1 **Tapping the archives: The sterol composition of marine sponge species, as determined**

2 **non-invasively from museum preserved specimens, reveals biogeographical features**

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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.

37 **Introduction**

38 The number of species in the phylum Porifera (sponges) is rapidly rising with over 8,600
39 currently recognized species, and suggestions that there could be more than twice as many
40 species globally (Van Soest *et al.*, 2012). Sponges are widespread in many shallow and deep
41 water reef systems, and, as filter feeders, they occupy a key role in the carbon cycle of marine
42 ecosystems (Van Soest *et al.*, 2012). The phylum is deeply branching in the Metazoa and
43 their phylogeny is of great interest to evolutionary biologists (Wörheide *et al.*, 2012).
44 Sponges are known to produce a vast number of highly diverse natural products (Genta-Jouve
45 & Thomas, 2012), including over 200, often unusual triterpenoids and steroids (Bergmann,
46 1949; D'Auria *et al.*, 1993).

47 Djerassi and Silva (1991) concluded that the composition of most sponges consists of fairly
48 common sterols, while some contain unusual sterols. This was a result of their analyses of
49 sponge sterols (the most common types of steroids, with a hydroxyl group on C-3, Fig. 1) by
50 mass spectrometry and nuclear magnetic resonance (NMR) in different specimens (De Rosa
51 *et al.*, 1973; Bergquist *et al.*, 1980; Kerr & Baker, 1991). Unusual sterols include the
52 cyclopropyl-side chain containing sterols found in sponges of the order Haplosclerida
53 (Proudfoot & Djerassi, 1987; Gauvin *et al.*, 1998; Giner *et al.*, 1999), the unusual 19-
54 *nor*sterols present in some members of the genus *Axinella* (Minale & Sodano, 1974; Crist &
55 Djerassi, 1983), or the multiply alkylated side chains produced by members of the order
56 Halichondrida (Stoilov *et al.*, 1986a, 1986b). Of particular interest to geobiologists is 24-
57 *iso*propylcholesterol, which was isolated first from *Pseudaxinyssa* sp. (Hofheinz &
58 Oesterhelt, 1979), now accepted as *Axinyssa* sp., family Halichondriidae, order Suberitida.
59 Sponges are the only known extant organisms where this compound is present in large
60 amounts (McCaffrey *et al.*, 1994; Love & Summons, 2015). This finding resulted in the
61 interpretation of high abundances of its geologically stable derivative, 24-*iso*propylcholestane

62 compared to 24-*n*-propylcholestane in the rock record as a proxy for the abundance of
63 Demosponges (Love *et al.*, 2009; Kelly *et al.*, 2011). However, the validity of this biomarker
64 is debated (Antcliffe, 2013; Love & Summons, 2015) and should be applied with caution as
65 small amounts of it are also produced by marine algae. Molecular clock studies of the
66 biosynthetic genes though have recently shown that pelagophyte algae evolved the gene for
67 the synthesis of this particular sterol later than the Cryogenian, when the first massive
68 occurrence of this molecular fossil is observed (Gold *et al.*, 2016). It provides a tantalizing
69 possibility for determining the rise of animal life.

70 As only a select number of sponge species and specimens has been analysed so far, the
71 relationship of sterol composition with phylogeny is not entirely clear: Bergquist *et al.* (1991)
72 reported a correlation, but others such as Fromont *et al.* (1994), concluded that sterol
73 composition was not necessarily related to phylogeny. Future opportunities lie in combining
74 DNA based phylogeny and elucidation of biosynthetic pathways, but in order to provide
75 comprehensive results, a representative number of species and specimens needs to be
76 analysed (Erpenbeck & van Soest, 2007). This is particularly important when considering that
77 sponges do not only employ *de novo* biosynthesis of sterols, but are also capable of
78 modifying dietary sterols (Bergquist, 1978; Silva *et al.*, 1991; Silva & Djerassi, 1992).
79 Consequently, the determining factors on the sterol composition of sponges are of high
80 interest to geochemists and geobiologists.

81 Analysis of sterols usually entails the extraction of collected or cultivated sponge tissue,
82 followed by purification through a gravity column or high performance liquid column
83 chromatography (HPLC) procedures (Popov *et al.*, 1976). Analysis by gas chromatography-
84 mass spectrometry (GC-MS) is then usually carried out on the derivatized sterols, carrying
85 either a trimethylsilyl group or an acetyl group (Goad & Akihisa, 1997). These
86 derivatizations have been reported to affect the distribution of measured sterols (Mitrevski *et*

87 *al.*, 2008). However, sponge tissue can be difficult to obtain due to the necessity of sampling
88 permits, as many locations are marine protected areas, and due to logistical reasons for
89 sampling in deep waters (trawls, remote operated vehicles have to be employed).
90 Identification of these sponge samples requires a taxonomist, is very time consuming and
91 presents one of the main bottlenecks in sponge research. Therefore, analyzing large numbers
92 of identified sponge samples for sterols would be useful for investigating and comparing
93 sterol distributions with respect to phylogenetic relationships, identifying unusual sterols of
94 potential biomedical interest, and of biomarker potential as a chemotaxonomy tool e.g. in the
95 field of paleontology (Erpenbeck & van Soest, 2007).

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

109 In order to determine the suitability of ethanol, used to preserve sponge specimens, for the
110 analysis of sterols, we analyzed ethanol from a preserved specimen of *Agelas* sp. collected off
111 the Western Australian coast and subsequently stored in the dark and at 18 °C and compared

112 it to an extract of samples of the same specimen, one of which was freeze dried and stored at

113 18 °C and one frozen at –20 °C. We also analyzed two other species preserved by the above

114 three methods, however, the specimens had been collected from various locations at different

115 times. Here, we resolve these sterols by GC×GC-TOFMS; demonstrate that the ethanol

116 collections of museum specimens can be a valuable resource for lipid and potentially other

117 natural products research, or for large scale studies in marine chemical ecology, and discuss

118 the differences between sponges of the same species but collected at different locations.

119 **Methodology**

120 *Sampling*

121 A specimen of the sponge *Agelas* sp. MF1 (family Agelasidae, order Agelasida) was

122 collected off the south-western Australian coastline during cruises and surveys as specified

123 (Table 1, Fig. S1). A part of the sponge was wet frozen at - 20°C, one part was preserved in

124 75% ethanol on board, and one part was wet frozen on board and lyophilized at the Western

125 Australian Museum. One specimen of *Petrosia* sp. 1 (family Petrosiidae, order Haplosclerida)

126 and one specimen of *Ecionemia* sp. SS1 (family Ancorinidae, order Tetractinellida) were

127 collected at Ningaloo (Table 1, Fig. S1) and stored in ethanol, and two specimens of each

128 were collected at Kalbarri (Table 1, Fig. S1) and stored wet frozen at -20°C and freeze dried,

129 respectively.

130 The ethanol preserved (6 to 9 years, analysis in 2014, see date of collection in Table 1) and

131 lyophilized tissue was stored in the dark at 18°C, while the frozen tissue was stored in the

132 dark and at -20°C. The frozen, freeze dried and ethanol preserved tissue was extracted as

133 outlined in section 2.2. 10 – 20 mL of the ethanol was sampled, dried under a stream of N_2,

134 dissolved in dichloromethane (DCM) / methanol (MeOH) 1:1 (*v/v)* and dried over $MgSO_4$,

135 dissolved to a concentration of 10 mg/mL and purified as detailed below.

136 *Tissue extraction*

137 Analysis steps are summarized in Fig. 2. Ethanol preserved tissue was dried under
138 atmospheric pressure at 22°C, wet frozen tissue was lyophilized, and lyophilized tissue
139 obtained from the museum was used without modification. The extraction protocol for the
140 aliquots of the *Agelas* MF1 specimen are also represented in Fig. 1. Dry tissue (0.5 – 1 g) was
141 ground with a pestle and mortar and sonicated in 10 mL DCM/MeOH 1:1 (v/v) (10 min).
142 After centrifugation at 3,000 rpm (5 min), the supernatant was collected. This procedure was
143 repeated twice; the combined supernatant was dried under N_2 and over anhydrous $MgSO_4$,
144 and constituted the total lipid extract (TLE).

145 For analysis of free sterols, 2.5 mg of TLE was subjected to gravity column chromatography,
146 and the polar fraction was eluted from 0.8 g activated 60 mesh SiO_2 with 4 mL DCM/MeOH
147 1:1 after the apolar and aromatic compounds had been eluted with 4 mL hexane and 4 mL
148 Hex/DCM 3:7 (v/v). The polar fraction was dried under a stream of N_2 and dissolved in *n*-
149 hexane prior to analysis by GC-MS and GC×GC-TOFMS. For some of the extracts, free
150 hydroxyl groups were converted to trimethylsilyl (TMS)-ethers by reaction with 50 µL
151 pyridine and 50 µL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 70°C for one hour,
152 and evaporated to dryness under a stream of N_2 before dissolving in hexane.

153 In addition, for the *Agelas* samples, the total extracted, i.e. free and bound, sterols were
154 determined. For this, 2.5 mg of the TLE was saponified: it was dissolved in 2 mL 1N KOH in
155 MeOH and refluxed for 2 h at 80°C. 2 mL of water and 2 mL of cyclohexane were added,
156 shaken, and the cyclohexane containing the sterols was collected. This was repeated twice
157 and the combined cyclohexane fractions were dried, dissolved in diethylether/ethylacetate 1:1
158 (v/v), eluted over SiO_2 and dissolved in hexane for analysis.

159 In order to determine the sterols bound in the biomass residue of the ethanol preserved
160 *Agelas*, the residue of the extracted biomass was dried and refluxed in 25 mL 1N KOH in
161 MeOH (1 h). The pH was adjusted to 6 with 2N HCl in MeOH, water was added in equal
162 amounts to the MeOH, and the aqueous phase was extracted three times with 10 mL DCM.
163 The combined DCM phases were dried under N_2 and over $MgSO_4$, dissolved in
164 diethylether/ethylacetate 1:1 (v/v) and purified by elution from a silica (SiO_2) column.

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

166 For GC-MS, an Agilent 5973 mass-selective detector coupled to a 6890 gas chromatograph
167 was employed, using a 30 m x 0.25 mm ID x 0.25 um film capillary column of type DB5-
168 MS, with a temperature programmed from 40 to 325°C at 10°C . min^{-1} and held at the final
169 temperature for 20 min. Samples were injected in ethylacetate on a split/splitless injector in
170 pulsed splitless mode at 320°C. The carrier gas was He at a constant flow of 1.1 mL/min.
171 Ionization was carried out at 70 eV, with an electron multiplier voltage of 1800 V and the
172 source kept at 230°C. Masses scanned ranged from 50 to 750 Da. Data analysis of GC-MS
173 data was carried out using Wsearch32 (www.wsearch.com.au).

174 For GC×GC-TOFMS, splitless injection at 310°C inlet temperature was employed, on an
175 7890 Agilent GC modified for GC×GC, coupled to a Pegasus 4D TOF-MS with linear
176 modulation (LECO Corporation, St. Joseph, MI) employing electron ionization (EI). Primary
177 column was a 30 m Restek CP5-Sil of 0.25 mm inner diameter and 0.25 μm film thickness
178 and secondary column a 1.5 m 17Sil-MS (equivalent to 50 % phenyl) of dimensions 0.18 mm
179 / 0.18 μm with helium as a carrier gas at a flow rate of 1.05 mL. min^{-1}. Modulation was
180 carried out directly on the secondary column and modulation time was 5s (0.8 s hot jet, 1.70 s
181 cold jet). The temperature was ramped from 40°C to 300°C at a rate of 3°C . min^{-1}, with the
182 modulator at a 15°C and the secondary column at a 40°C offset. The Pegasus 4D was

183 operated at 100 Hz and at a mass range of 50-650 Daltons, with the transfer line at 290°C and
184 the ion source at 230°C. This configuration and program was optimized on the secondary
185 column separation, as using the small differences in polarity of the sterols analyzed allowed
186 for separation of several co-eluting compounds under these conditions. Data analysis was
187 conducted using ChromaTOF automatic peak detection with a signal to noise ratio and peak
188 width of 20 and 0.1 s, respectively, for small peaks and 300/0.4 s for larger peaks, areas of
189 TIC were used in order to calculate the area percentages of individual sterols of the total
190 sterol area.

191 **Results and Discussion**

192 *Sterol abundances*

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

195 The ethanol from *Agelas* sp. MF1 contained sterols with 27, 28 and 29 carbon atoms (Fig. 3,
196 Fig. 4, Table 2). The sterols from the cholestane series consisted of **2**, **3**, **4** and **7**, the sterols
197 from the ergostane series of **6**, **10**, **13**, **14** and **17**, and the ones from the stigmasterane series
198 **9, 15**, **16**, **17**, **23** and **26**. The two sterols present in largest abundance were **4** and **20**, other
199 major sterols included **2**, **6**, **10**, **13**, **23** and **26**.

200 The ethanol from *Ecionemia* sp. SS1 contained a large variety of sterols, including **1 - 8**, **11**,
201 **12**, **15**, **18**, **19**, and **22-25** (Fig. 3, Fig. 5, Table 3). However, the sterols of both the frozen and
202 the freeze dried samples consisted mainly of **22**, with some other minor constituents (Fig. 5).
203 The sterols obtained from the ethanol of the *Petrosia* specimen consisted of **2**, **4**, **6**, **11**, **15**,
204 **19**, **21** and **22**, in a distribution largely similar to the preserved specimen. The wet frozen and
205 lyophilized *Petrosia* sp. 1 contained a slightly larger variety of sterols, with also **1**, **3**, **5** and **8**

206 present in considerable proportions (Table 3). It is possible that some of these were not
207 detected in the ethanol due to a very large peak of **4** and **17**. The ethanol of *Petrosia* sp. 1 also
208 contained a number of 3-oxosterols, which were not observed in the wet frozen and
209 lyophilized specimens. It is possible that these were degradation products, however, they
210 were not observed in any of the other ethanol preserved samples and it is thus more likely
211 that they were present in the sponge. No sterols of less than 27 and more than 29 carbon
212 atoms were detected, but it is possible that these were present in minor amounts.

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

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

223 In order to analyze the completeness of extraction achieved by storage in ethanol, we also
224 extracted some of the sponge tissue that had been preserved in ethanol, and had leached the
225 sterols. This resulted in similar sterol compositions to those observed in the ethanol (Fig. 4B).
226 However, we obtained a slightly larger amount of sterol **20** (22.1 / 25.7 %), which, in
227 conjunction with the larger amounts present in the wet frozen and lyophilized samples,
228 suggests that ethanol might not completely extract **20**. No $\Delta^{5,7}$ sterols were detected in any of
229 the samples. Whilst these sterols are known to be chemically rather labile, they were not

230 detected in the frozen and the lyophilized samples either, therefore this is probably not an
231 artefact of the preservation method.

232 When the residue of the ethanol preserved sponge after tissue extraction was subjected to
233 saponification in order to release the more strongly bound sterols, a similar distribution to the
234 ethanol extracted sterols was observed (Fig. 4B). However, interestingly, it was also observed
235 that a large number of (unidentified) triterpenoids were released, as exemplified by the
236 extracted ion current (EIC) for *m/z* 191, a common ion observed in many triterpenoids (Fig.
237 S4). These compounds were thus present as more strongly bound, non-extractable lipids, or
238 potentially were associated with symbionts. Many sponges are able to source carbon and
239 energy from a number of symbionts they harbor within their tissue (Webster & Blackall,
240 2009; Thacker & Freeman, 2012), many of which are known to produce bacteriohopanoids
241 (Ourisson & Albrecht, 1992).

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

251 Our results show that the ethanol taken from preserved museum specimens contains sterols
252 that can be representative in type and distribution for an individual sponge. This technique
253 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; Naeher *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

279 analysis (Fig. S2, S3) as the former exhibit a higher retention time in the second dimension
280 (polar column; Rt_2). This results in additional confidence in structural identifications.
281 Moreover, a number of different isomers were detected, such as compounds **6** / **7**, which were
282 co-eluting in one dimensional analysis (Fig. S2, S3). If some of these compounds are present
283 in trace amounts, the signals could be difficult to deconvolute. GC×GC chromatograms also
284 allow for sophisticated untargeted comparison of samples, thus potentially allowing
285 untargeted cross sample comparison (Reichenbach *et al.*, 2011; Marney *et al.*, 2013).
286 Further, whilst here, analysis was conducted following simple gravity column
287 chromatography separation, GC×GC also allows the analysis of an untreated extract, thereby
288 removing any possibilities of bias and loss of compounds present in low concentrations
289 during the workup. With appropriate derivatization, it could also be possible to determine a
290 range of other compounds of interest in these extracts, and of potential interest, such as
291 alkaloids or terpenoids (cf. Erpenbeck and van Soest, 2007; Genta-Jouve and Thomas, 2012).

292 *Differences in specimens from different locations*

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

304 responsible for the conversion of the sterols to ketones, but are unlikely to have caused all of
305 these differences.

306 A more likely reason for these differences is that the results are not directly comparable as
307 they were not derived from the same specimen, but rather from three different specimens
308 (Table 1), of which the one preserved in ethanol was obtained from a completely different
309 location (Fig. S1). This is in contrast to previous studies, where sterol composition was found
310 to be species specific and independent of location (Bergquist *et al.*, 1980; Fromont *et al.*,
311 1994). As sponges employ both *de novo* biosynthesis along with uptake and modification of
312 dietary and symbiont produced sterols (Bergquist, 1978) these sterol differences between
313 specimens of one species are not surprising. Habitat, depth, or times of collection are unlikely
314 to have caused these differences: for example, all specimens of *Ecionemia* sp. were collected
315 around 100 m depth in the same year. In the case of *Petrosia*, both the freeze dried and the
316 ethanol preserved specimens were collected at a similar depth (around 100 m depth), while
317 the wet frozen specimen was from 253 m depth, yet it was the ethanol preserved specimen
318 that contained different sterols from the other two. The collection time was austral summer
319 for all specimens (Table 1). Subtle differences were seen in the sponge color and spicule
320 dimensions of the ethanol preserved specimen of *Petrosia*, which was darker brown and had
321 thinner spicules that the wet frozen and freeze dried samples (260 x 12 μm compared to 270 x
322 20 μm for the largest size category of oxeas). It is possible that *Petrosia* sp. 1 is a species
323 complex (i.e. a group of two or more closely related cryptic species), but this could only be
324 determined with more detailed morphological analyses and molecular data.

325 However, in both *Petrosia* sp. and *Ecionemia* sp., the wet frozen and lyophilized samples,
326 which had differing sterol complements, had been collected at the same location, while the
327 ethanol preserved sponge had been collected in a different area (Fig. S1). It is thus most
328 likely that the sterol distributions are related to geographical or ecosystem differences, and

329 that the sterol composition varies moderately between species across their biogeographic
330 distributions. Ethanol preserved specimens of *Petrosia* and *Ecionemia* were collected in the
331 tropics at Ningaloo Reef (Carnarvon Shelf, NW Australia, 22°S) and the wet frozen and
332 lyophilized specimens of these species were collected from Kalbarri/Zuytdorp (Dirk Hartog
333 Shelf, Central Western Australia). The latter region is subtropical (27°S) and to ≤ 250 m
334 depth exposed to the Leeuwin current, potentially a rich source of particulates for filter
335 feeders such as sponges (Fromont *et al.*, 2012), that could influence the dietary sterol uptake
336 *via* organic matter supply (Silva *et al.*, 1991; Silva & Djerassi, 1992). It is thus possible that
337 sterol and sterane biomarker distributions derived from sponges can be indicative of
338 environmental factors such as their diet. Also other factors varying between localities and
339 individuals (nutrient regimes, a difference in symbionts, or microbial defense) could play a
340 major role in activating *de novo* biosynthesis or modification after uptake. This could explain
341 observations made by Kerr *et al.* (1991), who saw a strong variation in the sterol composition
342 of *Xestospongia muta* specimens collected in close proximity, although this could also reflect
343 cryptic speciation.

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

352 *Comparison of sterol compositions with the literature*

353 Sponges of the genus *Agelas* have been investigated for sterol composition on many

354 occasions: Santalova *et al.* (2004) analyzed *A. mauritiana*, and reported 20 sterols, including

355 **1** (trace amounts = tr), **2** (4.48 %), **3** (2.51 %), **4** (28.72 %), **5** (tr), **6** (tr), **7** (9.45 %), **8** (tr), **11**

356 (tr), **13** (9.03 %), **15** (tr), **16**(1.46 %), **17** (6.01 %), **19** (2.23 %), **20** (2.23 %), **23** (tr) and **26**

357 (20.57 %), in addition to a number of other sterols, including 5α-25-desmethyl-ergost-22-en-

358 3β-ol (tr), 5α-cholesta-7,22-dien-3β-ol (tr), 5α-ergosta-7,22-dien-3β-ol (5.22 %), 5α-23-

359 methyl-ergost-22-en-3β-ol (2.04 %). This profile resembles the one for the *Agelas* species

360 analyzed here, but differed slightly in relative amounts (Fig. 4). Also, sponges of the order

361 Petrosiidae have been extensively investigated for sterol composition, and were found to

362 contain a number of unusual, often cyclopropyl-containing sterols (Wahid Khalil *et al.*, 1980;

363 Gauvin *et al.*, 1998; Giner *et al.*, 1999; Reddy *et al.*, 1999), which were not detected in this

364 study. This was in agreement with Fromont *et al.* (1994) and Bergquist *et al.* (1980), who

365 examined various species of the genus *Petrosia*, but could not detect any of these unusual

366 sterols. Instead, *P. pigmentosa* and *P. australis* contained **1** (0.7 / 2.4 %), **3** (4.2 / 10 %), **4** (12

367 / 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

368 %), **13** (0.4 / 0 %), **15** (1.3 / 2.6 %), **19** (31 / 2.3 %), **22** (0.3 / 13 %), **23** (1.6 / 0 %) and **26** (13

369 / 0 %). Other sterols detected in these specimens were (*E*)-stigmasta-5,24(24^1)-dien-3β-ol

370 (0.1 / 7.6 %), 26-desmethyl-cholesta-5,22-dien-3β-ol (0.7 / 0.5 %), 26-desmethyl-cholest-22-

371 en-3β-ol (1.4 / 0 %), and a number of $\Delta^{5,7}$ sterols, which were not detected in our study.

372 These are known to be particularly labile and it is thus possible that they had been present in

373 the live sponge, but could not be detected in our samples. Similarity between the sterol

374 compositions of the two *Petrosia* species reported by Fromont *et al.* (1994) was not high, and

375 the samples investigated here also show little similarity to these species (Fig. 4). No sterol

376 composition for the genus *Ecionemia* has been reported.

377 A literature comparison of the sterols from the same genera as the species analyzed here
378 demonstrates the similarity of *Agelas* sp. MF1 to *A. mauritiana,* and confirms the
379 comparability of our method with results gained by more traditional methods. It is possible
380 that sponges of the genus *Agelas* are so similar to each other because they rely more strongly
381 on *de novo* biosynthesis, while sponges of the genus *Petrosia* (and *Ecionemia*) rely on
382 modified dietary sterols, which causes greater variation in sterols at the genus and to a lesser
383 extent, species level. This is in agreement with Silva *et al.* (1992), who attributed the unusual
384 sterols of *P. ficiformis*, which were not detected in the specimens investigated here, to dietary
385 modification in line with biosynthetic observations. However, contrastingly, Gold *et al.*
386 (2016) suggested that this species does possess all the genes necessary for their production.

387 The lack of unequivocal resolution of the phylogeny of the Haplosclerida further complicates
388 comparison of *Petrosia* sp. 1 sterols with other species. Recent advances in sponge
389 phylogeny have suggested that *Petrosia* is indeed a paraphyletic group (Redmond *et al.*,
390 2011), which might also cause the strong differences in sterol composition when comparing
391 our results to the literature, and explain the absence of the unusual sterols of *P. ficiformis* in
392 *Petrosia* sp. 1 and other *Petrosia* species (Fromont *et al.*, 1994). In accordance with ongoing
393 advances in sponge phylogeny, more detailed analysis of sterol complements, combined with
394 molecular analysis, with replicates of the same species from the same and different locations
395 or oceanographic and ecological conditions could provide valuable information for the
396 interpretation of sterol distributions, the sterane geological record and the evolution of
397 Porifera and the Metazoa.

398 **Conclusions**

399 The sterol composition obtained from the ethanol of museum voucher specimens presents a
400 new method for non-invasive sampling of archived, identified sponge specimens. This can

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

412

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421

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565

566 Figure captions

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

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

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

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

581 Figure 5. Sterol composition of the three sponges analyzed, in comparison to published
582 species from the same genera. *Agelas* sp. MF1 is compared to *A. mauritiana*, for *Petrosia* sp.
583 1, the composition determined from the three differently preserved specimens is shown, and
584 compared to *P. australis* and *P. pigmentosa*. For *Ecionemia* sp. SS1, sterol composition is as
585 determined from the three differently preserved specimens. [1] Composition as determined by
586 Santalova *et al.* (2004), [2] composition as determined by Fromont *et al.* (1994).

Tables

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).

Species	Museum registr. numbers	Preservation method	Location			Station	Depth [m]	Date collected	Cruise/Survey
				Start of trawl	End of trawl				
Agelas sp. MF1 Agelasidae, Agelasida, Demospongiae	Z49312	Ethanol	Broke Inlet	35°08'23"S 116°16'10"E	35°08'03"S 116°16'14"E	Trawl 1	65	2007/10/15	WA-MFBPS
	Z49312	Wet frozen	Broke Inlet	35°08'23"S 116°16'10"E	35°08'03"S 116°16'14"E	Trawl 1	65	2007/10/15	WA-MFBPS
	Z49312	Freeze dried	Broke Inlet	35°08'23"S 116°16'10"E	35°08'03"S 116°16'14"E	Trawl 1	65	2007/10/15	WA-MFBPS
Petrosia sp. 1 Petrosiidae Haplosclerida, Demospongiae	Z45259	Ethanol	Ningaloo Reef	22°36'53"S 113°34'55"E	22°36'52"S 113°34'55"E	RVS4545/2008	100	2008/02/05	AIMS-WAM III
	Z35817	Wet frozen	Kalbarri	27°55'42"S 113°08'16"E	27°56'01"S 113°08'38"E	SS1005/099	253.5	2005/12/04	CSIRO SS1005
	Z35811	Freeze dried	Kalbarri	27°48'48"S 113°18'39"E	27°49'05"S 113°18'39"E	SS1005/102	97	2005/12/05	CSIRO SS1005
Ecionemia sp. SS1 Ancorinidae, Tetractinellida Demospongiae	Z35069	Ethanol	Ningaloo South	22°04'00"S 113°48'40"E	22°04’15"S 113°48'54"E	SS1005/144	103.5	2005/12/10	CSIRO SS1005
	Z35949	Wet frozen	Zuytdorp	27°03'07"S 113°04'51"E	27°02'52"S 113°04'37"E	SS1005/110	106	2005/12/06	CSIRO SS1005
	Z35808	Freeze dried	Zuytdorp	27°03'06"S 113°06'03"E	27°02'56"S 113°05'59"E	SS1005/104	97	2005/12/05	CSIRO SS1005

Table 2. Percentages of identified sterols in Agelas samples. Pres. BM –sponge biomass preserved in ethanol. n.d. = not detected.

Nr.	Sterols	Free sterols					Total sterols			
		Ethanol	**Frozen**	**Lyophil.**	**Pres. BM**		**Ethanol**	**Frozen**	**Lyophil.**	**Pres. BM***
1	Cholesta-5,22-dien-3β-ol									
2	5α-Cholest-22-en-3β-ol	7.3	5.8	4.2	3.6	1.6	12.5	7.3	5.8	4.2
3	Cholest-5-en-3β-ol	3.7	2.5	n.d.	n.d.	n.d.	2.7	3.7	2.5	n.d.
4	5α-Cholestan-3β-ol	18.7	16.9	10.7	10.4	7.0	22.1	18.7	16.9	10.7
5	Ergosta-5,22-dien-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	5α-Ergost-22-en-3β-ol	8.4	7.8	6.1	5.9	4.5	7.3	8.4	7.8	6.1
7	5α-Cholest-7-en-3β-ol	2.0	2.1	2.0	1.5	1.9	2.4	2.0	2.1	2.0
8	5α-Ergosta-5,24(24^1)-dien-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9	C_{29}Δ-Sterol	3.6	4.8	7.1	5.7	7.4	5.9	3.6	4.8	7.1
10	23,24^1-Cycloergost-5-en-3β-ol	4.8	6.0	n.d.	n.d.	n.d.	n.d.	4.8	6.0	n.d.
11	Ergost-5-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	5α-Ergost-24(24^1)-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
13	5α-Ergostan-3β-ol	6.5	8.0	2.9	2.1	1.5	3.9	6.5	8.0	2.9
14	Ergostatrien-3β-ol	0.3	0.6	3.0	1.3	0.1		0.3	0.6	3.0
15	Stigmasta-5,22-dien-3β-ol	4.0	3.6	7.5	2.2	1.7	4.2	4.0	3.6	7.5
16	5α-Stigmast-22-en-3β-ol	0.7	0.8	0.5	0.6	0.7	0.9	0.7	0.8	0.5
17	5α-Ergost-7-en-3β-ol	3.1	3.5	4.2	3.1	4.0	4.7	3.1	3.5	4.2
18	23,24^1-Cyclostigmast-5-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
19	Stigmast-5-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20	5α-Stigmasta-7,22-dien-3β-ol	21.9	22.0	34.5	51.0	54.0	24.8	21.9	22.0	34.5
21	(E)-Stigmast-24(24^1)-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22	Stigmasta-5,24($24^{1)}$-dien-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
23	5α-Stigmastan-3β-ol	6.1	6.7	7.1	3.3	5.8	4.2	6.1	6.7	7.1
24	(Z)-Stigmast-24(24^1)-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
25	5α-Stigmast-8-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
26	5α-Stigmast-7-en-3ß-ol	9.0	9.0	10.1	9.5	9.8	4.4	9.0	9.0	10.1

*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.

		***Ecionemia* sp. SS1**			***Petrosia* sp. 1**		
Nr.	**Sterol name**	**Ethanol**	**Frozen**	**Lyophil.**	**Ethanol**	**Frozen**	**Lyophil.**
1	Cholesta-5,22-dien-3β-ol	2.0	1.3	2.0	n.d.	1.1	2.3
2	5α-Cholest-22-en-3β-ol	2.7	n.d.	0.3	1.2	n.d.	n.d.
3	Cholest-5-en-3β-ol	34.1	4.1	3.5	n.d.	3.2	3.7
4	5α-Cholestan-3β-ol	10.1	1.0	0.9	10.1	3.5	8.7
5	Ergosta-5,22-dien-3β-ol	3.2	6.4	7.5	n.d.	3.4	8.2
6	5α-Ergost-22-en-3β-ol	1.3	n.d.	0.5	0.9	n.d.	0.6
7	5α-Cholest-7-en-3β-ol	1.1	n.d.	n.d.	n.d.	n.d.	n.d.
8	5α-Ergosta-5,24(24^1)-dien-3β-ol	1.9	n.d.	0.3	n.d.	21.9	18.7
9	C_{29}Δ-Sterol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	23,24^1-Cycloergost-5-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
11	Ergost-5-en-3β-ol	1.4	n.d.	0.4	68.7	37.3	50.3
12	5α-Ergost-24(24^1)-en-3β-ol	3.4	n.d.	n.d.	n.d.	n.d.	n.d.
13	5α-Ergostan-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
14	Ergostatrien-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15	Stigmasta-5,22-dien-3β-ol	1.7	1.5	1.2	1.8	2	2.8
16	5α-Stigmast-22-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
17	5α-Ergost-7-en-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18	23,24^1-Cyclostigmast-5-en-3β-ol	1.6	n.d.	0.4	n.d.	n.d.	tr
19	Stigmast-5-en-3β-ol	13.4	3.4	1.8	11.3	24.6	n.d.
20	5α-Stigmasta-7,22-dien-3β-ol	n.d.	n.d.	n.d.	n.d.	n.d.	4.2
21	(E)-Stigmast-24(24^1)-en-3β-ol	n.d.	n.d.	n.d.	2.1	n.d.	n.d.
22	Stigmasta-5,24($24^{1)}$-dien-3β-ol	19.8	79.0	81.3	4.0	3	0.0
23	5α-Stigmastan-3β-ol	0.2	n.d.	n.d.	n.d.	n.d.	n.d.
24	(Z)-Stigmast-24(24^1)-en-3β-ol	1.8	n.d.	n.d.	n.d.	n.d.	n.d.
25	5α-Stigmast-8-en-3β-ol	0.3	3.3	n.d.	n.d.	n.d.	n.d.
26	5α-Stigmast-7-en-3ß-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Figure 1.

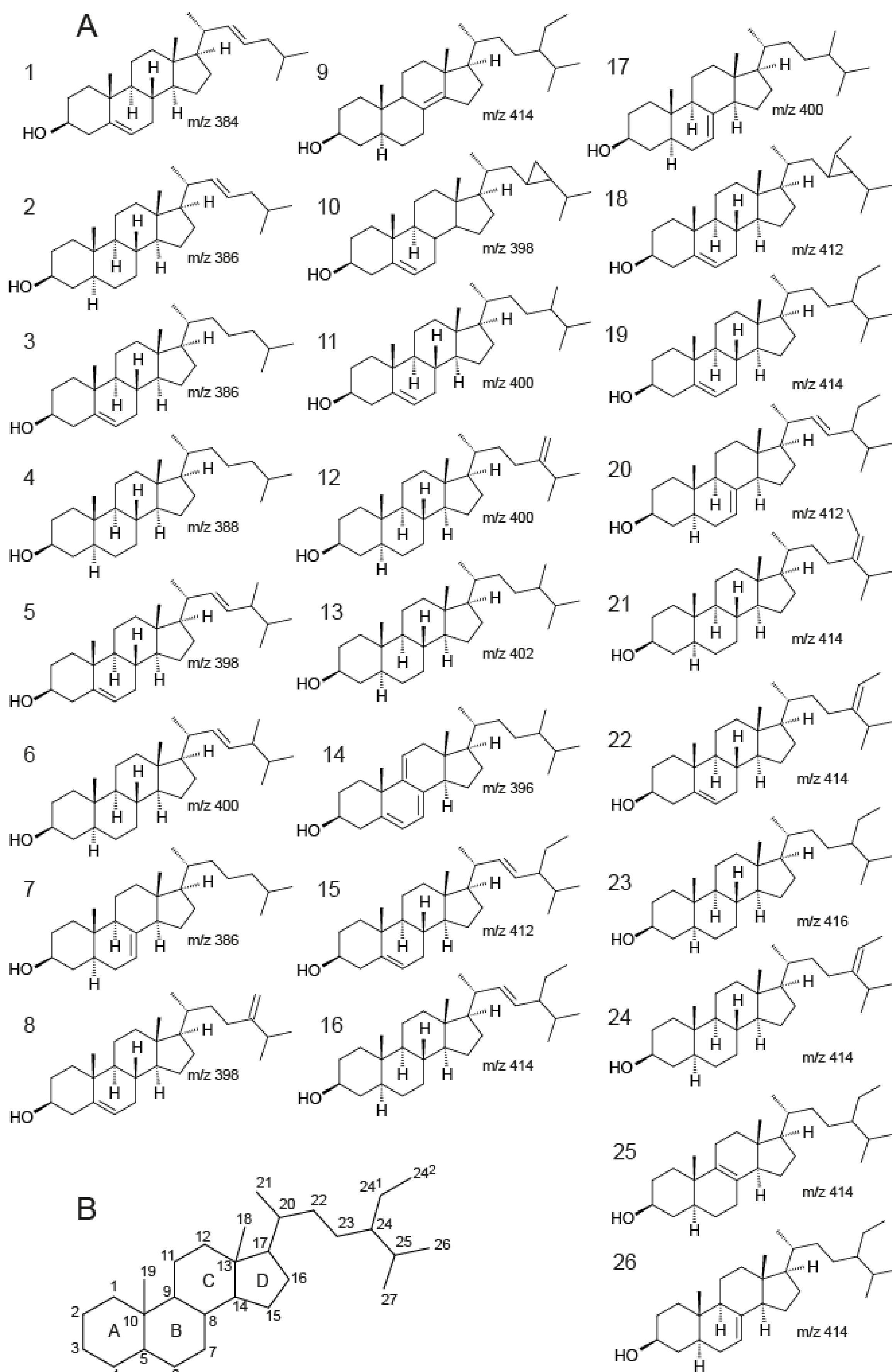

Figure 2.

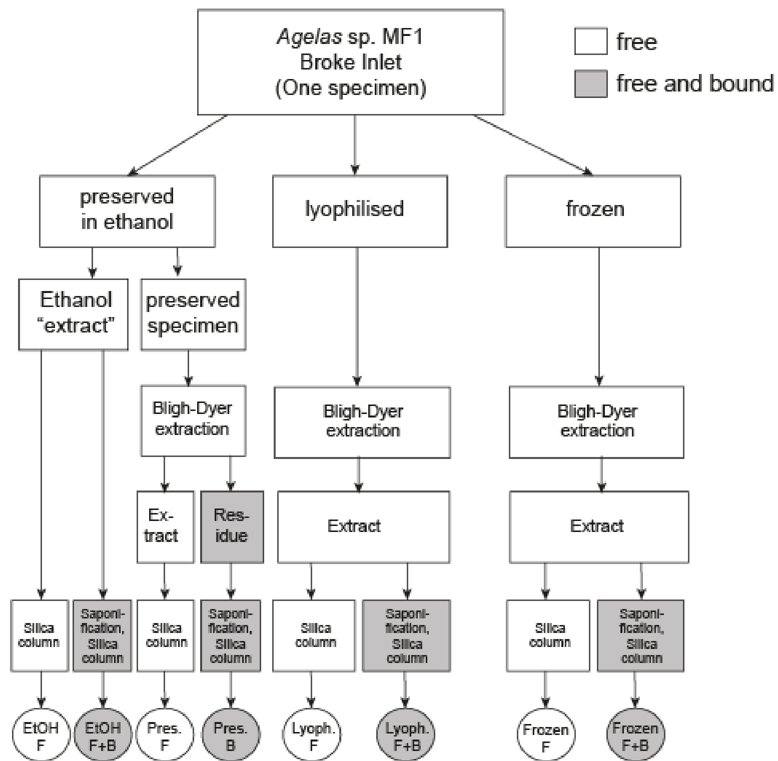

Figure 3.

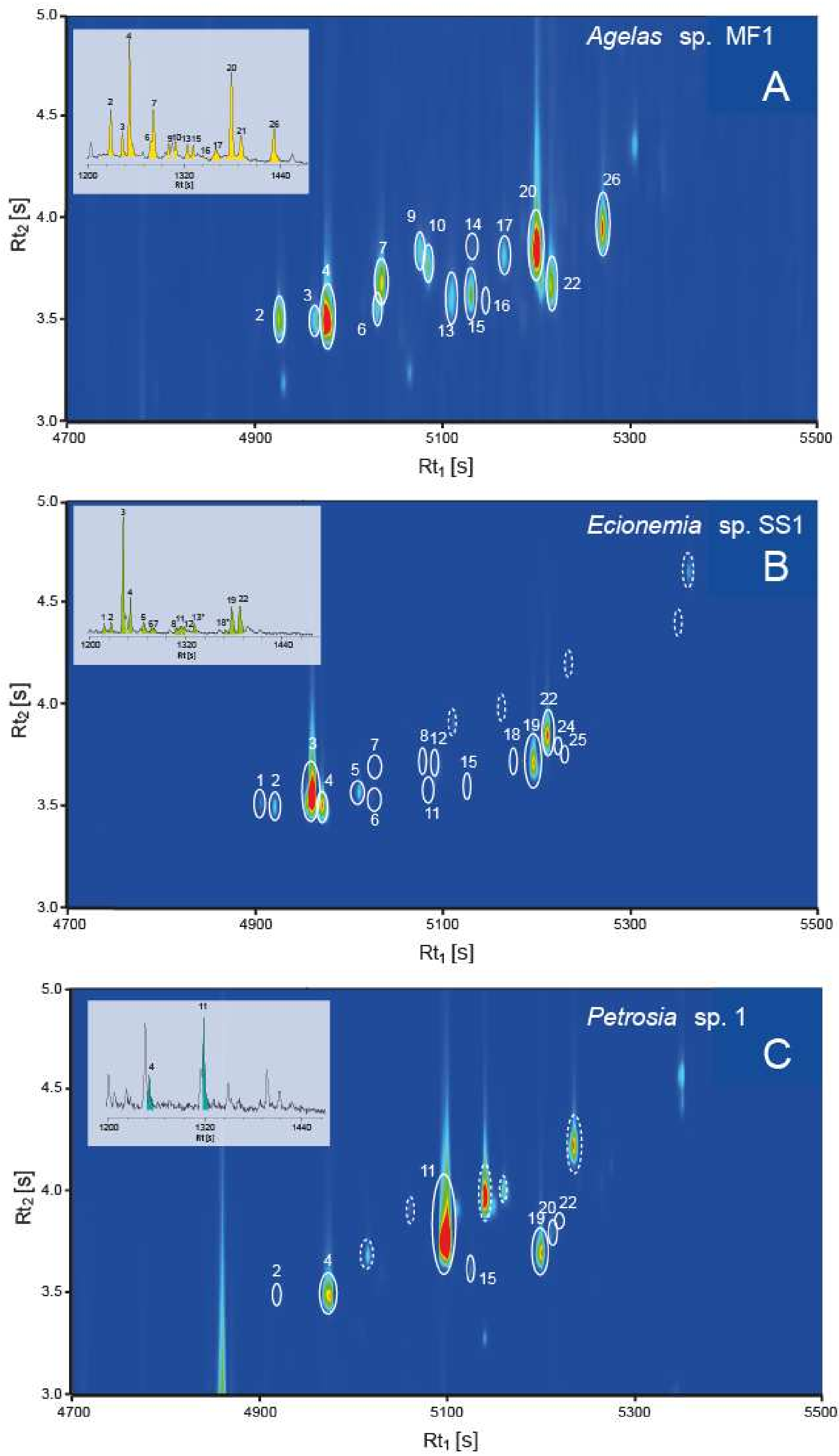

Figure 4.

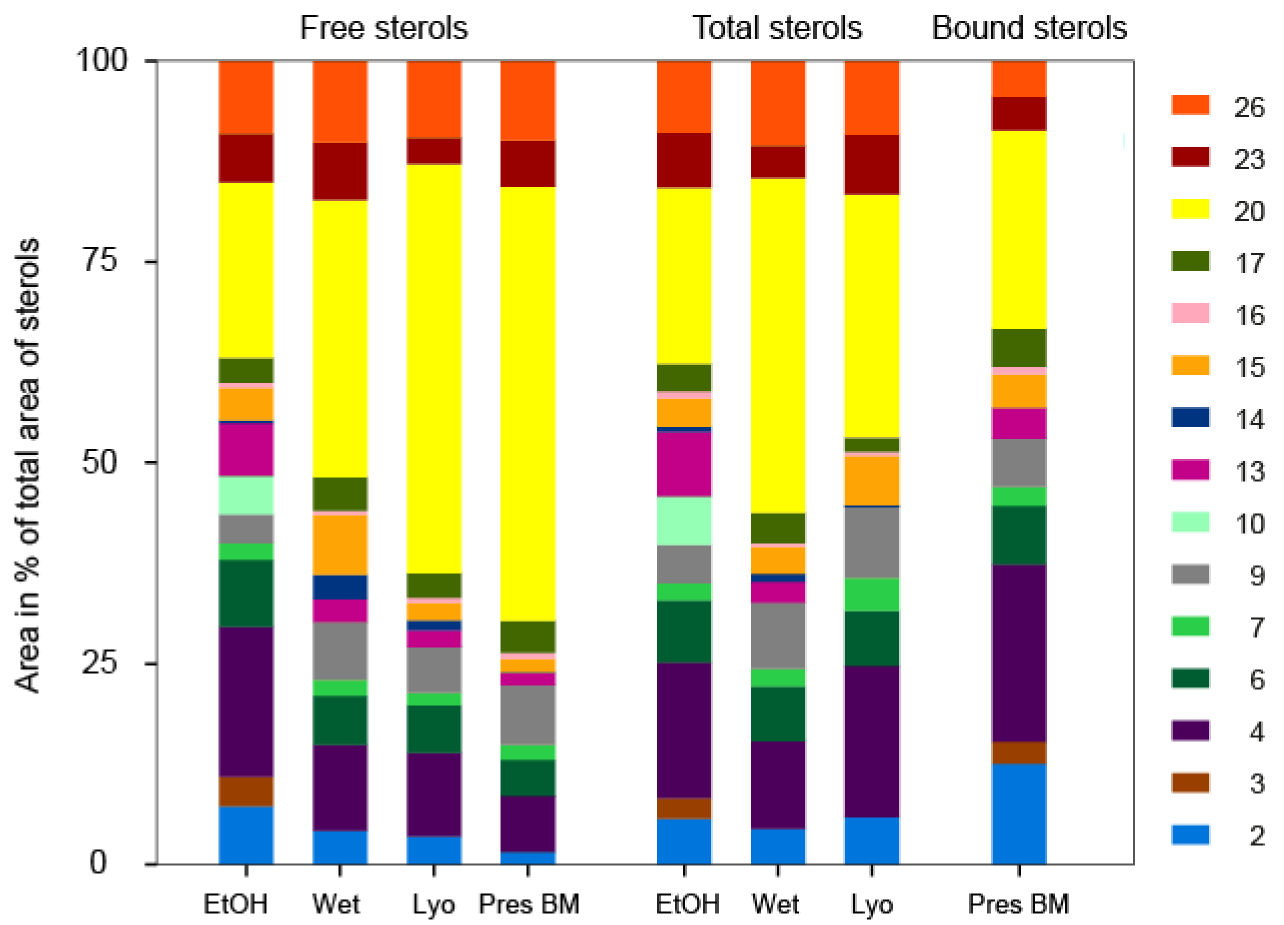

Figure 5.

Area in % of total area of sterols

0 25 50 75 100

Agelas sp. MF1 Ethanol

Agelas mauritania

other

Petrosia sp. 1

EtOH

Wet

Lyophilised

Petrosia pigmentosa

other

Petrosia australis

other

Ecionemia sp. SS1

EtOH

Wet

Lyophilised

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26