



Ecological and taxonomic characterisation of *Trentepohlia umbrina* (Kützing) Bornet growing on stone surfaces in Lazio (Italy)

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Received: 4 February 2019 / Accepted: 8 April 2019 / Published online: 15 May 2019
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Abstract

Purpose The colonisation of building material by photosynthetic organisms is highly dependent on the environmental conditions and the nature of the substrate. The growths of red-orange phototrophic biofilms have been widely reported in the literature and have commonly been associated with the order Trentepohliales, whose ecological and taxonomical information needs to be improved. Considering the recurrent presence of such biofilms throughout the Lazio region, we would identify the occurring species and define their favourable environmental conditions, through morphological, genetic and ecological analyses.

Results Biofilms were collected across an altitude range of 0 to 860 m asl, occurring from the coast to 60-km inland. A dominant presence of the filamentous terrestrial green alga *Trentepohlia umbrina* (Kützing) Bornet was confirmed in all sites sometimes mixed with cyanobacteria of the genus *Gloeocapsa*. The distribution of Trentepohliales is generally given as pan-tropical, but some species, such as *T. umbrina*, are also distributed in temperate climates. Here, it is reported for the first time a Mediterranean occurrence of the species. Low humidity and light conditions, such as those occurring on vertical surfaces with mainly northern aspects, are the preferred environmental conditions. Coastal areas were more favourable for its growth, probably due to the higher nocturnal condensation that occurs here. Concrete and mortars were particularly bioreceptive to *T. umbrina*, but marble and trachyte were also colonised under favourable conditions.

Conclusions The findings better define the ecological range of this species, suggesting a wider biogeographic distribution, and adding information on morphological features and genetic data.

Keywords Biodeterioration patterns · Building material · Biofilm · Reddish patinas · Green algae · *Trentepohlia umbrina*

Introduction

Green subaerial microalgae can colonise natural and artificial substrata to form biofilms (Ricci and Pietrini 1994; Bellinzoni et al. 2003; Tomaselli et al. 2000; Rindi and Guiry 2004). Advanced growths of these biofilms are visible to the naked eye, forming patinas of different colours, such as green, grey and reddish, and giving rise to characteristic biodeterioration patterns (BP) (Caneva et al. 2016). They can often be considered as aesthetical biodeteriogens, but the potential for structural deterioration, caused by physical and biochemical

process, may have started before biofilms are even visible (Eggert et al. 2006; Scheerer et al. 2009; Bartoli et al. 2014). The occurrence of green algae and cyanobacteria biofilms on outdoor stone monuments and other artificial substrata, such as roof tiles, concrete or building facades, can exacerbate natural weathering phenomena (Caneva et al. 1992; Albertano 1995; Gaylarde and Morton 1999; Tomaselli et al. 2000; Gaylarde et al. 2004). Apart from the aesthetical impact of phototrophic biofilms, in some cases, they can also be considered as bioprotective of stone materials, even if this role has not been comprehensively demonstrated (Cutler and Viles 2010; Ramirez et al. 2010; Pinna 2014).

The occurrence of red-orange biofilms is often associated with the green algal order Trentepohliales, whose colour is resulting from their large production of β -carotene and haematochrome pigments. The order includes one family (Trentepohliaceae) and five genera (*Trentepohlia*, *Printzina*, *Phycopeltis*, *Cephaleuurus*, *Stomatochroon*), and originally had a pan-tropical distribution. The genus *Trentepohlia* comprises an

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accepted 48 species that are either subaerial or the phycobionts of several lichen species (Guiry and Guiry 2018). It has a global distribution and occurs on a wide range of substrates, such as tree bark, leaves, rock and several types of artificial substrata (Rindi and Guiry 2002; Rindi et al. 2005; Unković et al. 2017). Species of this genus can produce extensive yellow to red growths that can cover building walls, inscriptions and monument details, making necessary expensive active interventions, such as repainting and cleaning. Their growths have already been well documented and detailed described for some tropical areas, such as East Asia (Wee and Lee 1980; Lee and Wee 1982; Ho et al. 1983; Bartoli et al. 2014) and Mesoamerica (Caneva et al. 2005; Gaylarde and Gaylarde 2005; Gaylarde et al. 2006; Ramirez et al. 2010; Ortega-Morales et al. 2012). Moreover, their occurrence has also been identified especially in North Europe, e.g. in Scotland (Wakefield et al. 1996), Ireland (Rindi and Guiry 2002) and in some Mediterranean area, such as Portugal (Pereira de Oliveira 2008) and Spain (Ariño and Saiz-Jimenez 1996).

Trentepohlia umbrina (Kützinger) Bornet also seems to have a global distribution and is frequently defined as corticolous, found growing on the bark of deciduous trees and conifers, but it has also been found growing on woodwork, rocks, carved stone, concrete walls and pillars (see references in Guiry and Guiry 2018). Most of the available ecological information on this species is rudimentary and often based on the lichen-associated, rather than the free-living form. Rindi and Guiry (2002) gave the first report of the ecology of this species looking at its distribution in Galway (Ireland), where they found small isolated red-brown patches on north facing of old limestone walls only, with no observable seasonal variations.

Regardless of their desiccation tolerance, all terrestrial green algae require liquid water, or an atmosphere saturated with humidity, to be metabolically active. It should be therefore plausible to have a widespread distribution in Northern Europe, where humidity levels and rainfalls are usually higher, and by the same chain of thought, we expect a restricted distribution in Southern Europe. However, in central Italy, numerous observations of red-orange biofilms, presumably referred to *Trentepohlia*, at several archaeological and urban sites, suggest there is a need to re-determine the distribution and the ecological range of this species.

As robust ecological information regarding this species is lacking, there is a need for a more widespread study of its distribution, ecology and identification. Also, according to Rindi and Guiry (2002) and John (2002), the taxonomic characterisation of *T. umbrina* has not been satisfactorily defined, because of confusion with other species of the same genus. Some of the doubt regarding this species is from a morphological perspective. Therefore, the aims of this paper were to characterise in detail the ecology and the morphological features of *T. umbrina* colonising highly diverse environments and substrate types in the Lazio region, and, where possible, to support the identification of this species by molecular methods.

Materials and methods

Study area

The study area is the Lazio region (Central Italy), which has a surface of 17, 227 km², made up of various landforms, comprising hills (54%), mountains (26%) and plains (20%) (Anzalone et al. 2010). The heterogenic orography, lithology and climatic gradient extending from the Tyrrhenian Sea to the central Apennines of Lazio region result in a series of different environment and vegetation types, representing 15 phytoclimate units (Blasi 1993; Anzalone et al. 2010; <http://www.pcn.minambiente.it>). To define sites, numerous outings were organised based on the ecological information gathered from the literature (John 2002; Guiry and Guiry 2018), previous sightings and observations of satellite imagery available in GIS-based websites.

Ecological data sampling

Sampling sites were identified by the presence of visual red-orange biofilms on stone surfaces, and field observations refer to a data set of 100 red-orange biofilms. For each red-orange biofilm, several ecological parameters were considered, such as the height of biofilm from the ground, aspects, sheltering, evidence of potential percolation, rising damp and shadowing of the surfaces, following usual protocols (Danin and Caneva 1990; Caneva et al. 2015; Traversetti et al. 2018). In evaluating the cover and the distribution of *T. umbrina*, the robust and repeatable phytosociological method of Braun-Blanquet (1964) was applied in the field. The cover-abundance of the biofilm was estimated according to the scale of Braun-Blanquet [+ (very few individuals)=0.5; 1 (numerous individuals)=1–5%; 2 = 5–25%; 3 = 25–50%; 4 = 50–75%; 5 = 75–100%]. These values were then converted in the van der Maarel abundance scale for statistical analyses (van der Maarel 1979).

For each site, covering the period from 2013 to 2017, to have an homogeneous dataset for all sites and considering also the conditions of a climatic change, meteorological data were collected from the Lazio region official website (<http://www.idrografico.roma.it>). In order to discern the bioclimatic preferences of *T. umbrina* (regarding pluviometry and temperatures), a bioclimatic map of the region was used (Blasi 1993; Anzalone et al. 2010; <http://www.pcn.minambiente.it>), and then thermo-pluviometric diagrams were constructed of the two localities at the extremities of the site climatic gradient, according to methods proposed by Bagnouls and Gaussen (1963).

In order to obtain general ecological information on lighting conditions, autumnal sun paths were superimposed over satellite images of each site (www.suncalc.org; www.sunearthtools.com). Using the autumnal sun path meant that

we gave conservative light load values, this was done to even out seasonality because some aspects receive more light in the afternoon/evening and some aspects in the morning.

Ground observations were made during sampling to understand the sources of shading that may not be noticed on older satellite images. Values of light exposure (in min day^{-1}) were divided into two diverse types, full and speckled exposure. The latter type was identified as many sites were surrounded and therefore shaded, by tree canopies, and the biofilms can be subjected to flecks of full solar irradiance.

Morphological and molecular analysis

For the morphological identification, sampling of biofilms involved either scraping of biomass from the substrate using clean razor blades or using adhesive fungi tape, a non-destructive method, for sites of cultural value (Bruno et al. 2014; Bruno and Valle 2017).

Microscopic observations (Zeiss Axio Scope) of fresh un-fixed samples were observed on return to the laboratory or the day after (samples stored in the dark at 4 °C) for cell measurement and identification following UNI 10923. Fresh un-fixed samples were always observed and photographed within 48 h for identification using a light microscope (Zeiss AxioScope). Initial identifications were made using the identifying morphological traits proposed by John (2002) and following the analytic keys of Guiry and Guiry (2018). To confirm the morphological characterisation, cell measurements were made using calibrated digital images. Measurements of length (L), width (W) and $L:W$ of 50 intercalary cells (the smaller apical cells were excluded) for 12 representative populations that covered the full range of substrate types were carried out.

Samples from the site Grassi Hospital were used for molecular identification of the species. Around 100 mg of fresh-weight samples were repeatedly frozen and thawed (ca. 20 cycles) and DNA was extracted using the GeneMATRIX Plant and fungi DNA purification kit (EURx). The chloroplast gene *rbcL* was amplified using the following primers: TrerbcL_mos_for (5'-GAAGCWATTCCRGGAGAAG-3') and TrerbcL_mos_rev (3'-CATCCATTCTTGAGWAAAGAATAC-5') (Eurofins Genomics, Ebersberg, Germany). Amplifications involved an initial denaturation at 94 °C for 3 min; then 30 cycles of denaturing at 94 °C for 30 s, annealing at 51 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 7 min. Amplified fragments were sequenced on both strands at the BMR-Genomics Sequencing Service (Padua, Italy). The *rbcL* gene sequence obtained was analysed using the BLAST function of GenBank (www.ncbi.nlm.nih.gov/blast), and phylogenetic analysis was carried out with the most similar sequences. The selected sequences were aligned with the Clustal Omega program (www.ebi.ac.uk/Tools/msa/clustalo/). Maximum likelihood and neighbour-joining trees were generated using the PAUP*4.0 software

package. Bootstrap values were obtained from 500 replicates with one random sequence addition to jumble the data using the PAUP. A neighbour-joining tree was constructed using MODELTEST v. 3.7. The *rbcL* sequences of *Ulva australis* Areschoug (EF372236) and *Ulva linza* L. (AB097620) were used as outgroup, and the tree was edited using TREEVIEW version 0.5.0. (R.D.M. Page).

Statistical analyses

Tests for normality and variance were carried out on morphological and environmental data using Q-Q-plots and Levene's test before ANOVA. Tukey's pair-wise Q post hoc tests were carried out in case of significant differences realised by the ANOVA. For non-normal data, in this case cellular L and W , Kruskal-Wallis tests were used, followed up with Dunn's post hoc analysis with Bonferroni correction of p values to identify sites significantly different from each other. The cover values were used to evaluate the influence of several ecological parameters on the growths of *T. umbrina*, such as (i) aspects, (ii) daily sunlight loads and (iii) substrate type. All statistical analyses were carried out using PAST3 and the Real Statistics Resource Pack software for excel (Release 5.4).

Results

Ecological characterisation

The sampling campaign highlighted nine different sites with distinctly diverse characteristics and a total of six substrate types: basalt, brick, concrete, white marble and two magmatic effusive rocks as peperino and trachyte. The sites, where the red-orange biofilms resulted more widespread, were located in three different provinces of Lazio (Rome, Viterbo and Frosinone), (Table 1).

The area covers a wide range of altitude, that is from sea level to 860 m asl (Fig. 1), and across diverse phytoclimates, ranging through a Mediterranean bioclimate (Fig. 2a), close to the coast, to a temperate bioclimate (Fig. 2b) in sub-mountain areas. Site altitude was positively and negatively correlated with 5-year mean values of precipitation ($p < 0.01$) and temperature ($p < 0.05$), respectively (Fig. 2c, d), whilst RH remained relatively unchanged (ca. 60–73%; $p > 0.05$). Annual precipitation (5-year mean) ranged from 550 mm at Santa Severa to 1550 mm at Trevi nel Lazio, whilst temperature (5-year mean) ranged from 12 (Trevi nel Lazio) to 19 °C (Palatine Hill).

The field observations highlighted that, in favourable environmental conditions, this BP is very widespread and showing also an abundant cover (Fig. 3a). Moreover, it was evident how this BP is one of the first colonisers of the stone substrate

Table 1 Site characteristics with detailed geographical and physical information and sites specific meteorological data

Prov.	Station	Acronyms map	Coordinates	Substrate	Altitude (m asl)	Inclination	Methereological data (2013–2017)			
							Mean annual temperature (°C)			Annual rainfall (mm)
							Min	Mean	Max	
RM	Palatine Hill, Rome	PL	41°53' 26" N 12°29'16" E	Basalt/brick	30	90°	13.46	18.84	24.22	800
RM	Grassi Hospital, Ostia	GH	41° 43' 49" N 12° 18' 13" E	Concrete	10	90°	11.57	16.48	21.06	785.86
RM	Ostia Antica archaeological park	OA	41° 45' 11" N 12° 17' 17" E	Marble	10	90°	11.71	16.31	21.14	785.86
RM	Non-Catholic Cemetery, Rome	CA	41° 52' 31" N 12° 28' 43" E	Marble	10–20	90°	11.82	16.39	21.18	683.04
RM	Santuario Madonna del Tufo, Rocca di Papa	RP	41° 75' 63" N 12° 70' 68" E	Brick	693	90°	9.88	13.05	16.21	1165.72
RM	Santa Severa/S. Marinella	SS	42° 2' 17" N 11° 50' 11" E	Concrete	0–10	90°	12.31	16.56	20.81	553.76
VT	Villa Lante, Bagnaia	LB	42° 25' 35" N 12° 09' 20" E	Peperino	420–470	90°	11.25	16.42	21.6	810.14
VT	Bomarzo Garden	BZ	42° 29' 29" N 12° 14' 43" E	Trachyte	200–210	90°	11.71	15.12	18.44	867.52
FR	Trevi nel Lazio	TL	41° 51' 1" N 13° 12' 4" E	Concrete	860	90°	6.22	11.74	17.27	1548.92

(Fig. 3b), changes in cover in relation to the aspect and that it is usually associated to black patinas (Fig. 3c).

The changes in the cover of these biofilms in relation to the aspects of surfaces are shown in Table 2. A preference for northern and western aspects is clearly evident (Fig. 4a), and the cover of such BP on the north and

north-west aspects accounted for nearly 49% (27.4% N and 21.6% NW) of the total mean cover for all aspects. Substrates with other aspects were also colonised, although to a lesser extent, with cover values of 10% or less of the total, and minimum values for south-facing aspects (1.5%).

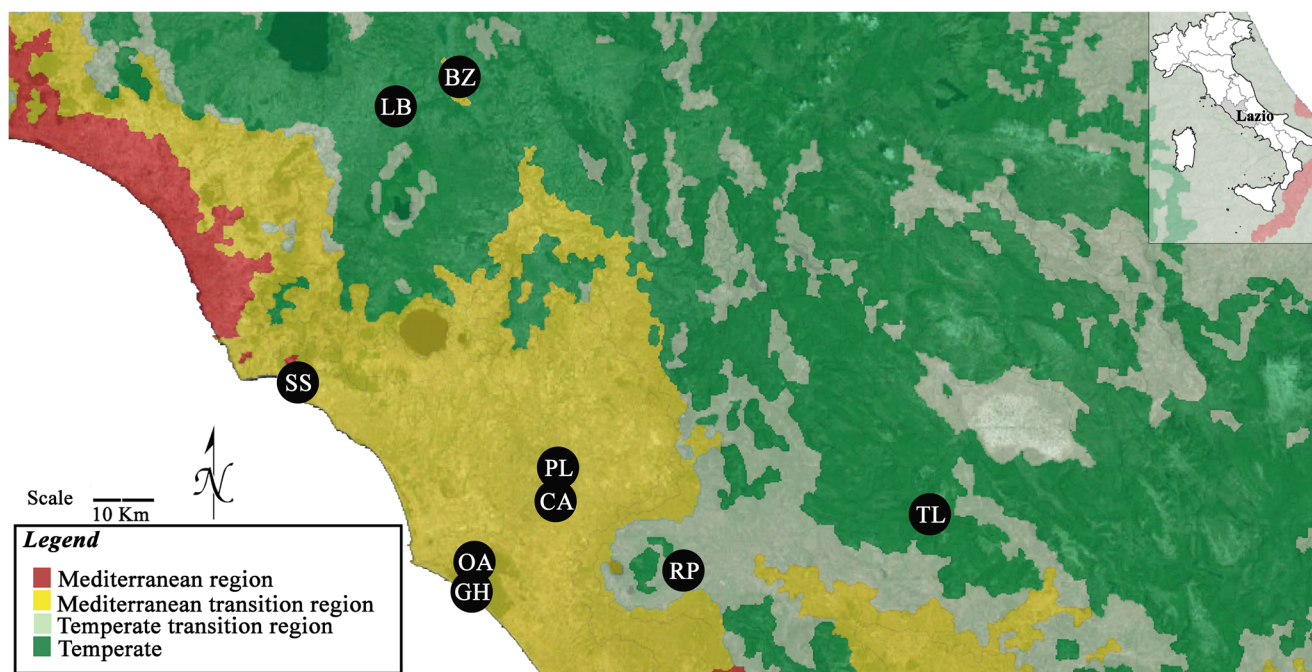


Fig. 1 Bioclimatic sites map: distribution of the analysed sites in the different bioclimatic region of the Lazio. The acronyms of the site are specified in Table 1

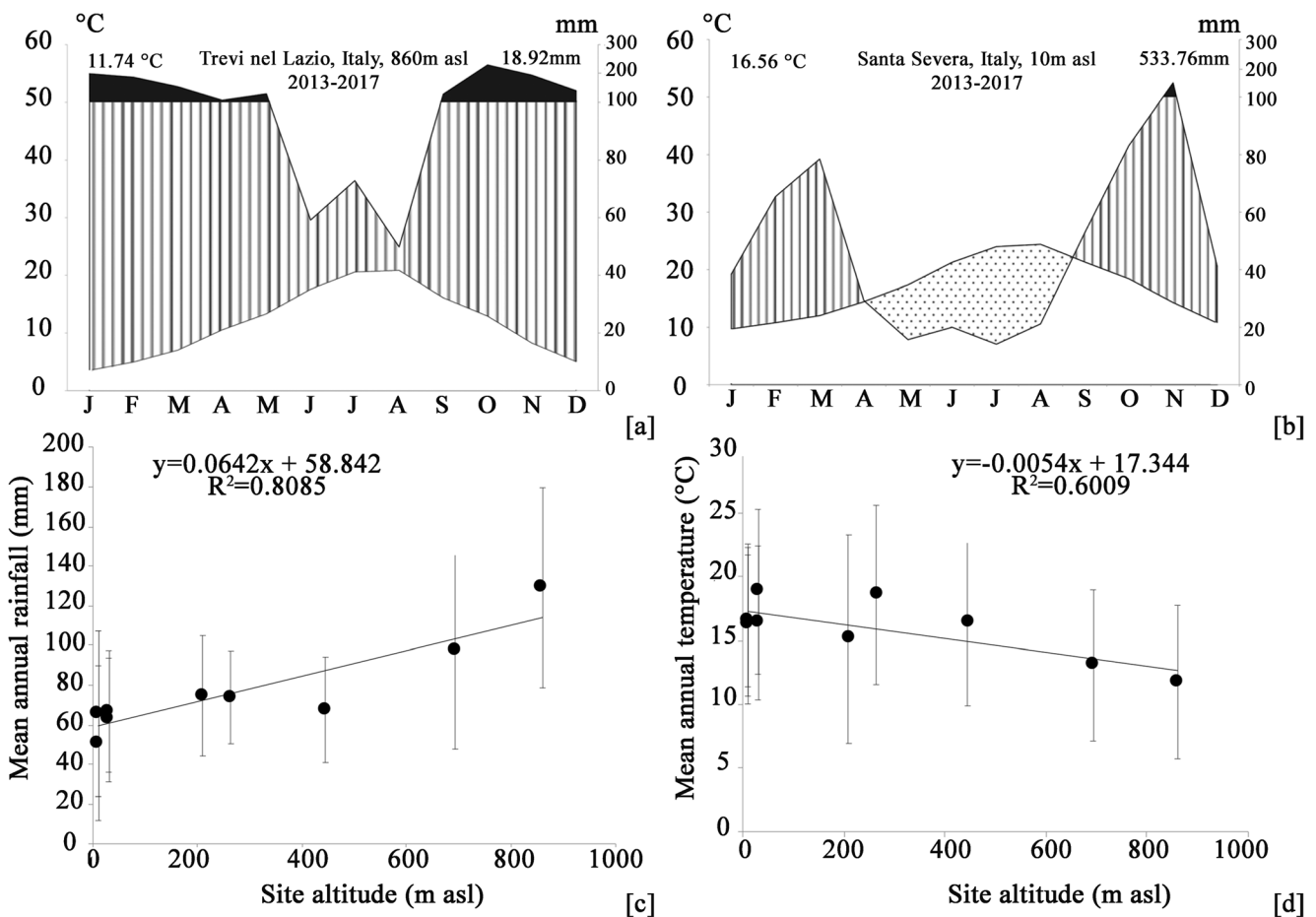


Fig. 2 Bagnoul-Gaussen graphs of the bioclimatic extremes. **a** Santa Severa, **b** Trevi nel Lazio sites; and **c** mean annual rainfall and **d** relative humidity correlation with altitude of the sites

A strong negative correlation between the diurnal light loads/aspects and biofilm cover ($R^2 = 0.816$; $p < 0.005$) was detected. The estimations of the daily light load at each site showed that *T. umbrina* had a preference for N and NW aspects that received low light loads (Fig. 4b).

Different substrates were colonised, but based on the number of observations, there seemed to be a preference for concrete and mortars (Fig. 4c), reaching the highest values for the concrete posts.

Morphological and molecular characterisation

All the collected biofilms resulted primarily composed of *T. umbrina* colonies. Confident identification of *T. umbrina* based on morphological analysis was possible even if no sexual structures were found in any of the samples (Fig. 5). Samples taken toward the centre of the red biofilms were composed almost entirely of *T. umbrina* cells with only a scarce presence of meristematic fungi. Closer to the edge of the red-orange biofilms, other phototrophs were present, including cyanobacteria such as *Gloeotheca* sp., *Gloeocapsa* sp. or *Scytonema* sp., and a few small pennate diatoms.

The Kruskal-Wallis analysis of morphological measurements showed a high degree of variability, with significant differences in cell length and width among populations. Dunn's post hoc test identified around 50% out of a total of 60 comparisons were significantly different ($p < 0.05$) for both *L* and *W* data. Comparing the means of *L:W* of intercalary cells of *T. umbrina* using an ANOVA revealed there was a significant difference between sites. Post hoc investigation, however, showed only RP1 was significantly different from OA1, OA2 and CA3 ($p < 0.01$); otherwise, this descriptor was relatively conservative. Scatter plots of the site-specific *W* and *L:W* mean values show the range of cell size within and among populations (Fig. 6) but also reveal the large overlap in the ranges among sites. Based on the 95th percentile values of all 600 cell measurements, we report an intercalary cell size range of (8.1) 13.5–27.5 (41.8) μm wide (Fig. 6a) and *L:W* of 1.1–1.6, and a mean of 1.25 times longer than wide (Fig. 6b).

The *rbcL* sequence determined in this study (610 bp) for strain GRAS2 (sample taken from Grassi Hospital) was submitted to the GenBank database with accession no. MK281583 and showed high sequence identity (97.2%) with a sequence of *T. umbrina* voucher UNA00068462. The

Fig. 3 Widespread and distribution of BP. **a** The abundance of the red-orange biofilm in the favourable condition of Grassi hospital. **b** The differences of colonisation related to the aspects (Santa Severa). **c** The ecological transition from red-orange BP to black BP (S. Marinella)



neighbour-joining tree (Fig. 7) obtained with a data set of 12 *rbcL* gene sequences had the same topology of trees inferred with by the maximum likelihood and parsimony methods (data not shown). Strain GRAS2 clustered with the strain of *T. umbrina* voucher UNA00068462 isolated from the bark of the tree on banks of river Ticino (Rindi et al. 2009) supported by a bootstrap value of 100%. Another strain of *Trentepohlia* cf. *umbrina* isolated from a concrete wall in Ireland (Rindi et al. 2009) grouped in a different cluster along with other species of Trentepohliales. On the basis of morphological observations and molecular analyses, the strain GRAS2 investigated in this study can be assigned to the species *T. umbrina*.

Discussion

This study clarifies and improves the ecological and morphological characterisation of the species *T. umbrina*, also highlighting that this species, in its free-living, non-lichenized form, is much more widespread than the available literature suggests. *T. umbrina* is the most widespread species of the genus (Rindi and López-Bautista 2007), but records of its free-living form refer only to countries of tropical (Rindi et al. 2005, 2006; Gaylarde et al. 2006) and temperate climates (Rindi and Guiry 2002; Lüttge and Büdel 2010; Marmor and Degtjarenko 2014; Unković et al. 2017). There are Mediterranean records for the genus in Portugal (Pereira de Oliveira 2008; Macedo et al. 2009) and Spain (Alvarez et al. 1994; Ariño and Saiz-Jimenez 1996) but not specifically for the species *T. umbrina*.

Also, in Italy, the most records refer to the genus *Trentepohlia* (Tomaselli et al. 2000; Zucconi et al. 2012) or to the species *T. aurea* (Rizzi Longo et al. 1980), instead the only records of *T. umbrina* are from Pavia (Rindi et al. 2005) and Lucca (Ricci and Bartoli 2017). Therefore, this study shows for the first time the presence of *T. umbrina* in the Lazio region and more specifically in the area with Mediterranean bioclimate. This study shows many *T. umbrina* populations across a relatively small area (Lazio region), but due to the altitude differences among sites, a relatively large ecological gradient is covered (12 to 19 °C and 554 to 1549 mm y⁻¹ rainfall, 5-year means), giving further data to the ecological characterisation of the species. Moreover, among the surveyed areas, six are considered to have cultural heritage value: *Domus Augustana* (Palatine hill, RM), the Non-Catholic Cemetery (RM), *Ostia Antica* archaeological park (RM), *Bomarzo* historic garden (VT), *Villa Lante di Bagnaia* (VT), *Santuario Madonna del Tufo* (Rocca di Papa). Three sites were considered as modern urban, such as facades of buildings (in Santa Severa and S. Marinella), concrete fence posts (Grassi Hospital) and concrete electricity poles (Trevi nel Lazio). The majority of literature on *Trentepohlia* as a biodeteriogen of monuments is related mainly to the genus *Trentepohlia* (Tomaselli et al. 2000; Caneva et al. 2005; Macedo et al. 2009; Zucconi et al. 2012; Ortega-Morales et al. 2012; Bartoli et al. 2014) and *T. aurea* (Noguerol-Seoane and Rifon-Lastra 1996; Ramirez et al. 2010), whereas records of *T. umbrina* are only limited to Tropical areas (Gaylarde et al. 2006; Samad and Adhikary 2008). Given the biodeteriogenic potential of this genus (Wakefield et al. 1996; Cutler and Viles 2010), the newly

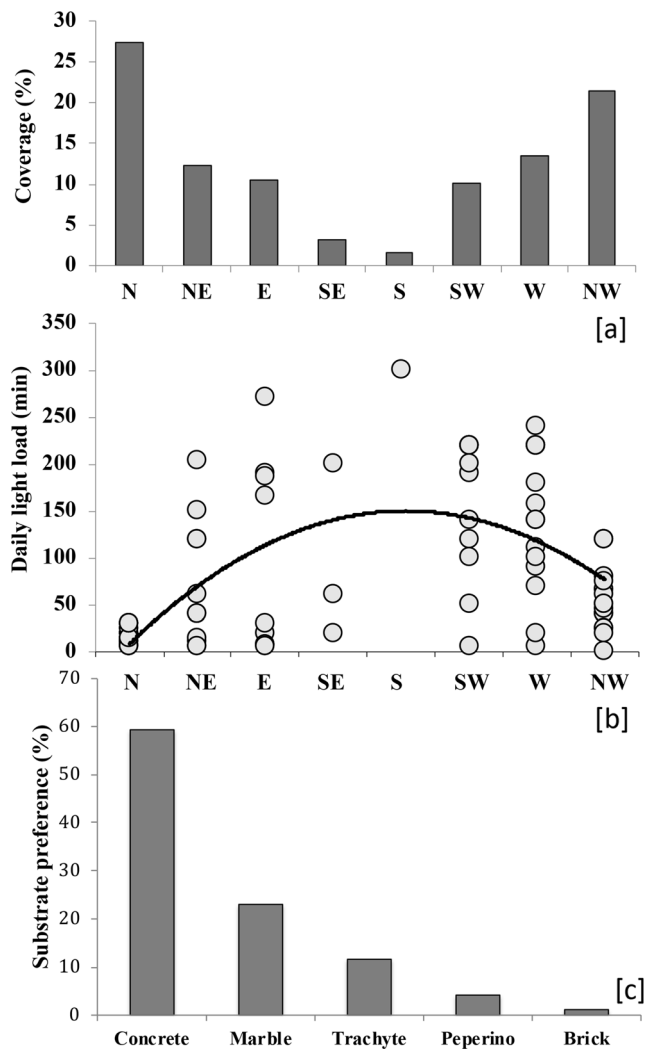


Fig. 4 Cover of BP (% of total cover based on the mean for each aspect) in relation to **a** the aspects, **b** the light availability for each aspect and **c** the substrate

discovered presence of *T. umbrina* on sites of cultural heritage in Lazio is relevant for the conservation of monuments in these sites.

The most significant micro-climatic factors controlling algal growth on the lithic surface are generally moisture, light

and temperature (Ariño and Saiz-Jimenez 1996; Tran et al. 2014; Caneva et al. 2016), which are also related to the surface chemico-physical conditions and aspects. In the case of *T. umbrina*, the need for substrate moisture was relatively low, considering that it occurs almost entirely on vertical, lithic surfaces. It is highly probable its water requirement is derived from atmospheric humidity and dew formation. Its main occurrence on northern aspects and in coastal areas supports this theory. The unusual presence of *T. umbrina* on a southern facing substrate at the Grassi Hospital seems associated with the nature of the substrate, which was very porous and in relatively humid conditions. In this particular south aspect, the humidity content related to the high substrate permeability will result in it holding on for longer to the minimum moisture level, higher than a classic southern aspect. If this is so, it would allow *T. umbrina* to be photosynthetically active for longer after sunrise than if the substrate was less adsorptive. This hypothesis could also go some way to explain the apparent preference for concrete within this survey, that has been shown to be particularly bioreceptive due to their combined roughness and porosity (Tran et al. 2014).

Humidity is normally higher in warmer air, but not when exposed to high solar radiation, so the main presence of *T. umbrina* on substrates with northern aspects would be expected. This can also help explain why *T. umbrina* seems to tolerate a wide temperature range (annual mean from 12 to 19 °C), was not found on southern aspects in the higher altitude sites and why growth/biomass was more abundant at the warmer coastal areas; perhaps growth in the uplands simply continues but slower in comparison with that in the lowlands.

In the case of *T. umbrina*, it has been shown that light is among the most relevant growth limiting factors (Odum et al. 1971). The negative correlation between *T. umbrina* cover and light, and that light loads were mainly determined by substrate aspect, further underlines the low light preference. Toward the outer lower edge of the *T. umbrina* biofilms observed here, cyanobacteria, and other green algae and in some instances, diatoms were observed. These data would confirm the ecological model proposed by Caneva et al. 2016 showing the strong relationship between the ecological features of this species



Fig. 5 *T. umbrina* from a macroscopic to a microscopic point of view

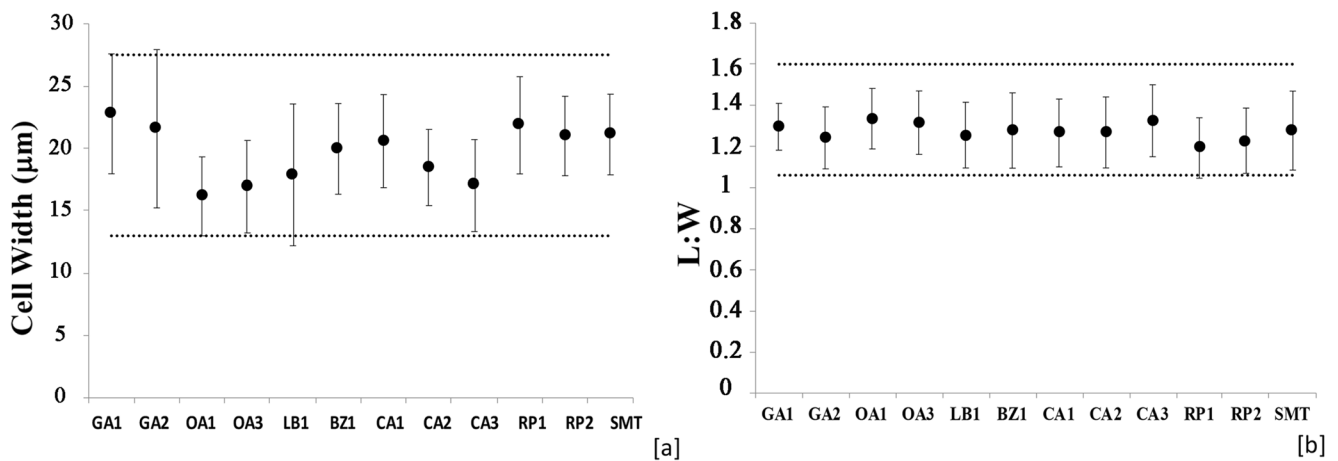


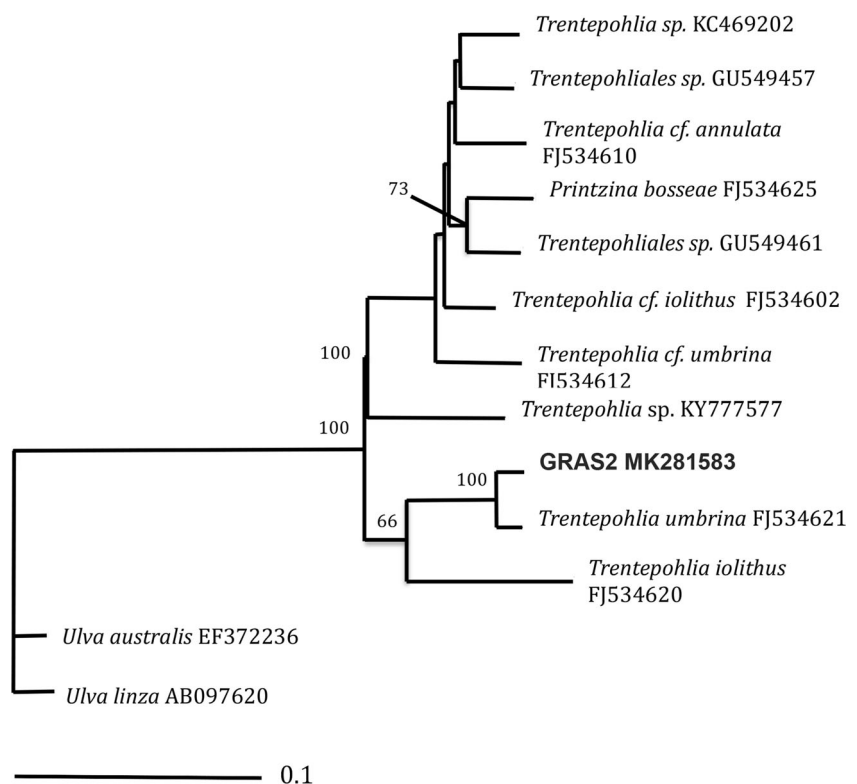
Fig. 6 Morphological measurements of intercalary cells of *T. umbrina*. Data are mean values with standard deviations for the sites (acronyms listed in Table 1). Dotted lines are the 95th percentiles of the whole dataset (600 cells)

and cyanobacteria: it seems that situation in tropical and Mediterranean climates is similar, showing a transition from *Trentepohlia*-dominated community to a cyanobacteria community with an increase in light availability.

The cell dimension of all the populations included in this study was always close to the ranges set out by John (2002). Rindi and Guiry (2002), however, gave a smaller cell width range than in John (2002) at 8–20 µm compared with 15–27 µm. In total, there were 600 cells measured to give robust cell width range of 13 to 27.5 with a mean of 19.6. The *L:W*

range of 1 to 1.7 with a mean of 1.25 was a more restricted range than those previously reported of one to two times longer than wide. Cell width is recognised as an important morphological character in the taxonomy of *Trentepohlia*, and in this study, there was some variation among sites, but it was rare that it was significant, and all the widths within one standard deviation of the mean fell between the grouped 95th percentile range. Other morphological features worth noting include short irregular filaments frequently composed of two to four bulbous cells; however, John (2002) reported

Fig. 7 Neighbour-joining tree inferred from 12 *rbcL* gene sequences (610 bp). The numbers at the nodes indicate bootstrap values as a percentage greater than 50% obtained using distance as an optimality criterion with 500 replicates. The sequence determined in this study is indicated in bold. The sequences of *Ulva australis* and *Ulva linza* were designated as outgroup. The GenBank accession numbers are reported for each sequence. The scale marker represents 0.1 nucleotide substitution per sequence position



that these short filaments are possibly broken as a result of sample processing during sampling and slide preparation. There was no obvious distinction between prostrate and erect structures. Filaments are sometimes branched; this feature is possibly more common as this is likely the point where filaments break when being processed. Terminal cells are smaller at around 10 µm and seem more regular in form than the intercalary cells. No sexual structures were noted during the period of the study nor during the numerous attempts to culture this species. Based on these morphological data and knowledge of the closely related species within this genus, it was possible to identify with some security the species observed as *T. umbrina*.

However, as previously established by Rindi et al. (2009), in the order Trentepohliales, the species concept cannot be based exclusively on morphological features. Thus, we carried out a molecular investigation in order to determine its taxonomic position using the *rbcl* gene, which has a better resolution than the 18S rRNA analysis in this species (Rindi et al. 2009). The results showed the high base similarity of our strain with another Italian strain of *T. umbrina* and so confirming the morphological identification of strain GRAS2 as the species *T. umbrina* with some certainty. Species of *T. umbrina* resulted as polyphyletic taxa, and in a previous study, some strains of *T. umbrina* clustered together with other species of *Trentepohlia* and *Pritzina* with a widespread geographical distribution (Rindi and Guiry 2002). In particular, different strains of *T. umbrina* clustered with strains of *T. iolithus*. However, it is worth of note that our strain from Ostia (Roma, Italy) clustered with the Italian (Pavia, Italy) strain of *T. umbrina* possibly indicative of environmental influence in the distribution of this species.

Conclusions

Free-living *T. umbrina* distribution is more widespread than previously thought. It is widely distributed within the Lazio region colonising a diverse range of substrates occurring across a wide ecological range from the Mediterranean to temperate climates. *T. umbrina* has a clear preference for vertical substrates with northern aspects, often associated with low light and humid conditions. Our research also refined the morphological features that describe the species and supported this with molecular techniques.

The importance of understanding the ecology of this species is becoming increasingly evident, as the information of its possible biodeteriogenic nature comes to the fore.

Funding Financial support to the research arises from the cooperation agreement among the Universities of Tor Vergata and Roma Tre (2016/2019).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Research involving human participants and/or animals No humans or animals were used in this work.

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