

Characterization of *Leucetta prolifera*, a calcarean cyanosponge from south-western Australia, and its symbionts

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The biology and ecology of calcarean sponges are not as well understood as they are for demosponges. Here, in order to gain new insights, particularly about symbiotic relationships, the calcarean sponge Leucetta prolifera was sampled from south-western Australia and examined for its assumed photosymbionts. Pulse amplitude modulated fluorometry and extraction of photopigments established that the sponge was photosynthetic. Molecular analysis of the bacterial symbionts via sequencing of the V1–V3 region of the 16S rDNA gene confirmed that between 5 and 22% of all sequences belonged to the phylum Cyanobacteria, depending on the individual sample, with the most dominant strain aligning with Hormosquilla spongeliae, a widely distributed sponge symbiont. Analysis of fatty acids suggested that the sponge obtains nutrition through photosynthates from its symbionts. The relationship is assumed to be mutualistic, with the sponge receiving dietary support and the cyanobacteria sheltering in the sponge tissues. We list all Calcarea presently known to harbour photosymbionts.

Keywords: Porifera, Calcarea, Cyanobacteria, *Hormosquilla spongeliae*, photosymbiosis, chlorophyll, 16S gene sequencing, membrane fatty acids, stable isotopes, photosynthate translocation

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INTRODUCTION

While many demosponges are known to harbour photosymbionts of various origins (e.g. Schönberg & Loh, 2005; Díaz *et al.*, 2007; Lemloh *et al.*, 2009; Thacker & Freeman, 2012), many calcareous sponges do not appear to be photosynthetic, frequently having pale colours such as yellow or white (e.g. Rossi *et al.*, 2011), implying they may lack this type of symbiont. There is minimal information on any calcarean symbionts, and what is available is usually restricted to reporting their presence (Thacker & Freeman, 2012), and suggesting the photosymbioses are almost exclusively with cyanobacteria. For example, macrolides of cyanobacterial origin were isolated from New Caledonian specimens of *Leucascandra caveolata* Borojevic & Klautau, 2000 (D'Ambrosio *et al.*, 1999; Kartika, 2008), although there was uncertainty whether these were present in the living sponge or associated with dead parts of the sampled material. Endocytobiotic, unicellular cyanobacteria were described from *Sycon* sp. from the Mediterranean (Feldmann, 1933; Wilkinson, 1980; Díaz *et al.*, 2007). Burlando *et al.* (1988, as *Clathrina*) observed Gram-negative bacteria in

Borojevia cerebrum (Haeckel, 1872), and Wilkinson (1980) and Díaz *et al.* (2007) mentioned endocytobiotic, unicellular cyanobacteria in *Clathrina* sp. from the Mediterranean, citing the thesis of Duclaux (1977). In the southern hemisphere, Díaz *et al.* (2007) listed a *Leucetta* sp. from Papua New Guinea as a possible cyanosponge. *Pericharax heterorhaphis* Poléjaeff, 1883, from the Australian Great Barrier Reef has been reported to contain cyanobacteria distributed in a superficial layer in the sponge, yielding low concentrations of chlorophyll and low rates of carbon fixation (Wilkinson, 1982, 1983). This species appears to depend on supplies from the symbionts and has a distribution restricted to shallow water (Wilkinson, 1978a, b, 1980; Díaz *et al.*, 2007). It can distinguish between symbiont and non-symbiont bacteria digesting only the latter (Wilkinson *et al.*, 1984). In south-western Australia two calcarean sponges produced fluorescence when scanned with pulse-amplitude modulated fluorometry (Lemloh *et al.*, 2009), with the symbiont identified as *Synechococcus* sp.

Apart from the study in south-western Australia (Lemloh *et al.*, 2009) all of the incidences of light requiring symbionts in Calcarea have been reported from tropical regions, largely in the northern hemisphere. Moreover, all of the symbionts in Calcarea were found to be cyanobacteria, except for *Clathrina* sp. from Puerto Rico, which was reported to contain diatoms as a response to a bleaching episode (*Pleurosigma* sp.; Vicente, 1990).

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Here we provide an account of *Leucetta prolifera* (Carter, 1878) from south-western Australia, a dark olive-green sponge, a colour which suggests it may be photosynthetic by association with microbial symbionts (Appendix 1). As information about phototrophic Calcarea is very limited to date, in particular about species from Western Australia, we analysed this sponge for pigments, and used molecular taxonomy to identify its putative photosymbionts. We also analysed the membrane fatty acid composition based on the phospholipid derived fatty acids in order to investigate carbon transfer between the symbionts and the sponge.

Leucetta prolifera was first described from Fremantle by Carter (1878) and had not been recorded in the literature until recently (Fromont *et al.*, 2013). It is a common local species in south-western Australia, accounting for 32% of sponge individuals recorded in the Jurien area, ~200 km north of Fremantle (Fromont *et al.*, 2013).

MATERIALS AND METHODS

Sample collection

Three specimens of *Leucetta prolifera* were collected from Three Mile Reef (31°46'32.16"S 115°40'29.9994"E) in Marmion Marine Park north of Perth at 10 m depth by scuba diving. Sponges were placed in clip seal plastic bags underwater. At the sea surface seawater was poured off, and samples were rinsed with 0.2 µm autoclaved seawater to remove loosely associated microbes. Samples were divided in two, and one subsample was prepared for fatty acid analysis by rinsing with autoclaved and 0.2 µm filtered MilliQ water, then being wrapped in aluminium foil. The other subsample was kept in the original clip seal plastic bag and later used for pulse-amplitude modulated fluorometry, chlorophyll extraction and DNA analysis. Subsamples were kept on ice in the dark. On return to the laboratory (~6 h later) they were frozen at -80°C until further analysis.

Pulse-amplitude modulated fluorometry and chlorophyll extraction

Frozen subsamples of each specimen were screened with pulse-amplitude modulated fluorometry in a maxi iPAM (Walz, Iffeltrich, Germany) at default settings to rapidly confirm whether they were photosynthetic. Pigment extractions were then performed following the protocol of Wellburn (1994). During these tasks all material was handled with gloves and all weights were obtained in triplicate at room temperature (20°C), using a desiccator to cool tubes and samples after drying for 24 h at 80°C.

For chlorophyll extraction the samples of sponge tissue were fully defrosted to reach room temperature, then further subsamples of ~1 × 1 × 0.5 cm were taken to represent parts with more colour than others that were pale (Appendix 1). Tissue was immediately placed into pre-dried and pre-weighed 15 mL falcon tubes and weighed on analytical scales (Shimadzu AUW 220D, VWR International, Murrarie, Australia; accuracy to 0.0001 g) to obtain sample wet weight (WW). Depending on the size of the subsamples, tissue was then immersed in 2–4 mL of laboratory grade methanol for 2 h in darkness at 20°C, shaking the solution

at the beginning and end of the extraction period. After 2 h, turbidity in the extraction solutions was removed by 5 min centrifugation at 300 rpm (Eppendorf 5810R centrifuge, VWR International, Murrarie, Australia). Using micropipettes, aliquots of solutions were taken from the falcon tubes and diluted depending on the strength of the colouration. Transmission properties of the solutions were measured across 450 to 700 nm in triplicate in disposable cuvettes against clean methanol in a UV 1800 Shimadzu spectrophotometer (VWR International, Murrarie, Australia; 1 cm bandwidth). Cuvettes were closed with parafilm to reduce rapid methanol evaporation possibly causing sample bias or risk of inhalation. If necessary, solutions were further diluted with methanol, always taking note of volumes used. After determining that measurements were successful, cuvettes and remaining solutions were decanted for evaporation, without loss of bottom sediment. Open falcon tubes, paired with each respective lid, were left to evaporate, and then dried 24 h at 80°C, closed, allowed to cool and weighed to obtain the dry weight after extraction (DW, dry weight minus pigments in solution). The dry weight is considered to be the more accurate reference than wet weight. This process was repeated with a second set of the remaining material, duplicating the process.

Pigment concentrations for chlorophyll *a* and *b* and for carotenoids were quantified according to Wellburn (1994) referring to the extraction volumes and dilutions and using the post-freezing wet weights and the post-extraction dry weights as reference. Respective values are considered to be underestimates and conservative, because samples were not directly snap-frozen after sampling. Means of means were calculated, then recalculated omitting all original values that remained significantly lower than 80% of the highest values, thus reducing the bias due to delayed freezing.

Genomic DNA extraction

Approximately 0.5 g of each of the three specimens was extracted for genomic DNA using the ammonium acetate bead beating method as described by Peterson *et al.* (2004). Briefly, 1 mL of extraction buffer (400 mL 6.25 M ammonium acetate; 100 mL Tris with pH 8.0; 40 mL 0.5 M EDTA; 460 mL molecular grade water) was added to a polypropylene tube containing 200 mL of silica beads (Daintree Scientific Products, St. Helens, Australia), 0.015 g PVPP, 300 mL of chloroform:isoamyl alcohol (24:1), and the sample. Bead-beating was performed in an Oscillating Mill MM 200 (Retsch, Haan, Germany), followed by centrifugation (30 min, 15,000 g, 4°C) and collection of the supernatant. This was then precipitated overnight (4°C) with 0.3 M sodium acetate and isopropanol, followed by centrifugation for 30 min (15,000 g, 4°C). Pellets were washed twice with 70% ethanol, dried, and resuspended in 30 µL molecular grade water.

16S gene targeted PE Illumina sequencing

Extracted DNA was quantified and adjusted to 20 ng µL⁻¹. All samples were PCR amplified with universal bacterial primers (27F/519R with barcode on the forward primer) targeting the 16S rRNA gene V1–V3 variable region. PCR primers were used in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following

conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. After amplification, PCR products were checked on a 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples (100 samples) were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. The pooled and purified PCR product was used to prepare DNA libraries by following the Illumina TruSeq DNA library preparation protocol. Sequencing was performed at Molecular Research Laboratories (Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. The r1 and r2 files for amplicons >300 bp and <570 bp were joined at Molecular Research Laboratories (Shallowater, TX, USA). Resulting data were subsequently denoised and processed using the MOTHUR MiSeq standard operating procedure <http://www.mothur.org> (Kozich *et al.*, 2013). In summary, sequences <463 bp and sequences containing ambiguous base calls or homopolymer runs above 8 bp were removed. Sequences were aligned with the SILVA 16S rRNA alignment (Pruesse *et al.*, 2007), unique sequences identified and operational taxonomic units (OTUs) (97% similarity) were defined. Chimeric artefacts were also removed using UCHIME (Edgar *et al.*, 2011). Any sequences that were classified as mitochondria or eukaryotic as well as any sequences of unknown origin were filtered out of the dataset. Final OTUs were taxonomically classified using BLASTn against a curated database derived from greengenes, RDP II and NCBI (<http://www.ncbi.nlm.nih.gov>; DeSantis *et al.*, 2006; Wang *et al.*, 2007). Validity for the identification of photosynthetic microbial taxa was confirmed in Guiry & Guiry (2014). The MiSeq dataset was deposited in the NCBI Sequence Read Archive (SRA) database with the accession number SRR1802581.

Phylogenetic analysis

All sequences were further analysed by comparison with the sponge ARB database (Ludwig *et al.*, 2004; Simister *et al.*, 2012) which contains over 7500 16S rDNA sequences obtained from sponges. Prior to importing into ARB, sequences were aligned using the SINA sequence aligner (Pruesse *et al.*, 2012). Alignments were manually corrected within the ARB environment, and phylogenetic analyses were done using 880 unambiguously aligned nucleotide positions. Neighbour joining, maximum likelihood and maximum parsimony methods were performed, and bootstrap proportions from 1000 resamplings were calculated using the maximum likelihood/rapid bootstrapping tool within the CIPRES science gateway (http://www.phylo.org/sub_sections/portal/) and viewed using the interactive tree of life (<http://itol.embl.de>) (Letunic & Bork, 2006, 2011).

Analysis of phospholipid derived fatty acids (PLFAs)

PLFAs were extracted from 1.9 g of lyophilized, defrosted sponge tissue by the Bligh–Dyer method (modified; Bligh & Dyer, 1959; Lengger *et al.*, 2012), which, different from methods using only organic solvent, allows extraction of polar lipids by using phosphate buffer and organic solvents

in miscible amounts. For this, 15 mL of a mix of methanol/dichloromethane were added to the ground tissue (DCM/phosphate buffer 100 mM, pH 7.4, 2:1:0.8), ultrasonicated for 15 min and centrifuged at 2000 rpm for 3 min. The supernatant was collected, two more extractions were performed and the supernatants united. The solvent ratio was adjusted to methanol/DCM/phosphate buffer (100 mM, pH 7.4) 1:1:0.9 in order to separate the mixture into an aqueous and a DCM phase, with the DCM phase containing the lipids. The DCM phase was collected, and the liquid/liquid extraction on the methanol/phosphate buffer phase was conducted twice more. The combined DCM phases were reduced by rotary evaporation, filtered over cotton wool in DCM/methanol 9:1 and dried under a stream of nitrogen. The phospholipids (PL) were separated from other lipids by gravity column chromatography on 0.8 g of activated (130°C) 60 mesh silica (Guckert *et al.*, 1985; Heinzelmann *et al.*, 2014). Elution was performed with 4 mL of DCM, 4 mL of acetone and 10 mL of methanol (containing the PL). This fraction was blow-dried under a gentle nitrogen purge and saponified in order to release the fatty acids bound to the glycerol in methanolic KOH (2 N) for 2 h at 80°C under reflux. 2 mL of MilliQ water were added, the pH adjusted to 3, and the PLFA derived fatty acids were extracted from the aqueous phase with hexane, followed by DCM (three times). Fatty acids were methylated for 20 min in 2 mL 30% BF₃-methanol at 70°C. The reaction was stopped by adding 1 mL of MilliQ water. The fatty acid methyl esters were extracted from the methanol/water phase three times with 2 mL of hexane and diluted to an approximate concentration of 1 mg mL⁻¹ for gas chromatography-mass spectrometry (GC-MS), 100 (δ²H) and 10 mg mL⁻¹ (δ¹³C) for GC coupled to isotope ratio mass spectrometry (GC-IRMS).

Phospholipid-derived fatty acid methyl esters (PLFAME) were identified by GC-MS on a Hewlett Packard 6890 gas chromatograph (Palo Alto, CA, USA) equipped with a 6890 autosampler and a splitless injector, interfaced to a Hewlett Packard 5973 mass selective detector (Palo Alto, CA, USA). The gas chromatograph was fitted with a 60 m DB5-MS column with 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA). Helium was used as a carrier gas at a constant flow rate of 1.1 mL min⁻¹. One μL sample injections were added and heated to 320°C, with the oven temperature being raised from 40 to 320°C at a rate of 3 °C min⁻¹. The transfer line was maintained at 290°C with mass selector operating with a solvent delay of 3 min in full scan mode for a mass range of 50 to 500 Daltons and held isothermally at 320°C (15 min). Quantification was achieved by GC coupled to flame ionization detection (FID) on a Hewlett Packard 6890 gas chromatograph fitted with FID detection under the same chromatographic conditions as GC-MS (with exception of the He flow which was increased to 1.8 mL min⁻¹). δ¹³C and δ²H values were determined on a ThermoScientific Trace GC Ultra (Waltham, MA, USA), equipped with a GC IsoLink with a combustion furnace at 1000°C (δ¹³C) or a high-temperature conversion reactor at 1400°C (δ²H), and the produced CO₂/H₂ was analysed in a ThermoScientific Delta V Advantage isotope ratio mass spectrometer (Waltham, MA, USA). Chromatographic conditions were identical to GC-MS conditions. The isotopic values were calculated by integrating the mass 44, 45 and 46 ion currents (δ¹³C) and 1, 2 and 3 ion currents of the peaks (δ²H), and comparison to CO₂/H₂ reference gas spikes with a known

$^{13}\text{C}/^{12}\text{H}$ content (Dawson *et al.*, 2007). Performance was monitored by regular injections of an n-alkane standard (A. Schimmelmann, University of Indiana, IL, USA), and values were reported in comparison to the references Vienna Mean Standard Ocean Water (V-SMOW) and Vienna Pee Dee Belemnite (V-PDB) and were corrected for the added carbon during methylation, however, $\delta^2\text{H}$ values of the added methyl could not be determined. Standard deviations were between 0.1 and 1.1‰ for $\delta^{13}\text{C}$ (four analyses) and between 1 and 51‰ for $\delta^2\text{H}$ (in duplicate). Fatty acids are reported with their carbon number, followed by a colon and the number of double bonds. Methyl branched fatty acids are preceded by *i* for *iso* and *a* for *anteiso* acids, referring to the position of the branching (terminal/second-to-terminal).

PLFA analysis isolates the phospholipids and other intact polar lipids (Heinzelmann *et al.*, 2014), and provides the lipid profile of the lipids present in active, live cell membranes only. Symbionts and sponge tissue, in this case, were not separated before extraction, thus the lipid profile represents a mixture of these organisms.

RESULTS

Photopigments

Leucetta prolifera was associated with photosynthetic symbionts. Frozen sponge tissue generated weak fluorescence when checked with pulse-amplitude modulated fluorometry. Methanol extractions and photometry resulted in curves typical for photosynthetic organisms, with clear peaks at 666 nm for chlorophyll *a* (chl; Figure 1). Pigment concentrations varied widely, as expected when tissue samples are not immediately frozen (Table 1, Figure 1B). After correcting the data by omitting all values significantly below 80% of the highest measures per pigment, we concluded that *L. prolifera* had average pigment concentrations of $112.7 \mu\text{g g}^{-1}$ WW or $406.2 \mu\text{g g}^{-1}$ DW for chl *a*, $12.5 \mu\text{g g}^{-1}$ WW or $47.2 \mu\text{g g}^{-1}$ DW for chl *b*, and $9.2 \mu\text{g g}^{-1}$ WW or $42.8 \mu\text{g g}^{-1}$ DW for carotenoids, or 0.48, 0.06 and 0.04% of the DW, respectively (these values may represent underestimates).

Olive-green colour in life, a pink aqueous solution from the samples and a light green solution and light pink tissue and sediment at the end of the methanol extraction (Appendix 1) suggested an association with cyanobacteria, and the samples were further subjected to molecular analysis of the symbionts.

Molecular analysis of the microbial community

A total of 102,229 high-quality sequences (> 300 bp, median length 521 bp) were obtained from the three sponge specimens, with an average of $34,076 \pm 7854$ sequences per sample. *Leucetta prolifera* contained representatives from 25 different bacteria phyla, with 61.5% ($\pm 7.6\%$) of sequences belonging to the Proteobacteria, 17.5% ($\pm 3.5\%$) to the Bacteroides, 16.7% ($\pm 9.1\%$) belonging to the Cyanobacteria and 1.5% ($\pm 1.5\%$) to the Actinobacteria. All other phyla contributed with less than 1% to the total sequences (Figure 2). 20 OTUs (97% sequence similarity) fell into the phylum Cyanobacteria, with the two most abundant cyanobacterial OTUs (OTU54: 7549 sequences, $32.7 \pm 22.0\%$ of the Cyanobacteria; and OTU836: 4665 sequences, $22.8 \pm 11.2\%$ of the Cyanobacteria) most closely related to sequences within the order Oscillatoriales and family Rivulariaceae (Table 2). Phylogenetic analysis placed both of these sequences adjacent to sequences from the cyanobacterium *Hormosilla spongelliae* (Gomont) Anagnostidis & Komárek, 1988 (frequently published as *Oscillatoria*; Figure 3). Cyanobacterial sequences were also acquired from the Chroococcales, Pleurocapsales, Cyanobacteriaceae, and Nostocales. 3114 chloroplast sequences were also obtained. These were placed into 24 OTUs that fell within the Bacillariophyceae (diatoms; 13 OTUs) and the three phyla Chlorophyta (green plants; 6 OTUs), Streptophyta (land plants, 3 OTUs) and Cryptophyta (2 OTUs) (flagellated phytoplankton; Table 2).

Phospholipid derived fatty acids and their isotopic values

The main fatty acids in the *L. prolifera* sample making up the PLFA fraction were identified and semi-quantified in their

Table 1. *Leucetta prolifera* pigment concentrations for three specimens measured twice.

Specimen no.	Measurement	Total chl (μg) per WW sponge (g)			Total chl (μg) per DW sponge (g)			% chl per DW sponge (g)		
		chl <i>a</i>	chl <i>b</i>	Carotenoids	chl <i>a</i>	chl <i>b</i>	Carotenoids	chl <i>a</i>	chl <i>b</i>	Carotenoids
1	1	(78.083)	(8.693)	9.765	(326.737)	(36.376)	40.862	(0.307)	(0.034)	0.038
	2	107.508	11.633	10.697	449.404	48.628	44.716	0.503	0.054	0.050
	Mean	(92.796)	(10.163)	10.231	(388.070)	(42.502)	42.789	(0.405)	(0.044)	0.044
2	1	117.856	14.866	(3.360)	362.995	45.786	(10.348)	(0.311)	(0.039)	(0.009)
	2	(79.652)	11.926	(0.000)	(212.147)	(31.764)	(0.000)	0.459	0.069	(0.000)
	Mean	(98.754)	13.396	(1.680)	(287.571)	(38.775)	(5.174)	(0.385)	(0.054)	(0.004)
3	1	(67.344)	(7.287)	(7.004)	(233.456)	(25.261)	(24.281)	(0.181)	(0.020)	(0.019)
	2	(77.808)	(8.663)	8.110	(255.724)	(28.470)	(26.655)	(0.188)	(0.021)	(0.020)
	Mean	(72.576)	(7.975)	(7.557)	(244.590)	(26.866)	(25.468)	(0.185)	(0.020)	(0.019)
1–3	Overall mean	(88.042)	(10.511)	(6.489)	(306.744)	(36.048)	(24.477)	(0.325)	(0.039)	(0.023)
1–3	Recalculated	112.682	12.514	9.171	406.199	47.207	42.789	0.481	0.062	0.044
	overall means									

Means were recalculated omitting values in parentheses that were significantly below 80% of the highest individual pigment concentrations measured (in bold).

Chl, chlorophyll; WW, sponge wet weights after freezing at -80°C ; DW, sponge dry weights after extraction.

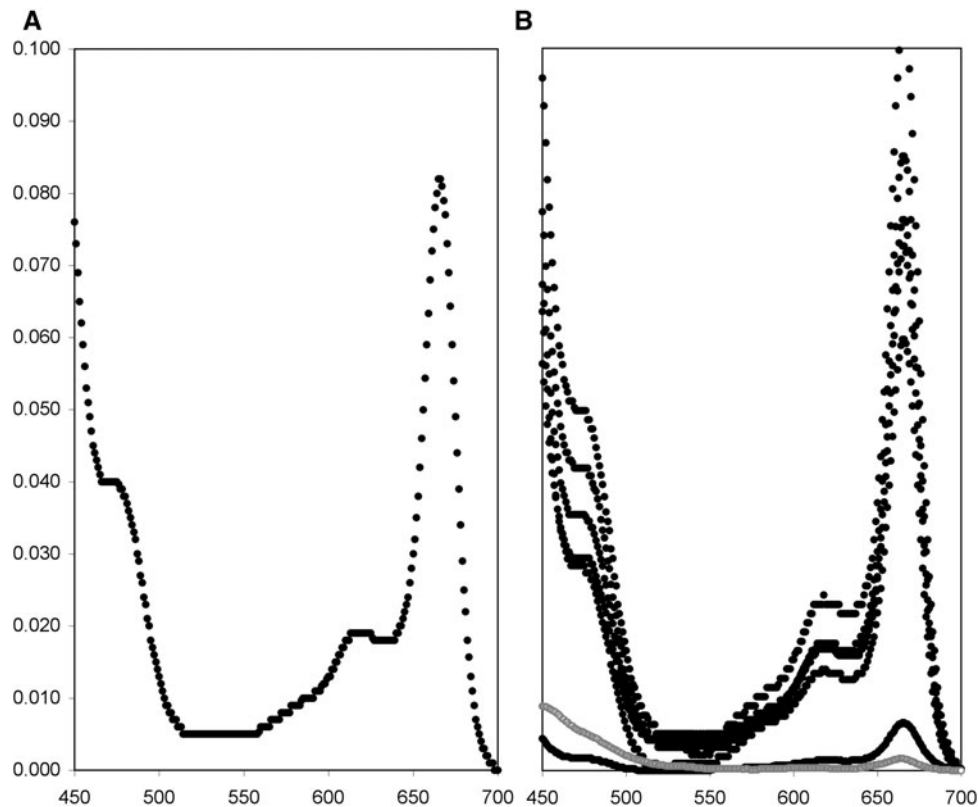


Fig. 1. Transmission spectra for pigments extracted from Western Australian *Leucetta* spp. (A) *Leucetta prolifera* spectrum for one of the darkest pieces of tissue. (B) After multiplying all transmission data with the extraction volume and dividing by dry weight, spectra from different measurements become comparable but are not realistic, so the scale on the y-axis was omitted. Black markers are for *L. prolifera* (two extractions for three specimens), while grey markers represent results from *Leucetta* sp. from the Onslow area in north-west Australia (Schönberg *et al.*, unpublished data).

esterified form. The majority consisted of straight chain, saturated hexadecanoic acids (C_{16:0}, 33.1%) and the terminally methylated *i*-C_{17:0} (19.7%; Figure 4A). Other PLFA present in considerable amounts were 4,8,12-trimethyltridecanoic acid (TMTD), *i*C_{15:0}, C_{16:1} (mix of double bonds at C-6 and C-9), *a*-C_{17:0}, and *a*-C_{19:0} (all between 2–9%), and traces of other branched and straight chain, unsaturated and saturated fatty acids. Isotope values of the identified, specific fatty acids were determined: $\delta^{13}\text{C}$ values ranged from -21.5 to -39.4‰ (Figure 4B), with C_{16:0} and C_{16:1} being the most strongly depleted fatty acids (-32.3 and -36.1‰ , respectively). C_{18:1} also showed depleted values (-34.6‰), but was eluting close to many other peaks, and its values might have been compromised. Observed $\delta^2\text{H}$ values ranged from strongly depleted (-420‰) to enriched (70‰ , Figure 4B), with the most depleted fatty acid being TMTD (-420‰).

DISCUSSION

Identification of the photosymbiont

In the present study we confirmed by pigment, molecular and isotopic analyses that *Leucetta prolifera* is associated with photosynthetic cyanobacteria. Sequencing results showed that over 16% of all retrieved bacterial sequences belonged to the phylum Cyanobacteria, with the two dominant OTUs most closely related to *Hormoscilla spongelliae*. *Hormoscilla spongelliae* has previously been reported from the chondrosid demosponge *Chondrilla australiensis* Carter, 1873 from

south-western Australia, as well as from many sponges in other regions around the globe (e.g. Lemloh *et al.*, 2009). Interestingly, while the phylogenetic analysis associated both of the two abundant OTUs (OTU54 and OTU836) with *H. spongelliae*, the greengene comparison placed only the most abundant taxon OTU54 with *H. spongelliae*, and OTU836 within the genus *Calothrix*, another cyanobacterium genus. Members of this genus are capable of nitrogen fixation (Eichner *et al.*, 2014) and have previously been reported from other sponges, e.g. in diseased tissue of *Carteriospongia foliascens* (Pallas, 1766) (Gao *et al.*, 2014).

It appears that photosymbiosis is more common and symbiont diversity is higher in demosponges than in calcareous sponges (Díaz, 1999; Díaz *et al.*, 2007; Thacker & Freeman, 2012). Apart from Vicente's (1990) account of diatoms in *Clathrina* sp., all other records on photosymbionts in Calcareia are of cyanobacteria (see dot points below). Occurrence of diatoms in sponge tissue is common, but may represent parasitism rather than mutualism, or a supplement to the sponge diets during times of stress or limitation (Vicente, 1990; Bavestrello *et al.*, 2000; Cerrano *et al.*, 2000, 2004; Cárdenas & Rapp, 2013).

Díaz (1999) recognized three of 17 families of the Calcareia to contain cyanosponges (Thacker & Freeman, 2012). Díaz (1999) was later incorrectly cited that cyanobacteria are found in 17 families of Calcareia (Hentschel *et al.*, 2006; Usher, 2008; Zhu *et al.*, 2008; Caroppo *et al.*, 2012). Presently, with reports of cyanobacterial metabolites in a jenkinsid sponge (D'Ambrosio *et al.*, 1999; Kartika, 2008), this number of three families in the Calcareia with cyanosponges

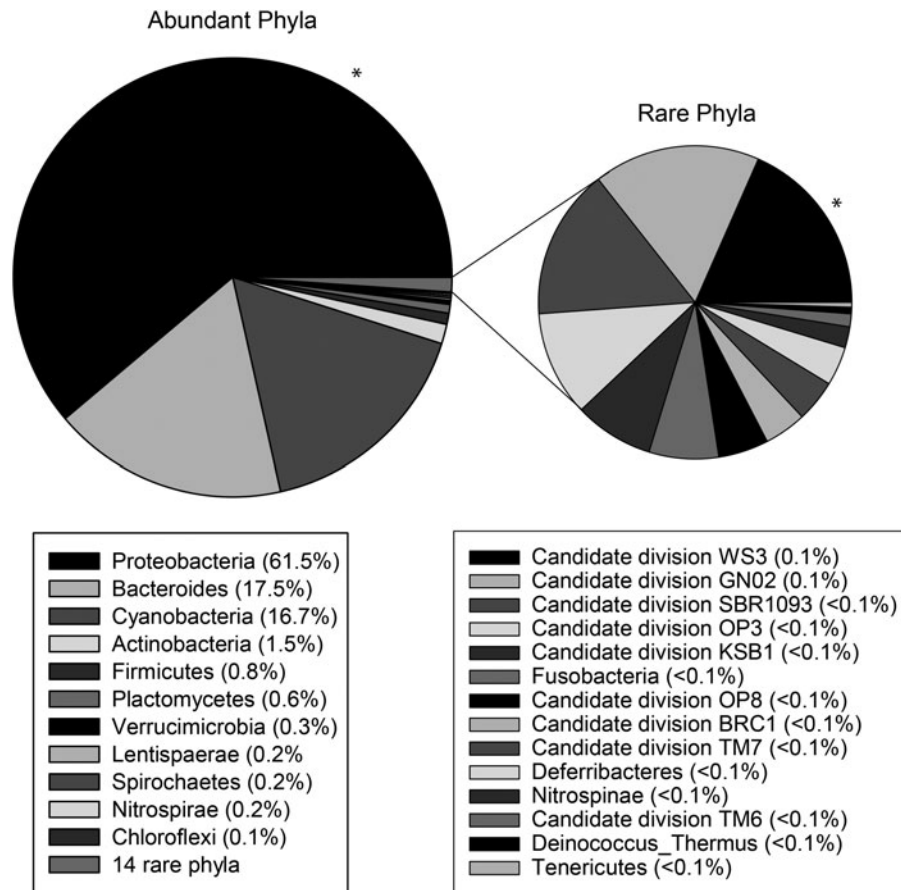


Fig. 2. Mean relative abundance (% of all sequences) of bacterial phyla within *Leucetta prolifera*. Phyla are listed in the legend from most abundant to least abundant and are displayed in the graph in an anticlockwise direction, with most abundant (black, indicated with *) shown in the top right.

can be extended to four for sponges identified at minimum to genus:

- Sycettidae (*Sycon* sp.; Feldmann, 1933)
- Clathrinidae (*Borojevia cerebrum* in Burlando *et al.*, 1988, as *Clathrina*; *Clathrina* sp. *sensu* Wilkinson, 1980, may be *B. cerebrum* as well, as also sampled from the Mediterranean)
- Leucettidae (*Leucetta* sp. *sensu* Díaz *et al.*, 2007; *Leucetta prolifera*, present publication; possibly *Leucetta* sp. *sensu* Schönberg *et al.*, unpublished data, see below – this species differs from *L. prolifera*; *Pericharax heterorhaphis* in Wilkinson, 1978a, b, 1980, 1982, 1983; Wilkinson *et al.*, 1984)
- Jenkinidae (*Leucascandra caveolata*; D'Ambrosio *et al.*, 1999; Kartika, 2008)

Photosynthetic activity in *L. prolifera*

Leucetta prolifera occurs in full light or shaded positions on shallow, exposed rocky temperate reefs. It is common beneath macroalgal canopies, and is incompressible and brittle with a growth form of low vertical ridges with apical oscules (Fromont, personal observation). The presence of photosymbionts in this sponge may aid its ability to coexist with macroalgae, and its very resistant low growth form may protect it from wave surge and macroalgal movement on exposed reefs. Western Australia is renowned for high biomass, high productivity temperate shallow benthic

ecosystems, yet is bathed in the relatively low nutrient, southward flowing Leeuwin Current (Fromont *et al.*, 2013). Photosymbiosis in *L. prolifera* may increase the ability of the sponge to survive in this low nutrient environment, with the clear, warm Leeuwin current waters assisting the growth of the light requiring symbionts. Indeed, Webster & Taylor (2012) noted that sponges with symbiotic cyanobacteria may become net primary producers.

Fluorescence generated by the frozen samples was low and not further quantified, but pigment analyses confirmed that *L. prolifera* contained photopigments. Corrected mean values for chl *a* concentrations in *L. prolifera* reached 113 µg per g wet sponge tissue, a value considered intermediate compared with a wide range of other sponges (Erwin & Thacker, 2007). The only other study available on pigment extraction from calcarean sponges found much lower values in *P. heterorhaphis*, i.e. 2.8 µg per g sponge tissue (Wilkinson, 1982).

Coincidentally, another *Leucetta* species was recently sampled during a thermal event in March 2013 (Lafratta *et al.*, unpublished data; Schönberg *et al.*, unpublished data), in 11.2 m depth near Onslow, north-western Australia. This sponge was almost white in life with a green tinge throughout and was assumed not to be photosynthetic. Yet, when the fresh sample was screened post dive with pulse-amplitude modulated fluorometry as described above, the sponge contour and area were faintly visible as red dots in the image obtained for maximum fluorescence yield. The low signal was initially interpreted as background noise, or

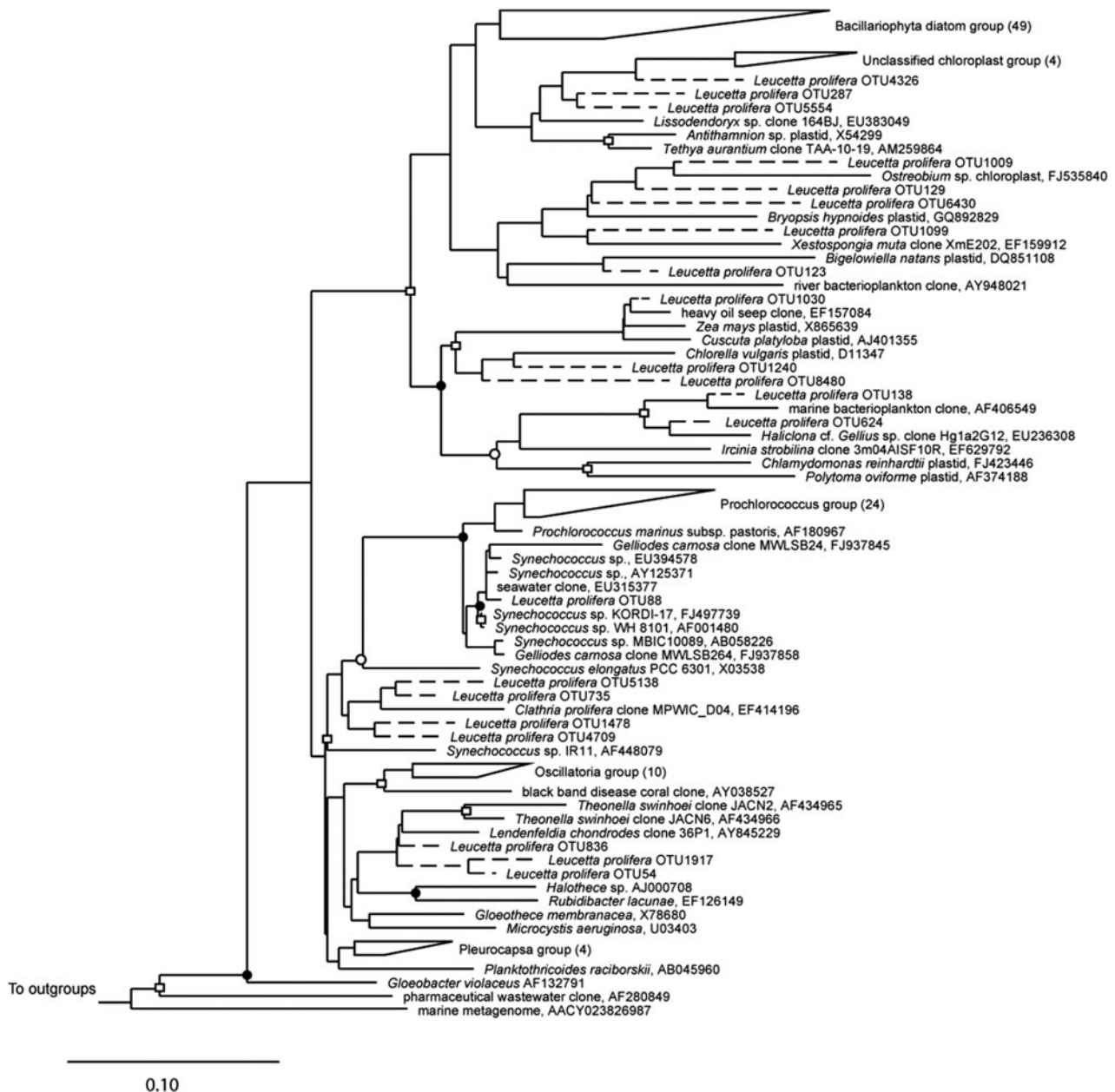


Fig. 3. 16S rRNA phylogeny of sequences that fell within the phylum Cyanobacteria and neighbouring chloroplasts. Sequences were aligned with selected sequences from the Cyanobacteria tree from the sponge ARB database (Simister *et al.*, 2012). The tree shown is a maximum likelihood tree based on long sequences (>1000 bp); shorter sequences were added using the parsimony interactive tool in ARB and are indicated with a dashed line, following Simister *et al.* (2012). Bootstrap values were calculated using the CIPRES science gateway (<http://www.phylo.org/subsections/portal/>). Symbols indicate bootstrap support as follows: filled circles 60–70, open circles 80–90, open squares >90. Bar signifies 10% divergence. Numbers in parentheses represent number of sequences within wedges.

reflections off the sponge surface. When extracting a subsample, this sponge still yielded pigment concentrations of $1.61 \mu\text{g g}^{-1}$ WW or $6.84 \mu\text{g g}^{-1}$ DW for chl *a*, $0.46 \mu\text{g g}^{-1}$ WW or $1.96 \mu\text{g g}^{-1}$ DW for chl *b*, and $1.91 \mu\text{g g}^{-1}$ WW or $8.12 \mu\text{g g}^{-1}$ DW for carotenoids, or 0.01, <0.01 and 0.01% of the DW respectively, and the respective transmission spectrum had a very low but discernible peak at 666 nm, suggesting remnant presence of chl *a* (Figure 1). Because the green sheen was uniform, the sponge may have been bleached rather than the results being due to contamination, e.g. through algal material held in choanocyte chambers.

Many symbioses develop as a result of feeding activities, and photosymbiotic calcarean sponges such as *Leucetta* spp. may

originally have obtained their symbionts by filtering them from seawater. Perea-Blázquez *et al.* (2010) found that *Leucetta* sp. from New Zealand feeds on bacterioplankton, of which the photosynthetic *Prochloron* and *Synechococcus* spp. were retained at higher efficiency than heterotrophic bacteria. Lemloh *et al.* (2009) determined that symbionts in the unidentified south-western Australian *Calcarea* species had 98.8% similarity to a free-living *Synechococcus*. Wilkinson (1980) demonstrated that sponges can differentiate between non-symbiotic and symbiotic bacteria offered as food, and Hill & Hill (2012) suggested that where sponges receive nutrients from symbiotic associations, the symbionts have significant control over invading a host, and preventing host digestion.

Table 2. Taxonomic classification of sequences from *Leucetta prolifera* used in Figure 3. Classifications were assigned using the RDPII, NCBI Taxonomy and greengenes browser (<http://www.ncbi.nlm.nih.gov>, DeSantis et al., 2006; Wang et al., 2007).

OTU identifier	Phylum	Genus	Number of sequences per OTU per sample (3 samples)		
OTU54	Cyanobacteria	<i>Hormoscilla</i>	232	5948	1369
OTU10	Cyanobacteria	<i>Hormoscilla</i>	61	11	7
OTU4326	Cyanobacteria	<i>Hormoscilla</i>	0	24	0
OTU19179	Cyanobacteria	<i>Hormoscilla</i>	0	1	2
OTU4298	Cyanobacteria	<i>Hormoscilla</i>	1	0	0
OTU735	Cyanobacteria	<i>Leptolyngbya</i>	0	3	0
OTU836	Cyanobacteria	<i>Calothrix</i>	238	3302	1125
OTU5554	Cyanobacteria	<i>Calothrix</i>	0	0	18
OTU88	Cyanobacteria	<i>Prochlorococcus</i>	1077	249	706
OTU311	Cyanobacteria	<i>Prochlorococcus</i>	20	18	1
OTU235	Cyanobacteria	<i>Prochlorococcus</i>	0	0	1
OTU9203	Cyanobacteria	<i>Synechococcus</i>	0	9	0
OTU1478	Cyanobacteria	<i>Pleurocapsa</i>	0	2	0
OTU5138	Cyanobacteria	<i>Chroococcidiopsis</i>	0	1	0
OTU4709	Cyanobacteria	<i>Pleurocapsa</i>	0	0	1
OTU17782	Cyanobacteria	<i>Crocospaera</i>	0	4	0
OTU17282	Cyanobacteria	<i>Pleurocapsa</i>	0	3	0
OTU1028	Cyanobacteria	<i>Pleurocapsa</i>	0	0	1
OTU8012	Cyanobacteria	<i>Anabaena</i>	0	0	10
OTU15126	Cyanobacteria	<i>Desmonostoc</i>	5	0	0
OTU2105	Bacillariophyta	<i>Bacillariophyta</i>	0	58	0
OTU20387	Bacillariophyta	<i>Bacillariophyta</i>	25	2	0
OTU3604	Bacillariophyta	<i>Bacillariophyta</i>	0	31	0
OTU6431	Bacillariophyta	<i>Bacillariophyta</i>	0	15	0
OTU5190	Bacillariophyta	<i>Bacillariophyta</i>	0	13	0
OTU2702	Bacillariophyta	<i>Bacillariophyta</i>	0	7	0
OTU13957	Bacillariophyta	<i>Bacillariophyta</i>	0	0	5
OTU15712	Bacillariophyta	<i>Bacillariophyta</i>	0	0	4
OTU5565	Bacillariophyta	<i>Bacillariophyta</i>	4	0	0
OTU2728	Bacillariophyta	<i>Bacillariophyta</i>	0	1	0
OTU5093	Bacillariophyta	<i>Bacillariophyta</i>	1	0	0
OTU6297	Bacillariophyta	<i>Bacillariophyta</i>	0	0	1
OTU129	Bacillariophyta	<i>Chlorarachniophyceae</i>	0	1	1
OTU1099	Streptophyta	<i>Chaetosphaeridium</i>	91	33	15
OTU1240	Streptophyta	<i>Spirogyra</i>	71	18	22
OTU1030	Streptophyta	Unidentified	0	16	46
OTU138	Chlorophyta	Unidentified	42	0	51
OTU624	Chlorophyta	Unidentified	0	80	0
OTU6430	Chlorophyta	<i>Sherffelia</i>	0	16	0
OTU8480	Chlorophyta	<i>Sherffelia</i>	0	10	0
OTU123	Chlorophyta	<i>Sherffelia</i>	1	1	0
OTU1009	Chlorophyta	<i>Sherffelia</i>	1	0	0
OTU72	Cryptophyta	Unidentified	0	21	1
OTU74	Cryptophyta	Unidentified	1	2	0

Possible transfer of photosynthate as determined by stable isotope analysis of PLFAs

The lipid profile generated from *L. prolifera* was in close agreement with other profiles from *Calcarea*, and specifically *Leucetta* species (Schreiber et al., 2006). Different classes of fatty acids can be specific to the organisms in which they occur. The terminally branched methyl fatty acids (*iso* and *anteiso*) found in our specimens are unusual in eukaryotes, and are generally considered to be of bacterial provenance. However, they appear to be absent from the often sponge-associated α -proteobacteria (Kaneda, 1991; Pape, 2004; Schreiber et al., 2006; Grice et al., 2008), are only present in trace amounts in cyanobacteria (Cohen & Vonshak, 1991) and can thus be considered to be sponge-specific. This was confirmed by Schreiber et al. (2006), who

found *iso* and *anteiso* fatty acids in segments of sponge tissue without symbionts (in particular *i*-C17:0 and *a*-C19:0 fatty acids). C16:0, C16:1, C18:0 acids, which occurred in large amounts, are rather unspecific. However, cyanobacteria such as the confirmed symbiont in *L. prolifera* are known to produce these (as well as other very common fatty acids with various double bonds, Cohen & Vonshak, 1991). In addition, some of these major fatty acid constituents could also stem from proteobacteria, a large and diverse bacterial group with members capable of autotrophy, and confirmed to be present in *L. prolifera* by sequencing (Figure 2). C16:0, C16:1 and C18:0 fatty acids are thus likely to represent a mixture of sponge and symbiont material.

Stable carbon isotopic composition ($\delta^{13}\text{C}$) of fatty acids has previously been used to investigate food webs and symbioses as it is linked to biosynthetic pathways and to the carbon

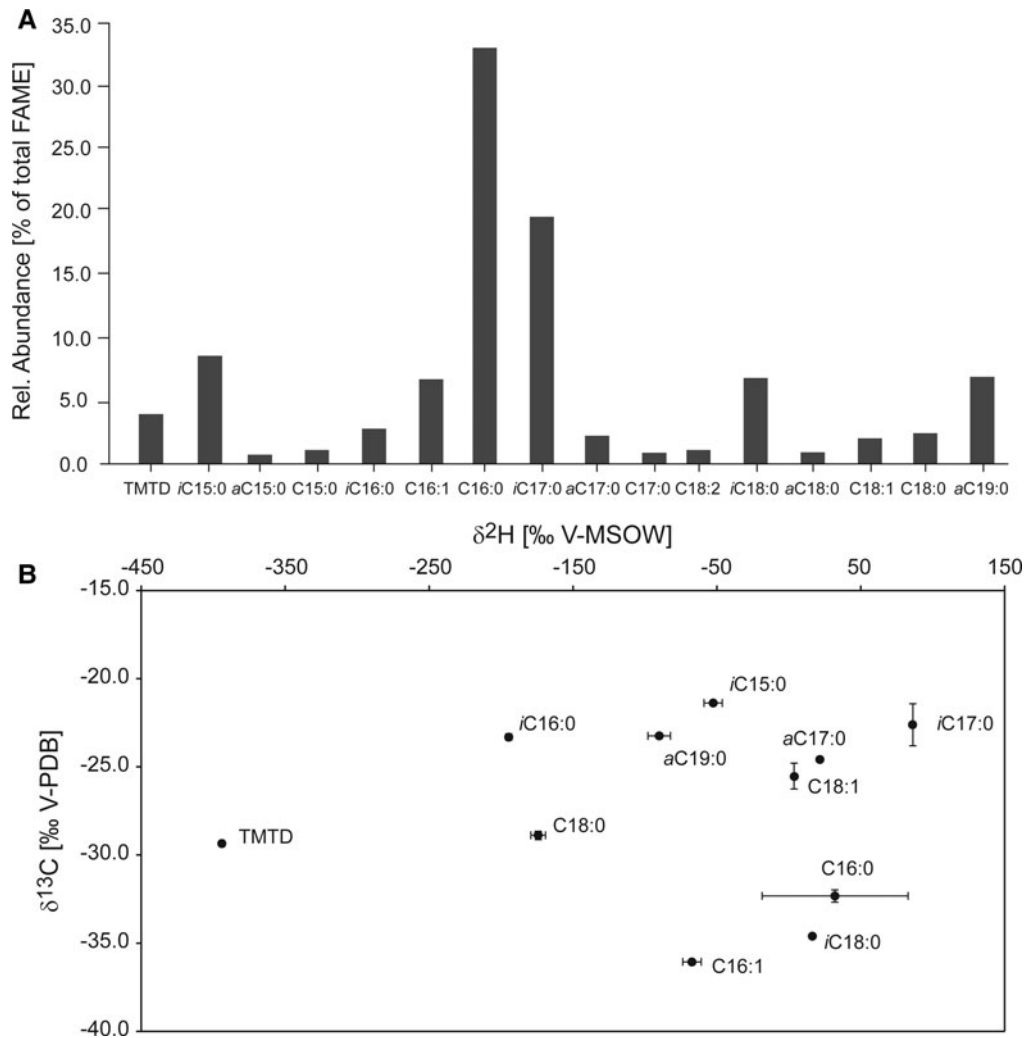


Fig. 4. Relative abundances and stable carbon and hydrogen isotopic composition of the PLFA extracted from *Leucetta prolifera*. (A) Relative abundances in per cent of total of the individual fatty acids analysed as methyl esters. (B) $\delta^{13}\text{C}$ in ‰ V-PDB vs $\delta^2\text{H}$ in ‰ V-MSOW show the distinct isotopic signatures of the individual PLFA. Standard deviations of triplicate measurements are displayed unless smaller than the data points.

metabolism of the producing organism (Schouten *et al.*, 1998). Here, the carbon isotope values of the methyl-branched fatty acids, expected to stem from the sponge, exhibited $\delta^{13}\text{C}$ values of around -22 to -25‰ . The carbon isotope values of the fatty acids which are probably partially derived from the symbionts were more depleted, with C16:0 at -32.3‰ and C16:1 at -36.1‰ , as well as C18:0 at -28.9‰ . While the $\delta^{13}\text{C}$ values of the biomass of heterotrophs are in general very similar to that of their carbon source (Grice *et al.*, 1998 and references therein), in a symbiosis where carbohydrates are expected to be transferred, such as here, the fatty acids in the host are isotopically heavier than the ones of the carbon-translocating cyanobacteria (Van der Meer *et al.*, 2003). This is due to differences in biosynthetic pathways, where carbohydrates are usually isotopically up to 15‰ heavier than fatty acids (Van Dongen *et al.*, 2002). This suggests that, while C16:0 and C16:1 may largely consist of autotrophically produced fatty acids, C18:0 most likely represents a mixture of the same compound produced by the sponge and by the cyanobacteria, and suggests that the sponge receives carbohydrates from its symbionts.

Transfer of organic compounds from the cyanobacteria to *L. prolifera* could only be partially confirmed by the hydrogen

isotopic composition of fatty acids ($\delta^2\text{H}$). It is highly influenced by the energy source of the producing organism (NADPH pathways), but has previously been shown to exhibit large variability between compounds even within one organism (Zhang *et al.*, 2009; Osburn *et al.*, 2011). In general, photoautotrophs exhibit fatty acids more depleted in hydrogen (-150 to -250‰), while heterotrophic growth produces comparatively ^2H -enriched fatty acids (-150 to $+200\text{‰}$). The $\delta^2\text{H}$ values of the sponge-derived fatty acids with methyl branching ranged from $+100$ to -200‰ , while the fatty acids of suspected cyanobacterial, or mixed, origin exhibited very variable $\delta^2\text{H}$ values (C16:0, C16:1 and C18:1, $+6$, -58 and -175‰ , respectively). However, factors influencing the hydrogen isotopic composition of fatty acids in symbiotic relationships are presently inadequately understood and did not allow definite conclusions. In addition, the presence of proteobacteria in the sponge could be responsible for some of the variation found in the isotope values.

A particularly intriguing isotopic composition was that of TMTD. It has been found before in the genus *Leucetta* and frequently occurs in other sponges of different classes (Calcarea, Demospongiae, Hexactinellida; Schreiber *et al.*, 2006).

However, it remained unclear whether its presence reflects the incorporation of chlorophyll degradation products or *de novo* biosynthesis by the sponge. The carbon and particularly the hydrogen isotopic composition measured here ($-29.4/-394\%$, respectively) clearly indicate that the source of TMTD in *L. prolifera* is the phytol of chlorophyll, which has been reported to be extremely depleted in ^2H (Chikaraishi *et al.*, 2009). Its $\delta^2\text{H}$ value is considerably lower than is typical for isoprenoid synthesis, suggesting that *L. prolifera* uses a chlorophyll derivative such as phytol from either ingested bacterioplankton or from its cyanobacterial symbiont, again indicating a possible transfer of compounds from the cyanobacteria to *L. prolifera*. Overall, the carbon and hydrogen isotopic composition of the phospholipid derived fatty acids indicate that *L. prolifera* uses carbohydrates produced photosynthetically by its cyanobacterial symbiont and that the TMTD present as a membrane lipid in this and other sponges is a chlorophyll degradation product, derived from ingested phytoplankton or the symbiont.

This study of *L. prolifera* provides new insights into temperate water sponge-symbiont relationships, when most such data are available from the tropics. To date and for calcarean sponges, Wilkinson's classic studies on sponge-microbial associations provide the only data on functional aspects of these photosymbioses (Wilkinson, 1978a, b, 1982; Wilkinson *et al.*, 1984). This highlights how understudied Calcareous species are in this regard in comparison to demosponges, an imbalance that needs to be addressed for species that are locally as common as *L. prolifera*.

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APPENDIX 1

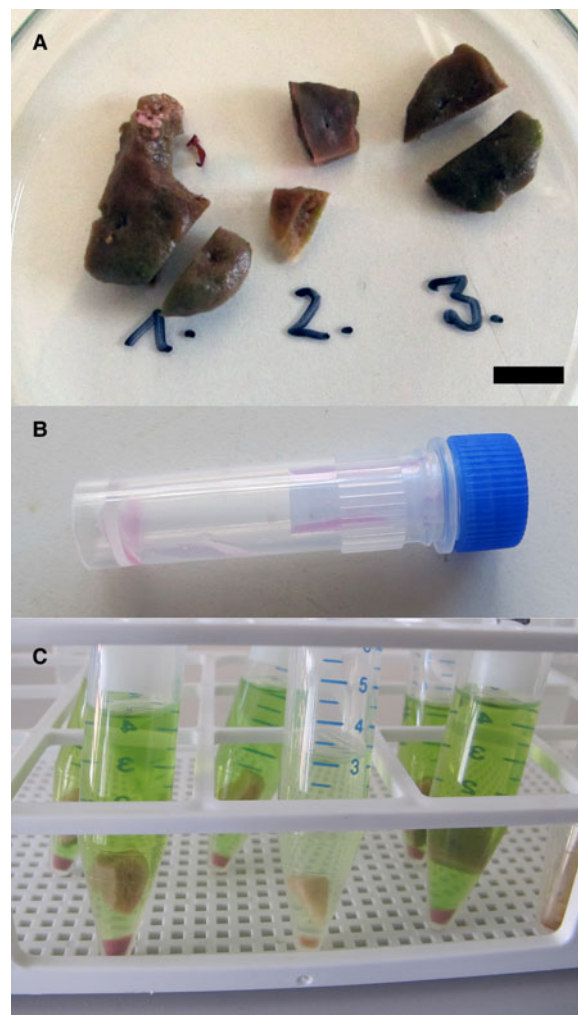


Fig. A1. Pre-analysis evidence for *Leucetta prolifera* harbouring cyanobacteria. (A) Olive-green colour of tissue, with pinkish hues. Note that the second piece of the second sponge is paler than the other pieces and obviously experienced pigment loss after sampling. (B) Aqueous solution in the cryovial shows the typical neon-pink colour of fluids that seep out of cyanosponges. (C) Samples after pigment extraction and centrifugation. The solutions are light green, while the tissue is now light pink, as is the sediment.