Supplementary Information

Influence of Intra-Skeletal Coral Lipids on Calcium Carbonate Precipitation

M. Reggi,[a] S. Fermani,[a] C. Samorì,[a, b] F. Gizzi,[c] F. Prada,[c] Z. Dubinsky,[d] S. Goffredo,[*, c] and G. Falini[*, a]

[a] *Dipartimento di Chimica "Giacomo Ciamician", Alma Mater Studiorum- Università di Bologna, via Selmi 2, 40126 Bologna, Italy.* [b] *Centro Interdipartimentale di Ricerca in Scienze Ambientali, Sede di Ravenna-Università di Bologna, via S. Alberto 163, 48100 Ravenna, Italy.* [c] *Marine Science Group, Department of Biological, Geological and Environmental Sciences, Alma Mater Studiorum - Università di Bologna, Via Selmi 3, I-40126 Bologna, Italy.* [d] *The Mina & Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 5290002, Israel*

Materials and Methods

Extraction of organic matrix and lipids

The samples of *B. europaea*, *A. calycularis* from Palinuro (Italian coast, North-Western Mediterranean Sea) and *S. pistillata* from the Gulf of Eilat (Red Sea, Israel) were randomly collected by scuba diving at 6, 9, 10 m depth respectively. Coral skeletons were cleaned, ground, and whole organic matrix (*w*OM) was extracted through decalcification using 0.1 M acetic acid solution as previously reported.[1] All *w*OMs were characterized by Fourier transform infrared spectroscopy (FTIR) using a Bruker Alpha FTIR spectrometer equipped with a single- reflection diamond ATR accessory working in the range of wavenumbers 4000-400 cm^{-1} at a resolution of 4 cm^{-1}.

After characterization, the lipid components (*lip*OM) were extracted from the *w*OM. To extract lipids, the *w*OM was dissolved in a DCM:MeOH solution (1:1 *v/v*) under stirring for four days. Then the solution was centrifuged and the supernatant was allowed to evaporate under a fume hood.

Lipid analysis

Powder of coral skeleton samples (about 50 mg) were extracted under reflux with chloroform/methanol mixture (2:1 v/v, 4 mL) for 1.5 h; the solvent phase was then removed and the procedure was repeated three times. The solvent phases were collected and concentrated by evaporation. The total fatty acids content was determined as follows: the lipid extracts were dissolved in dimethylcarbonate (0.4 mL), 2,2-dimethoxypropane (0.1 mL) and 0.5 M NaOH in MeOH (0.1 mL), and then placed in an incubator at 90 °C for 30 min. After cooling for 5 min to room temperature, 1.3 M BF3-methanol 10% (w/w) reagent (0.7 mL) was added before repeating the incubation for 30 min. After cooling for 5 min to room temperature, saturated NaCl aqueous solution (2 mL) and hexane (1 mL) containing methyl nonadecanoate (20 μg) were added and the samples were centrifuged at 4000 rpm for 1 min. The upper hexane-dimethylcarbonate layer, containing fatty acids, was transferred to vials for GC-MS analysis. The analyses were performed on 3 replicates of each coral.

GC-MS analyses were performed using a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP quadrupole mass spectrometer. The injection port temperature was 280 °C. Analytes were separated by a HP-5 fused-silica capillary column (stationary phase poly [5% diphenyl/95% dimethyl] siloxane, 30 m, 0.25 mm i.d., 0.25 μm film thickness), with helium as carrier gas (at constant pressure, 33 cm s^{-1} linear velocity at 200 °C). Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan s-1 within the 12-600 m/z range. The temperature of the column was increased from 50°C up to 180 °C at 50 °C min^{-1}, then from 180 °C up to 300 °C at 5 °C min^{-1}. Methyl nonadecanoate was used as internal standard for the quantification of each fatty acid, by assuming a unitary response factor.

All *lip*OMs were characterized by Fourier transform infrared spectroscopy (FTIR) using a Bruker Alpha FTIR spectrometer equipped with a single- reflection diamond ATR accessory working in the range of wavenumber 4000-400 cm^{-1} at a resolution of 4 cm^{-1}.

Dynamic light scattering was used for determination of the lipids particles size distribution employing a Malvern Nano ZS instrument with a 633 nm laser diode according to a reported procedure.[2]

Calcium carbonate crystallization experiments

Calcium carbonate ($CaCO_3$) *in vitro* crystallization experiments were carried out by vapour diffusion in a desiccator containing $(NH_4)_2CO_3$ (Carlo Erba) and anhydrous $CaCl_2$ (Sigma Aldrich). Microplates for cellular culture (MICROPLATE 24 well with Lid, IWAKI), containing a round glass cover slip in each well, were used as previously reported.[1] In each well, 750 µL of 10 mM $CaCl_2$ solutions ($CaCl_2$·dihydrate, Sigma-Aldrich) were poured. 0.2 mg of whole OM were added into wells. Lipid components (100 µg/mL) were dispersed directly in a 10 mM $CaCl_2$ solution. To obtained a homogeneous dispersion, the solutions were sonicated with a Falc ultrasonic UTA bath for 20 minutes. After that 750 µL of $CaCl_2$/lipids dispersion were added into wells. Microplates were located in desiccator for 4 days at room temperature. The obtained crystals were washed with milli-Q water and dried. $CaCO_3$ crystallization experiments were replicated at least four times. Lower concentrations of lipid dispersions did not affect the crystallization of $CaCO_3$.

Characterization of $CaCO_3$ precipitates was carried out by FTIR spectroscopy and microscopic observations. FTIR spectra of samples in KBr disks were collected at room temperature by using a Bruker Alpha FTIR spectrometer equipped with a single- reflection diamond ATR accessory working in the range of 4000-400 cm^{-1} at a resolution of 4 cm^{-1}. Optical microscope observations of $CaCO_3$ precipitates were carried out using a Leica microscope equipped with a digital camera. SEM observations were conducted using a Hitachi FEG 6400 scanning electron microscope after sample coating with gold.

Table ESI1: Assignment of organic compounds to bands on the FTIR spectra of *lip*OM; ν-stretching vibration, δ-bending vibrations, s-symmetric and as-asymmetric.

Wave number (cm^{-1})	Assignment
3422	ν(O–H) and ν(N–H): water molecules and choline
2956	$\nu_{as}(CH_3)$: lipids, cholesterol esters, fatty acids
2921	$\nu_{as}(CH_2)$: lipids, long-chain fatty acids
2872	$\nu_{s}(CH_3)$: lipids, fatty acids
2851	$\nu_{s}(CH_2)$: lipids, long-chain fatty acids
1735	ν (C=O): lipids, cholesterol esters, fatty acids oxides
1720	ν (C=O): COOH group of fatty acids
1685-1634	Amide I: ceramides
1578	$\nu_{as}(C=O)$: COO^- group of fatty acids
1540	$\nu_{s}(C=O)$: COO^- group of fatty acids
1470	$\delta(CH_2)$: aliphatic chains of fatty acids
1384	$\delta(CH_3)$: aliphatic chains of fatty acids
1206	$\nu_{as}(C–O)$: lipid ester bonds
1112	$\nu_{s}(PO_2)$: phospholipids
1026	$\nu_{s}(PO_2)$: phospholipids

The broad band detected around 3422 cm^{-1} was assigned to ν(O–H) and ν(N–H) of water molecules and choline, respectively. The bands assigned to asymmetric and symmetric stretching vibrations of the methyl and methylene groups were observed from 3050 to 2750 cm^{-1} and can be associated to fatty acids, lipids and cholesterol esters.[3-6] The maxima of the asymmetric, $\nu_{as}(CH_3)$, and symmetric bands, $\nu_{s}(CH_3)$, of the methyl groups were at 2956 and 2872 cm^{-1}, respectively; whereas asymmetric and symmetric bands of methylene groups, $\nu_{as}(CH_2)$ and $\nu_{s}(CH_2)$, occurred at 2921 and 2851 cm^{-1}. At 1735 cm^{-1} is located the band associated with stretching vibrations of the carbonyl group ν(C=O), usually due to ester bonds between fatty acids and glycerol. However, this bond can also be formed by peroxidation of fatty acid chains.[7] Subsequent bands 1685-1634 cm^{-1} were associated to amide vibration modes of ceramids.[8] The doublet at 1578 cm^{-1} and 1540 cm^{-1} corresponds to the asymmetric stretching vibration of carboxyl functional group. The bands at 1470 and 1384 cm^{-1} are assigned to bending vibrations of the methyl and methylene groups of fatty acids. The bands at 1206, 1112 and 1026 cm^{-1} can be assigned to a stretching vibration of the C–O group of lipid ester bond and to a stretching vibration of the phosphate group of phospholipids.[9]

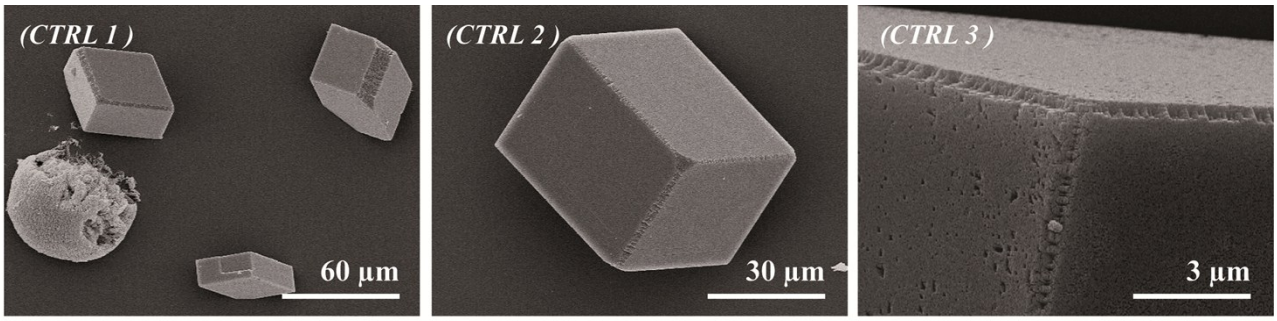

Figure ESI1. Scanning electron microscopy pictures at increasing magnification (1-3) of calcite crystals precipitated by the vapor diffusion method in the absence of additives.

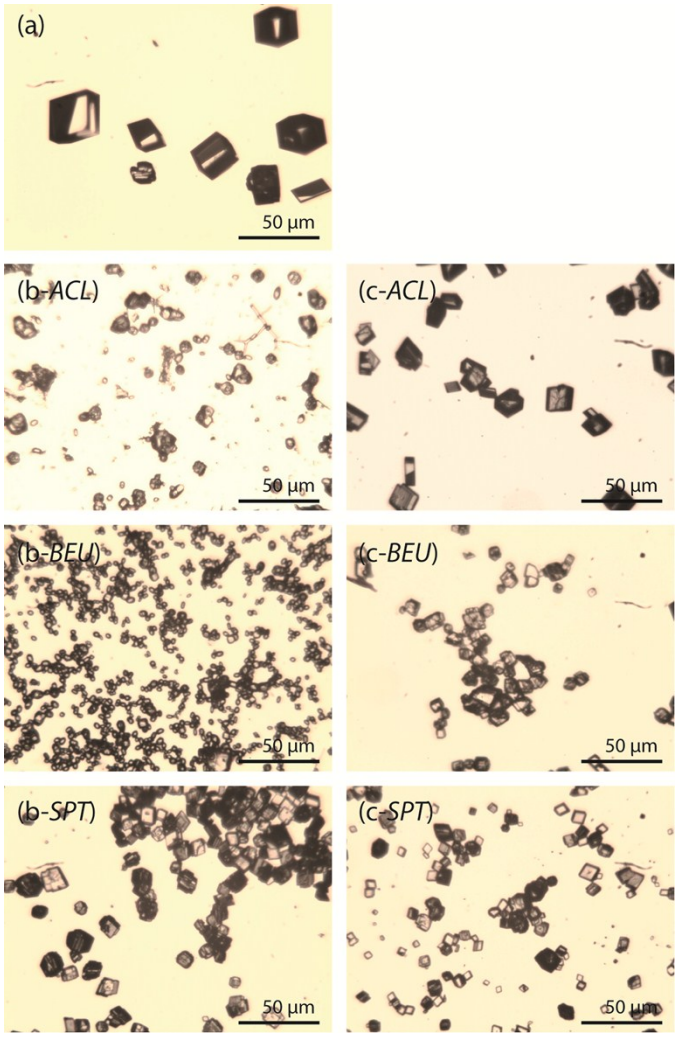

Figure ESI2. Optical microscope pictures of calcium carbonate particles precipitated in absence of additives (a), in the presence of *w*OM (b) and in the presence of *lip*OM (c) for *A. calycularis* (ACL), *B. europaea* (BEU) and *S. pistillata* (SPT).

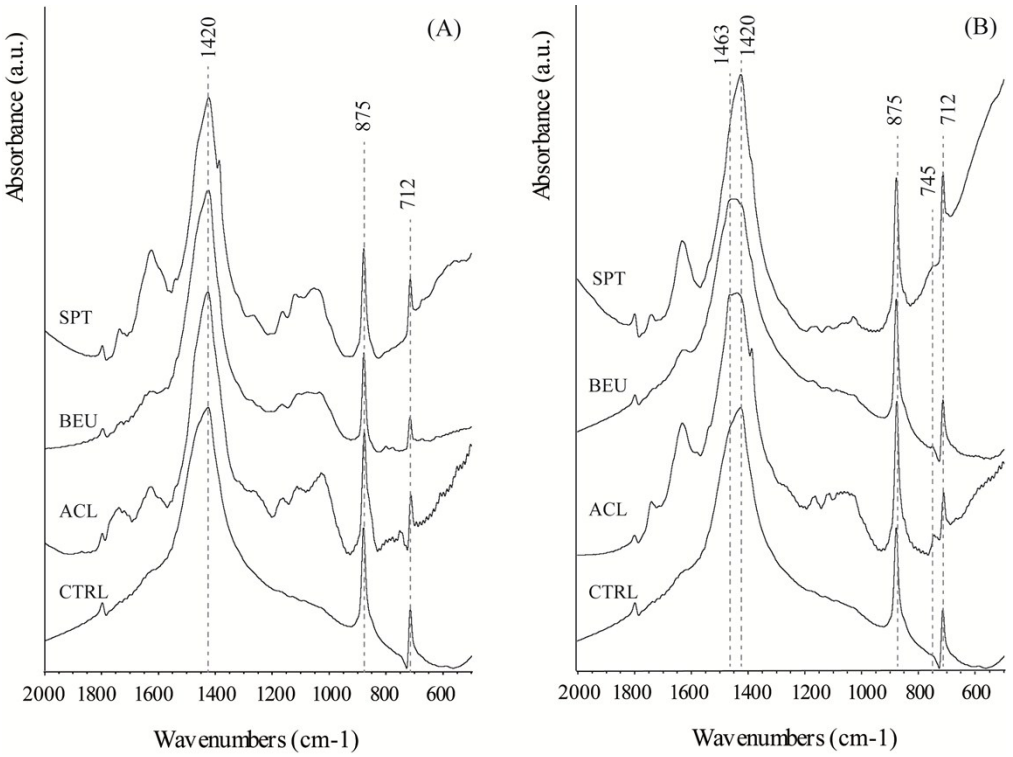

Figure ESI3. FTIR spectra of calcium carbonate precipitated from 10 mM $CaCl_2$ solutions in the presence of *w*OMs (A) and *lip*OM (B) which were extracted from the skeletons of *A. calycularis* (ACL), *B. europaea* (BEU), *S. pistillata* (SPT). FTIR spectrum of calcium carbonate precipitated from 10 mM $CaCl_2$ solution without additives is also reported (CTRL). The wavenumbers of the main absorption bans are indicated.

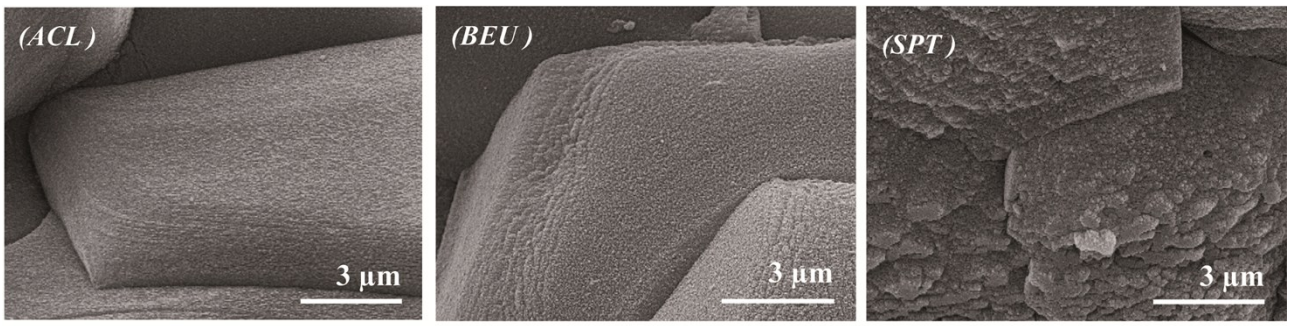

Figure ESI4. SEM images of the calcium carbonate precipitated in the presence of *w*OM from *A. calycularis* (ACL), *B. europaea* (BEU) and *S. pistillata* (SPT). Smoothed edges and corner of rhombohedral calcite crystals are showed. These pictures are the most representative of the populations of observed particles.

References

1. G. Falini, M. Reggi, S. Fermani, F. Sparla, S. Goffredo, Z. Dubinsky, O. Levi, Y. Dauphin and J.-P. Cuif, *J. Struct. Biol.*, 2013, **183**, 226–238.
2. C. Beato, M. S. Fernandez, S. Fermani, M. Reggi, A. Neira-Carrillo, A. Rao, G. Falini and J. L. Arias, *CrystEngComm*, 2015, **17**, 5953–5961.
3. A. Rohman and Y. B. Che Man, *Vibr. Spectrosc.*, 2011, **55**, 141–145.
4. J. L. R. Arrondo and F. M. Goñi, *Chem. Phys. Lipids*, 1998, **96**, 53–68.
5. I. Dreissig, S. Machill, R. Salzer and C. Krafft, *Spectrochim. Acta A: Mol.Biomol. Spectr.*, 2009, **71**, 2069–2075.
6. J.-C. Tsai, Y.-L. Lo, C.-Y. Lin, H.-M. Sheu and J.-C. Lin, *Spectroscopy*, 2004, **18**, 423–431.
7. B. Fuchs, K. Bresler and J. Schiller, *Chem. Phys. Lipids*, 2011, **164**, 782-795.
8. M. E. Rerek, D. Van Wyck, R. Mendelsohn and D. J. Moore *Chem. Phys. Lipids,* 2005, **134**, 51–58.
9. J. M. Nzai and A. Proctor, *JAOCS*, 1998, **75**, 1281–1289.