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1 **Rapid formation of mature microbialites in Lake Alchichica, Mexico**

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18 **Running title:** Rapid microbialite formation in Lake Alchichica

19 **Originality-Significance Statement**

20 Microbialites are rocks formed by the activity of complex microbial communities that sequester CO₂ in
21 the form of biomass and mineral carbonates. However, little is known about microbialite growth rate
22 and the steps that take place early during their formation. Here, we show that nascent, mm-sized
23 microbialites form on Nylon mesh after two years of exposure in the alkaline Lake Alchichica, which
24 harbors extensive hydromagnesite and aragonite microbialites. The associated prokaryotic and
25 eukaryotic microbial communities were not significantly different of those associated to mature
26 microbialite of the lake. However, only thin microbial biofilms developed on aragonite and
27 hydromagnesite crystals, despite the fact that their microbial communities were similar to those
28 associated to mature microbialites. Our results show that mature microbialites can form very rapidly
29 (up to 1 mm/year) on some surfaces but not on others and open intriguing questions about carbonate
30 nucleation that deserve future exploration.

31 Microbialites are emblematic sedimentary rocks formed by phylogenetically and metabolically complex
32 microbial communities thriving under specific physicochemical conditions. Most microbialites are
33 photosynthesis-based ecosystems frequently formed by carbonates, thereby capturing inorganic carbon
34 in the form of both, organic matter and mineral precipitates. However, little is known about the amount
35 of sequestered carbon and the kinetics of the process, i.e. microbialite growth rate. To assess microbialite
36 growth rate and the influence of substrates on carbonate formation in Alchichica, an alkaline crater lake
37 harboring well-developed carbonate microbialites, we incubated in situ sterilized Nylon mesh,
38 hydromagnesite and aragonite crystals, and bleached-coral aragonite for two years. We observed the
39 rapid formation of nascent hydromagnesite and aragonite-containing microbialites on Nylon mesh, with
40 an average growth rate of ~ 0.6 (and up to 1) mm/year. By contrast, only thin (< 0.2 mm) biofilms developed
41 on exposed hydromagnesite and aragonite crystals and bleached-coral aragonite, suggesting decoupled
42 microbial colonization and biomineralization and/or potential interference of those mineral surfaces with
43 new carbonate nucleation. Microbial communities associated with 2-year-old microbialites and biofilms
44 were fully comparable to mature communities populating Lake Alchichica indigenous microbialites.

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48 **Keywords:** 16S/18S rRNA metabarcoding; stromatolite; carbonate precipitation; biomineralization;
49 colonization; cyanobacteria; anoxygenic photosynthesis

50

51 Introduction

52 Microbialites are organosedimentary structures formed under the influence of phylogenetically and
53 functionally diverse microbial communities in particular physicochemical environments (Riding, 2000;
54 Dupraz and Visscher, 2005). Microbialites are not only emblematic as the oldest undisputed traces of
55 life on Earth (~3.5 Ga-old laminated microbialites or stromatolites)(Tice and Lowe, 2004; Allwood et al.,
56 2006) but also constitute poorly understood carbon traps (Ahrendt et al., 2014; McCutcheon et al.,
57 2014; McCutcheon et al., 2019). Most marine and freshwater microbialites are formed by
58 photosynthesis-based microbial communities that favor carbonate precipitation by locally increasing
59 the alkalinity (Dupraz and Visscher, 2005; Dupraz et al., 2009) and/or upon an alkalinity threshold (Zeyen
60 et al., 2017; Iniesto et al., 2020). Consequently, they constitute carbon reservoirs in the form of both,
61 biomass and solid carbonates. To ascertain if microbialite systems are efficient carbon sinks, more
62 quantitative measurements are needed. However, relatively little is known about early microbialite
63 formation and growth rate. Short-term $^{13}\text{C}/^{14}\text{C}$ -labeling experiments suggest that carbonate
64 precipitation occurs, albeit with low rates (0.05 mm/year) in Lake Pavilion (Brady et al., 2009), but other
65 systems seem to have much higher rates, in the 0.2-0.6 mm/year range (Chivas et al., 1990; Carvalho et
66 al., 2017).

67 Recent comparative studies in lakes from the Trans-Mexican volcanic belt showed that microbialite
68 size and community structure significantly correlate with alkalinity (Zeyen et al., 2017; Iniesto et al.,
69 2020), suggesting that local biotic and abiotic factors result in different growth rates. In particular, Lake
70 Alchichica, an alkaline (pH~9; $[\text{HCO}_3^-] \sim 40$ mM) and relatively Mg-rich ($[\text{Mg}^{2+}] \sim 17$ mM) crater lake
71 located at high altitude (2,300 m above sea level) displays massive (meter-sized) microbialites, mostly
72 non-laminated (thrombolites), composed of hydromagnesite ($\text{Mg}_5(\text{CO}_3)_4(\text{OH})_2 \cdot 4(\text{H}_2\text{O})$) and aragonite
73 (CaCO_3) that precipitate in situ (Kazmierczak et al., 2011; Couradeau et al., 2013; Gerard et al., 2013).
74 Microbial communities associated to these structures have been previously studied using 16S/18S rRNA
75 gene metabarcoding and metagenomic approaches (Couradeau et al., 2011; Saghaï et al., 2016;
76 Valdespino-Castillo et al., 2018). Interestingly, core microbial communities shared by microbialites
77 across various lakes accounted for higher proportions of the total microbial community in the highest-
78 alkalinity sites, notably Alchichica (Iniesto et al., 2020). Various indicators, such as active carbonate
79 precipitation observed in the water column and the presence of more or less developed microbialites
80 on plants growing on the rim of the lake as well as plastic bottles and tires abandoned on the lake shore,
81 suggested that microbialite formation is an active and relatively fast process in Lake Alchichica.
82 However, details about the first steps, including growth rate, of microbialite formation in this lake were
83 missing.

84

85 Results and discussion

86 To evaluate the capacity of calcifying microbial communities to colonize different surfaces and form new
87 microbialites as well as to assess microbialite growth rate, we placed sterilized crystals of
88 hydromagnesite and aragonite as well as bleached-coral fine-grained aragonite in extensively
89 perforated 50-ml Falcon tubes that were capped with 200 μm -pore diameter Nylon mesh pieces
90 (Fig.1A). These colonization devices were immersed at 0.5 m depth in the North shore of Lake Alchichica
91 from January 2012 to May 2014. Nascent microbialites and biofilms growing on the incubated substrates
92 were then collected, as well as, for comparison, fragments from large older microbialites growing at
93 equivalent depth (0-3 m) below the water surface on the North and West shores (Supplementary
94 Methods, Supplementary Fig.1 and Supplementary Table 1) at sampling points previously studied
95 (Couradeau et al., 2011; Saghai et al., 2015; Saghai et al., 2016; Iniesto et al., 2020).

96 After the 28 month-incubation period, small, distinct microbialite structures of up to approximately
97 2 mm in diameter could be observed growing on the Nylon mesh that was used as permeable barrier
98 capping the Falcon tubes (Fig.1A-C). These formed discrete structures that tended to merge, and not a
99 continuous calcifying biofilm, highlighting the importance of local nucleation. Individual measurements
100 allowed an estimation of an average microbialite growth of ~ 0.6 mm/year (and up to 1 mm/year),
101 although this growth rate might be different for already growing structures. No macroscopically visible
102 stratification or lamination was observed. To determine the overall mineral composition of these
103 incipient structures, we applied attenuated total reflection Fourier transform infrared (ATR-FTIR)
104 spectroscopy (Supplementary Methods). The obtained spectra highlighted their composite nature,
105 unambiguously showing the occurrence of two distinct carbonate phases: hydromagnesite and
106 aragonite (Fig.1D). In addition, we detected a silica-rich phase, possibly associated with diatom frustules
107 (ochrophytes; Fig.2C and D). This mineral composition is consistent with previous observations made on
108 mature microbialites from this lake site (Zeyen et al., 2019), indicating that nascent microbialites have a
109 similar overall mineral composition to well-established microbialites. Furthermore, since
110 hydromagnesite is the carbonate mineral spontaneously precipitating in the lake (Couradeau et al.,
111 2011; Kazmierczak et al., 2011) and aragonite has been shown to be spatially associated with particular
112 microbial groups, notably some cyanobacteria (Gerard et al., 2013; Saghai et al., 2015), the observed
113 composition strongly suggests active biomineralization in nascent microbialites. By contrast, only a thin
114 biofilm (a few tens up to 1-200 μm) but no apparent microbialitic structures formed on any of the
115 incubated mineral fragment surfaces, whatever their orientation, suggesting that microbial colonization
116 of surfaces can be uncoupled from biomineralization. The absence of microbialite formation on the
117 exposed mineral surfaces is unlikely to be strongly influenced by slight differences of physicochemical
118 conditions within the perforated Falcon tube compared to the surface of the Nylon mesh. First, mineral
119 fragments occupied around 5% of the inner 50-ml Falcon volume and water could circulate via the many

120 ~3-5 mm diameter holes suggesting that, if there were chemical differences between the inside and
121 outside of the tube, these were minor. In any case, these differences would be comparable to those
122 found between small protected pockets in the very irregular cauliflower-like native microbialites, with
123 limited water circulation, and the open lake waters. Second, small pocket zones and irregular shapes in
124 native growing microbialites result in the existence of areas protected from direct light incidence. This,
125 together with the fact that microbialites actively develop from the surface of the lake to at least 40 m
126 depth (personal observations), implies that the observed patterns cannot be easily explained by
127 differences in light intensity and quality. Therefore, although these initial observations of biofilm-only
128 formation on aragonite and hydromagnesite crystals after two years of exposure need to be confirmed
129 by more extended experiments, the observed patterns might suggest some type of nucleation
130 interference or inhibition caused by those mineral surfaces.

131 To determine the microbial community structure associated with these samples, we studied the
132 prokaryotic and eukaryotic components of two nascent microbialites growing on the Nylon mesh and
133 the 2-year-old biofilms growing on the mineral substrates by a metabarcoding approach. We amplified
134 16S and 18S rRNA gene fragments and massively sequenced the resulting amplicons using paired-end
135 Illumina MiSeq. After quality trimming, we built operational taxonomic units using SWARM and Cd-hit
136 at 97 and 98% identity thresholds, which yielded comparable prokaryotic OTU numbers (Supplementary
137 Methods; Supplementary Table 2). We retained SWARM clustering for subsequent analyses due to the
138 resulting lower, more tractable, number of eukaryotic OTUs. OTUs were assigned to phyla and/or high-
139 rank taxa by sequence similarity and, for divergent OTUs, phylogenetic placement in reference
140 phylogenetic trees (Supplementary Tables 3-4). The community structure of nascent microbialites but
141 also of the biofilms was comparable to that of mature Alchichica microbialites in terms of relative
142 abundance and, most especially, OTU diversity (Fig.2A-D). This was further shown by the scattering of
143 mature and young samples in a non-metric multidimensional scaling (NMDS) ordination analysis (Fig.2E;
144 Supplementary Fig.5). Prokaryotic communities were rich in Cyanobacteria and their associated
145 hydrocarbon-scavenging taxa (Bacteroidetes, Planctomycetes, Verrucomicrobia). Anoxygenic
146 photosynthetic bacteria were also abundant, notably Chloroflexi and some Alphaproteobacteria
147 (Rhodobacterales, Rhodospirillales), but also some Gammaproteobacteria (Chromatiaceae) and minor
148 proportions of Chlorobi. Green algae and diatoms (ochrophytes), together with ciliate and fungi were
149 the most relatively abundant eukaryotes. However, because they represent a minor part compared to
150 the prokaryotic component (Saghai et al., 2016), the relative abundance of eukaryotic taxa is likely
151 affected by higher stochasticity. Only minor differences appeared in terms of relative abundance of
152 major groups; the most notable being the presence of a higher proportion of the *Thermus-Deinococcus*
153 clade in the sampled aragonite-colonizing biofilm (Fig.2A). Despite these minor differences, the two
154 nascent microbialites and the three mineral-associated biofilms shared over 900 OTUs with mature

155 Alchichica samples collected at the north (Alchichica N) and west (Alchichica W) shorelines (Fig.2F).
156 These shared OTUs represented 80-90% of the total prokaryotic OTUs, and 87-97% of the total
157 eukaryotic OTUs, in Alchichica N and W samples, nascent microbialites and mineral-associated biofilms
158 (Fig.2G-H; Supplementary Table 5). PERMANOVA pairwise comparisons showed that differences
159 between mature, nascent microbialite communities or 2-year-old biofilms growing on incubated
160 minerals were not significant or very marginally so (p-value 0.05 - 0.83; Supplementary Table 6). Biofilm
161 and microbialite communities were very different from planktonic communities along the water column
162 of the lake (Iniesto et al., submitted). This can be easily visualized in an NMDS plot, where microbialite-
163 associated and planktonic communities clearly clustered apart (Supplementary Fig. 6).

164 Collectively, our results show a rapid colonization dynamics of certain newly available inert surfaces
165 (Nylon mesh, but also plastic bottles or plants on site) in Lake Alchichica, with the formation of mature-
166 like microbialites in terms of mineral and microbial community compositions in only two years. By
167 contrast, although biofilms growing on incubated hydromagnesite and aragonite crystals, including coral
168 aragonite, have similar microbial composition to that of mature microbialites, carbonate precipitation
169 does not occur at visible scale. This suggests an uncoupling between community assembly and induced
170 biomineralization on these surfaces and/or a potential interference of hydromagnesite and aragonite
171 crystalline surfaces with microbialite carbonate nucleation, opening intriguing questions about
172 carbonate nucleation in these natural ecosystems.

173

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238 **Figure legends**

239

240 **Fig.1.** Rapid growth of nascent microbialites in Lake Alchichica. **A**, photograph of colonization devices
241 deposited in the North shore of Lake Alchichica in January 2012 and collected in May 2014. **B-C**, detail
242 of the incipient microbialites growing on Nitex 200 μm -mesh sized membranes in the corresponding
243 areas framed in (A). **D**, ATR-FTIR spectrum showing the presence of hydromagnesite and aragonite
244 peaks.

245

246 **Fig.2.** Prokaryotic and eukaryotic community composition in mature and nascent microbialite samples
247 as well as biofilms growing on 2-year exposed mineral substrates in Lake Alchichica, Mexico. **A**,
248 histograms showing the phylogenetic diversity and relative proportion of 16S rRNA gene amplicon
249 sequences. **B**, phylogenetic diversity and relative abundance of prokaryotic operational taxonomic units
250 (OTUs) across phyla. **C**, phylogenetic diversity and relative proportion of 18S rRNA gene amplicon
251 sequences. **D**, phylogenetic diversity and relative abundance of eukaryotic OTUs across phyla. Detailed
252 histograms of the categories Cyanobacteria, Alphaproteobacteria and 'Other Bacteria' are provided in
253 Supplementary Fig.2, those of Archaea and 'Other eukaryotes' are provided in, respectively,
254 Supplementary Figs.3A and 4. **E**, Non-metric multidimensional scaling (NMDS) biplot of the different
255 microbial communities associated to nascent microbialites and large mature microbialites growing on
256 the North and West shores of Lake Alchichica, as well as 2-year old biofilms growing on coral aragonite
257 (CA), aragonite (A) and hydromagnesite (HM). **F**, Venn diagram showing the number of OTUs shared by
258 the different groups of microbialite and mineral-associated 2-year old biofilm samples. **G-H**, relative
259 abundance and phylogenetic distribution of prokaryotic (G) and eukaryotic (H) core shared OTUs (as
260 shown in F). Detailed sample descriptions are provided in Supplementary Table 1; nasc. mic., nascent
261 microbialites.

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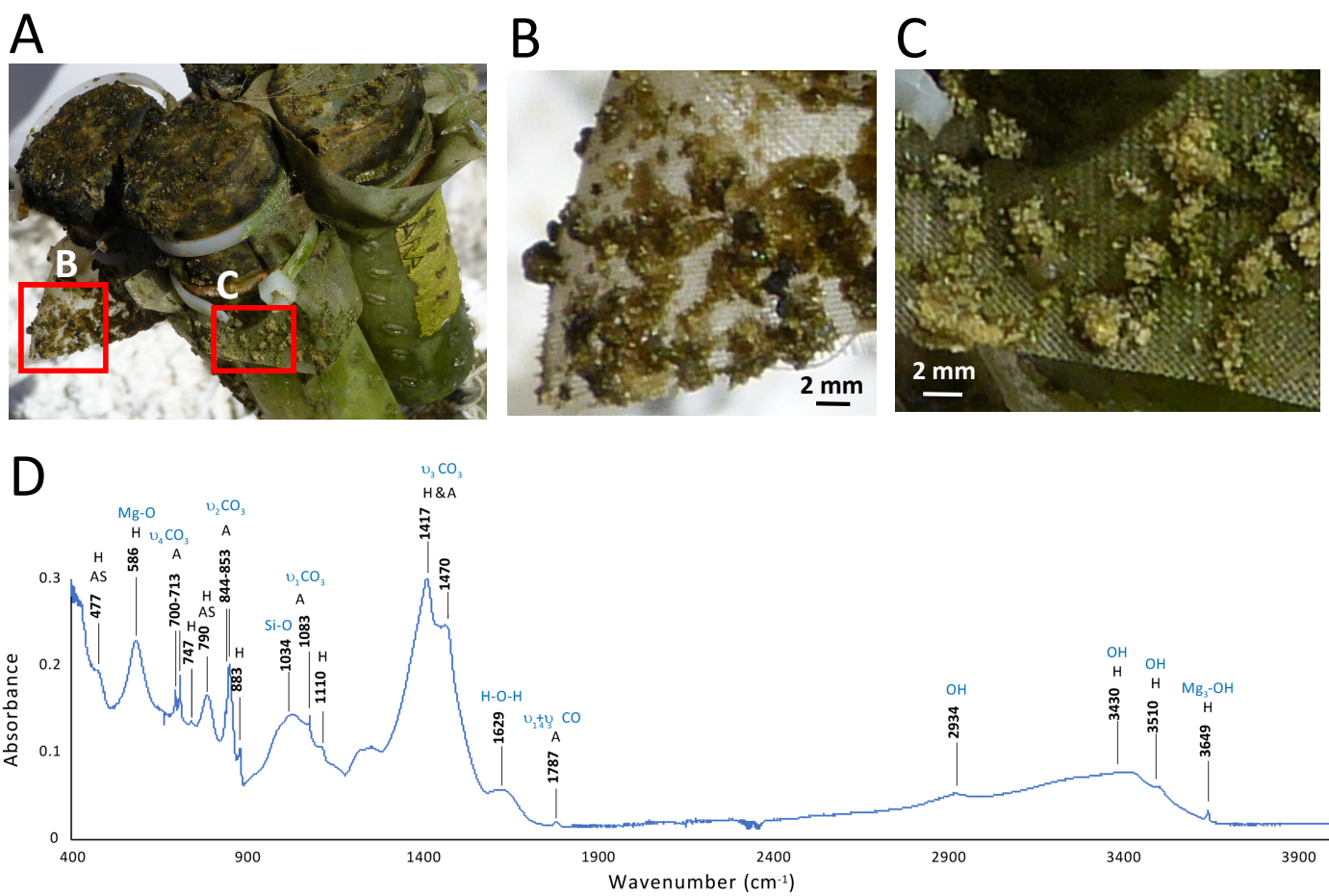


Figure 1. Iniesto et al.

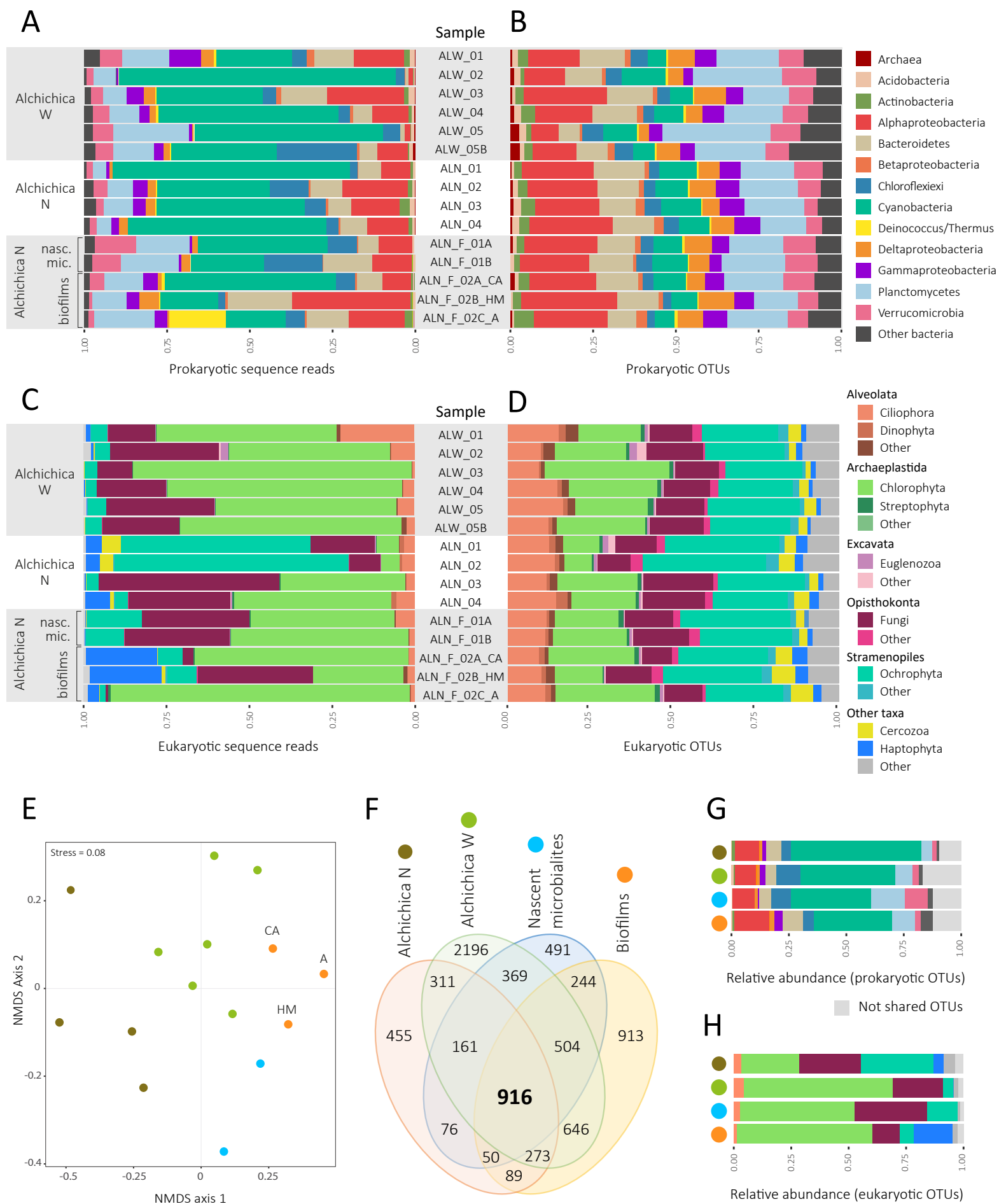


Figure 2. Iniesto et al.