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On the dual nature of lichen-induced rock surface weathering in contrasting micro-environments

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23	

24 Abstract

25

26 Contradictory evidence from biogeomorphological studies has increased the debate on the extent 27 of lichen contribution to differential rock surface weathering in both natural and cultural settings. This study, undertaken in Côa Valley Archaeological Park, aimed at evaluating the effect of rock 28 29 surface orientation on the weathering ability of dominant lichens. Hyphal penetration and oxalate 30 formation at the lichen-rock interface were evaluated as proxies of physical and chemical 31 weathering, respectively. A new protocol of pixel-based supervised image classification for the 32 analysis of periodic acid-Schiff stained cross-sections of colonized schist revealed that hyphal 33 spread of individual species was not influenced by surface orientation. However, hyphal spread was significantly higher in species dominant on north-west facing surfaces. An apparently 34 opposite effect was noticed in terms of calcium oxalate accumulation at the lichen-rock interface, 35 36 detected by Raman spectroscopy and complementary X-ray microdiffraction on south-east facing 37 surfaces only.

38 These results suggest that lichen-induced physical weathering may be most severe on north-west 39 facing surfaces by means of an indirect effect of surface orientation on species abundance, and 40 thus dependent on the species, whereas lichen-induced chemical weathering is apparently higher on south-east facing surfaces and dependent on micro-environmental conditions, giving only 41 42 weak support to the hypothesis that lichens are responsible for the currently observed pattern of 43 rock-art distribution in Côa Valley. Assumptions about the drivers of open-air rock-art 44 distribution patterns elsewhere should also consider the micro-environmental controls of licheninduced weathering, to avoid biased measures of lichen contribution to rock-art deterioration. 45

47 Keywords

48

49 Biodeterioration, Biogeochemistry, Biomineralization, Raman spectroscopy, XRMD, Image50 analysis, Schist

51

52 Introduction

53

54 The last three decades have been extremely rich in contributions to the knowledge of the various 55 aspects of lichen-induced rock weathering, as seen by the number of reviews available (Adamo 56 & Violante 2000, Chen et al. 2000, Seaward 2015, St. Clair & Seaward 2004). Alternative approaches to this subject have been focusing on: i) identifying individual species and species 57 assemblages colonizing rock surfaces and making assumptions on their impact based on previous 58 59 knowledge about their ecological requirements (e.g. Carballal et al. 2001); ii) determining the 60 climatic constraints and habitat preferences of colonizing species based on field observations (e.g. Steinbauer et al. 2013, Viles & Cutler 2012) or controlled experiments (Adamson et al. 61 62 2013, Carter & Viles 2003, Kidron & Termina 2010) thus contributing to the knowledge of 63 environmental factors that are also important for rock conservation; iii) detecting geophysical and geochemical changes at the lichen-rock interface associated with the growth of individual 64 species including the occurrence of organic and mineral by-products of lichen activity (e.g. 65 Arocena et al. 2007, Favero-Longo et al. 2005); iv) addressing the influence of human activities 66 67 on such changes (e.g. Cámara et al. 2015) and v) developing methods to quantify the weathering rates induced by individual species or by a limited set of the most representative ones on the 68

surface of interest (e.g. Aghamiri & Schwartzman 2002, Bartoli et al. 2014, Gazzano et al.
2009a, b, Mcllroy de la Rosa et al. 2014).

The majority of work has been applied at characterizing the biodeterioration of a range of
stonework in Europe and only sporadically in other regions of the globe. Few papers have dealt
with the relationship between lichen growth and the weathering of schist (Aghamiri &
Schwartzman 2002, Cann et al. 2012, Galvan et al. 1981, Fry 1924, 1927, Sanders et al. 1994)
despite the increasing demand of schist as a building stone.

76 The processes of schist weathering, including those induced by lichen activity, are a primary concern in the Côa Valley Archaeological Park (Vila Nova de Foz Côa, north-east Portugal) 77 78 where one of the world's most important sets of Prehistoric rock-art is located, almost invariably 79 on schistose surfaces. Efforts are being made there to understand the weathering dynamics acting on the schist outcrops that support the rock-art, integrating biological, geophysico-chemical and 80 81 environmental data in order to prevent major damages to the engraved surfaces (Aubry et al. 82 2012). There is general consensus about the combination of physical (mechanical) and chemical 83 changes brought about by lichens to rock surfaces but the extent and relative contribution of their weathering action is a fundamental, yet still unanswered question, both in the Côa Valley and in 84 85 the field of rock-art conservation in general. Adding to the debate over the threats of licheninduced processes is the contradictory evidence for lichen protection of rock surfaces against 86 other deteriogenic agents (Carter & Viles 2005, McIlroy de la Rosa et al. 2013). 87 Recently, Aubry et al. (2012) suggested that aspect-related differences in the extent of lichen 88 89 (and bryophyte) colonization of vertical schist surfaces in the Côa Valley could be partly responsible for the differential weathering of those surfaces and resulting pattern of rock-art 90 91 distribution in the Côa Valley, which is currently more concentrated at south-east facing than at

92 north-west facing slopes (Fernandes 2012). Provided that the interaction between lichens and the 93 rock surface is species-dependent (e.g. Favero-Longo et al. 2005), a key uncertainty in the 94 assumptions on the relationship between lichen colonization and rock weathering is precisely in 95 the way that lichen species act under different weathering environments. Studies aimed at evaluating the response of individual species to changes in environmental conditions have 96 97 typically demonstrated a shift in oxalate production (thus in the biodeteriorative action) in 98 response to environmental variation (Caneva 1993, Edwards et al. 1995, Prieto et al. 2000). The 99 extreme environments of cold and hot deserts have been particularly interesting for research (e.g. Wierzchos et al. 2013) since, besides acting differently in distinct weathering contexts, species 100 101 are also expected to change their performance under the influence of environmental change. 102 However, although useful first approximations, the existing studies commonly assume species 103 neutrality, with substrate and climate as the primary controls. As a result, knowledge about 104 lichen-induced rock weathering is mostly based on static views of the influence of 105 environmental, geological or climatic parameters such as rock porosity, permeability and 106 mechanical properties, or temperature, solar radiation and humidity. 107 The variation of the diversity and composition of lichen assemblages with rock surface 108 orientation is a well-known phenomenon in both natural and cultural contexts (Adamson et al. 109 2013) as orientation acts as a proxy of those environmental variables, mainly temperature and 110 humidity, which affect the structure and dynamics of saxicolous lichen communities. Some 111 components of that variation in the Côa Valley have been previously addressed from an 112 ecological perspective (Marques et al. 2014). The present study directly assessed the effect of rock surface orientation on lichen-induced 113 114 physical and chemical weathering in order to determine the contribution of lichen action to

115 differential rock weathering in the Côa Valley Archaeological Park. Hyphal penetration and 116 calcium oxalate formation at the lichen-rock interface were confidently assumed as proxies for 117 physical and chemical weathering, respectively. Although how much hyphae penetrate through 118 existing pores and fissures or actively produce discontinuities in the rocks is not definitely clear, 119 microscopical observations have shown that penetrating hyphae induce mineral breakage and 120 rock surface disaggregation by mechanical action (Ascaso & Wierzchos 1995, Favero-Longo et 121 al. 2005). The close relationship between the chemical composition of colonized rocks and the 122 type of oxalates accumulating immediately beneath or within the thalli of some lichen species have generally indicated an involvement of the mycobiont-secreted oxalic acid in lichen-induced 123 124 chemical weathering of rock substrata (Adamo & Violante 2000 with refs. therein).

125 Material and methods

126

127 The study area

128

The Côa Valley Archaeological Park is a UNESCO world heritage site located in the confluence 129 130 of River Côa with River Douro, approximately 200 km upstream of the mouth of the Douro, in 131 the city of Porto (Portugal). Lithology in this part of the Côa Valley is dominated by meta-132 sedimentary rocks of the schist-greywacke complex ranging in age from the Precambrian to the 133 Ordovician. River Côa and its tributary streams have cut deeply through the schist and 134 greywacke basement, taking advantage of pre-existing major faults roughly NE-SW oriented 135 (Aubry et al. 2012) and forming numerous steep-walled valleys that play a major influence on regional landscape. A special feature resulting from the down-cutting of the Côa Valley is the 136 137 occurrence of massive vertical schist surfaces arranged in layers along the valley's slopes, which

138	have been gradually exposed by rock toppling, i.e. a sequence of gravity-induced block				
139	displacement after splitting of vertically orientated joints, along the schistosity plane.				
140	Climate is predominantly dry meso-Mediterranean, sheltered from the Atlantic influence by				
141	mountains to the north and the west, but thermo-Mediterranean microclimates are usually				
142	produced in the bottom of the valleys, where temperature is frequently above 40°C in late spring				
143	and summer (June-August), daily thermal amplitudes may reach 10° to 15° C and mean annual				
144	precipitation is often below 300 mm (Fernandes 2012).				
145					
146	Geological and mineralogical characterization of the studied lithotype				
147					
148	The target lithotype is a relatively low-grade metamorphic (greenshist facies) phyllite consisting				
149	of thin alternating layers of whitish psammitic and dark pelitic components (Aires et al. 2011,				
150	Búrcio 2004, Sousa 1982). The psammitic component is sometimes more abundant and the rock				
151	is then classified as a metaquartzwacke instead of a phyllite. For a matter of simplicity, this				
152	phyllite-metaquartzwacke sequence will be addressed under the broad term schist. This schist is				
153	mainly composed of quartz, sericite and/or muscovite, chlorite and biotite minerals, as well as				
154	plagioclase feldspars (mostly albite) in variable amounts depending of the psammitic				
155	contribution. Calcite is usually present in the matrix of these rocks in sufficient amounts to				
156	produce effervescence when treated with dilute hydrochloric acid. Magnetite and, more				
157	sporadically, pyrite crystals are present in both the psammitic and pelitic levels as accessory				
158	constituents (Sousa 1982). Additional accessory minerals including illite, kaolinite,				
159	montmorillonite, graphite, turmaline, zircon, apatite, epidote, hematite, leucoxene and alkali				

- 160 feldspars, such as microcline and orthoclase, were detected by polarized light (petrographic)
- 7

161	microscopy and X-ray diffraction (Aires et al. 2011, Búrcio 2004, Gomes & Almeida 2003,				
162	Sousa 1982). The general strike of the target fracture/joint surfaces is NE-SW, which is sub-				
163	parallel to the NNE-SSW sinistral strike-slip fault system that crosses the study area and formed				
164	by the same tectonic stress (Aubry et al. 2012). The plane of schistosity is consistently vertical				
165	and oriented subperpendicular to the fracture/joint surfaces.				
166					
167	Microclimatic characterization of vertical schist surfaces in the Côa Valley				
168					
169	To characterize the thermal and hydric contrasts of opposite slopes in the Côa Valley,				
170	Hygrochron iButton data-loggers (Maxim Integrated Products Inc., Sunnyvale (CA), USA) were				
171	placed on 12 vertical schist surfaces of varying orientations. The results were then grouped in the				
172	two aspect classes of interest for this study: north-west (NW) and south-east (SE). Data-loggers				
173	were synchronized and set to record both temperature (°C) and relative humidity (%), at hourly				
174	or half-hourly intervals during a 3-year period from late September 2010 to late September 2013.				
175					
176	Target species				
177					
178	The study dealt with the physical and chemical weathering driven by four locally-common				
179	lichens: Aspicilia contorta subsp. hoffmanniana S. Ekman & Fröberg (Aspicilia hoffmanniana				
180	hereafter), Caloplaca subsoluta (Nyl.) Zahlbr., Lecanora pseudistera Nyl. and Peltula euploca				
181	(Ach.) Poelt. Taxa selection was based on higher frequency and abundance (cover) on the				
182	vertical schist surfaces of the Côa Valley Archaeological Park (Marques et al. 2014). Aspicilia				
183	hoffmanniana is a crustose lichen varying in colour from greenish-grey in shaded NW facing				

184 surfaces to light brown in exposed SE facing surfaces, where it is more abundant. Caloplaca 185 *subsoluta* is a deep orange coloured crustose species that is common on siliceous rocks 186 throughout the Mediterranean. In the Côa Valley it was found equally abundant on the vertical 187 schist surfaces of the two opposing slopes. Lecanora pseudistera is a white crustose species 188 proliferating on NW facing surfaces, although it can also be found less abundantly on SE facing 189 schist surfaces. *Peltula euploca* is a widespread squamulose epilith characteristic of the rain-190 track communities of vertical schist surfaces and exclusive of SE facing exposures (Marques et 191 al. 2014). Each squamule is attached to the substrate by a central umbilicus and its lower surface 192 is pale to dark brown. The algal partner is *Trebouxia* spp. in the three crustose lichens and 193 unicellular cyanobacteria (Chroococcidiopsis spp.) in Peltula euploca. The three crustose lichens 194 are able to reproduce sexually, although Aspicilia hoffmanniana is most frequently sterile. *Peltula euploca* is often fertile in the study area but its characteristic mode of dispersal is through 195 196 vegetative propagules (soredia). Secondary compounds in Aspicilia hoffmanniana are either 197 lacking or include only aspicilin. Caloplaca subsoluta produces anthraquinones. The major secondary metabolites produced by Lecanora pseudistera are atranorin and 2'-O-methylperlatolic 198 199 acid. Peltula euploca lacks secondary metabolites.

200

201 Sampling strategy and in-field sample collection

202

203 Colonized rock samples, with lichen thallus kept intact, were taken at random from non-

204 engraved NW and SE facing schist surfaces, located at representative rock-art sites, namely

205 Canada do Amendoal, Foz do Côa, Quinta das Tulhas, Vale do Forno and Vale de José Esteves,

and are therefore very similar to the surfaces bearing rock-art in terms of their macro- and micro-

207 environmental constraints. Since Peltula euploca is virtually exclusive of SE facing surfaces, 208 appropriate samples of rock colonized by this particular species could only be found and 209 collected at SE. Uncolonized rock samples were also taken from the source outcrops to be used 210 as controls. The use of bare-rock controls is a necessary condition for isolating the lichen-211 induced effects from the ones induced by other weathering agents (either biotic or abiotic). 212 The collected samples were cut perpendicular to the colonized or previously exposed surface, up 213 to a depth of 3 to 4 cm and width of 5 cm, with an Isomet 1000 Precision Saw (Buehler, 214 Düsseldorf, Germany). The surfaces of the resulting cross-sections were polished with sandpaper attached to an Ecomet 3000 (Buehler, Düsseldorf, Germany) polisher machine. Nine replicates 215 216 were prepared for each combination of species vs orientation, and respective control 217 (uncolonized) in order to evaluate if weathering associated with lichen colonization differs from the weathering produced on identical but lichen-free surfaces. Three subsets of three replicates 218 219 each were taken from the initial sample set, and processed accordingly for Periodic acid-Schiff 220 (PAS) staining, X-ray microdiffraction and FT-Raman spectroscopy. Almost all samples had 221 been included in polyester resin (*Recapoli* 2196 styrene and phthalic anhydride, Methyl Ethyl 222 Ketone peroxide as catalyst) before cutting, to avoid excessive loss of material due to the fragile 223 nature of the schist samples, except for those used in Raman analysis, since resin inclusion 224 would preclude from taking measures directly on the lichen thallus.

225

226 Periodic acid-Schiff staining

227

This procedure aimed at highlightingthe Hyphal Penetration Component (HPC) of the target
lichen species, as a proxy of lichen-induced physical weathering, following Favero-Longo *et al.*

230 (2005). Microphotographs of the stained cross-sections were then acquired at a $\times 10$

magnification using a Nikon SMZ1000 stereomicroscope equipped with a Nikon DS Fi1 digital
camera, at three random locations of each cross-section. Data on the depth of hyphal penetration
was obtained by visual inspection of all hyphal bundles highlighted in the stained cross-sections
under the same stereomicroscope. The mean and the maximum of measured data were calculated
for each species at different aspects to provide the average and maximum depth of hyphal
penetration sensu Favero-Longo *et al.* (2011).

237 In order to quantify the extent of hyphal penetration, as well as the size of other important weathering-related features (e.g. weathering rind), the acquired images were submitted to a new 238 239 protocol of pixel-based supervised classification using colour and texture features, which can be 240 described briefly as follows: 1) Image pre-processing in ImageJ (http://imagej.nih.gov/ij/), 241 including the resize of original images to 40% of the initial size using bilinear resampling (to 242 increase computation speed and efficiency) and contrast and sharpening enhancement to allow a 243 better discrimination of the HPC and weathering rind (when present), from the rock core; 2) 244 Feature extraction, including a total of 162 texture features based on run-length, co-occurrence, 245 image histogram and gradient matrices in MaZda (http://www.eletel.p.lodz.pl/programy/mazda/) 246 and colour features based on several colour spaces namely RGB, HIS, YUV, YIQ and XYZ 247 using 'adimpro' package (Polzehl & Tabelow 2007) in R; 3) Generation of training input data in 248 ImageJ, by manually assigning points (i.e., XY coordinates of pixels) in the original images to 249 the corresponding structures of interest, namely 'lichen thallus', 'hyphae', 'weathering rind' and 250 'rock core'; 4) Combination of the extracted features and training data, to calibrate a Random 251 Forest (RF) classifier (Breiman 2001, Liaw & Wiener 2002) in R with 'ntree'= 200, 'mtry'= 6, 252 'nodesize'= 5 and the remaining parameters kept as default; the feature set included only a

reduced subset containing the 20 top colour and texture features according to their relative predictive importance (Boulesteix et al. 2012, Oppel et al. 2009); 5) Evaluation of classifier's performance through Monte-Carlo cross-validation (Xu et al. 2004) with 70% for training and 30% for testing and a total of 100 replicates; 6) Prediction of the labels of the structures of interest for the whole image, using the RF classifier with highest overall test accuracy. The extent of the HPC and weathering rind (given in mm²) in each sample are among the descriptive statistics retrieved by the classifier.

260

261 FT-Raman spectroscopy

262

263 Spectroscopic analyses were performed in three locations of each colonized schist sample: 1) the upper layer (cortex) of the lichen thalli as in Prieto et al. (2000); 2) the surface of the rock in 264 265 contact with the lichen thallus, named lichen-rock interface (Ascaso et al. 1976), where mineral 266 neoformation, if taking place at all, is most likely to be due to lichen-rock interactions; and 3) the rock interior (at least 2 cm away from the surface) which is used as a specific control for each 267 sample since lichen-induced oxalate formation is assumed not to reach such deeper areas beneath 268 269 the lichen-rock interface (Adamo & Violante 2000). Control FT-Raman spectra were also 270 recorded on non-colonized schist samples collected from the same schist outcrops, including 1) 271 the exposed surface (taken from the top as in colonized samples); 2) a 5 mm deep virtual 272 interface; and 3) the rock interior. A Bruker RFS 100/S FT-Raman spectrometer was used with a Nd:YAG laser operating at 1064 nm as the excitation light source and a resolution of 4 cm⁻¹. 273 Spectral data were acquired after 1024 laser scans of 20 mW in lichen thallus, to minimize lichen 274 275 damage, and 64 laser scans of 250 mW in rock interior and lichen-rock interface.

277 X-ray microdiffraction (XRMD)

278

279 XRMD measurements were performed on an Empyrean (PANalytical, Almelo, The Netherlands) 280 diffractometer, equipped with a five-axis Chi-Phi-x-y-z stage goniometer, a copper sealed anticathode X-ray tube (*Empyrean Tube Cu Lff Hr*) and a PIXcel^{3D} (PANalytical, Almelo, The 281 Netherlands) X-ray detector. Each sample was first submitted to a set of five random line scan 282 283 readings along the polished cross-section in order to obtain a preliminary depth profile of the mineralogical composition of the studied schist. Yet no significant differences were detected 284 285 between the surface of the sample and its interior so priority was given to the lichen-rock 286 interface as in FT-Raman spectroscopy. The final measurements are based on ten random readings along the lichen-rock interface. Bragg angles were scanned between 4 and 47°, with 287 steps of 0.02° (12 minutes time length) for exploratory measurements and between 3.5 and 60°, 288 289 with steps of 0.02° (2 hours time length) for the final measurements, with a laser diameter of 0.6 290 mm. 291 292 Statistical analysis 293 294 The effects of species and orientation on the depth and extent of the Hyphal Penetration 295 Component (HPC) was tested by means of two-way analysis of variance (ANOVA) and post-hoc Tukey HSD for pair-wise comparisons with 'agricolae' package (de Mendiburu 2013) in R. 296

- 297 ANOVA's assumption of normality of residuals was checked graphically using qq-plots and that
- 298 of homoscedasticity tested by means of the Levene test, also in R.

- 300 Results and discussion
- 301

302 Lichen-induced physical weathering

303

304 The pattern of hyphal penetration into the rock substrate is highly influenced by the 305 characteristics of the rock (Sanders et al. 1994) since hyphae tend to follow paths of least 306 resistance. Depth of hyphal penetration of the four lichens analysed was on average 3.4 mm in 307 samples coming from NW facing surfaces (n = 12), 4.6 mm in samples coming from SE facing 308 surfaces (n=9), and varied between 0.1 to 37 mm when considering all samples. Maximum 309 depth of hyphal penetration is apparently higher at SE than at NW facing surfaces (Table 1). 310 However, within sample variation, as depicted by the standard-deviation, was extremely high 311 and there was no statistically significant effect of orientation or species in the depth of hyphal 312 penetration. Additionally, although maximum depth of hyphal penetration detected among the analysed samples was 37 mm (in Aspicilia hoffmanniana from SE facing surfaces), hyphal 313 314 bundles frequently reached the lower extremity of the samples, which were roughly between 30 315 and 40 mm long, and measure of real maximum depth of penetration could not be accurately 316 estimated. As suggested by previous works (e.g. Favero-Longo et al. 2005, Wierzchos & Ascaso 317 1996, 1998) schistose rocks may be susceptible to greater lichen-induced weathering than 318 igneous rocks due to their higher predisposition to break and the easier progression of hyphae 319 along the planes of weakness, parallel to the schistosity (Fig. 1). Additional planes of weakness come from the intense tectonically-related fracturing that characterizes the studied rock. One 320 321 feature observed in almost every sample, and also commonly referred in literature (e.g. Fry

322 1927), is the extremely penetrative type of hyphal bundles associated with the occurrence of 323 apothecia, which are able to reach much deeper into the schist than adjacent hyphae (Fig. 1). 324 Analysis of the effects of penetrating hyphae have long been relying on the use of scanning 325 electron microscopy (SEM) and other high-resolution laboratory techniques (e.g. Jones et al. 326 1981, Strech & Viles 2002, Wierzchos & Ascaso 1994). Although extremely useful for 327 examining the very specific changes occurring in rock minerals by direct contact with individual 328 hyphae, the scale of these approaches is often too small to answer questions related with the 329 performance of the entire lichen or lichen community at the scale of the whole surface (micro-330 scale) or site where these surfaces are located (meso-scale). Additionally, these techniques may 331 only be considering worst (or best) case scenarios resulting from chance unless they are based on 332 large sample sizes and use full random sampling as in Strech & Viles (2002) to achieve statistical 333 robustness.

Many studies have determined the maximum or average depth of hyphal penetration for a wide range of lithotypes, but usually ignore hyphal spread, with few exceptions (Bartoli et al. 2014, Casanova-Municchia et al. 2014, Gazzano et al. 2009a). Image analysis of colonized crosssections after PAS staining retrieved accurate values for the hyphal spread of each species inside the rock (Table 1; Fig. 1). These values could then be used to determine if surface orientation had any effect on species ability to spread into the rock interior and induce mineral breakdown.

341 [Fig. 1 approximately here]

342

343 The studied lichens differed significantly in the spread of the HPC irrespective of surface

orientation (F-value= 10.974, p-value< 0.001). These differences were caused by a significantly

345 higher hyphal spread in Lecanora pseudistera than in any other of the lichens analysed, whereas 346 Aspicilia hoffmanniana, Caloplaca subsoluta and Peltula euploca were very similar among each 347 other (Table 1). The effect of interaction between species and surface orientation was not 348 statistically significant (F-value= 0.867, p-value= 0.364) neither was the effect of orientation 349 itself (F-value= 1.339, p-value= 0.262), which means that even if there were differences between 350 species in terms of their ability to spread inside the rock, there are no statistically significant 351 differences between NW and SE oriented surfaces in any of the studied species. 352 353 Weathering is quite obvious to the naked eye in most samples, where the black to more usually 354 dark-grey rock core corresponding to unweathered parent rock gradually changes into the light 355 brown to greyish-brown coloured weathering rind (Fig. 1b). All samples from NW facing surfaces showed such features, against only 13% of the samples from SE facing surfaces. The 356

dimension of the weathering rind was unrelated with the extent of the HPC (Table 1: Spearman= -0.012).

359

360 [Table 1 approximately here]

361

Although the extent of the HPC is unrelated to surface orientation, as species differ in their ability to spread inside the rock, an indirect effect of orientation on lichen-induced physical weathering, as depicted by the proxy HPC, could be produced by means of the abundance of individual species. Since *Lecanora pseudistera* is the most effective in terms of hyphal spread among the four lichens analysed (Table 1), and having been the most frequent and abundant on NW facing slopes (Marques *et al.* 2014) one might assume a more intensive lichen-induced

368	physical weathering happening at NW than at SE facing surfaces in the Côa Valley. Among the				
369	four lichens analysed, Peltula euploca was probably the most surprising in terms of physical				
370	performance since the attachment of this peltate (shield-like) growth form is far from being				
371	limited to superficial layers, penetrating up to 3.9 mm into the rock.				
372					
373	Lichen-induced chemical weathering				
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375	FT-Raman and complementary X-ray microdiffraction analyses of bare-rock controls confirmed				
376	the presence of quartz, chlorite, muscovite and albite as the major minerals in all analysed				
377	locations of the presumably unweathered schist (Fig. 2 and Supporting Information Tables S1				
378	and S2).				
379					
380	[Fig. 2 approximately here]				
380 381	[Fig. 2 approximately here]				
380 381 382	[Fig. 2 approximately here] The wavenumber region 100-1700 cm ⁻¹ of FT-Raman spectra contains useful spectroscopic				
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391 easier to differentiate from those related to other substances (see below). According to Wang et 392 al. (2002) di-octahedral phyllosilicates such as muscovite produce a strong FT-Raman peak at 260 cm⁻¹, which is depicted in the FT-Raman spectra of the rock interior and lichen-rock 393 394 interface of almost all colonized samples. Mg-bearing phyllosilicates, such as chlorite, peak strongly at approximately 350 cm⁻¹. The peak at 356 cm⁻¹ visible in the FT-Raman spectra of the 395 rock interior and lichen-rock interface of schist samples colonized by Aspicilia hoffmanniana 396 (Fig. 2), can therefore be assigned to chlorite. Peaks at 200 cm⁻¹ are characteristic of tri-397 398 octahedral phyllosilicates (Wang et al. 2002) and most probably indicate the presence of either 399 chlorite or biotite. Quartz is very resistant to weathering and persists even at the surface of bare rock and at the lichen-rock interface of all samples (Fig. 2). It was detected in every sample by a 400 sharp band at 464 cm⁻¹ in FT-Raman spectra and at 3.33 Å in X-ray microdiffraction (Supporting 401 402 Information Tables S1 to S10). The presence of the same band in the FT-Raman spectra of Aspicilia hoffmanniana and Caloplaca subsoluta (Fig. 2a) indicates an incorporation of quartz 403 particles by the thallus of these lichens. Evidence for the ability of Aspicilia hoffmanniana to 404 incorporate phyllosilicate particles is also seen in its FT-Raman spectra, with characteristic bands 405 at 200 and 260 cm⁻¹ (Wang et al. 2002). However, the occurrence of quartz and phyllosilicates in 406 407 lichen thalli and lichen-rock interfaces can also have an exogenous origin from airborne dust, as 408 suggested by Vingiani et al. (2013) after detecting the same kind of mineral incorporation in 409 lichens growing on quartz- and phyllosilicate-free vulcanic rocks. Incorporation of quartz and 410 phyllosilicate minerals by lichens is not exclusive of crustose lichens as can be inferred by the FT-Raman spectra of *Peltula euploca* showing a band at 432 cm⁻¹ (Fig 3), which is assignable to 411 412 either of these silicate minerals. This would require further confirmation through higher 413 resolution techniques such as scanning electron microscopy (SEM).

414 Besides those minerals that are known to characterize the unweathered rock (see materials and 415 methods section), X-ray microdiffraction and FT-Raman spectroscopy confirmed the occurrence 416 of halloysite at both the virtual interface of bare rock samples and the lichen-rock interfaces, 417 irrespective of the species and the orientation of the parent outcrop (Figs 2). Hallovsite is a 418 common product of schist weathering, resulting from the transformation of chlorite, biotite, 419 muscovite and feldspars (Banfield & Eggleton 1990, Kretzschmar et al. 1997, Parham 1969). 420 Despite the differences stated above in terms of weathering rind formation, no differences were 421 observed in terms of the occurrence of halloysite between NW and SE facing surfaces. Peak at 260 cm⁻¹ in FT-Raman spectra could also correspond to kaolinite, another product of schist 422 423 weathering, but differentiation of kaolinite minerals from muscovite, and the latter from 424 vermiculite, can be problematic because peaks shared by the three minerals are not fully differentiable by X-ray microdiffraction or FT-Raman spectroscopy (Wang et al. 2002). 425 426

FT-Raman and complementary X-ray microdiffraction analyses also allowed detecting the
occurrence of neoformation minerals commonly attributed to lichen activity, namely calcium
oxalates, at the lichen-rock interface and thalli of some of the target species, and confirmed their
absence at the rock interior of all samples. The pattern of occurrence of such minerals is,
however, variable among the considered species, their origin and analysed location within
samples.

Key molecular signatures for oxalates occur in the 1400-1500 cm⁻¹ region of FT-Raman spectra
(Edwards *et al.* 2003b) where 1476 cm⁻¹ is considered distinctive for weddellite (Fig. 2b). Other
distinguishing bands for weddellite occur at 912 and 1634 cm⁻¹. The signature of weddellite was
found either completely or partially in the thalli of *Aspicilia hoffmanniana*, *Lecanora pseudistera*

and *Peltula euploca* from south-east facing surfaces (Fig 3a), but not in the thalli of *Caloplaca subsoluta* neither in any of the specimens taken from north-west facing surfaces (Fig 2a). In fact,
previous experimental works had indicated that microclimatic factors could be important in
determining the state of hydration of calcium oxalate in lichens (Edwards *et al.* 1995) and have
associated the occurrence of the dyhidrate form with lichen's strategy for maintaining its water
balance in dry exposed surfaces (Prieto *et al.* 2000, Prieto & Silva 2003).

443 Calcium oxalate monohydrate, known as whewellite, has been detected in the thalli of Aspicilia

444 *hoffmanniana* and *Lecanora pseudistera* on samples from NW facing surfaces, peaking in FT-

445 Raman spectra at 1463 and 1631 cm⁻¹, respectively (Fig. 2).

446 Variation in measured temperature and relative humidity between NW and SE facing surfaces is

summarized in Table 2. There are similarities in the general pattern of annual rock surface

temperature and relative humidity regimes. Both NW and SE facing surfaces exhibited a

seasonal pattern of high temperature and low relative humidity values from around June to

450 September followed by a much colder and moist period between November and February.

451 Variation in temperature was huge at both orientations, but nevertheless higher at SE than at NW

452 facing surfaces. The same happened with relative humidity, although relative humidity was

453 always higher at NW facing surfaces than at SE facing surfaces.

454 The occurrence of oxalates in the lichen thallus of *Aspicilia hoffmanniana* and *Lecanora*

455 *pseudistera* is not completely unexpected since the fruiting bodies of the former are well known

456 for being pruinose (*i.e.* covered by oxalate crystals) and the later belongs to a group of lichens

457 that are characterized precisely by the presence of large amphithecial crystals. The occurrence of

458 the monohydrate form in specimens that grow under less contrasting humidity and temperature

459 regimes is consistent with the physiological role attributed to calcium oxalate.

The origin of Ca ions for calcium oxalate production in lichens, however, is still an unsolved matter. Calcite (CaCO₃) is usually present in the matrix of the studied rock type in sufficient amounts to produce effervescence when treated with dilute hydrochloric acid. Calcite is highly alterable and a potential source of Ca ions for calcium oxalate formation promoted by biological colonization. However, calcite, with characteristic features in FT-Raman spectra being a strong, sharp band at 1086 and weaker bands at 712 and 286 cm⁻¹ (Edwards *et al.* 1995, 2003b), is missing in all schist samples.

467 Although it has been proved that lichens are able to uptake Ca ions from calcareous rocks such 468 as limestones and marbles for calcium oxalate production (e.g. Seaward & Edwards 1995), the 469 rock is definitely not the only source of this element as the occurrence of both forms of calcium 470 oxalate has been reported in lichens colonizing non-calcareous substrates such as granites (e.g. Prieto & Silva 2003), serpentinites (Favero-Longo et al. 2005) or even tree-bark (Edwards et al. 471 472 2005) and tree leafs (de Oliveira et al. 2002). The presence of calcium oxalates inside the lichen 473 thallus is therefore not necessarily indicative of its biodeteriogenic activity and, when the 474 calcium source is exogenous, calcium oxalate patinas were even indicated as bioprotective (McIlroy de la Rosa et al. 2013). Occurrence of calcium oxalates in lichens growing on iron- and 475 476 magnesium-rich siliceous rocks instead of the most expected ferrous oxalate dehydrate (humboldtine) and magnesium oxalate (glushinskite), respectively, has also been reported before 477 (e.g. Prieto et al. 1997, 2000, Prieto & Silva 2003, Favero-Longo et al. 2005). This phenomenon 478 479 can, according to Prieto & Silva (2003) and Favero-Longo et al. (2005), be explained by the 480 higher water solubility of ferrous and magnesium oxalates as well as their higher susceptibility to oxidation. The detection of calcium oxalates at the lichen-rock interface by X-ray 481 482 microdiffraction (Table 3 and Supporting Information Table S7, S9 and S10), however, is not as

483 easily assignable to external sources of Ca. Only weddellite has been detected at the lichen-rock 484 interface and that was by X-ray microdiffraction exclusively on samples taken from SE facing 485 surfaces, colonized by Caloplaca subsoluta, Lecanora pseudistera and Peltula euploca (Table 486 3). Weddellite was apparently absent from the lichen-rock interface of Aspicilia hoffmanniana 487 although present in the thallus (Table 4). The opposite was observed in the samples colonized by 488 *Caloplaca subsoluta*, with weddellite detected at the lichen-rock interface by X-ray 489 microdiffraction (Table 3) and no form of calcium oxalate detected inside the thallus by FT-490 Raman (Table 4). These results suggest that calcium oxalates at the lichen-rock interface and 491 inside the lichen thallus may have different origins and/or functions. Except for the occurrence of 492 weddellite, FT-Raman spectroscopy and X-ray microdiffraction of the lichen-rock interface 493 retrieved quite similar results to that of the virtual interface in bare rock controls. 494 The relevance of these results in the search for the causes of differential weathering of schist 495 surfaces in the Côa Valley is opposite to those already mentioned for lichen-induced physical 496 weathering. Assuming that calcium oxalate at the lichen-rock interface is being produced from Ca taken from the rock and considering its formation as a proxy of lichen-induced chemical 497 498 weathering, such type of weathering might be more intense on SE facing surfaces. The 499 possibility that due to differences in micro-environmental conditions, calcium oxalates on SE 500 surfaces are subject to less dissolution and are consequently more stable than those on NW 501 surfaces cannot be ruled out by present evidence. However it seems unlikely because the poorly 502 soluble Ca oxalates were found to persist at the lichen-rock interface of thalli exposed to 503 significantly higher precipitation regimes (Favero-Longo et al. 2005). Other bands present in FT-Raman spectra include 1158, 1552 and 1612 cm⁻¹, which are 504 505 characteristic of parietin and therefore present in the FT-Raman spectra of Caloplaca subsoluta.

The resemblance of the FT-Raman spectra of *Caloplaca subsoluta* and that of other members of
the lichen family Teloschistales (Jorge-Villar *et al.* 2004) is quite obvious mainly due to the
profile of parietin.

509 The FT-Raman spectra of the thallus of Aspicilia hoffmanniana and Lecanora pseudistera on 510 samples taken from NW and SE facing surfaces, respectively, contain a series of bands that 511 could not be assigned to any substance for the time being, but are probably related with other 512 secondary metabolites produced by these lichens, which may further act as biodeteriogenic 513 factors (Adamo & Violante 2000, with refs. therein). Also unknown were the peaks at 5.96 and 9.09 Å in X-ray microdiffraction of samples colonized by Aspicilia hoffmanniana and 2.32 Å in 514 samples colonized by Aspicilia hoffmanniana and Caloplaca subsoluta (Supporting Information 515 Table S7 and S9). The very broad bands at 1332 and 1595 cm⁻¹ of all FT-Raman spectra are due 516 to amorphous carbon. 517

518

519 Conclusions

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521 Variation in microclimatic factors related to surface orientation produces different effects 522 depending on the nature of lichen-induced weathering. Analysis of stained polished cross-523 sections of schist samples colonized by the crustose Aspicilia hoffmanniana, Caloplaca 524 subsoluta, Lecanora pseudistera and the squamulose Peltula euploca showed that hyphae 525 originating from the medulla of these lichens penetrate more than 30 mm and follow a 526 unidirectional pattern along the depth rock profile. The lamellar nature of schist minerals offering pathways of least resistance along intermineral voids, probably favours this deep 527 528 penetration. Therefore the effects of hyphal penetration on schist should go far beyond the

529 surface and also involve the minerals in the deeper layers. The spread of the hyphal penetration 530 component in the analysed species was similar on NW and SE facing surfaces, but may turn out 531 to be more severe at NW facing surfaces, due to the higher frequency and abundance of species 532 with higher penetrative ability, such as Lecanora pseudistera. Orientation is thus likely to have 533 an indirect effect on lichen-induced physical weathering by means of the abundance patterns of 534 individual species, highlighting the importance of accurate estimates of the relative abundances 535 of colonizing species, stemming from community ecology approaches, for rock-art condition 536 assessments.

537 Other evidences of lichen-induced weathering produced in these rocks are related with the 538 incorporation of quartz and phyllosilicate particles by the thalli of all the lichens studied, 539 including the squamulose Peltula euploca. The external origin of these particles cannot be ruled out, however, as the mechanisms of airborne mineral incorporation by the lichen thallus are not 540 541 fully understood. Also of interest for the purpose of this study is the presence of kaolinite and 542 halloysite, two common products of schist weathering, at the lichen-rock interface and on the 543 surface of bare rock controls, irrespective of surface orientation. Variations in the amount of these minerals depending on the colonizing species and microclimatic factors remain to be 544 545 tested.

Evidence for the occurrence of metabolic by-products of lichen activity in the analysed samples
is limited to calcium oxalates. Specimens of *Aspicilia hoffmanniana* from dry SE facing surfaces
produced weddellite exclusively, while those from moist NW facing surfaces produced a mixture
of weddellite and whewellite. Specimens of *Lecanora pseudistera* produced weddellite on SE
facing surfaces and whewellite on NW facing surfaces. Weddellite was also detected inside the
thalli of squamulose *Peltula euploca*, occurring only on SE facing surfaces. None of these forms

552 of calcium oxalate were detected inside the thalli of Caloplaca subsoluta. These results indicate, 553 as others beforehand, that there is some preference among the studied lichens for the production 554 of the dehydrate form of calcium oxalate under the highly variable microclimate conditions 555 provided by SE facing surfaces, while on the slightly less variable NW facing surfaces, a mixture 556 of both monohydrate and dehydrate forms can occur. Given the possibility of calcium uptake 557 from airborne particles, it is impossible to state unequivocally whether calcium ions used in the 558 formation of calcium oxalates were acquired from the substrate, but the probability that this 559 might have happened is higher in those cases where calcium oxalates were also detected at the 560 lichen-rock interface. Weddellite was detected at the interface of all species except Aspicilia 561 hoffmanniana from SE facing surfaces.

562 This study therefore suggests that lichen-induced physical weathering in the Côa Valley is species-specific and may be stronger on north-east facing surfaces, whereas lichen-induced 563 564 chemical action is microclimatically controlled and may be more severe on SE facing surfaces. 565 There is probably some variation in the relative abundance of alteration minerals and calcium 566 oxalates at different portions of the samples but according to present evidence, the lichens currently dominant on the vertical schist surfaces in the Côa Valley are unlikely to be responsible 567 568 for the differential weathering (and presumably consequent distribution pattern) of engraved 569 schist surfaces.

Furthermore, calcium oxalate production by lichens not attributable to any kind of lichen
activity, as it happens with *Aspicilia hoffmanniana*, adds to the doubts concerning its importance
in lichen-induced weathering, especially since this seems to be limited to a few species, and, as
demonstrated by this study, changes with microclimatic conditions. Assumptions about the
drivers of open-air rock-art distribution elsewhere should therefore also consider the micro-

environmental controls of lichen-induced weathering and associated deteriogenic activity in
order to avoid biased measures of the influence of lichen activity in the deterioration process, or
superficial decisions in terms of rock-art conservation practices.

578

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580

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773	Tables	
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Table 1. Hyphal penetration: measures taken out of colonized cross-sections after PAS staining.
Depth: estimated depth of hyphal penetration in mm; HPC: area occupied by the hyphal
penetration component in mm²; WR: area occupied by the weathering rind in mm². The area
analysed in each cross-section was 57.5 mm². Different letters in brackets indicate statistically
significant pair-wise comparisons (p-value< 0.05).

Species	Aspect	Depth	Depth	HPC (mean ±	WR
		(mean ± sd)	(max)	sd)	
Aspicilia	South-east	5.7 ± 7.6	37.0	2.96 ± 2.03 (a)	0.00
hoffmanniana					
	North-west	3.5 ± 3.5	12.0	2.37 ± 0.81 (a)	0.00
Caloplaca subsoluta	South-east	4.1 ± 5.3	30.0	2.09 ± 1.71 (a)	4.07
	North-west	1.8 ± 1.9	6.8	1.74 ± 0.71 (a)	7.56
Lecanora pseudistera	South-east	5.1 ± 6.6	31.0	8.01 ± 2.02 (b)	0.00
	North-west	4.1 ± 3.8	13.0	5.79 ± 2.97 (b)	30.36
Peltula euploca	South-east	2.4 ± 1.0	3.9	0.69 ± 0.60 (a)	6.72
				Spearman: – 0.01	2

		North-west	South-east	
Temperature	Average	17 °C	20 °C	
	Minimum	13 °C	15 °C	
	Maximum	23 °C	30 °C	
Relative humidity	Average	70 %	63 %	
	Minimum	55 %	45 %	
	Maximum	81 %	77 %	

Table 2. Data on rock surface microclimate at opposite orientations in the Côa Valley.

Table 3. Summary of the neoformed calcium minerals detected in the lichen-rock interface (FT-

Raman spectroscopy and X-ray microdiffraction).

	Aspicilia		Caloplaca subsoluta		Lecanora		Peltula
	hoffmanniana				pseudistera		euploca
	North-	South-	North-	South-	North-	South-	South-east
	west	east	west	east	west	east	
Whewellite							
Weddellite				Х		Х	Х

	Aspicilia hoffmanniana		Caloplaca subsoluta		Lecanora pseudistera		Peltula
							euploca
	North-	South-	North-	South-	North-	South-	South-east
	west	east	west	east	west	east	
Whewellite	Х				Х		
Weddellite	Х	Х				Х	Х
Quartz		Х	Х				X
Phyllosilicates		Х				Х	

Table 4. Summary of the minerals detected in lichen thalli (FT-Raman spectroscopy)

793 Figure legends

794

795 Figure 1. On the top, cross-sections of schist samples colonized by Lecanora pseudistera from a 796 north-west facing surface (a) and by Aspicilia hoffmanniana from a south-east facing surface (b); 797 and on the bottom, cross-section of a schist sample colonized by Aspicilia hoffmanniana from a 798 south-east facing surface after PAS staining (c) and respective image classification (d) with 799 medium grey areas corresponding well with the area occupied by the hyphal penetration 800 component (in purple in the original image). 801 802 Figure 2. FT-Raman spectra of bare rock control samples (first row) and colonized samples 803 (remaining rows). On the first row: external surface (thick line), internal surface (thin line) and core (dotted line) of bare rock control samples from north-west (a) and south-east (b) facing 804 805 vertical schist surfaces. On the remaining rows: thallus surface (thick lines), rock-lichen interface 806 (thin lines) and rock core (dotted lines) of, from top to bottom, Aspicilia hoffmanniana, Caloplaca subsoluta, Lecanora pseudistera and Peltula euploca from north-west (a) and south-807 808 east (b) facing vertical schist surfaces. Conditions as explained in material and methods. Wavenumber region: 100-1700 cm⁻¹. Wavenumber assignments are based on Freeman et al. 809 810 (2008), Frost (1997), Wang et al. (2002), Jorge-Villar et al. 2004, Edwards et al. (1997, 2000, 811 2003a). Additional information on the wavenumbers for the Raman spectra of all species and 812 respective wavenumber assignment is given in Supporting Information Tables S1-S5. 813

814

817 Figure 1







A

820

B