Dear Steven,

We agree that both reviewers provided constructive suggestions, we are therefore happy to submit a revised version of our manuscript complying to their suggestions and based on the replies we previously posted.

We notably revised the manuscript by making a more careful use of the term “preference”, providing a more detailed methods section, adding or rephrasing all ambiguous lines of discussion and fixed all the minor edits that were suggested to improve wording clarity.

We hope that you will now find our manuscript suitable for publication in your journal.

Sincerely,

Estelle Couradeau

Diversity and mineral substrate preference in endolithic microbial communities from marine intertidal outcrops (Isla de Mona, Puerto Rico).

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Running title: endolithic cyanobacteria substrate preference

Abstract

Endolithic microbial communities are prominent features of intertidal marine habitats, where they colonize a variety of substrates, contributing to their erosion. Almost two centuries worth of naturalistic studies focused on a few true-boring (euendolithic) phototrophs, but substrate preference has received little attention. The Isla de Mona (Puerto Rico) intertidal zone offers a unique setting to investigate substrate specificity of endolithic communities since various phosphate rock, limestone, and dolostone outcrops occur there. High-throughput 16S rDNA genetic sampling, enhanced by targeted cultivation, revealed that, while euendolithic cyanobacteria were dominant OTUs, the communities were invariably of high diversity, well beyond that reported in traditional studies, and implying an unexpected metabolic complexity, potentially contributed by secondary colonizers. While the overall community composition did not show differences traceable to the nature of the mineral substrate, we detected

specialization among particular euendolithic cyanobacterial clades towards the type of substrate they excavate, but only at the OTU phylogenetic level, implying that close relatives have specialized recurrently into particular substrates. The cationic mineral component was determinant in this preference, ~~calling for~~suggesting the existence in nature of alternatives to the boring mechanism described in culture that is based exclusively on transcellular calcium transport.

Introduction

In shallow and intertidal marine habitats, endolithic microbes colonize a variety of carbonaceous and phosphatic substrates, such as bone, shell, coraline carbonate, ooliths, as well as limestones, dolostone and phosphorite outcrops (Campbell, 1983). Some of these microbes take advantage of the natural pores or crevices in the solids, but some have the ability to actively bore their way into the substrate. Such microborers, also known as euendoliths (Golubic et al., 1981), build communities that can cover as much as 50% of the exposed solid surface (Golubic et al., 2000) with full colonization times of virgin substrate on the order of months (Gektidis, 1999; Grange et al., 2015). Several long-term geological phenomena are driven by microborers, from the erosive morphogenesis of coastal limestones (Purdy and Kornicker, 1958; Schneider, 1983; Torunski, 1979; Trudgill, 1987) and the destruction of coral reefs and other biological carbonates (Le Campion-Alsumard et al., 1995; Ghirardelli, 2002) to ~~the cementation of loosely bound~~the formation of lithified laminae of welded carbonate grains in coastal stromatolites (MacIntyre et al., 2000; Reid et al., 2000). Additionally, phototrophic euendoliths can cause significant damage and shell weakening to bivalve populations (Kaehler and McQuaid, 1999). Long-term rates of microborer-driven carbonate dissolution, the "bioerosion" process, range between 20 and 930 g $CaCO_3$ m^{-2} d^{-1}~~,~~ and are of clear geologic significance (Grange et al., 2015; Peyrot-Clausade et al., 1995; Tudhope and Risk, 1985; Vogel et al., 2000), and may increase under future scenarios of increased atmospheric CO_2 and ocean acidification (Tribollet et al., 2009).

There exists a very large body of descriptive literature spanning 18 decades, largely based on microscopic observations, documenting the biodiversity of microborers, with contributions in the microbiological, ecological, sedimentological and paleontological fields (Acton, 1916; Al-Thukair et al., 1994; Bachmann, 1915; Batters, 1892; Bonar, 1942; Bornet and Flahault, 1888; Budd and Perkins,

1980; Le Campion-Alsumard et al., 1995; Chodat, 1898; Duerden, 1902; Duncan, 1876; Ercegovic, 1925, 1927, 1930, Frémy, 1936, 1941; Ghirardelli, 2002; Golubic, 1969; Kölliker, 1859; Lehmann, 1903; May and Perkins, 1979; Nadson, 1927; Pantazidou et al., 2006; Perkins and Tsentas, 1976; Wisshak et al., 2011). Euendoliths have been reported among eukaryotes (fungi, green and red algae) and prokaryotes (cyanobacteria), taxa where it may have been selected as a strategy to escape predation from grazers, protect from UV radiation or acquire nutrients as a tradeoff for the boring energetic cost (Cockell and Herrera, 2008). The most common genera of phototrophic eukaryotic euendoliths are *Ostreobium* and *Phaeophila* in the green algae, as well as the red algal genus *Porphyra* (in its filamentous diploid generation, known also as *Conchocelis* stage). In the cyanobacteria, the pseudofilamentous genera *Hyella* and *Solentia* are quite common (Al-Thukair, 2011; Al-Thukair et al., 1994; Al-Thukair and Golubic, 1991; Brito et al., 2012; Campion-Alsumard et al., 1996; Foster et al., 2009; Golubic et al., 1996) , as are some forms in the simple filamentous genus *Plectonema* (Chacón et al., 2006; Pantazidou et al., 2006; Tribollet and Payri, 2001; Vogel et al., 2000). Morphologically complex cyanobacteria such as *Mastigocoleus testarum* (Golubic and Campion-Alsumard, 1973; Nadson, 1932; Ramírez-Reinat and Garcia-Pichel, 2012a) complete the list of common euendoliths. Less common genera of euendolithic cyanobacteria include: *Cyanosaccus* (Pantazidou et al., 2006), *Kyrtuthrix* (Golubic and Campion-Alsumard, 1973) and *Matteia* (Friedmann et al., 1993). To date, tThese genera were all assigned based upon morphological criteria and could represent morphological variations of the same types (Le Campion-Alsumard and Golubic, 1985), highlighting the need to re-assess the diversity of euendolithic cyanobacteria using a combination of characters including genetic markers, a task yet to be undertaken with any breadth.

Modern genomic methods for community fingerprinting have, more recently, been applied to provide an alternative complementary and more comprehensive, comprehensive description of endolithic communities. Some studies, focused on phototrophs from marine carbonates, revealed that, while some

biodiversity had been missed by deploying ~~merely~~ morphological studies, there was also congruency between DNA-based surveys, and the traditional literature (Chacón et al., 2006; Ramírez-Reinat and Garcia-Pichel, 2012b). DNA-based studies ~~brought to our attention~~have revealed that the endolithic habitat at large can harbor complex communities of microbes, ~~not just composed~~in addition to ~~of~~ euendoliths, particularly when the substrate rocks are naturally porous, or when they have been rendered porous by the action of euendoliths themselves. Horath and Bachofen 2006, for example, investigating terrestrial endolithic communities in dolomite outcrops in the Alps, found a large diversity of presumably chemotrophic bacteria and archaea, in addition to expected green algae and cyanobacteria. Similar conclusions could be drawn from the work of de la Torre et al. (De la Torre et al., 2003) on Antarctic sandstone cryptoendoliths, those of Walker and colleagues (Walker et al., 2005; Walker and Pace, 2007) on terrestrial limestones, sandstones and granites or the recent contribution of (Crits-Christoph et al., 2016) who used a metagenomic approach to investigate the chasmoendolithic communities of the hyper-arid Atacama desert. However, no high throughput sequencing studies are available on the globally significant intertidal endolithic communities. ~~no studies are yet available on the globally significant intertidal endolithic communities that have used the power of high-throughput sequencing techniques.~~

Tribollet (2008) provided an account of the dynamic changes in microborer community composition taking place after coral death, which obviously constitute a true succession in the ecological sense, with pioneer euendoliths (such as *Mastigocoleus testarum*) and secondary colonizers such as *Ostreobium quekettii* and *Plectonema terebrans,* as well as fungi (Grange et al., 2015; Tribollet, 2008). During laboratory studies with the cultivated strain of *Mastigocoleus testarum* strain BC008, used as a model to understand the physiology of cyanobacterial boring (Garcia-Pichel et al., 2010; Guida and Garcia-Pichel, 2016; Ramírez-Reinat and Garcia-Pichel, 2012b), we ~~could show~~found that, among the carbonates, this strain excavated ~~fastest~~ most rapidly into various types of calcite and aragonite

minerals ($CaCO_3$). It could bore slowly into strontianite ($SrCO_3$), but was unable to penetrate into magnesite ($MgCO_3$), dolomite ($CaMgCO_3$), witherite ($BaCO_3$), rhodochrosite ($MnCO_3$), siderite ($FeCO_3$) or ankerite ($CaFe(CO_3)_2$)(Ramírez-Reinat and Garcia-Pichel, 2012a). However, literature reports do exist detailing microborings in modern and fossil dolomitic substrates (see e.g. (Campbell, 1983; Golubic and Lee, 1999). Similar ~~arguments~~ substrate preferences have also been observed ~~can be made~~ for phosphates: *M. testarum* strain BC008 did not bore into calcophosphatic substrates, including hydroxyapatite, vivianite or dentine; yet, the literature is replete with reports of cyanobacterial microborings on biotic and abiotic phosphatic rocks (Soudry and Nathan, 2000; Underwood et al., 1999; Zhang and Pratt, 2008)). The expression of such a mineral substrate preference among the pioneer euendolithic cyanobacteria could principally drive the whole community towards a different successional sequence with distinct mature community assemblages and metabolic potentialities. We wanted to ask the question if evolutionary specialization has resulted in a highly adapted endolithic flora for each type of mineral substrate, and if there exist specialized apatite-borers, dolomite-borers, or carbonate-borers in nature. ~~Surprisingly, this aspect of endolithic microbiology had not been directly addressed yet.~~

In order to answer these questions, we investigated in depth the marine endolithic communities of Isla de Mona (PR), a small, uninhabited Caribbean island offering a variety of coastal cliffs composed of dolomite and limestone, as well as raised aragonitic and phosphatic reefs, with the dual purpose to (i) describe the microbial diversity of intertidal endolithic community at high resolution and (ii) to test the effects of substrate composition on community structure in a single geographic location with common bathymetry (the intertidal notch), controlling for other known major determinants of community composition.

Materials and Methods

Sampling site and procedure

Samples were obtained from Isla Mona (18.0867° N, 67.8894° W), a small (11 km by 7 km) carbonate island 66 km W of Puerto Rico. Isla Mona is a protected habitat and all necessary permits were acquired from the Departamento de Recursos Naturales y Ambientales prior to arrival. The present study did not involve endangered or protected species. Endolithic communities were obtained by sampling different locations from nine separate island localities. Rock samples containing endolithic biomass, verified using a digital field microscope, were chipped off from large boulders and rock walls using a standard geological hammer. The hammer was thoroughly washed with surrounding sea water at each sampling point. Material was predominantly collected within the boring notch of the intertidal zone. Bathymetric samples were collected via SCUBA diving at sample site K at depths of 3.5, 4.6, 7, and 9.1 meters. ~~Three replicates were~~Each sample was broken into three pieces~~-~~, each biological replicate was stored in a ~~taken per sample which consisted of~~ sterile 50 mL falcon tubes~~ filled with material~~, one replicate was air dried for mineralogical analysis, one was kept viable in seawater for strain isolation and another was preserved *in situ* in 70% ethanol for DNA extraction. Air drying and alcohol preservation were done in the field. Samples were shipped at room temperature, in the dark for 5 days, and, upon arrival in the lab, the preserved samples were immediately stored at -20^{o}C until extractions were performed. Aliquots of local seawater were collected at sample site K and filtered through 0.22 µm syringe filters into sterile 50 mL falcon tubes. After 5 days of transit at room temperature in the dark, the seawater sample was stored at 4 ^{o}C in the dark for an additional week before being processed for physico-chemical analysis.

Bulk powder X ray diffraction and elementary analyses

A fragment of each sample was ground down to powder in 100% ethanol. XRD patterns were collected using Panalytical X'Pert Pro diffractometer mounted in the Debye-Scherrer configuration with a CuKα monochromatic X-Ray source. Data were recorded in continuous scan mode within a 10–90° 2θ range. X'Pert High Score plus software was used to identify mineral phases and ~~retrieved~~ their relative concentration using the automatic Rietveld refinement method implemented in the software under default parameters. The elementary composition of the rocks and water sample analyses were performed by the Goldwater Center at Arizona State University using a Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) - Thermo iCAP6300.

Total genomic DNA purification

The surface of the ethanol fixed samples was brushed vigorously with a sterile toothbrush and sterile MilliQ water to remove epilithic material. A chip of 8 cm^3 was further ground~~ed~~ in a sterile mortar as recommended by (Wade and Garcia-Pichel, 2003). 0.5 g of the obtained coarse powder was then transferred into the bead tube of the MoBio PowerPlant Pro kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). The first lysis step of the kit was modified ~~as follow~~by homogenizing bead tubes ~~were homogenized~~ horizontally at 2,200 rev/min for 10 minutes and 7 freeze-thaw cycles ~~were applied~~ (Wade and Garcia-Pichel, 2003). The next steps of the extraction were conducted following the MoBio PowerPlant Pro kit following manufacturer's guidelines.

16s rRNA gene library preparation and sequencing

The 16S rRNA gene V3 - V4 variable region was targeted using PCR primers 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACVSGGGTATCTAAT) with a barcoded forward primer. The PCR amplification was performed using the HotStartTaq Plus Master Mix Kit (Qiagen,

USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, followed by a final 5min elongation step at 72°C. PCR product were further purified and pooled into a single DNA library using the Illumina TruSeq DNA library preparation protocol. This library was further sequenced on a MiSeq following the manufacturer's guidelines. The library preparation, sequencing paired ends assembly and first quality trimming (with phred score of Q25 cutoff) ~~was~~ were performed by MR DNA (www.mrdnalab.com, Shallowater, TX, USA).

16S rDNA sequences from the newly cultured euendolithic strains were retrieved using the PCR condition and primers described by (Nübel et al., 1997) followed by sanger sequencing. Briefly, the primers used were the forward Cya106F (CGG ACG GGTGAGTAACGCGTGA) and an equimolar mixture of the Cya781R(a) (GACTACTGGGGTATCTAATCCCATT) and Cya781R(b) (GACTAC AGGGGTATCTAATCCCTTT) as reverse. The PCR amplification was performed using the GoTaq enzyme and master mix (Promega, Madison, USA) at 1X concentration. The amplification conditions were as follow: after an initial denaturation step 94°C for 5 min, 35 PCR amplification cycles were performed, each consisting of 1 min denaturation step at 94°C, 1 min annealing step at 60°C, and 1 min elongation step at 72°C.

OTU table building and analysis

Sequences were further processed using the Qiime version 1.9 (Caporaso et al., 2010). The sequences were first run through the *split_libraries.py* script under the default parameter that includes barcodes removal, quality filtering (sequences of less than 200bp or with homopolymer runs exceeding 6bp were removed) and split of the dataset per sample. The output file was further processed through the *pick_open_reference_otus.py* script using the default parameters except for the taxonomic assignment that was done by the RDP classifier (see parameter file in supplementary information for more details).

This step clustered the sequences at a similarity threshold of 97% (Edgar, 2010) to build Operational Taxonomic Units (OTUs), assign their taxonomy and further reported their specific abundance in for each sample into an OTU table. Because in this case we were not interested into the rare biosphere but focused on the most abundant OTUs and how they vary, we filtered the OTU table to remove the rare OTUs. The OTUs retained were those that occurred in at least 5 samples among the 34 analyzed, or that represent more than 0.1% of the total sequences found in a particular sample. By doing this, we eventually analyzed 90% of all the single sequences but only 11% of the initial OTUs. The Qiime script *summarize_taxonomy_through_plots.py* was run on the final OTU table for all the prokaryotes and for the Cyanobacteria only (filtering out the chloroplasts) in order to build the summarized microbial community composition bar graphs displayed on the figure 2.

Accession numbers

One representative sequence per OTU was deposited to genebank under the accession numbers KT972744-KT981874. The 16S rDNA sequences of the new euendolithic strains described in this article received the following accession numbers: *Ca.* PleuronemaPleurinema perforans IdMA4 [KX388631], *Ca.* Mastigocoleus perforans IdM [KX388632], *Ca.* PleuronemaPleurinema testarum RPB [KX388633].

Meta-analysis of microbial communities

For comparison, rRaw sequences from datasets ID 662/678/809/627/713/925 were retrieved from the Qiita repository along with their mapping table. All these studies used comparable sequencing depth, technology and targeted the same region of the 16 rRNA gene compared to the present study. Two samples from Alchichica cyanobacteria dominated microbialites communities (Couradeau et al., 2011) were processed in parallel to the Isla de Mona samples (same extraction methodology, sequenced in the

same MiSeq run), and also ~~they were~~ included in this analysis ~~as well~~. The sequences were all aggregated into a masterfile that was processed in Qiime version 1.9 (Caporaso et al., 2010). The same exact procedure than the one described above was used to pick OTUs. Again we retained the OTUs that occurred at least in 5 samples. We ran the *jackknifed_beta_diversity.py* pipeline using the Bray Curtis metrics under default parameters. The obtained distances were used to cluster samples under a UPGMA hierarchical clustering method and 5000 sequences were included in each jackknifed subset in order to generate nodes support.

Differential abundance of OTUs analyses

To determine if some OTUs were more associated to certain type of substrates we ~~run~~ ran the *differential_abundance.py* of the Qiime 1.9 package (Caporaso et al., 2010) using the DESeq2 method (Love et al., 2014), under a negative binomial generalized linear model. This method was initially developed to assess the differential gene expression from RNA seq data but can be applied to any count matrix data such as OTU tables (Love et al., 2014). It was recently implemented for the treatment of 16S rDNA OTU table and has been widely used since (e.g. (Debenport et al., 2015; Pitombo et al., 2015)) because it (i) is a sensitive and precise method, (ii) controls the false positive rate (Love et al., 2014) and (iii) it uses all the power of the dataset without the need to rarefy the OTU table (McMurdie and Holmes, 2014). After checking the good agreement between the fit line and the shrinked data on the dispersion plot, a Wald test was applied to each OTU to reject the null hypothesis ($p<0.05$) being that the logarithmic fold change between treatments (i.e. in our case type of mineral substrate) for a given OTU is null.

Phylogeny reconstruction

In order to determine which of the cyanobacterial OTUs of the dataset were possible euendolithic organisms, we built a phylogeny to assess their proximity to proven boring cultured strains. The maximum-likelihood phylogenetic reconstruction was performed using TREEFINDER (Jobb et al., 2004) under a general time reversible (GTR) and a four-category discrete approximation of a Γ distribution. Bootstrap values were inferred from 1000 replicates. The sequence dataset used for the reconstruction was first aligned with MAFFT (Katoh et al., 2005) and then manually checked and trimmed using the MUST package (Philippe, 1993).

Results & Discussion

Geological setting of Isla de Mona outcrops.

The island is an 11 by 7 km emerged platform of Miocene Isla de Mona Dolomite (up to 80 m thick) topped by a thinner (up to 40 m) layer of Miocene Lirio limestone (Briggs and Seiders, 1972; Frank et al., 1998). It is partially surrounded in its Southern and Southwestern shores by a Pleistocene raised reef flat, mostly composed of biogenic carbonates (Fig. 1). The island also harbors secondary phosphorite deposits formed by the diagenetic alteration of guano, most typically associated with an extensive system of karstic caves at the interface of limestone and dolostone (Briggs, 1959). Isla de Mona was never continuously inhabited~~,~~. The island was mostly used as a guard post over the Mona Passage throughout the 20th century, and declared a Nature Preserve in 1993 (National Parks Register, USA). The coastal area has been protected from disturbance ever since. We took advantage of this unique and pristine geological setting to sample dolostones, limestones and phosphorites exposed to similar environmental conditions. We analyzed a set of 34 samples consisting of pieces of exposed rock, in most cases taken directly at the intertidal notch. Location of sampling sites are in the simplified geological map in Figure 1a. The mineralogical composition of each sample (Fig. 2), determined using

282 bulk powder X-Ray diffraction, confirmed the presence of apatite ($Ca_5(PO_4)_3(OH,Cl,F)$), dolomite
283 ($CaMg(CO_3)_2$), calcite ($CaCO_3$) and aragonite($CaCO_3$) in various proportions depending of the
284 sampling site (Fig. 2a).
285
286 *The endolithic microbial communities*
287
288 We studied the endolithic community composition by analyzing the 16S rDNA diversity present in total
289 genomic DNA extracted from the rock after aggressively brushing away epilithic growth from the
290 external sample surface. The 16S rDNA sequences were obtained after specific PCR amplification and
291 Illumina-based high-throughput sequencing, with one library per sample (Table S2). We clustered
292 sequences into OTUs (Operational Taxonomic Units) based on a 97% similarity criterion, and further
293 filtered the dataset to remove the rare OTUs, focusing our study on OTUs that occurred in at least five
294 separate samples, or those that made up more than 0.1% of all sequences in any one sample. Bacterial
295 OTU richness in these samples was 4058 ±1252, as given by the chao1 metric (Figure 2c). Thus,
296 comparatively our endolithic communities are of rather low diversity, an order of magnitude lower than
297 current estimates assigned to bulk soil bacterial communities (Roesch et al., 2007), but similar to other
298 microbial communities such as biological soil crusts (Couradeau et al., 2016), microbial mats
299 (Hoffmann et al., 2015) or stromatolites (Mobberley et al., 2011), that are dominated by cyanobacterial
300 primary producers. This suggests that endolithic habitat nurtured by the presence of cyanobacterial
301 primary producers can support the development of a high diversity of microorganisms even if this type
302 of habitat is expected to be nutrient limited due to its low connectivity with sea water (Cockell and
303 Herrera, 2008). Taxonomic assignment of the OTUs on the basis of the Greengene database (McDonald
304 et al., 2012), allowed us to reconstruct the endolithic prokaryotic communities from Isla de Mona at
305 various level of taxonomic resolution. At the phylum level (Figure 2b), the analysis revealed complex
306 microbial communities with numerically very significant populations of bacteria other than

Cyanobacteria: *Proteobacteria, Chloroflexi, Actinobacteria* and *Bacteroidetes*. In fact, the contribution of cyanobacteria to the total sequence richness was only 12 ± 3%. These communities clearly host not only a large number of bacterial types, but also a wide diversity of phylogenetic and metabolic potential beyond oxygenic photosynthesis. Clearly, mature endolithic cyanobacterial communities in this study are much more complex than the ~~overwhelming~~ majority of the ~~traditional~~ literature ~~would suggest~~to date (for example, the exhaustive descriptive literature review in the introduction does not report beyond cyanobacteria and eukaryotic algae). While it is proven ~~by the use of model organisms~~that some axenic ~~in culture that~~ cyanobacteria ~~alone~~ are able to initiate excavation on virgin substrate (Ramírez-Reinat and Garcia-Pichel, 2012a), it is interesting to entertain that in such complex communities, other metabolic activities (of co-occurring microorganisms), particularly those that result in pH changes might play a significant role on the determination of the local saturation index of the carbonate mineral (Baumgartner et al., 2006; Dupraz et al., 2009; Dupraz and Visscher, 2005), and in this way influence the overall mineral excavation yield or rates. At this level of taxonomic resolution, we did not detect any significant association of substrate mineralogy and community composition (as judged by non significant Spearman's ρ when comparing each phylum's relative abundance to mineralogical composition, not shown).

Because endolithic communities have not received much attention, we integrated our dataset into a meta-analysis of various cognate microbial communities, for which technically comparable datasets were publicly available (http://qiita.microbio.me.). To do so, we aggregated all the sequences from the selected Qiita datasets into a single file that was used to pick and cluster 16S rDNA OTUs anew, and conducted similarity analyses. The meta-community analysis revealed that endolithic communities clustered together, and apart from other types of phototrophic microbial communities in terms of composition (beta-diversity). The fact that they clustered together indicates that their microbial assemblages are recognizable and distinct beyond just their belonging to the marine habitat itself, in a

microbiological and presumably adaptive way. However at this stage ~~A cautionary alternative reading,~~we cannot exclude that the observed pattern ~~however,~~ could ~~be that this pattern~~ represent~~s~~ a biogeographical island effect. Further studies involving a larger dataset of endolithic communities will be necessary to disentangle the local signature controlled by environmental parameters from the endolithic signature presumably universal to all endolithic communities. ~~, in that all of our samples come from a relatively small geographical area. This alternative explanation is unlikely given the cosmopolitan nature of marine cyanobacteria (Garcia-Pichel et al., 1996; Lodders et al., 2005)~~ Interestingly, our endolithic community samples could be separated into 2 self-similar clades (A and B Figure 3) but so far we cannot ascertain a factor that would drive the observed separation beyond the fact that it is not substrate type. While it would be of interest to compare our communities to other endolithic communities, such as those studied by (Chacón et al., 2006; Crits-Christoph et al., 2016; Horath and Bachofen, 2009; De la Torre et al., 2003) this is not technically possible, given that all of those studies used alternative methods for community analyses (Clone libraries, DGGE, metagenomes) that do not allow direct comparisons.

A diverse cyanobacterial community dominated by likely euendoliths

Because they comprise the pioneer microborers and primary producers within many endolithic communities, cyanobacteria are of particular interest in this study. We therefore analyzed cyanobacteria at a higher resolution. The cyanobacterial community appeared quite diverse with a specific chao1 richness of 484 ±184, certainly much more genetic diversity among this group than could be surmised from the wealth of microscopically based accounts in the botanical literature (Chazottes et al., 1995; Pantazidou et al., 2006; Sartoretto, 1998; Tribollet et al., 2006). In these studies typically one finds reports of anywhere from 1 to 5 morphotypes. Even accounting for the fact that morphotypes typically underestimate genetic diversity by a significant fraction (Nübel et al., 1999) this is a very large

underestimation of oxygenic phototroph diversity. Phylotypes assignable to the orders *Pseudanabaenales, Chroococcocales, Nostocales* and *Stigonematales* were most common and widespread. Again no pattern linking mineralogy to microbial community composition arose at this taxonomic level, as judged by the non-significant Spearman's ρ when comparing the relative abundance of each cyanobacterial to mineralogical composition (not shown). A combination of literature search and additional efforts of cultivation and genetic characterization of isolates, allowed us to attempt the assignment of a true-boring (euendolithic) role to some of our cyanobacterial OTUs (Table 1 and Figures S2-S3). Interestingly, out of the five most abundant OTUs in our combined dataset, four (NR_OTU741, OTU 842393, NR_OTU193 and OTU 351529) could be deemed as likely euendoliths, given their close phylogenetic affiliation to cultivated isolates proven in the laboratory to be able to bore. The fifth most abundant OTU (OTU 186537) fell between *Mastigocoleus testarum* BC008 (a proven euendolith) and *Rivularia atra* (not described as boring in the literature), so its capacities remain unclear. Notably, the most abundant OTU, NR_OTU741 in our set is virtually indistinguishable from one of our isolates obtained from the same samples, the boring strain *Ca.* ~~Pleuronema~~Pleurinema perforans IdMA4 (similarity > 99%), which is not only the most abundant cyanobacterial OTU but also the second most abundant bacterial OTU overall in our dataset. Overall the 7 OTUs that could be assigned as possible euendolith based on their phylogenetic proximity to known microborers account for 0.8% to 73% (average value 29%) of the total number of sequences depending on the sample considered. These results suggest that eudendoliths compose a major fraction of the community, one that ~~does~~ not only represents an initial set of pioneers, but one that maintains relevance even after bioerosive degradation and reworking of the mineral substrates allow the colonization of newly made pore spaces by non-boring endoliths.

On analyzing the diversity of the possible euendoliths detected in this dataset, we realized that while many of the most common known genera of cyanobacterial microborers are represented and abundant,

the thin, filamentous *Plectonema terebrans* is not. This was surprising because *Plectonema terebrans* has always been described as an important member of the euendolithic community ~~who can account for~~accounting for up to 80% of the total of microborer biomass (Tribollet, 2008) and is found associated to *Mastigocoleus testarum*. This apparent paradox is likely not due to the absence of the organism, but to failure to properly identify it molecularly, due to the lack of reference sequences in the databases. Indeed morphotypes resembling *Plectonema terebrans* ~~was~~ were visually recognized, but not detected molecularly in the extensive study of euendolithic cyanobacteria from various locations by (Ramírez-Reinat and Garcia-Pichel, 2012b). In the present dataset *Plectonema* could have been assigned to another member of the Oscillatoriales, such as *Phormidium* or *Halomicronema,* which represent 10 and 4.6% , respectively, of the cyanobacterial sequences. A *bona fide* isolate proven to bore in the lab will be needed before we can advance regarding the presence and abundance of simple filamentous euendolithic cyanobacteria anywhere. Among the cyanobacterial taxa detected, the following have never been reported to be true borers: Gloeobacterales, Nostocaceae, Acaryochlorales, Cyanobacteriaceae, Spirulinaceae, Pseudanabaenales. In all, these cyanobacteria contribute at least to some 43 ±20 % indicating that a significant proportion of the community is likely made up of adventitious endoliths. A study of the temporal dynamics of colonization could help understand the true role of each taxon.

Substrate preference among cyanobacteria

We knew from the experimental study of the model euendolith *Mastigocoleus testarum* strain BC008*,* that this particular organism exhibits a clear boring substrate preference. It bores into Ca-carbonates (like aragonite and calcite) and to a lesser extent Sr-carbonate (strontianite), but not into CaMg-carbonate like dolomite (Ramírez-Reinat and Garcia-Pichel, 2012a). This strain remains the single case where the boring preference has been directly tested, but it is unknown if this preferential behavior is

representative of euendoliths at large. Only a few studies examined endolithic communities colonizing dolostone, (Jones, 1989) provided the first comparison of endolithic communities from dolostones and limestones from Grand Cayman Ironshore. He observed that dolostones were less colonized by endoliths than limestones and concluded that the bioerosion of limestones was faster due to the more abundant endolithic flora while the erosion pattern of the dolostone was slower and allowed the development of more epiliths. When looking at the endolithic microbial diversity of terrestrial dolostones (Horath et al., 2006) found the same cyanobacterial genera than the ones typically described on freshwater limestones substrates (Norris and Castenholz, 2006) while (Sigler et al., 2003) concluded that the endolithic dolostone phototrophic community resembled other desiccation-tolerant endolithic communities. The question of whether there really exists a specialized community associated to dolostone *vs.* limestone remained clearly open.

Our own data showed no specificity for substrate at family level, highlighting the need to analyze this at a phylogenetically deeper resolution. To do so, we analyzed how cyanobacterial OTUs where differentially represented in sample subsets from contrasted mineralogical substrates using the DESeq2 method (Love et al., 2014). This method was developed to analyze RNA-seq datasets but can be used on any count matrix such as an OTU table. This statistical framework is sensitive and precise and does not involve rarefying the dataset to an even sampling depth, so that the entire statistical power of the data is accounted for (McMurdie and Holmes, 2014). We used it to determine whether any given OTU is significantly differentially represented in a particular subset of samples sharing a common mineralogical substrate compared to another set. In comparing OTU detected in samples were mineralogically dominated by Ca-carbonates (calcite or aragonite, n=13) with those that were dolomitic in nature (CaMg-carbonate, n=14), we found 31 OTUs to be were significantly enriched in Ca-carbonate substrates ($p<0.05$; corresponding to $\log_2$ fold difference > |2.83|), while 22 preferred dolomite with $p<0.05$, out of 1039 cyanobacterial OTUs considered. ~~It becomes clear~~Results suggest

that substrate preferences are ~~indeed~~ found when one looks at fine taxonomic resolution, and that some likely euendoliths show such preference: *Mastigocoleus testarum* close relative NR_OTU193 prefers the Ca-carbonate pole (log_2 fold difference = |3.4|) while another possible euendolith NR_OTU741 belonging to the *Pleurocapsales* clearly prefers dolomite (log_2 fold difference = |1.7|). It is also clear that for most of the OTUs, either there is not sufficient resolution at the 16S rDNA level to detect it, or, more parsimoniously, these OTUs represent taxa that can colonize various substrates. Many in this group of OTUs ~~showing no~~are not ~~preference~~ differentially represented on a particular substrate type, suggesting that they may be adventitious endoliths that do not bear the burden of boring into the substrate and can potentially colonize any substrate~~,~~. However,~~but~~ at least some of these represent most likely euendoliths (NR_OTU4, OTU 351529 and OTU 842393), and still ~~they do not seem to show preference at this level of genetic resolution~~are not differentially represented with respect to the mineral phase they colonize.

Using the same method, we then compared Ca-carbonate dominated samples (n=14) to Ca-Phosphate dominated samples (n=3). Although t~~T~~he paucity of phosphate samples ~~certainly~~ restricted our statistical power, ~~but even then~~ we were still able to identify 81 OTUs that were statistically significantly enriched on the phosphatic substrate ($p<0.05$) side, while only 21 were enriched in carbonates ($p<0.05$) (Figure 5). This suggests an asymmetrical effect of carbonate *vs.* phosphate substrate types, the latter being a more powerful driver of differential abundance among cyanobacteria. But again, in this case, the majority of OTUs, including some of the most abundant, were ~~promiscuous~~widespread across different substrate types. *Mastigocoleus sp.* (NR_OTU193) appeared clearly enriched in the carbonates (log_2 fold difference = |3.8|), while the other potential borers including the Pleurocapsales OTUs did not exhibit statistically significant differential abundance with substrate ~~preference~~.

In all, these results suggest that some cyanobacteria do have a substrate preference, and that these preferences sometimes occur among closely related clades (like NR_OTU193 and NR_OTU4), which do exhibit differential occurrence. These comparisons highlight the differential role of the cationic *vs.* the anionic mineral component. NR_OTU193 for instance showed a higher rate of occurrence ~~preference for~~when testing for both components, suggesting that it prefers calcium over magnesium in terms of cation and carbonate over phosphate as an anion. On the other hand, NR_OTU741 only appeared differentially represented when the cationic part of the mineral varied. Finally, it is important to note that only a small fraction of the cyanobacterial community seems to be influenced by the substrate, 3.5% of the total number of species on average accounting for 16 ±4% of the total number of cyanobacterial sequences analyzed. These results are consistent with the idea that borers may be specialized, but ancillary endoliths are not. The substrate specialization of euendoliths may be due to the physiological requirements of excavation into specific mineral types. Future endolithic community metagenomic reconstructions and comparisons could aid in the identification of alternative pumps that may be specific to mineral types.

Implications for the diversity of the boring mechanism and substrate-driven evolution of euendoliths

A question that follows naturally from the previous findings is how such a substrate preference may relate to the physiological mechanism of boring. The model strain *Mastigocoleus testarum* BC008 is clearly specialized in the excavation of calcium carbonate through the uptake of calcium anions at the boring front and their active transport along the filament toward the surface (Garcia-Pichel et al., 2010; Guida and Garcia-Pichel, 2016). In culture, *M. testarum* strain BC008 could not bore into dolomite or magnesite. In agreement with this, the closest phylogenetic allies to this strain in our communities, (NR_OTU193) did also show a ~~preference~~ higher rate of occurrence ~~for~~ in calcium carbonates ~~over~~ as compared to magnesium carbonate. Experiments with natural endolithic communities using calcium

pump inhibitors have shown that the calcium-based mechanism is commonly at work in many localities but, at least in one case, boring was impervious to inhibition, pointing to the potential existence of mechanistic diversity (Ramírez-Reinat and Garcia-Pichel, 2012b). Because we could not detect preferential enrichment of *bona fide* euendoliths in the phosphate compared to the carbonate substrates, we must assume that the mineral anion is not a strong determinant of substrate choice in these communities. The boring mechanism described for *M. testarum BC008* is in fact only dependent on the nature of the cation, and could work in principle on calcium phosphates as well, and yet *M. testarum* strain BC008 did not bore into pure hydroxyapatite in the laboratory. These contrasted findings highlight that there must be factors other than the cationic part of the mineral determining the excavation ability of a particular strain and that the boring mechanism proposed for *M. testarum* strain BC008 might be only incompletely described. Other mechanisms have been suggested to explain boring mechanism which have been invalidated for the model organism *M. testarum* strain but may prove themselves valuable for oher taxa. The dissolution of carbonate mineral by acid excretion was proposed by (Haigler, 1969) and (Golubic et al., 1984). This mechanism could involve spatial and temporal separation of photosynthesis *vs.* respiration by Cyanobacteria or acid production as a byproduct of other heterotrophic bacteria activity (Garcia-Pichel, 2006). These hypotheses will need to be re-evaluated for other euendoliths as well as in natural communities.

Conclusion

An in depth survey of endolithic microbial communities associated to Isla de Mona intertidal outcrops revealed a high diversity of organisms, comparable to those ~~one~~ found in other benthic marine microbial communities such as the intertidal sediments and rock surfaces. These complex communities likely host various microbial metabolic guilds beyond oxygenic phototrophs described during more than a century of naturalist's descriptions. The analysis of the cyanobacterial community revealed the

prominence of possible euendolithic species belonging to all the known microborers genera except perhaps *Plectonema*. Contrasting with results obtained at higher taxonomical level, evidence of substrate preference could only be detected among cyanobacteria at the OTU level and close relatives have different distribution patterns, arguing for the existence of boring mechanisms somewhat different to the one described in the model strain *Mastigocoleus testarum*.

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758

759

760 **Figures Captions**

761

762 **Figure 1: Isla de Mona setting** (a) Simplified geological map modified from that of (Briggs and
763 Seiders, 1972) showing the locations of the sampling sites. (b) Sky view of Isla de Mona, the cliff is
764 composed of the Isla de Mona Dolomite topped by the Lirio limestone, the Isla de Mona lighthouse is
765 visible (c-d) Views of Isla de Mona coastal area, samples were taken from isolated boulders (c),
766 directly from the cliff (d) at the notch (white arrows c-d) or on the raised reef flat (c-d).

767

768

769 **Figure 2: Mineral composition and microbial community structure of Isla de Mona intertidal**
770 **outcrops** Each line corresponds to one sample. (a) Mineralogical composition as retrieved by bulk
771 powder XRD (b) Distribution of 16 rDNA OTUs taxonomically assigned at the phylum level and
772 associated chao1 richness metric (c). This reflect the total microbial community structure (d)
773 Distribution of the cyanobacterial 16 rDNA OTUs assigned at the phylum level, excluding chloroplasts
774 and associated chao1 richness metric for Cyanobacteria (e).

775

776

777 **Figure 3: Hierarchical clustering analysis (UPGMA) of bacterial community composition in**
778 **various settings based on pairwise Bray Curtis distance metrics.** The robustness of the topology
779 was assessed through jackknife repeated resampling of 5000 sequences. The number of samples in a
780 given collapsed tree branch are in parentheses, while the numbers in brackets are the Qiita dataset ID
781 number.

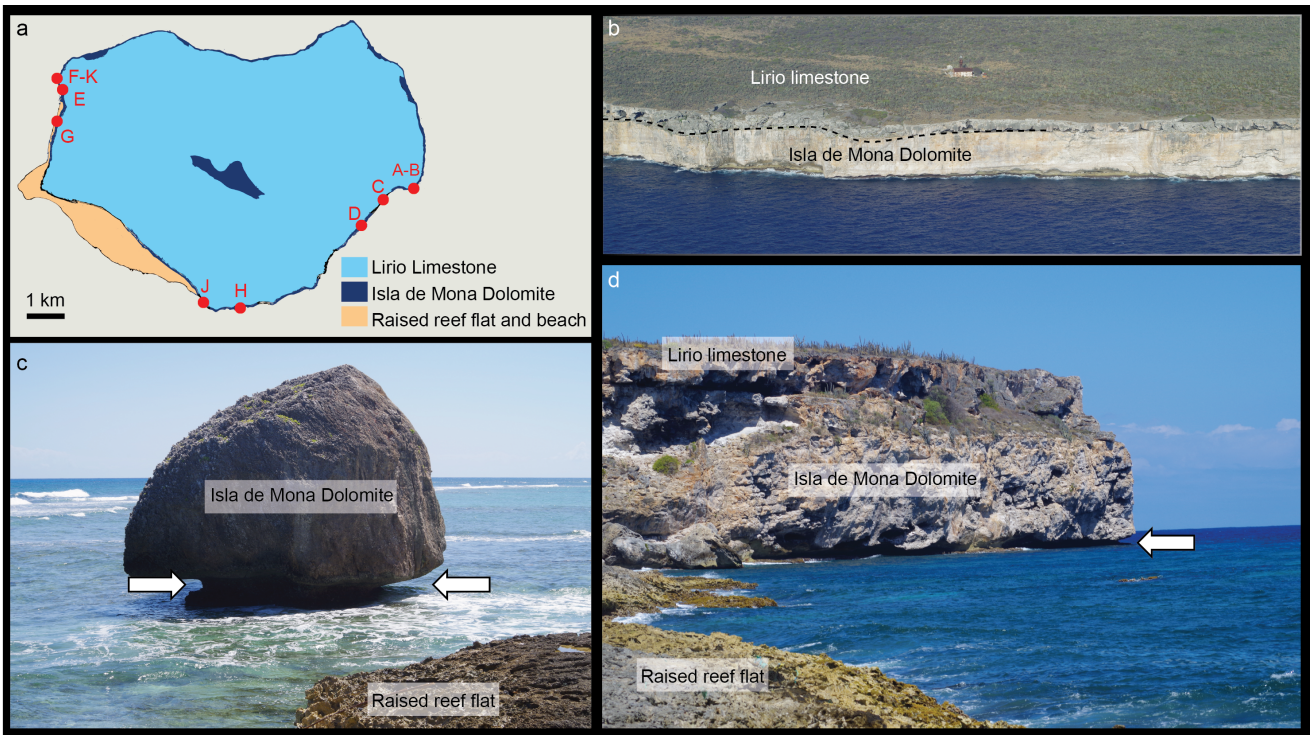
782

783

784 **Figure 4: Differential abundance of cyanobacterial OTUs in Ca-carbonates (calcite-aragonite)**

n=14 vs. CaMg-carbonate (dolomite) n=13 samples. This plot was constructed using the DESeq2 method. It displays the average normalized counts per OTU as a measure of abundance against the log2 fold difference. The OTUs that were significantly differentially abundant in the two conditions ($p<0.05$) are represented as open circles, the other ones are displayed as close symbols. Positive values indicate enrichment towards CaMg-carbonate and negative values indicate enrichment towards Ca-Carbonate. The OTU ID and taxonomical assignment of the most abundant OTUs is displayed on the right. The stars tag the possible euendolithic OTUs as determined by phylogenetic proximity to known microborers (Figure S3).

Figure 5: Differential abundance of cyanobacterial OTUs in Ca-carbonate (calcite-aragonite) n=14 vs. Ca-phosphate (apatite) n=3 samples This plot was constructed using the DESeq2 method. It displays the average normalized counts per OTU as a measure of abundance against the log2 fold difference. The OTUs that were significantly differentially abundant in the two conditions ($p<0.05$) are represented as open circles, the other ones are displayed as close symbols. Positive values indicate enrichment towards Ca-phosphate and negative values indicate enrichment towards Ca-Carbonate. The OTU ID and taxonomical assignment of the most abundant OTUs is displayed on the right. The stars tag the possible euendolithic OTUs as determined by phylogenetic proximity to known microborers (Figure S3).



806

807 **Figure 1**

808

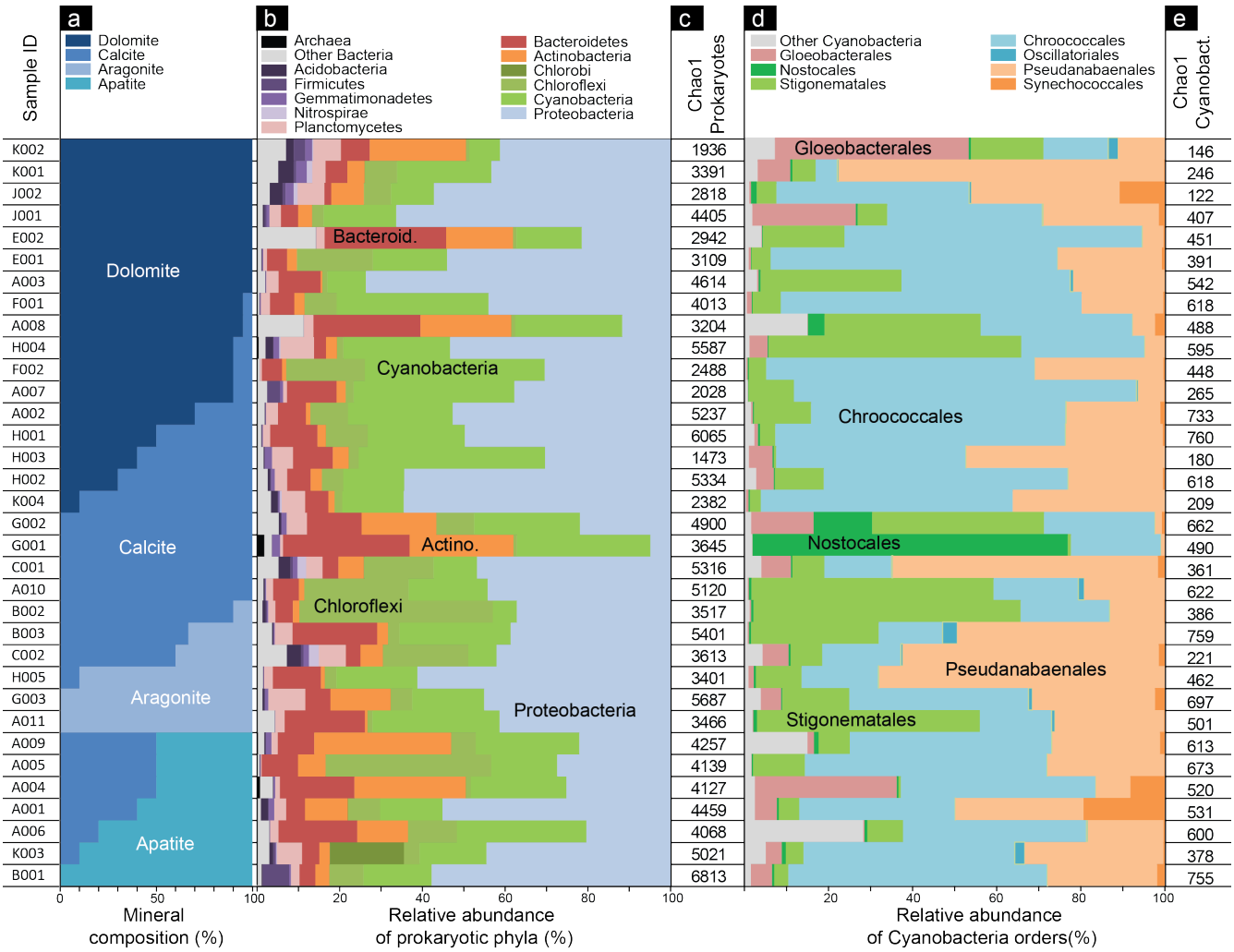
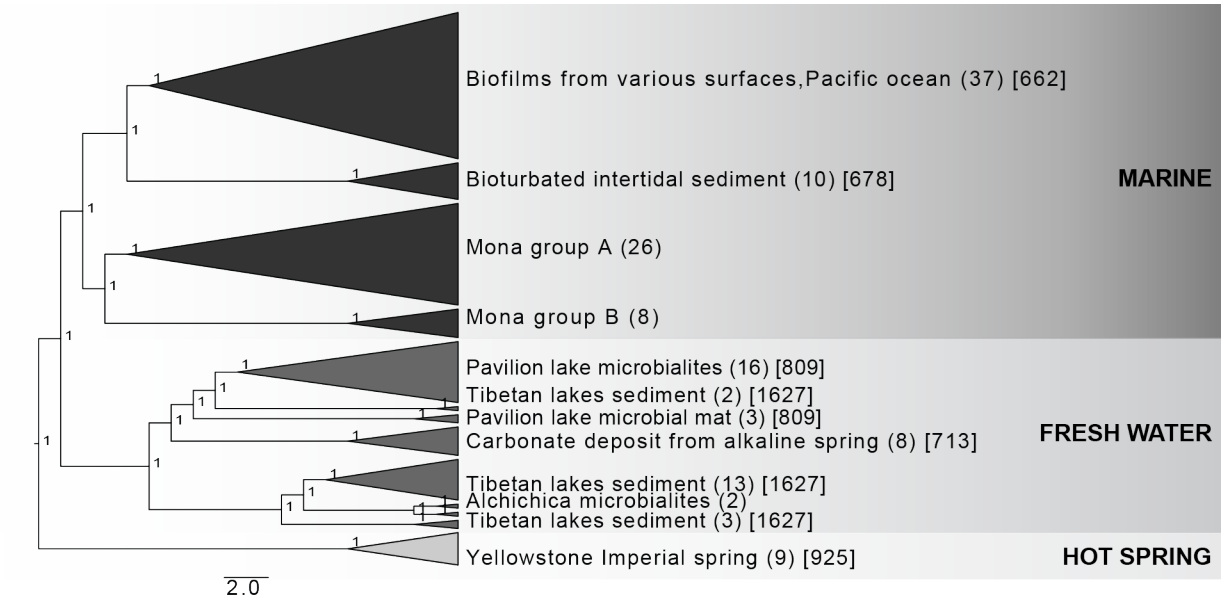

a
b
c
d
e
Sample ID
Dolomite
Calcite
Aragonite
Apatite
Archaea
Other Bacteria
Acidobacteria
Firmicutes
Gemmatimonadetes
Nitrospirae
Planctomycetes
Bacteroidetes
Actinobacteria
Chlorobi
Chloroflexi
Cyanobacteria
Proteobacteria
Chao1 Prokaryotes
Other Cyanobacteria
Gloeobacterales
Nostocales
Stigonematales
Chroococcales
Oscillatoriales
Pseudanabaenales
Synechococcales
Chao1 Cyanobact.
K002 1936 146
K001 3391 246
J002 2818 122
J001 4405 407
E002 2942 451
E001 3109 391
A003 4614 542
F001 4013 618
A008 3204 488
H004 5587 595
F002 2488 448
A007 2028 265
A002 5237 733
H001 6065 760
H003 1473 180
H002 5334 618
K004 2382 209
G002 4900 662
G001 3645 490
C001 5316 361
A010 5120 622
B002 3517 386
B003 5401 759
C002 3613 221
H005 3401 462
G003 5687 697
A011 3466 501
A009 4257 613
A005 4139 673
A004 4127 520
A001 4459 531
A006 4068 600
K003 5021 378
B001 6813 755
Dolomite
Calcite
Aragonite
Apatite
Bacteroid.
Cyanobacteria
Actino.
Chloroflexi
Proteobacteria
Gloeobacterales
Chroococcales
Nostocales
Pseudanabaenales
Stigonematales
0 50 100
Mineral composition (%)
20 40 60 80 100
Relative abundance of prokaryotic phyla (%)
20 40 60 80 100
Relative abundance of Cyanobacteria orders(%)

Figure 2



812

813 **Figure 3**

814

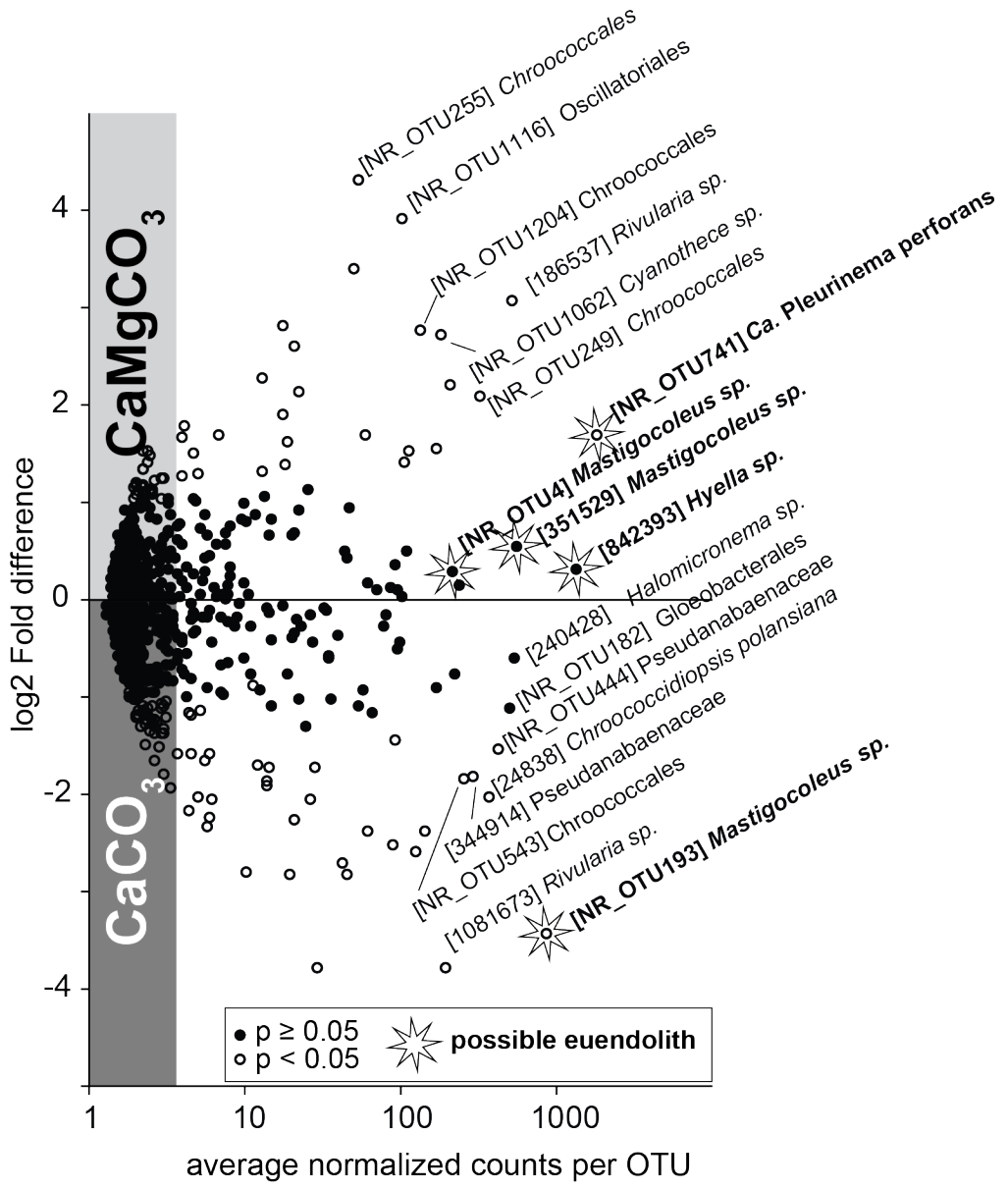
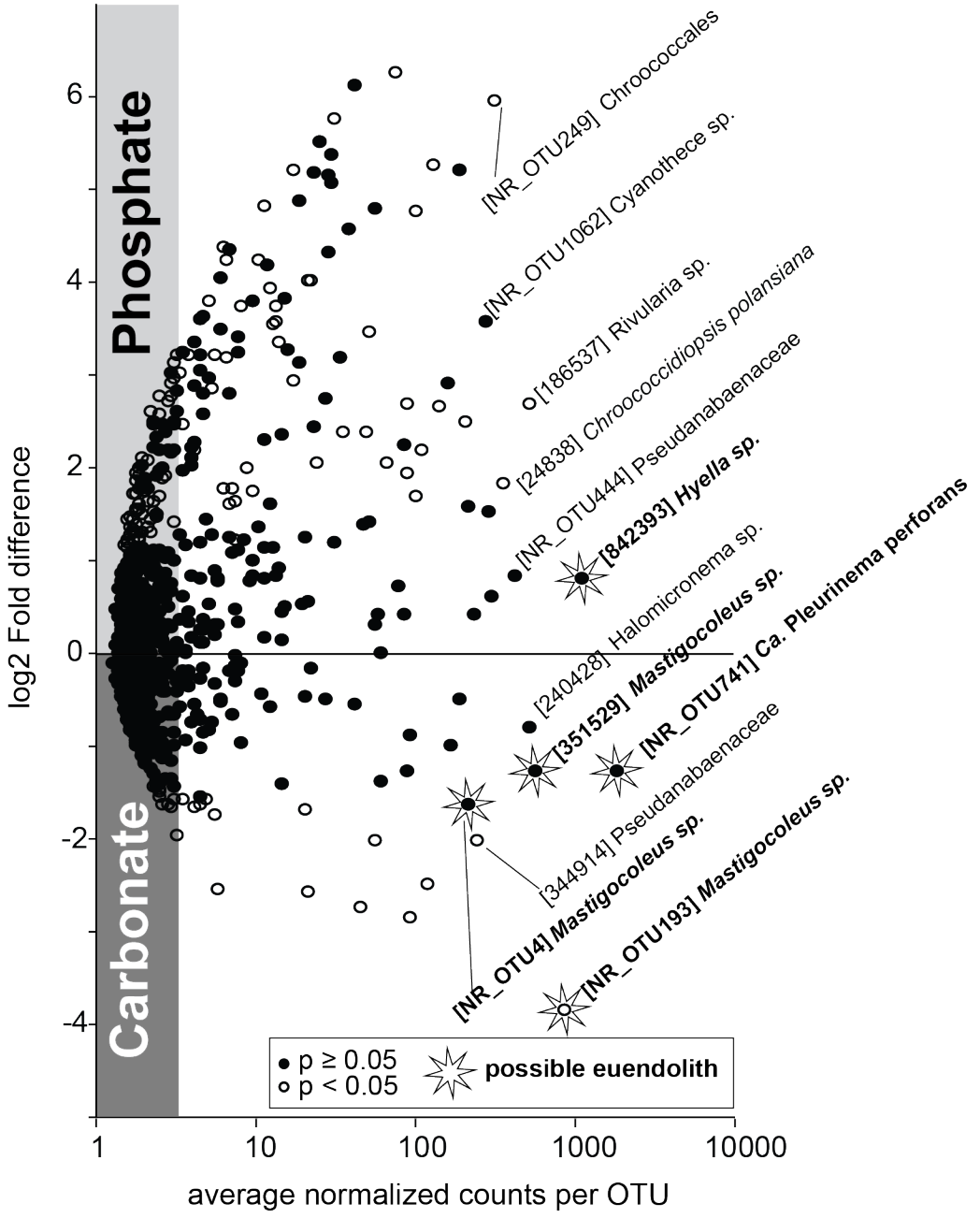

CaMgCO3
CaCO3
log2 Fold difference
average normalized counts per OTU
4
2
0
-2
-4
1
10
100
1000
[NR_OTU255] Chroococcales
[NR_OTU1116] Oscillatoriales
[NR_OTU1204] Chroococcales
[186537] Rivularia sp.
[NR_OTU1062] Cyanothece sp.
[NR_OTU249] Chroococcales
[NR_OTU741] Ca. Pleurinema perforans
[NR_OTU4] Mastigocoleus sp.
[351529] Mastigocoleus sp.
[842393] Hyella sp.
[240428] Halomicronema sp.
[NR_OTU182] Gloeobacterales
[NR_OTU444] Pseudanabaenaceae
[24838] Chroococcidiopsis polansiana
[344914] Pseudanabaenaceae
[NR_OTU543] Chroococcales
[1081673] Rivularia sp.
[NR_OTU193] Mastigocoleus sp.
p ≥ 0.05
p < 0.05
possible euendolith

Figure 4



818

819 **Figure 5**

820

Table 1: Euendolithic cyanobacterial strains used to assign potential roles to OTUs

Strain name	order	reference sequence	presence in this dataset	Isolation source	bores in culture	*reference*
Mastigocoleus testarum	Stigonematales	DQ380405	yes	Cabo Rojo carbonate, Puerto Rico	yes	(Chacón et al., 2006)
Solentia sp. HBC10	Pleurocapsales	EU249126	no	Stromatolite bahamas	yes	(Foster et al., 2009)
Hyella sp. LEGE 07179	Pleurocapsales	HQ832901	yes	Rocky Moledo do Minho beach (Portugal)	not tested*	(Brito et al., 2012)
Ca. **~~Pleuronema~~Pleurinema perforans IdMA4**	Pleurocapsales	KX388631	yes	Isla de Mona outcrop	yes	*this study*
Ca. **Mastigocoleus perforans IdM**	Stigonematales	KX388632	yes	Isla de Mona outcrop	yes	*this study*
Ca. **~~Pleuronema~~Pleurinema testarumRPB**	Pleurocapsales	KX388633	Yes	Puerto Peñasco Coquina reef	yes	*this study*

*Hyella sp. LEGE 07179 was isolated from inside a patella shell where it was identified as a true borer by the authors but its boring ability was never tested again in the lab