of other compounds of interest in these extracts, and of potential interest, such as alkaloids or terpenoids (cf. Erpenbeck and van Soest, 2007; Genta-Jouve and Thomas, 2012).

Differences in specimens from different locations

While Agelas sp. showed distributions which were unaffected by the preservation method, the sterol compositions obtained from Ecionemia sp. and Petrosia sp. specimens largely differed between the ethanol and the lyophilized and preserved specimens. In Ecionemia sp., the diversity of sterols was higher in the ethanol preserved sponge, while in Petrosia sp. the diversity was higher in the wet frozen and lyophilized sponges. This is in contrast to the results obtained from the Agelas sp. specimen. It is thus less likely that preservation methods were causing these differences, however it is possible that differences in the sponges such as proportions of spicules, and thus silica, in Ecionemia and Petrosia sp. compared to Agelas sp., (with comparatively fewer siliceous spicules) could have resulted in more pronounced changes in sterol composition in the two former species. In addition, sponges of the genus Petrosia are known to form reactive polyacetylenes (Cimino et al., 1989) which could be
responsible for the conversion of the sterols to ketones, but are unlikely to have caused all of these differences.

A more likely reason for these differences is that the results are not directly comparable as they were not derived from the same specimen, but rather from three different specimens (Table 1), of which the one preserved in ethanol was obtained from a completely different location (Fig. S1). This is in contrast to previous studies, where sterol composition was found to be species specific and independent of location (Bergquist et al., 1980; Fromont et al., 1994). As sponges employ both de novo biosynthesis along with uptake and modification of dietary and symbiont produced sterols (Bergquist, 1978) these sterol differences between specimens of one species are not surprising. Habitat, depth, or times of collection are unlikely to have caused these differences: for example, all specimens of Ecionemia sp. were collected around 100 m depth in the same year. In the case of Petrosia, both the freeze dried and the ethanol preserved specimens were collected at a similar depth (around 100 m depth), while the wet frozen specimen was from 253 m depth, yet it was the ethanol preserved specimen that contained different sterols from the other two. The collection time was austral summer for all specimens (Table 1). Subtle differences were seen in the sponge color and spicule dimensions of the ethanol preserved specimen of Petrosia, which was darker brown and had thinner spicules that the wet frozen and freeze dried samples (260 x 12 µm compared to 270 x 20 µm for the largest size category of oxeas). It is possible that Petrosia sp. 1 is a species complex (i.e. a group of two or more closely related cryptic species), but this could only be determined with more detailed morphological analyses and molecular data.

However, in both Petrosia sp. and Ecionemia sp., the wet frozen and lyophilized samples, which had differing sterol complements, had been collected at the same location, while the ethanol preserved sponge had been collected in a different area (Fig. S1). It is thus most likely that the sterol distributions are related to geographical or ecosystem differences, and
that the sterol composition varies moderately between species across their biogeographic distributions. Ethanol preserved specimens of *Petrosia* and *Ecionemia* were collected in the tropics at Ningaloo Reef (Carnarvon Shelf, NW Australia, 22°S) and the wet frozen and lyophilized specimens of these species were collected from Kalbarri/Zuytdorp (Dirk Hartog Shelf, Central Western Australia). The latter region is subtropical (27°S) and to ≤ 250 m depth exposed to the Leeuwin current, potentially a rich source of particulates for filter feeders such as sponges (Fromont *et al.*, 2012), that could influence the dietary sterol uptake via organic matter supply (Silva *et al.*, 1991; Silva & Djerassi, 1992). It is thus possible that sterol and sterane biomarker distributions derived from sponges can be indicative of environmental factors such as their diet. Also other factors varying between localities and individuals (nutrient regimes, a difference in symbionts, or microbial defense) could play a major role in activating *de novo* biosynthesis or modification after uptake. This could explain observations made by Kerr *et al.* (1991), who saw a strong variation in the sterol composition of *Xestospongia muta* specimens collected in close proximity, although this could also reflect cryptic speciation.

Our results suggest that inferences about *de novo* sterol biosynthesis from the sterol composition of a sponge sample can be difficult. Moreover, there are strong indications that the sterol composition of specimens of the same species of sponges could relate to their biogeographical and oceanographic environment. Regardless of whether *de novo* synthesis or dietary modification lead to the presence of a certain sterol in a sponge specimen, it appears that their sterol composition is shaped by additional factors which might also need to be taken into account when interpreting the sterane biomarker record, and could provide more information about depositional environments.

*Comparison of sterol compositions with the literature*
Sponges of the genus *Agelas* have been investigated for sterol composition on many occasions: Santalova *et al.* (2004) analyzed *A. mauritiana*, and reported 20 sterols, including 1 (trace amounts = tr), 2 (4.48 %), 3 (2.51 %), 4 (28.72 %), 5 (tr), 6 (tr), 7 (9.45 %), 8 (tr), 11 (tr), 13 (9.03 %), 15 (tr), 16 (1.46 %), 17 (6.01 %), 19 (2.23 %), 20 (2.23 %), 23 (tr) and 26 (20.57 %), in addition to a number of other sterols, including 5α-25-desmethyl-ergost-22-en-3β-ol (tr), 5α-cholesta-7,22-dien-3β-ol (tr), 5α-ergosta-7,22-dien-3β-ol (5.22 %), 5α-23-methyl-ergost-22-en-3β-ol (2.04 %). This profile resembles the one for the *Agelas* species analyzed here, but differed slightly in relative amounts (Fig. 4). Also, sponges of the order Petrosiidae have been extensively investigated for sterol composition, and were found to contain a number of unusual, often cyclopropyl-containing sterols (Wahid Khalil *et al.*, 1980; Gauvin *et al.*, 1998; Giner *et al.*, 1999; Reddy *et al.*, 1999), which were not detected in this study. This was in agreement with Fromont *et al.* (1994) and Bergquist *et al.* (1980), who examined various species of the genus *Petrosia*, but could not detect any of these unusual sterols. Instead, *P. pigmentosa* and *P. australis* contained 1 (0.7 / 2.4 %), 3 (4.2 / 10 %), 4 (12 / 0.4 %), 5 (7.9 / 8.1 %), 6 (0.5 / 0 %), 7 (4.5 / 0 %), 8 (0 / 47 %), 11 (2.2 / 0.7 %), 12 (0 / 1.8 %), 13 (0.4 / 0 %), 15 (1.3 / 2.6 %), 19 (31 / 2.3 %), 22 (0.3 / 13 %), 23 (1.6 / 0 %) and 26 (13 / 0 %). Other sterols detected in these specimens were (E)-stigmasta-5,24(241)-dien-3β-ol (0.1 / 7.6 %), 26-desmethyl-cholesta-5,22-dien-3β-ol (0.7 / 0.5 %), 26-desmethyl-cholesta-22-en-3β-ol (1.4 / 0 %), and a number of Δ5,7 sterols, which were not detected in our study. These are known to be particularly labile and it is thus possible that they had been present in the live sponge, but could not be detected in our samples. Similarity between the sterol compositions of the two *Petrosia* species reported by Fromont *et al.* (1994) was not high, and the samples investigated here also show little similarity to these species (Fig. 4). No sterol composition for the genus *Ecionemia* has been reported.
A literature comparison of the sterols from the same genera as the species analyzed here demonstrates the similarity of *Agelas* sp. MF1 to *A. mauritiana*, and confirms the comparability of our method with results gained by more traditional methods. It is possible that sponges of the genus *Agelas* are so similar to each other because they rely more strongly on *de novo* biosynthesis, while sponges of the genus *Petrosia* (and *Ecionemia*) rely on modified dietary sterols, which causes greater variation in sterols at the genus and to a lesser extent, species level. This is in agreement with Silva *et al.* (1992), who attributed the unusual sterols of *P. ficiformis*, which were not detected in the specimens investigated here, to dietary modification in line with biosynthetic observations. However, contrastingly, Gold *et al.* (2016) suggested that this species does possess all the genes necessary for their production.

The lack of unequivocal resolution of the phylogeny of the Haplosclerida further complicates comparison of *Petrosia* sp. 1 sterols with other species. Recent advances in sponge phylogeny have suggested that *Petrosia* is indeed a paraphyletic group (Redmond *et al.*, 2011), which might also cause the strong differences in sterol composition when comparing our results to the literature, and explain the absence of the unusual sterols of *P. ficiformis* in *Petrosia* sp. 1 and other *Petrosia* species (Fromont *et al.*, 1994). In accordance with ongoing advances in sponge phylogeny, more detailed analysis of sterol complements, combined with molecular analysis, with replicates of the same species from the same and different locations or oceanographic and ecological conditions could provide valuable information for the interpretation of sterol distributions, the sterane geological record and the evolution of Porifera and the Metazoa.

**Conclusions**

The sterol composition obtained from the ethanol of museum voucher specimens presents a new method for non-invasive sampling of archived, identified sponge specimens. This can
facilitate comparative studies in geochemistry, phylogeny, marine biogeography, and geobiology. While we cannot completely exclude the possibility that preservation method impacts sterol recovery, comparative analysis of different specimens of the same species of *Petrosia* sp. 1 and *Ecionemia* sp. SS1 most likely showed strong intraspecies variability, potentially due to differences in geographical location, nutrient regimes, microbial communities, the acquisition of sterols via their diet, or cryptic speciation. The relationship of biogeographical and oceanographic environment with sterol composition warrants further investigation in terms of the transfer of these features to the geological record. The sampling methodology presented here opens up the potential for non-destructive, non-invasive sampling of preserved museum specimens for analysis of sterols and potentially other compounds - currently an underutilized but vast resource for large scale biochemical studies.

**Acknowledgements:** The authors would like to thank Geoff Chidlow, Oliver Gomez and Mark Salotti for technical assistance; and the Australian Research Council (ARC) for providing a Discovery Outstanding Research Award (DORA) grant to Prof. K. Grice, which provided funding for the current project. The ARC is also thanked for funding allowing the acquisition of a GC×GC-TOFMS (ARC LIEFP Grant). All specimens were collected with the appropriate State and Commonwealth permits. Funding for expeditions was from the Western Australian Marine Science Institute (WAMSI, AIMS-WAM 111), CSIRO Wealth from Oceans Flagship (CSIRO SS1005) and Marine Futures (WA-MFBPS).
References


Marney LC, Christopher Siegler W, Parsons BA, Hoggard JC, Wright BW, Synovec RE  
(2013) Tile-based Fisher-ratio software for improved feature selection analysis of  
comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry data.  
McCaffrey MA, Michael Moldowan J, Lipton PA, Summons RE, Peters KE, Jeganathan A,  
Watt DS (1994) Paleoenvironmental implications of novel C$_{30}$ steranes in Precambrian to  
from the sponge *Axinella verrucosa*. *Journal of the Chemical Society, Perkin Transactions 1*  
2380.  
Mitrevski BS, Brenna JT, Zhang Y, Marriott PJ (2008) Application of comprehensive two-  
dimensional gas chromatography to sterols analysis. *Journal of Chromatography A* **1214**,  
134–142.  
Naeher S, Lengger SK, Grice K (2016) A new method for the rapid analysis of 1H-Pyrrole-  
2,5-diones (maleimides) in environmental samples by two-dimensional gas chromatography  
time-of-flight mass spectrometry (GC×GC-ToF-MS). *Journal of Chromatography A* 125–  
135.  
Popov S, Carlson RMK, Wegmann A, Djerassi C (1976) Minor and trace sterols in marine  
Proudfoot JR, Djerassi C (1987) Synthesis and stereochemistry of 23,24-dihydrocalysterol:  
implications for marine sterols of a unified biosynthetic scheme involving protonated  


Figure 1. Sterol structures. A - Identified sterols in the three species analyzed. Sterols were named according to IUPAC nomenclature and listed in Table 2. B – Numbering of the sterol skeleton.

Figure 2. Flow chart of the extractions of the different Agelas sp. MF1 aliquots. Steps for analysis of free sterols are shown in white boxes, and for analysis of free and bound or bound sterols (including saponification), in grey boxes.

Figure 3. GC×GC chromatograms of the ethanol extracts of the three sponges. A – Agelas sp. MF1, B – Ecionemia sp. SS1, C – Petrosia sp. 1; dotted lines indicate 3-oxosterols. Inserts show the 1D-GC-MS chromatograms.

Figure 4. Comparison of sterol distribution in extracts obtained from Agelas sp. MF1. Free sterols refers to sterols obtained from ethanol of the preserved specimen, from the wet frozen and the lyophilized sample. Total sterols includes sterols detected in the extracts after saponification, and bound sterols include those obtained from the preserved tissue after saponification of the extracted residue.

Figure 5. Sterol composition of the three sponges analyzed, in comparison to published species from the same genera. Agelas sp. MF1 is compared to A. mauritiana, for Petrosia sp. 1, the composition determined from the three differently preserved specimens is shown, and compared to P. australis and P. pigmentosa. For Ecionemia sp. SS1, sterol composition is as determined from the three differently preserved specimens. ¹ Composition as determined by Santalova et al. (2004), ² composition as determined by Fromont et al. (1994).
Table 1. Sample details. All sponges were collected during the following cruises and surveys: WA Marine Futures Biodiversity Project Survey Oct 2007 (WA-MFBPS), AIMS-WAM RV "Solander" Ningaloo Survey III Jan/Feb 2008 (AIMS-WAM III) and CSIRO RV "Southern Surveyor" Cruise SS1005 Nov/Dec 2005 (CSIRO SS1005).

<table>
<thead>
<tr>
<th>Species</th>
<th>Museum registr. numbers</th>
<th>Preservation method</th>
<th>Location</th>
<th>Station</th>
<th>Depth [m]</th>
<th>Date collected</th>
<th>Cruise/Survey</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agelas sp. MF1</strong></td>
<td>Z49312</td>
<td>Ethanol</td>
<td>Broke Inlet</td>
<td>Trawl 1</td>
<td>65</td>
<td>2007/10/15</td>
<td>WA-MFBPS</td>
</tr>
<tr>
<td><em>Agelasidae, Agelasida, Demospongiae</em></td>
<td>Z49312</td>
<td>Wet frozen</td>
<td>Broke Inlet</td>
<td>Trawl 1</td>
<td>65</td>
<td>2007/10/15</td>
<td>WA-MFBPS</td>
</tr>
<tr>
<td></td>
<td>Z49312</td>
<td>Freeze dried</td>
<td>Broke Inlet</td>
<td>Trawl 1</td>
<td>65</td>
<td>2007/10/15</td>
<td>WA-MFBPS</td>
</tr>
<tr>
<td><strong>Petrosia sp. 1</strong></td>
<td>Z45259</td>
<td>Ethanol</td>
<td>Ningaloo Reef</td>
<td>RVS4545/2008</td>
<td>100</td>
<td>2008/02/05</td>
<td>AIMS-WAM III</td>
</tr>
<tr>
<td><em>Petrosiidae Haplosclerida, Demospongiae</em></td>
<td>Z35817</td>
<td>Wet frozen</td>
<td>Kalbarri</td>
<td>SS1005/099</td>
<td>253.5</td>
<td>2005/12/04</td>
<td>CSIRO SS1005</td>
</tr>
<tr>
<td></td>
<td>Z35811</td>
<td>Freeze dried</td>
<td>Kalbarri</td>
<td>SS1005/102</td>
<td>97</td>
<td>2005/12/05</td>
<td>CSIRO SS1005</td>
</tr>
<tr>
<td><strong>Ecionemia sp. SS1</strong></td>
<td>Z35069</td>
<td>Ethanol</td>
<td>Ningaloo South</td>
<td>SS1005/144</td>
<td>103.5</td>
<td>2005/12/10</td>
<td>CSIRO SS1005</td>
</tr>
<tr>
<td><em>Ancorinidae, Tetractinellida Demospongiae</em></td>
<td>Z35949</td>
<td>Wet frozen</td>
<td>Zuytdorp</td>
<td>SS1005/110</td>
<td>106</td>
<td>2005/12/06</td>
<td>CSIRO SS1005</td>
</tr>
<tr>
<td></td>
<td>Z35808</td>
<td>Freeze dried</td>
<td>Zuytdorp</td>
<td>SS1005/104</td>
<td>97</td>
<td>2005/12/05</td>
<td>CSIRO SS1005</td>
</tr>
</tbody>
</table>
Table 2. Percentages of identified sterols in Agelas samples. Pres. BM –sponge biomass preserved in ethanol. n.d. = not detected.

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Sterols</th>
<th>Free sterols</th>
<th>Total sterols</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ethanol</td>
<td>Frozen</td>
</tr>
<tr>
<td>1</td>
<td>Cholesta-5,22-dien-3β-ol</td>
<td>7.3</td>
<td>5.8</td>
</tr>
<tr>
<td>2</td>
<td>5α-Cholest-22-en-3β-ol</td>
<td>3.7</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>Cholest-5-en-3β-ol</td>
<td>18.7</td>
<td>16.9</td>
</tr>
<tr>
<td>5</td>
<td>Ergosta-5,22-dien-3β-ol</td>
<td>8.4</td>
<td>7.8</td>
</tr>
<tr>
<td>6</td>
<td>5α-Ergost-22-en-3β-ol</td>
<td>2.0</td>
<td>2.1</td>
</tr>
<tr>
<td>8</td>
<td>5α-Ergosta-5,24(24(^1))-dien-3β-ol</td>
<td>3.6</td>
<td>4.8</td>
</tr>
<tr>
<td>12</td>
<td>5α-Ergost-24(24(^1))-en-3β-ol</td>
<td>6.5</td>
<td>8.0</td>
</tr>
<tr>
<td>13</td>
<td>5α-Ergostan-3β-ol</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>14</td>
<td>Ergostatrien-3β-ol</td>
<td>4.0</td>
<td>3.6</td>
</tr>
<tr>
<td>15</td>
<td>Stigmasta-5,22-dien-3β-ol</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>16</td>
<td>5α-Stigmast-22-en-3β-ol</td>
<td>3.1</td>
<td>3.5</td>
</tr>
<tr>
<td>20</td>
<td>5α-Stigmasta-7,22-dien-3β-ol</td>
<td>21.9</td>
<td>22.0</td>
</tr>
<tr>
<td>23</td>
<td>5α-Stigmastan-3β-ol</td>
<td>6.1</td>
<td>6.7</td>
</tr>
<tr>
<td>26</td>
<td>5α-Stigmast-7-en-3β-ol</td>
<td>9.0</td>
<td>9.0</td>
</tr>
</tbody>
</table>

*Abundances represent the bound sterols only, which were obtained by saponification of the residue from extraction.
Table 3. Percentages of identified sterols in Ecionemia and Petrosia specimens. Pres. BM – sponge biomass preserved in ethanol.

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Sterol name</th>
<th>Ecionemia sp. SS1</th>
<th>Petrosia sp. 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ethanol</td>
<td>Frozen</td>
</tr>
<tr>
<td>1</td>
<td>Cholesta-5,22-dien-3β-ol</td>
<td>2.0</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>5α-Cholesta-22-en-3β-ol</td>
<td>2.7</td>
<td>n.d.</td>
</tr>
<tr>
<td>3</td>
<td>Cholest-5-en-3β-ol</td>
<td>34.1</td>
<td>4.1</td>
</tr>
<tr>
<td>4</td>
<td>5α-Cholestan-3β-ol</td>
<td>10.1</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>Ergosta-5,22-dien-3β-ol</td>
<td>3.2</td>
<td>6.4</td>
</tr>
<tr>
<td>6</td>
<td>5α-Ergost-22-en-3β-ol</td>
<td>1.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>7</td>
<td>5α-Cholest-7-en-3β-ol</td>
<td>1.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>8</td>
<td>5α-Ergosta-5,24(24&lt;sup&gt;1&lt;/sup&gt;)-dien-3β-ol</td>
<td>1.9</td>
<td>n.d.</td>
</tr>
<tr>
<td>11</td>
<td>Ergost-5-en-3β-ol</td>
<td>1.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>15</td>
<td>Stigmasta-5,22-dien-3β-ol</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td>18</td>
<td>23,24&lt;sup&gt;1&lt;/sup&gt;-Cyclostigmast-5-en-3β-ol</td>
<td>1.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>19</td>
<td>Stigmast-5-en-3β-ol</td>
<td>13.4</td>
<td>3.4</td>
</tr>
<tr>
<td>20</td>
<td>5α-Stigmasta-7,22-dien-3β-ol</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>22</td>
<td>Stigmast-5,24(24&lt;sup&gt;1&lt;/sup&gt;)-dien-3β-ol</td>
<td>19.8</td>
<td>79.0</td>
</tr>
<tr>
<td>23</td>
<td>5α-Stigmastan-3β-ol</td>
<td>0.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>25</td>
<td>5α-Stigmast-8-en-3β-ol</td>
<td>0.3</td>
<td>3.3</td>
</tr>
</tbody>
</table>
Figure 2.
Figure 3.
Figure 4.
Figure 5.