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A synopsis of green-algal lichen symbionts with an emphasis on their free-living lifestyle

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ABSTRACT

We present a synopsis of studies reporting the free-living occurrence of green algal lichen symbionts. We give an overview of all known lichen photobiont genera together with comprehensive descriptions, taxonomical classification and occurrence data. Based on the analysis of 310 records, we discovered that at least 80% of lichen photobiont genera were observed in the free-living state. *Diplosphaera chodatii*, *Elliptochloris bilobata* and *Chloroidium ellipsoideum* represent both morphologically and genetically the most frequently reported free-living photobiont species. *Trebouxia*, the most prevalent genus of lichen photobionts, has frequently been reported to exist independently to fungal hyphae. Based on our literature survey, free-living photobionts of lichens are able to grow in a wide range of environments and substrates, with most records coming from soil and biological soil crusts.

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INTRODUCTION

Lichens are prime examples of symbiotic interactions formed by associations of heterotrophic ascomycete and basidiomycete fungi, i.e., the lichen-forming fungi (mycobiont), which makes up most of the lichen's thallus, with an algal and/or cyanobacterial partner (photobiont), which provides the fungus with carbohydrates. The dual nature of lichens was discovered by Schwendener (1869), making lichens the first observed mutualistic-symbiotic system. However, Schwendener's hypothesis was rejected by most lichenologists until the end of the 19th century (Honegger 2000). Today, a high diversity of other organisms (e.g., bacteria, other fungi, yeast) are known to be present within the lichen thallus as well (Farrar 1976; Grube *et al.* 2009; U'Ren *et al.* 2012; Spribille *et al.* 2016; reviewed in Morillas *et al.* 2022). For example, nitrogen-fixing bacteria can be very advantageous for lichens lacking a cyanobiont (Grube *et al.* 2009). Many fungi with functions different from the mycobiont, the so-called ‘endolichenic fungi’, were also found inside lichens (U'Ren *et al.* 2012; Spribille *et al.* 2016).

The species names of lichens refer to the species names of the mycobiont (Tschermak-Woess 1988). The number of mycobiont species is estimated to be more than 18,000 (Nelsen *et al.* 2011; Muggia *et al.* 2014; Morillas *et al.* 2022), thus greatly exceeding the number of lichen photobionts, belonging to about 50 genera (Sanders & Masumoto 2021). However, the diversity of photobionts is still largely unknown, and many new lineages are still awaiting discovery (Muggia *et al.* 2020).

Reproduction of lichens may involve the formation of asexual propagules (e.g., soredia, isidia, or other types of thallus fragments,

Sanders & Lücking 2002) containing both symbiotic partners, as well as the production of sexual or asexual spores by the mycobiont only. When the fungal spores germinate, they must encounter a suitable alga to form a new lichen (Armaleo *et al.* 2019). Thus, it must re-establish the symbiotic state at each reproductive cycle, selecting the photobiont from the algal pool available in the environment. The mycobionts can show various degrees of selectivity towards the photobiont species. Some can even switch their photobionts or may associate with multiple minor/secondary symbiotic partners in addition to the major biologically relevant photobiont (Moya *et al.* 2017, 2020). Moreover, it has been shown that mycobionts can form tightly interconnected communities by sharing a common pool of photobionts, i.e., photobiont-mediated guilds (Rikkinen 2003; Dal Grande *et al.* 2014; Kaasalainen *et al.* 2021; Peksa *et al.* 2022).

The nature of the relationship between the two symbionts remains unclear. It is usually described as a mutualism, i.e., a mutually beneficial coexistence between the two partners. Ahmadian (1993), however, refers to controlled parasitism, as the fungus usually completely envelopes the photobiont and often forms haustoria that penetrate the algal cell. In addition, the fungus seems to benefit more from the association since the free-living photobionts often achieve higher growth rates than the lichenized ones (Nash 2008).

Approximately 90% of lichens contain eukaryotic photobionts from the green algal lineage Chlorophyta. This includes many mostly terrestrial members of the class Trebouxiophyceae and a few belonging to the classes Ulvophyceae and Chlorophyceae (Sanders & Masumoto 2021). In addition to Chlorophyta, some lichens seem to associate with at least one

streptophyte algal species (Voytsekhovich *et al.* 2011). Photobionts also recruit from other eukaryotic lineages such as Xanthophyceae (Tschermaek-Woess 1988), and even Phaeophyceae (Sanders *et al.* 2004) and Rhodophyta (Kohlmeyer & Volkmann-Kohlmeyer 1998), where they form a major part of thalli of the so-called ‘borderline-lichens’. We must not omit a significant group of photobionts that recruit from an immensely important bacterial phylum, the Cyanobacteria (Kaasalainen *et al.* 2021).

Trebouxia and *Asterochloris* (Trebouxiophyceae, Chlorophyta) are the most common genera of lichen photobionts, associating mainly with fungi from the orders Lecanorales and Teloschistales (Ascomycota). *Nostoc* is the most common cyanobacterial photobiont of the order Peltigerales (Ascomycota); tropical orders of lichenized fungi (e.g., Arthoniales, Ostropales, Pyrenulales, and Trypetheliales) most commonly host photobionts from the Trentepohliales group. On the contrary, members of the family Verrucariaceae form a symbiosis with a multitude of different photobiont species, including non-Chlorophyta photobionts (Thüs *et al.* 2011), as well as various marine Ulvophyceae algae (Černajová *et al.* 2022; Malavasi *et al.* 2022). Green algae entering lichen associations usually possess a simple morphology (Figs 1–30) which can be categorized into the following types: coccoid, uniseriate filamentous, or sarcinoid. The morphological distinction of photobiont species, or even genera, is extremely difficult due to high similarity and high morphological plasticity (Sanders & Masumoto 2021). Moreover, the identification of these algae becomes even more difficult in the lichenized state, and cannot be done without cultivation, because their morphology is often heavily simplified in symbiosis (Nash 2008; Peksa & Škaloud 2008).

The existence of free-living lichen photobiont populations, i.e., without the presence of fungal hyphae, has been questioned for decades. Most attention has been paid to the genus *Trebouxia* in this respect. Vernon Ahmadjian, one of the world’s leading experts on lichen algae, claimed that representatives of the genus *Trebouxia* occur in the free-living state only rarely (Ahmadjian 1967, 1988, 2001). In fact, Ahmadjian considered lichen algae as ‘a source of food for lichen fungi’, absolutely dependent on the fungal partner, which cultivates them in a way ‘comparable to heads of lettuce’ (Ahmadjian 2001). He believed that all observations of free-living *Trebouxia* cells originated from asexual propagules produced by lichens or observations of leprose lichens (Ahmadjian 1967). This idea was later rejected by Tschermaek-Woess (1978) who reported numerous findings of free-living *Trebouxia* cells at various localities. Later on, free-living *Trebouxia* algae were reported by several other authors (e.g., Bubrick *et al.* 1984; Mukhtar *et al.* 1994; Sanders 2014). Today, most algae occurring in lichens are considered facultative photobionts, with the ability to live independently of the mycobiont (Sanders & Masumoto 2021). Ahmadjian, however, never stopped believing in his theory. His work is still being cited and the doubts concerning the independent existence of lichen photobionts are still present (Gärtner & Stoyneva 2003; Nash 2008; Novakovskaya *et al.* 2020).

There is a large body of literature on the diversity of both free-living terrestrial algae and photobionts living inside of lichen thalli (see Sanders & Masumoto 2021 and citations

herein) that discussed photobionts associated with lichen-forming fungi. The diversity of free-living lichen algal symbionts was already described by Ettl & Gärtner (2013), however, the subject matter went beyond what the cited book could cover. Accordingly, the aim of this paper is to present an updated list of lichen photobionts (including newly described genera, see Fig. 31 for reference) and, more importantly, provide a comprehensive overview of studies reporting their free-living occurrence. We focused on “green algae” (Viridiplantae or Chlorobionta), a monophyletic lineage consisting of two distinct groups, Chlorophyta and Streptophyta (Leliaert *et al.* 2012) that have important roles in all terrestrial and most aquatic ecosystems (Leebens-Mack *et al.* 2019). We analysed a total of 310 records of free-living green algal lichen symbionts reported from various habitats (Table S1) and summarized their occurrences and ecology.

RESULTS

Division Chlorophyta

Chlorophyta comprises most of the species diversity of green algae. Members of this group inhabit marine, terrestrial, and freshwater environments and are characterized by considerable morphological diversity and a characteristic arrangement of the flagellar apparatus (Moestrup 1978). The core Chlorophyta includes three large and diverse classes: Trebouxiophyceae, Ulvophyceae, Chlorophyceae (so called UTC-clade), and several small classes of generally small marine flagellates (Leliaert *et al.* 2012; Del Cortona *et al.* 2020).

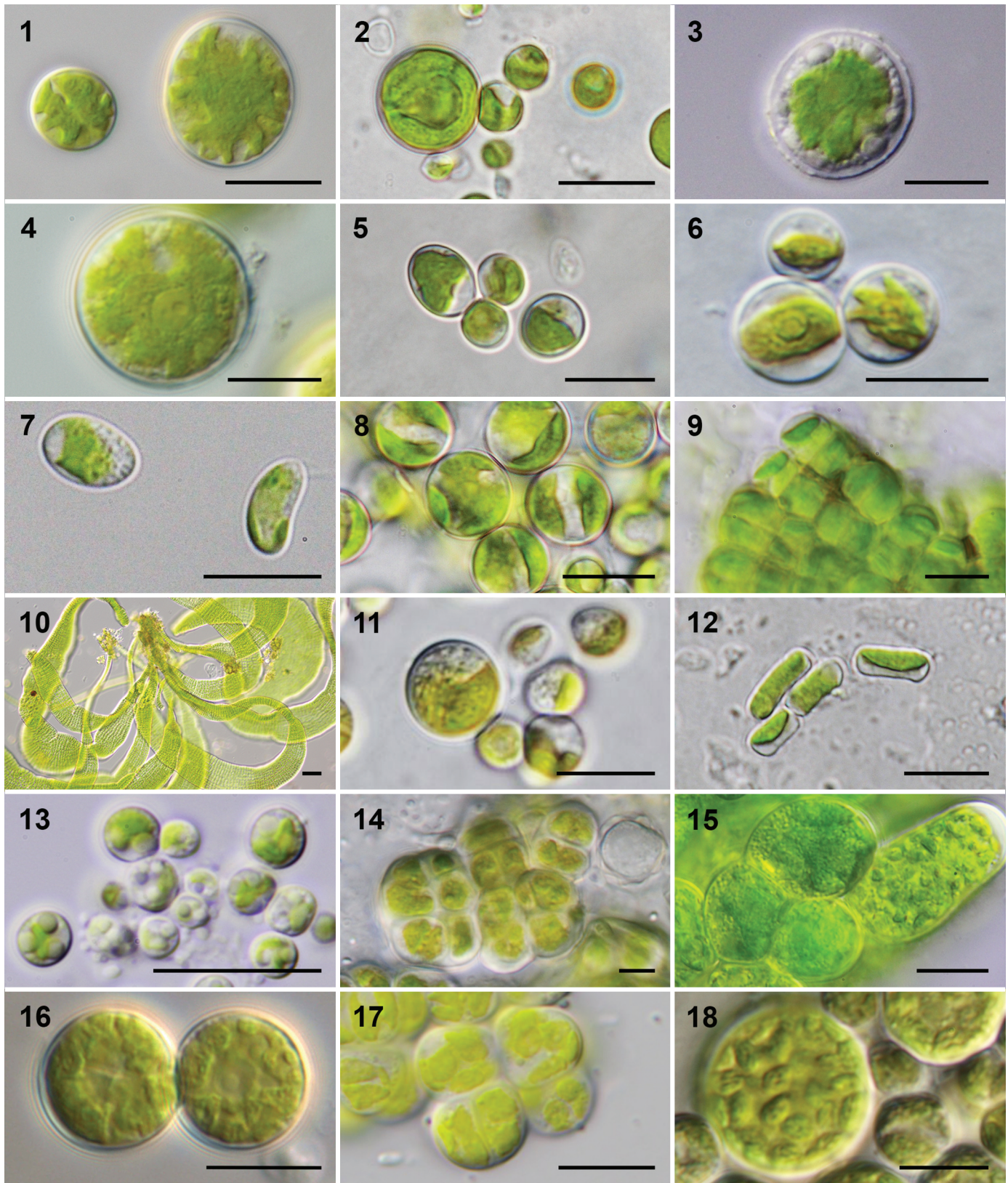
Class Trebouxiophyceae

The class Trebouxiophyceae was described based on the genetic similarity of investigated strains as a group of algae with non-flagellated vegetative cells, unicellular, sarcinoid, or filamentous thalli (Friedl 1995).

Phylogenetically the Trebouxiophyceae is the sister group to the Chlorophyceae and Ulvophyceae, and can be further subdivided into six main lineages: Trebouxiales, Watanabeales, *Elliptochloris*-clade, Prasiolales, Chlorellales and Microthamniales (Leliaert *et al.* 2012; Lemieux *et al.* 2014; Darienko *et al.* 2016; Li *et al.* 2021). Members of most of these lineages (except for the order Microthamniales) have been found to associate with lichens (e.g. Thüs *et al.* 2011; Škaloud *et al.* 2015). The nature of the relationships between these lineages remains unclear, and some studies even question the monophyly of the entire class (Leliaert *et al.* 2012).

TREBOUXIALES

***Asterochloris* Tschermaek-Woess.** *Asterochloris* is one of the most common, widespread and diversified genus of lichen photobionts (Škaloud *et al.* 2015). The morphology of the cell is simple, mostly spherical, sometimes oval or pear-shaped with a thin cell wall that may be thickened locally. The genus was named after the single star-shaped chloroplast with differently shaped lobes, extending to the edges of the cell (Fig. 1). The deeply lobed chloroplast is one of the main



Figs 1–18. Lichen photobiont genera (including nonlichenized species) belonging to classes Trebouxiophyceae and Chlorophyceae.

Fig. 1. *Asterochloris lobophora*.

Fig. 2. *Myrmecia incisa*.

Fig. 3. *Trebouxia impressa*.

Fig. 4. *Vulcanochloris symbiontica*.

Fig. 5. *Chloroidium saccharophilum*.

Fig. 6. *Jaagichlorella luteoviridis*.

Fig. 7. *Coccomyxa* sp.

Fig. 8. *Elliptochloris subsphaerica*.

Fig. 9. *Diplosphaera chodatii*.

Fig. 10. *Prasiola crispa*.

Fig. 11. *Pseudochlorella pringsheimii*.

Fig. 12. *Stichococcus* sp.

Fig. 13. *Auxenochlorella protothecoides*.

Fig. 14. *Apatococcus* sp.

Fig. 15. *Leptosira* sp.

Fig. 16. *Symbiochloris tschermakiae*.

Fig. 17. *Uvulifera verrucariae* (Lorenz 2016).

Fig. 18. *Bracteacoccus* sp. Scale bars: 10 μ m (Figs 1–9, 11–18), 200 μ m (Fig. 10).

morphological features that distinguish this genus from *Trebouxia*. The presence of one or more pyrenoids is also another important feature. Asexual reproduction is by aplanospores and zoospores. Sexual reproduction is very rare and has been observed only in a single species *A. woessiae* (Škaloud *et al.* 2015).

Currently, the genus includes 19 species (Guiry & Guiry 2022), a large proportion of which have only recently been discovered or separated from the genus *Trebouxia*. However, a number of cryptic species still remain to be formally described (Škaloud & Peksa 2010; Škaloud *et al.* 2015; Kosecka *et al.* 2021). Škaloud & Peksa (2010) formally delineated the genus *Asterochloris* and established new combinations for former *Trebouxia* species. *Asterochloris* associates mainly with mycobionts from the families Cladoniaceae and Stereocaulaceae (Muggia *et al.* 2018). All known members of this genus are capable of lichenization (Škaloud & Peksa 2010; Škaloud *et al.* 2015; Kosecka *et al.* 2021).

The presence of free-living *Asterochloris* species was genetically confirmed in two studies: the alga was detected on the hair of sloths in South and Central America (Suutari *et al.* 2010) and on the bark of trees growing in the sub-Mediterranean region (Fiesia, Piran, Slovenia and Cernizza, Duino, Italy; Kulichová *et al.* 2014). The authors of the second paper emphasize that they avoided habitats with lichens during the sampling and carefully removed all isolated pieces of lichen thalli. The species *A. excentrica* was isolated from soil in the mountain tundra of the Northern Urals. However, the sample came from a community dominated by the lichens *Cladonia rangiferina* and *Flavocetraria nivalis*, and thus it cannot be excluded that the alga was lichenized (Novakovskaya *et al.* 2020). Two other records of the occurrence of this species come from soil samples (Andreyeva & Chaplygina 2006, 2007). The species *A. italiana* (= *Trebouxia italiana*) was found on a granite rock outcrop of the left bank of the Ukrainian river Pivdennyi Bug. However, the collection sites showed a high percentage of lichen cover (40–90 %; Mikhailyuk *et al.* 2003). The presence of *A. magna* in soil was reported by Andreyeva (2005) and Andreyeva & Chaplygina (2006). Non-lichenized cells of *Asterochloris* were also isolated from the tree bark samples (Neustupa & Štifterová 2013; Štifterová & Neustupa, 2015). However, in both cases it could not be excluded that the observed cells belong to the genus *Trebouxia*.

Myrmecia Printz. *Myrmecia* is a coccoid alga with spherical, ovoid, or pear-shaped cells that can form multicellular aggregates. The cell wall may be locally thickened. The chloroplast is parietal, cup-shaped, wavy at the edges, and usually divided into 2–4 lobes (Fig. 2). It reproduces asexually by zoospores, aplanospores or autospores (Ettl & Gärtner 2013). From the nine taxonomically accepted species (Guiry & Guiry 2022) only two (*M. biatorellae* and *M. israelensis*) have so far been confirmed as photobionts of *Psora*, *Placidium* and *Heteroplacidium* from the family Verrucariaceae (Thüs *et al.* 2011; Moya *et al.* 2018).

Free-living *M. biatorellae* is often reported from arid regions (Flechtner *et al.* 1998; Vinogradova *et al.* 2004; Flechtner *et al.* 2008; Venter *et al.* 2015). However, there are

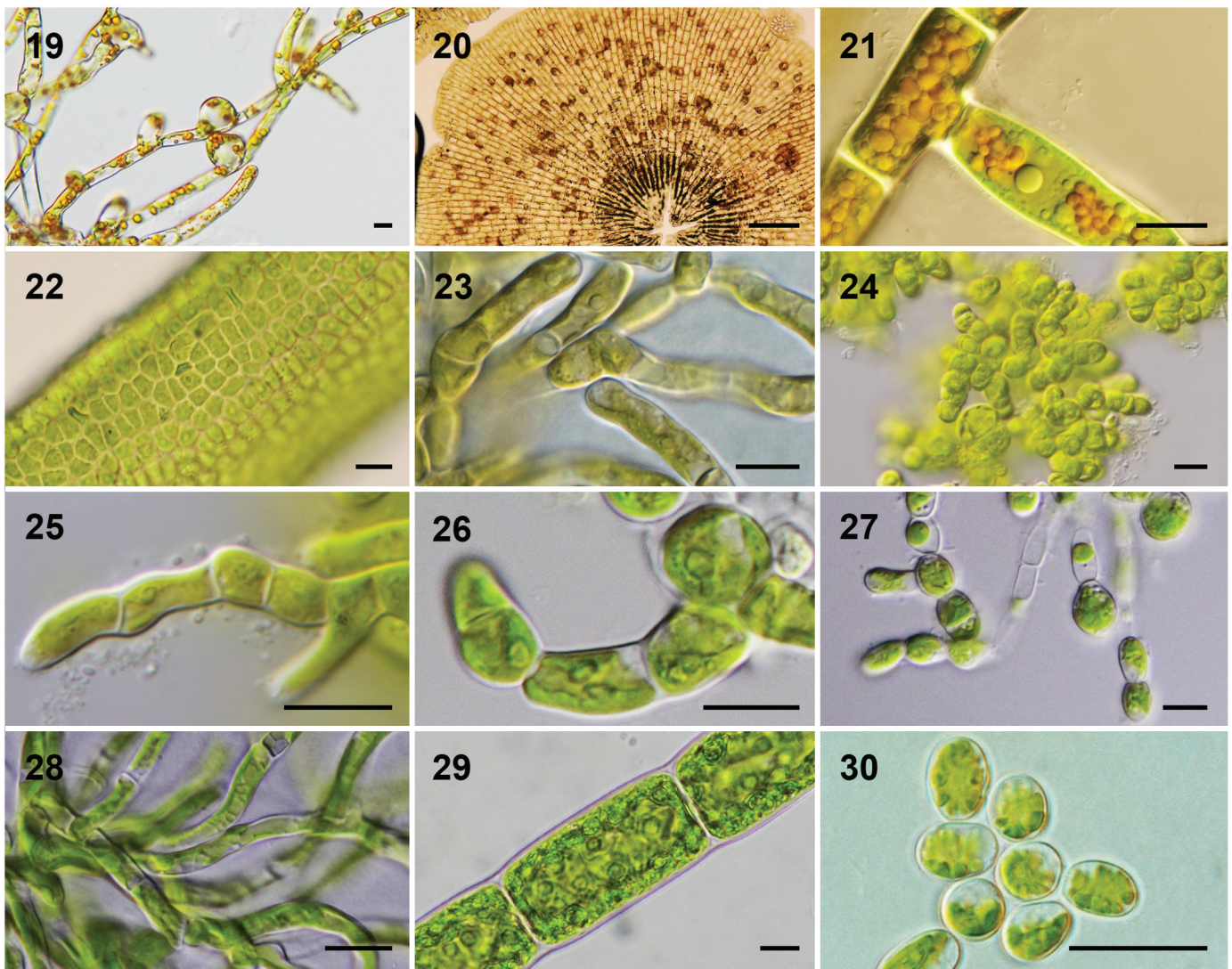
also records from the tundra in north-eastern Russia (Andreyeva 2005). This species has been reported quite frequently from caves (Roldán *et al.* 2004; Vinogradova *et al.* 2009). For instance, Roldán & Hernández-Maríné (2009) isolated *M. biatorellae* from a biofilm on the surface of stalactite in Collbató Cave (Spain). The granite walls of historic buildings in Galicia (Spain) are also a substrate inhabited by this alga (Rifón-Lastra & Noguerol-Seoane 2001). In addition to rocks (Vinogradova *et al.* 2004), this species often inhabits soil (Khaybullina *et al.* 2010; Bakieva *et al.* 2012), tree bark (Khaybullina *et al.* 2010), sand (Schulz *et al.* 2016) and survives on serpentinite soils (Venter *et al.* 2015).

Myrmecia israelensis (= *Friedmannia israelensis*), similarly to the previous species, is frequently recorded in arid (Friedmann *et al.* 1967; Grondin & Johansen 1993; Büdel *et al.* 2009) and semiarid (Johansen *et al.* 1993) areas and on rocky substrates (Friedmann *et al.* 1967) including granite (Vázquez-Nion *et al.* 2016). Friedmann *et al.* (1967) reported free-living cells of *M. israelensis* in the Negev desert, Israel. In addition, it has been detected in the Namib Desert soil (Büdel *et al.* 2009) and genetically confirmed on World Heritage sites in Santiago de Compostela (Vázquez-Nion *et al.* 2016).

Trebouxia Puymaly. *Trebouxia*, the most diverse and common genus of lichen photobionts (Nash 2008), has a large axial chloroplast with at least one pyrenoid (Fig. 3). It reproduces by zoospores with two flagella of equal length, or by autospores (Archibald 1975). Only immobile stages with a reduced chloroplast can be found in the lichen thallus (Nash 2008).

Representatives of the genus were initially spread across several genera: *Trebouxia*, *Pseudotrebouxia* and *Asterochloris* (Nash 2008). *Pseudotrebouxia* was separated because of differences in asexual reproduction (Archibald 1975) but was later rejected on the basis of morphological observations (Gärtner 1985) and, later, molecular data (Kroken & Taylor 2000). *Asterochloris* and *Trebouxia*, differing in chloroplast morphology (Škaloud *et al.* 2015), were separated in 2010 (Škaloud & Peksa 2010). These two genera also differ ecologically. While *Asterochloris* prefers mycobionts from the families Cladoniaceae and Stereocaulaceae, *Trebouxia* forms lichens more frequently with the families Parmeliaceae and Lecanoraceae (Muggia *et al.* 2018). The 27 taxonomically accepted species (Guiry & Guiry 2022) accompany about 20 % of all lichen species (Rambold & Triebel 1992). The real species diversity of this genus is likely to be much higher, as a large proportion of the lineages discovered have not been formally described, and, in addition, new unknown species-level lineages are still being reported (Muggia *et al.* 2020).

As mentioned before, the existence of free-living members of the genus *Trebouxia* has been questioned in the past (Ahmadjian 1967, 1988, 2001) and other authors have not denied its existence but considered it very rare (Bubrick *et al.* 1984; Zavada & Simoes 2001). However, an overwhelming number of studies reported direct observations of free-living *Trebouxia* (Friedmann *et al.* 1967; Tschermak-Woess 1978; Bubrick *et al.* 1984; Sanders 2001, 2005; Sanders & Lücking 2002; Roldán & Hernández-Maríné 2009; Kharkongor & Ramanujam 2014). More recent studies report this genus as



Figs 19–30. Lichen photobiont genera (including nonlichenized species) belonging to classes Ulvophyceae and Klebsormidiophyceae.

Fig. 19. *Cephaleuros virescens* (Darienکو 2017).

Fig. 20. *Phycopeltis arundinacea*.

Fig. 21. *Trentepohlia* sp.

Fig. 22. *Blidingia marginata*.

Fig. 23. *Halofilum ramosum* (Darienکو 2008).

Fig. 24. *Lithotrichon pulchrum* (Darienکو 2016b).

Fig. 25. *Paulbroadya prostrata* (Darienکو 2016a).

Fig. 26. *Pseudendoclonium* sp.

Fig. 27. *Rindifilum verrucariae*.

Fig. 28. *Undulifilum symbioticum*.

Fig. 29. *Urospora penicilliformis*.

Fig. 30. *Interfilum terricola*. Scale bars: 10 µm (Figs 19, 21–30), 50 µm (Fig. 20).

one of the most common genera of photobionts that can be encountered in nature, sometimes described as very frequent (Barberousse et al. 2006; Štifterová & Neustupa 2017) or even dominating (Ismail et al. 2019; Popović et al. 2019). Molecular genetic studies have confirmed the presence of unspecified members of this genus (related to *T. jamesii* and *T. asymmetrica*) in the fur of sloths from South and Central America (Suutari et al. 2010) and on the walls of a castle ruin in Germany (Hallmann et al. 2013). In addition to tropical rainforests (Suutari et al. 2010), this photobiont inhabits deserts (Friedmann et al. 1967; Samolov et al. 2020) and tundra environments (Elster et al. 1999; Garraza et al. 2011;

Novakovskaya et al. 2020; Stewart et al. 2021), where lichen-dominated communities are often found (Novakovskaya et al. 2020) and thus the algae found may have been lichenized. *Trebouxia* was also detected in marine environments (Metz et al. 2019), although one cannot be certain that the detected sequences represent free-living individuals (Sanders & Masumoto 2021).

Trebouxia is often found in anthropogenic environments. It lives in coal post-mining areas (Lukešová 2001), on trees in close proximity to air polluting power plants (Ismail et al. 2019) and is often part of biofilms covering the facades of buildings (Rindi & Guiry 2004; Barberousse et al. 2006;

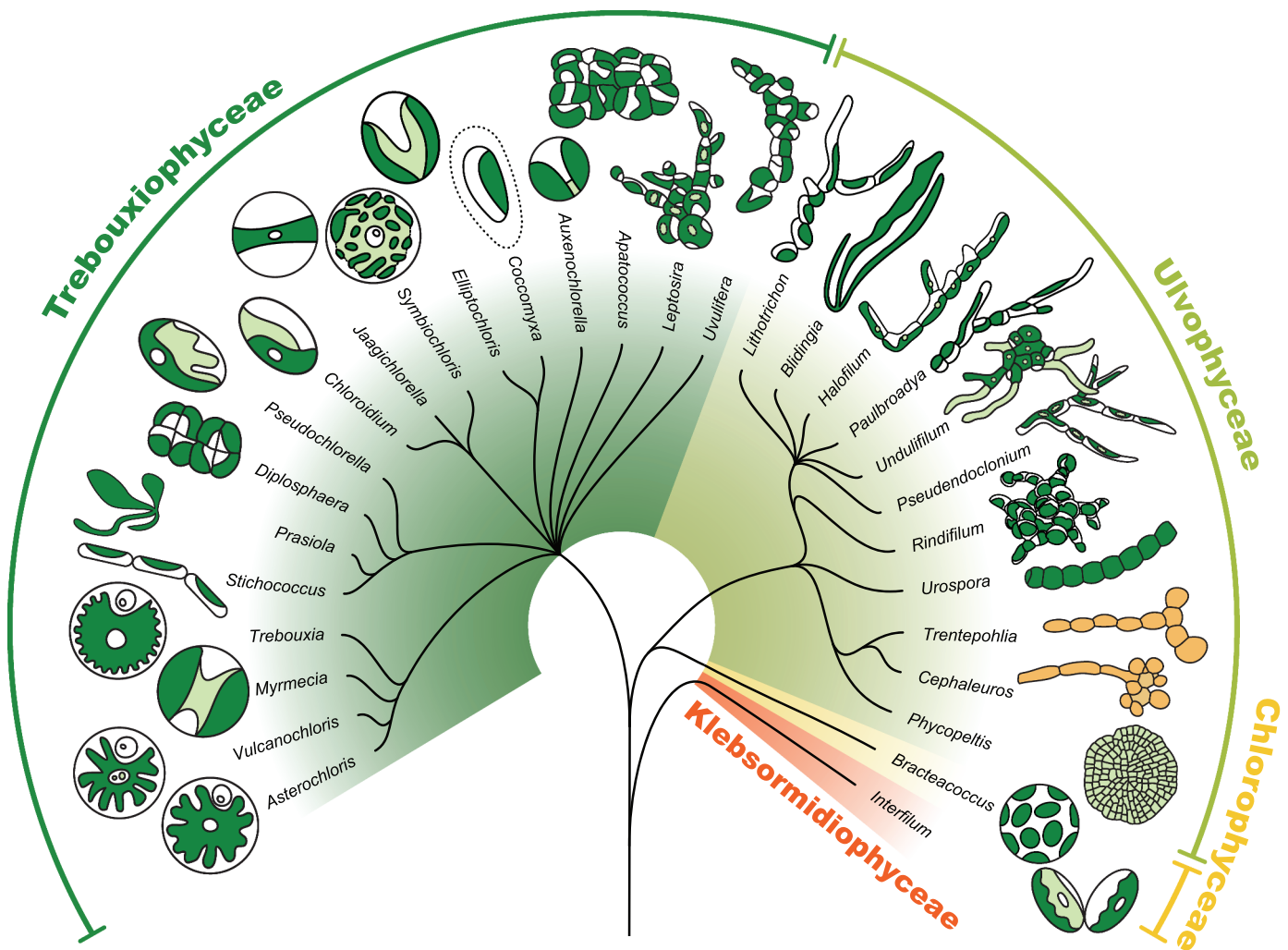


Fig. 31. Overview phylogeny of green algal photobiont genera.

Hallmann *et al.* 2013; Hofbauer & Gärtner 2021). It has also been found, for example, on historical buildings of the former concentration camp in Auschwitz (Nowicka-Krawczyk *et al.* 2014). Caves are also a frequent habitat (Roldán & Hernández-Mariné 2009; Vinogradova & Mikhailyuk 2009; Vinogradova *et al.* 2009; Popović *et al.* 2019; van Vuuren *et al.* 2019). This resilient alga tends to be one of the first organisms to colonize fire-sterilized environments (Grondin & Johansen 1993; Mukhtar *et al.* 1994). In addition to these substrates, unspecified *Trebouxia* species have also been found on trees (Wylie & Schlichting 1973; Kharkongor & Ramanujam 2014; Štifterová & Neustupa 2017), in soil (Macentee 1970; Macentee *et al.* 1972; Carson & Brown 1976), on rocks (Mikhailyuk *et al.* 2018a) and on moss (Škaloud 2009).

The most frequently observed species is *T. arboricola*. It has been found in soil (Andreyeva 2004, 2005, 2009; Büdel *et al.* 2009; Dirborne & Ramanujam 2017; Andreyeva & Chaplygina 2007), on rocks (Gärtner & Stoyneva 2003; Škaloud 2009; Stoyneva & Gärtner 2009), tree bark (Rindi & Guiry 2003; Gupta 2008; Kharkongor & Ramanujam 2014), on dead wood (Smith & Stephenson 2010), in caves (Stoyneva & Gärtner 2009), on facades (Hofbauer & Gärtner 2021) and

on unusual substrates such as basidiocarps of wood-decaying fungi (*Fomes fomentarius*; Stoyneva *et al.* 2014), or a tombstone in a historic cemetery in Bratislava (Uher 2008). The presence of this species has been genetically confirmed on a waste container lid, however, the cells observed were not necessarily free-living (Hallmann *et al.* 2016). Non-cultivated samples containing free-living *T. arboricola* have been examined in several studies (Rindi & Guiry 2003; Gupta 2008; Stoyneva & Gärtner 2009; Smith & Stephenson 2010; Kharkongor & Ramanujam 2014; Stoyneva *et al.* 2014).

Trebouxia aggregata has been detected on granite (Rifón-Lastra & Noguerol-Seoane 2001; Mikhailyuk 2013), in soil (Flechtner *et al.* 2008), in oak leaf litter (Maltsev & Maltseva 2018), and on *Trametes versicolor* basidiocarps (Videv *et al.* 2017). Zavada & Simoes (2001) isolated an unspecified representative of the genus *Trebouxia* from basidiocarps of the same fungal species and suggested that the two organisms might have a lichen-like relationship. However, they did not present any convincing evidence for this.

The species *T. corticola* is morphologically confirmed from tundra soil in north-eastern Russia (Andreyeva & Chaplygina 2006). The genetically confirmed finding of a lineage closely related to *T. corticola*, comes from air samples in Hawaii

(Singh et al. 2018). Genetic studies have also confirmed the presence of another species, *T. impressa*, in the air (Genitsaris et al. 2011) and on a waste container lid (together with several other *Trebouxia* clones), where, however, a significant number of fungal hyphae was present in close proximity to the algae (Hallmann et al. 2016). The records of a species similar to *T. gigantea* comes from rock (Mikhailyuk 2013) and soil (Andreyeva 2005; Andreyeva & Chaplygina 2006). *Trebouxia incrustata* was found on granite (Mikhailyuk et al. 2003) and in soil (Flechtner et al. 2008). *Trebouxia decolorans* on façade (Vojtková 2017), *T. anticipata* on granite (Rifón-Lastra & Noguerol-Seoane 2001), *T. potteri* in moss (Škaloud 2009), *T. jamesii* on granite (Mikhailyuk 2013) and *T. cladoniae* in desert soil (Cameron 1960).

***Vulcanochloris* Vančurová, Peksa, Němcová & Škaloud.**

Vulcanochloris is morphologically very similar to its sister genus *Asterochloris* (Fig. 4), but it differs in the presence of spherical incisions within the pyrenoid (Vančurová et al. 2015). *Vulcanochloris* is also distinct by its ecological preferences, tolerating warmer and drier climatic conditions (Vančurová et al. 2018, 2021). The genus comprises three species (*V. canariensis*, *V. guanchorum* and *V. symbiotica*) isolated from the lichen *Stereocaulon vesuvianum* growing on igneous rocks. As this genus has only recently been described, not many publications describing its free-living status. Moreover, it is quite possible that free-living *Vulcanochloris* species were mistaken as species of *Trebouxia* and *Asterochloris*. Environmental sequencing has revealed the presence of this genus in the soil of the French Alps (Stewart et al. 2021).

WATANABEALES

***Chloroidium* Nadson.** All eleven currently accepted *Chloroidium* species (Guiry & Guiry 2022) are characterized by an ellipsoidal, ovoid, or spherical cell shape bounded by a relatively thin cell wall that becomes thicker with age, a parietal lobed or lobe-less chloroplast (Fig. 5), and reproduction by unequally sized autospores. Representatives of this genus differ from the other *Chlorella*-like species in the production of ribitol, a metabolite typically produced in response to osmotic stress (Dariencko et al. 2010). *Chloroidium* accommodates species, which were previously attributed to the various *Chlorella* species, including *C. saccharophila*, *C. ellipsoidea*, *C. angusto-ellipsoidea*, and *C. viscosum*, and later moved to the genus *Chloroidium* based on phylogenetic analyses (Dariencko et al. 2010; Dariencko et al. 2018). *Chloroidium* is a widely distributed genus that inhabits a variety of environments, including water (Dariencko et al. 2018). *Chloroidium ellipsoideum*, *C. saccharophilum*, *C. lichinum* and *C. viscosum* were described as lichen symbionts (Thüs et al. 2011; Vančurová 2012; Dariencko et al. 2018).

Chloroidium ellipsoideum (= *Chlorella ellipsoidea*) is probably the most common free-living photobiont species. It is often one of the most abundant species of aerophytic algae (Lukešová 2001; Fathi & Zaki 2003; Hoffmann & Dariencko 2005; Štifterová & Neustupa 2015) and forms macroscopic growths (Mikhailyuk 2008). The species was genetically

confirmed from waste container biofilm in Germany (Hallmann et al. 2016) and from the bark of pine and oak from two different sub-Mediterranean sites (Kulichová et al. 2014). Several more molecular findings of *C. ellipsoideum* on different substrates are published in Dariencko et al. (2010). *Chloroidium ellipsoideum* can adapt to freezing (Broady 1984; Elster et al. 1999) as well as heat and drought (Flechtner et al. 1998; Fathi & Zaki 2003). It is very common in substrates affected by anthropogenic activities (Neustupa & Škaloud 2005; Škaloud et al. 2008a) and, possibly due to its ability to produce osmotically active substances (Dariencko et al. 2010), it can tolerate hypersaline soils (Sommer et al. 2020). Sometimes it even dominates in similar extreme anthropogenic habitats (Lukešová & Hoffmann 1996; Lukešová 2001). However, this alga cannot withstand severe air pollution caused by dust particles of a diameter of 10 µm or smaller (PM₁₀), whereas it seems to be very resistant to elevated ozone concentrations (Freystein et al. 2008).

Chloroidium ellipsoideum cells can attach to a wide variety of substrates. Indeed, they are frequently found on building facades and walls (Schlichting 1975; Rifón-Lastra & Noguerol-Seoane 2001; Rindi & Guiry 2004; Barberousse et al. 2006; Wasserbauer et al. 2014; Hofbauer & Gärtner 2021), on various tree species (Czerwik & Mrozinska 2000; Johansen et al. 2007; Khaybullina et al. 2010; Štifterová & Neustupa 2017), on granite (Rifón-Lastra & Noguerol-Seoane 2001; Mikhailyuk et al. 2003; Mikhailyuk 2008), sandstone (Hoffmann & Dariencko 2005), and sand (Schulz et al. 2016; Mikhailyuk et al. 2018a). The presence in soil is also quite common (Durrell 1964; Zancan et al. 2006; Škaloud et al. 2008a; Stoyneva & Gärtner 2009; Bakieva et al. 2012; Glaser et al. 2018). This species has also been recorded in air samples (North & Davis 1988; Chu et al. 2013) and in caves (Vinogradova & Mikhailyuk 2009; Vinogradova et al. 2009).

Molecular sequences confirm the presence of *C. saccharophilum* (= *Chlorella saccharophila*) on various hard substrates (Dariencko et al. 2010) and in soil (Vishnivetskaya 2009). Similarly to the first-mentioned species, it is often isolated from soils (Zancan et al. 2006; Andreyeva 2009; Dirborne & Ramanujam 2017), including those heavily anthropogenically impacted (Lukešová & Komárek 1987; Lukešová 2001; Škaloud et al. 2008a), from tree bark (Freystein et al. 2008; Neustupa & Škaloud 2010; Štifterová & Neustupa 2017), and from the air (Parrando & Davis 1972; North & Davis 1988). It also cannot tolerate high concentrations of airborne dust particles (Freystein et al. 2008). Although *C. saccharophilum* occurs frequently in the subtropics and tropics (Neustupa & Škaloud 2010; Kharkongor & Ramanujam 2014; Dirborne & Ramanujam 2017), many records of its presence also come from Antarctica (Broady 1984; Mataloni et al. 2000; Cavacini 2001), the Arctic permafrost (Vishnivetskaya 2009) and other cold regions (Elster et al. 1999). However, no records of the organism have been found in deserts.

Genetically confirmed records of *C. lichinum* (= *C. lichenum*, *C. angusto-ellipsoideum*, *Chlorella angustellipsoidea*) come from the lid of a container (Hallmann et al. 2016) and a variety of other substrates (Dariencko et al. 2010). This species occurs in very small numbers in some localities

(Neustupa & Albrechtová 2003; Štifterová & Neustupa 2017), elsewhere (rock) it can form visible growths (Mikhailyuk *et al.* 2003). It grows epiphytically on tree bark (Neustupa & Škaloud 2010; Štifterová & Neustupa 2017) and on spruce needles (Neustupa & Albrechtová 2003). A different species, *C. viscosum* (= *Chlorella viscosa*), was isolated from a biofilm collected from the bark (Darienکو *et al.* 2018).

Jaagichlorella Darienko & Pröschold. *Jaagichlorella* possesses uninucleate solitary, usually spherical cells with one saucer-like (in young cells) or band-like (mature cells) chloroplast containing a single pyrenoid (Fig. 6; Darienko & Pröschold 2019). Recently, the tropical genus *Heveochlorella* was synonymized with *Jaagichlorella* (Darienکو & Pröschold 2019), but this taxonomic treatment was overlooked by some authors (Lindgren *et al.* 2020; Sanders & Masumoto 2021). The genus is an important member of algal communities in the tropical phyllosphere (Zhu *et al.* 2018) and represents the most common genus of photobionts of *Sticta* (Lindgren *et al.* 2020). According to Sanders *et al.* (2016), *Jaagichlorella* can be easily cultivated and probably exhibits both lichenized and free-living lifestyles. Further support for this view is direct observations conducted on plastic coverslips (Sanders 2014; Sanders & Masumoto 2021).

ELLIPTOCHLORIS-CLADE

Coccomyxa Schmidle. *Coccomyxa* cells are ellipsoid or nearly spherical, and sometimes dorsoventrally flattened (Fig. 7). They form microscopic and macroscopic colonies that are connected by mucilage. In older colonies, mucilage forms concentric envelopes around the periphery of cells. Reproduction is usually by 2–4 autospores (Ettl & Gärtner 2013). The genus currently includes 34 recognized species (Guiry & Guiry 2022) and while described as early as 1901 (Schmidle 1901), it is still taxonomically poorly understood (Muggia *et al.* 2011; Malavasi *et al.* 2016).

It is a relatively abundant and widely distributed genus that inhabits almost all environments except the sea (Darienکو *et al.* 2015). However, it can occur as a parasite of mussels in marine environments (Rodríguez *et al.* 2008). Some species survive even in extreme environments such as those highly polluted by heavy metals (Malavasi *et al.* 2016), acidic water (Fuentes *et al.* 2016), and high-ionizing radiation habitats (Rivasseau *et al.* 2013; Malavasi *et al.* 2020). Even macroscopic mass development on grassland has been described (Gärtner & Ernet 1993). It occurs in lichens (Asco- and Basidiomycota) and has also been found inside of the cells of *Ginkgo biloba* (Trémouillax-Guiller & Huss 2007) and in association with carnivorous plants of the genus *Drosera* (Sciuto *et al.* 2019). In the lichenized state, mycobiont haustoria do not penetrate cells, but are only attached to the cell surface (Muggia *et al.* 2011). Many species (*C. glaronensis*, *C. icmadophilae*, *C. mucigena*, *C. solorinae*, *C. ovalis*, *C. thallosa*, *C. arvernensis*, *C. dispar*, *C. viridis*, and *C. subellipsoidea*) are recognized as lichen photobionts (Ettl & Gärtner 2013; Malavasi *et al.* 2016). However, lichenized and strictly free-living species are closely related and it can be very difficult to distinguish them from each other (Malavasi *et al.* 2016).

A genetic study confirmed the presence of free-living algae closely related to *C. mucigena* and *C. glaronensis* on a polyethylene lid of a container in Göttingen, Germany (Hallmann *et al.* 2016). Another genetic study clearly shows that *C. subellipsoidea* and *C. arvernensis* clades include both free-living and lichenized strains. Interestingly, the *C. subellipsoidea* clade includes only free-living epiphytic strains and photobionts of terricolous lichens but does not occur in the soil (Malavasi *et al.* 2016). Additionally, *C. dispar* was detected during environmental sequencing of soil in the French Alps (Stewart *et al.* 2021). Among morphology-based studies, free-living cells of the species *C. cf. solorinae-saccatae* and *C. cf. solorinae-croceae* have been detected in soil samples from an oak forest in the Ardennes, Belgium (Hoffmann *et al.* 2007). These species, however, represent one monophyletic lineage described as *C. solorinae* (Malavasi *et al.* 2016) and here we treat them as synonyms for simplicity. Other records of *C. solorinae* come from soil samples collected at various sites in Russia (Getsen *et al.* 1994; Andreyeva 2009). *Coccomyxa cf. thallosa* was recorded on the soil surface in Czech Republic (Neustupa *et al.* 2002).

Elliptochloris Tschermak-Woess. *Elliptochloris* forms ellipsoid to globose, sometimes slightly irregular cells (Fig. 8). Chloroplasts can vary greatly in shape: parietal, striate, or hollow-spherical, bilobed, reticulate, or lobe-less, with or without a pyrenoid. Reproduction is by two types of autospores, either 2–4 large spherical (S-type) or 16–32 smaller elongated (E-type) cells. Both types of autospores often occur simultaneously (Darienکو *et al.* 2016). The genus currently includes eight accepted species (Guiry & Guiry 2022). Half of them (*E. bilobata*, *E. perforata*, *E. reniformis* and *E. subsphaerica*) forms lichens (Voytsekhovich *et al.* 2011; Darienko *et al.* 2016; Masumoto 2020).

Elliptochloris bilobata is a widely distributed free-living species. After numerous records of this alga on granite rock outcrops (Mikhailyuk *et al.* 2003; Mikhailyuk 2008, 2013), where it often dominates (Mikhailyuk *et al.* 2003; Mikhailyuk 2008) and even forms macroscopic growths (Mikhailyuk 2008). Mikhailyuk (2008) hypothesized a preference for rocky substrates in this genus. However, *E. bilobata* is much more commonly found in soils of various climates, especially in forests (Hoffmann *et al.* 2007; Temraleeva *et al.* 2015; Dirborne & Ramanujam 2017; Glaser *et al.* 2018). The species is also abundant in the soil of tundra (Andreyeva 2004, 2005; Andreyeva & Chaplygina 2007; Novakovskaya *et al.* 2012; Novakovskaya & Patova 2013; Patova & Novakovskaya 2018; Novakovskaya *et al.* 2020), whereas no occurrence in the desert has been recorded. Similarly to other photobiont species (see *Diplosphaera chodatii* below), *E. bilobata* shows a broad tolerance to air pollution and thus thrives in the highly polluted centre of Leipzig, Germany (Freystein *et al.* 2008). Furthermore, this alga is encountered in Antarctica (Garraza *et al.* 2011; Borchhardt *et al.* 2017) and has been identified in caves (Vinogradova *et al.* 2009).

Another symbiotic member of *Elliptochloris* is *E. perforata*, which occurs free-living on bark, epilithic on tombstone (Darienکو *et al.* 2016), and in soil (Hoffmann *et al.* 2007;

Samolov *et al.* 2020). A different species, *E. reniformis*, is also common in soil (Lukešová 2001; Neustupa & Škaloud 2005; Khaybullina *et al.* 2010; Temraleeva *et al.* 2015; Darienko *et al.* 2016; Novakovskaya *et al.* 2020). Additionally, the species was reported from rocks (Johansen *et al.* 2007) and from building facades (Hofbauer & Gärtner 2021).

In addition, *E. subsphaerica* is a very versatile species confirmed from lichen thalli (Voytsekhovich *et al.* 2011; Masumoto 2020) as well as from many different types of substrates. It is frequently reported from soil (Zancan *et al.* 2006; Hoffmann *et al.* 2007; Takeshita *et al.* 2010; Schulz *et al.* 2016; Samolov *et al.* 2020) even from soil in heavily anthropogenically affected areas (Lukešová 2001; Neustupa & Škaloud 2005) and city centres (Rindi & Guiry 2003; Freystein *et al.* 2008). Other substrates include tree bark (Freystein *et al.* 2008; Neustupa & Škaloud 2010; Masumoto 2020), building facades (Hofbauer 2007; Hofbauer & Gärtner 2021) and rocks (Rifón-Lastra & Noguerol-Seoane 2001; Johansen *et al.* 2007; Mikhailyuk 2013; Mikhailyuk *et al.* 2018a). Furthermore, *E. subsphaerica* represents the dominant species in some studies (Mikhailyuk *et al.* 2003; Mikhailyuk 2008; Novakovskaya *et al.* 2020). It was also observed in pine litter (Maltsev & Maltseva 2018).

PRASIOALES

Diplosphaera Bialosuknia. To date *D. chodatii* is the only taxonomically accepted species of this genus (Guiry & Guiry 2022). Its globose to ellipsoid cells occur singly or form two- or four-celled packages (Fig. 9) and may be surrounded by mucilage. The chloroplast is cup-shaped, parietal, with regularly arranged thylakoid membranes. Pyrenoids are usually absent. The morphologically similar species *Nannochloris normandinae*, once taught to be a photobiont of *Normandina pulchella* (Tschermak-Woess 1988), has recently been synonymized with *D. chodatii* (Pröschold & Darienko 2020). The genus *Diplosphaera* is very difficult to distinguish from *Stichococcus* based on morphological characters (they differ only in forming two-celled clusters) and the exact delimitation of these two and other related genera was only published quite recently (Pröschold & Darienko 2020). Representatives of this genus are the most common photobionts of lichens in the family Verrucariaceae (Thüs *et al.* 2011).

Diplosphaera chodatii is one of the most common species of photobionts which can be encountered in free-living state. Molecular data reveal its presence in sand and on rocks (Mikhailyuk *et al.* 2018a), on tree bark and freshwater habitats (Pröschold & Darienko 2020). Morphologically, it has been reported from a variety of environments and substrates. Indeed, it can be found both in deserts (Vinogradova *et al.* 2004; Büdel *et al.* 2009), representing the only *Stichococcus*-related species that occurs in this habitat (Hodač *et al.* 2016), and in environments that are mostly covered by snow and ice (Elster *et al.* 1999; Borchhardt *et al.* 2017; Ilchibaeva *et al.* 2018). It also survives in soils significantly altered by anthropogenic activities (Lukešová & Hoffmann 1996; Lukešová 2001; Neustupa & Škaloud 2005; Škaloud *et al.* 2008a) and sometimes even belongs to the most abundant species in such places (Sommer *et al.* 2020). It does not even mind the air of big cities (Freystein *et al.* 2008) and inhabits facades

(Hofbauer 2007; Hofbauer & Gärtner 2021) as well as sand dunes (Schulz *et al.* 2016). It grows on a variety of substrates: soil (Zimonina 1998; Neustupa 2001; Škaloud *et al.* 2008b), tree bark (Johansen *et al.* 2007; Štifterová & Neustupa 2017), moss (Škaloud 2009) and rocks and caves (Vinogradova & Mikhailyuk 2009; Vinogradova *et al.* 2009). In some localities, it is a very abundant species (Elster *et al.* 1999; Mikhailyuk *et al.* 2003; Samolov *et al.* 2020).

Prasiola Meneghini. *Prasiola* is a cosmopolitan genus, which can be found in a wide range of habitats (Broady 1989; Heesch *et al.* 2012; Rindi *et al.* 2007). Unlike its relatives, *Prasiola* forms macroscopic, usually monostromatic thalli of various shapes (ribbon-shaped/sheet-like blades; Fig. 10) and sizes. The cells, arranged in groups of four, contain axial, asteroid chloroplast with one centrally-located pyrenoid. *Prasiola* reproduces asexually by thallus fragmentation or sexually oogamously (Ettl & Gärtner 2013).

Two of the 34 described species (Guiry & Guiry 2022), *P. borealis* and *P. delicata*, enter lichen-like symbiosis with the bipolarly distributed fungus *Mastodia tessellata* (Verrucariaceae; Garrido-Benavent *et al.* 2018). This unusual association, sometimes referred to as 'borderline-lichen', gives rise to a thallus, which is formed mainly by the algal partner, whose outer appearance is not changed, but its inner structure is significantly altered (Kováčik & Pereira 2001).

Free-living *P. borealis* and *P. delicata* specimens have been collected from coastal rock in Alaska (Garrido-Benavent *et al.* 2017). In addition, free-living *P. delicata* was observed in the intertidal of Hokkaido, Japan (Sutherland *et al.* 2016) and in Kamchatka, Russia (Klochkova *et al.* 2017).

Pseudochlorella J. W. G. Lund. *Pseudochlorella*, originally described as *Chlorellopsis* by Zeidler (1954), is characterized by a coccoid type of thallus with ellipsoid to globular cells surrounded by a thin and smooth cell wall (Fig. 11). The cells may form groups and can be enclosed in mucilage. They contain a grooved or plate-like chloroplast with a spherical pyrenoid (Ettl & Gärtner 2013).

Pseudochlorella is very difficult to distinguish from *Elliptochloris* since they share many morphological features, including the formation of S-type and E-type autospores. However, the two genera belong to distantly related clades, the Prasiolales and the *Elliptochloris*-clade (Darienکو *et al.* 2016). Some *Pseudochlorella* species are found in extreme environments. For example, a strain closely related to *P. pringsheimii* has been isolated from an extremely acidic environment of a mine in Japan (Hirooka *et al.* 2014). There are three accepted species names within the genus (Guiry & Guiry 2022). Two of them (the type species *P. pyrenoidosa* and *P. signiensis*) were isolated from *Lecidea* and *Trapelia* lichens, respectively (Zeidler 1954; Darienko *et al.* 2016).

Pseudochlorella pyrenoidosa has been found, for example, in soil in the Italian Dolomites (Ettl & Gärtner 2013) or in an abandoned field in north-eastern Italy (Zancan *et al.* 2006). The species *P. signiensis* occurs free-living in soil and on artificial hard substrates (Darienکو *et al.* 2016).

Stichococcus Nägeli. *Stichococcus* is a coccoid alga forming clusters of two cells, or short chains, which readily break into single cylindrical cells with a thin cell wall that may be curved and rounded at the edges (Fig. 12). Chloroplasts are parietal, sometimes with a naked pyrenoid. *Stichococcus* reproduces by filament disintegration or cell division (Ettl & Gärtner 2013).

The genus was recognized to be polyphyletic (Handa *et al.* 2003). However, representatives of this genus have recently been reassigned to several newly described monophyletic genera: *Protostichococcus*, *Deuterostichococcus*, *Tritostichococcus* and *Tetrastichococcus* (Pröschold & Darienko 2020). Since these genera are morphologically not recognizable from each other and from the genus *Stichococcus* s. str., we are treating them here together.

These algae inhabit both freshwater and terrestrial environments (Ettl & Gärtner 2013) and travel very long distances through the air, yet certain patterns can be traced in their distribution (Hodač *et al.* 2016). Moreover, some of them have the potential for biodiesel (Olivieri *et al.* 2011), and have been observed many times as photobionts of lichens (Tscherma-Woess 1988; Thüs *et al.* 2011). *Stichococcus mirabilis* is, based on morphology, a likely photobiont of the lichen *Staurothele* (Thüs *et al.* 2011). *Deuterostichococcus allas* is a photobiont of the lichen genus *Placopsis* (Beck *et al.* 2019), while *Tritostichococcus coniocybes* associates with *Chaenotheca*, *Chaenothecopsis* and *Coniocybe* (Pröschold & Darienko 2020). A genetically confirmed record of *S. mirabilis* comes from a castle wall in Thuringia, Germany (Hallmann *et al.* 2013). Its occurrence has also been confirmed in Antarctica soil (Cavacini 2001), in a granite canyon of the Teteriv River, Ukraine (Mikhailyuk 2008) and in air samples from northern Florida (Parrando & Davis 1972; North & Davis 1988). *Tritostichococcus coniocybes* occurs free-living in soil and on rocks (Pröschold & Darienko 2020).

CHLORELLALES

Auxenochlorella (I. Shihira & R. W. Krauss) T. Kalina & M. Punčochářová. The genus *Auxenochlorella* is a unicellular coccoid alga of minute cell size (Fig. 13), differentiated from other *Chlorella*-like coccoid algae by molecular genetic data (Huss *et al.* 1999). The cells are ellipsoidal to globose, bounded by a double-layered cell wall without mucilage cover. Chloroplasts are parietal without pyrenoids. The cells reproduce by autospores (Kalina & Punčochářová 1987). The type species *A. protothecoides* was previously considered closely related to the non-photosynthetic genus *Prototheca* and placed within the 'APH lineage' (Ueno *et al.* 2005). Thüs *et al.* (2011) places this genus in the Chlorellales.

An unspecified species-level lineage *Auxenochlorella* sp. related to free-living *A. protothecoides* was isolated from the lichen *Psoroglaena stigonemoides* (Nyati *et al.* 2007; Thüs *et al.* 2011) but has not yet been recorded free-living.

Chlorella-like algae. Members of the genus *Chlorella* are sometimes considered as photobionts of lichens (Tscherma-Woess 1988; Muggia *et al.* 2013). Nearly 100 different 'green balls' were previously included in this highly paraphyletic genus (Guiry & Guiry 2022). However, most of them were

later reassigned to other and often unrelated genera (apart from the aforementioned genera *Chloroidium* and *Auxenochlorella*, for example, the genera *Watanabea*, *Mychonastes*, *Muriella* and *Scenedesmus*; Huss *et al.* 1999). These shifts highlight how difficult it is to identify the genus *Chlorella* and similar genera on the basis of morphological characters alone. Therefore, the genus *Chlorella* was not considered when searching for articles focused on the diversity of free-living lichen symbionts.

CLASS TREBOUXIOPHYCEAE, INCERTAE SEDIS

Apatococcus F. Brand. *Apatococcus* typically has spherical cells, which can sometimes be angularly flattened (Fig. 14). During division, irregular packages or multi-layered clusters of cells are formed. The chloroplast is parietal, without a pyrenoid, and in older cells it partially detaches from the cell wall. It reproduces by aplanospores or zoospores (Ettl & Gärtner 2013). According to Li *et al.* (2021), *Apatococcus* is closely related to the order Watanabeales and to the genus *Symbiochloris*.

This genus consists of five taxonomically recognized species (Guiry & Guiry 2022). *Apatococcus lobatus* is the most common and widespread aerophytic algal species. Watanabe *et al.* (1997) obtained isolates morphologically similar to this species from several marine lichen taxa. However, Beck (2002), questions this identification and considers the isolated algae as contaminants. To date, the occurrence of *A. lobatus* in lichens has not received any support in the form of molecular data (Zahradníková *et al.* 2017). In contrast, a newly described species *A. fuscideae*, was shown to associate with lichens of the genus *Fuscidea* (Zahradníková *et al.* 2017). Probably due to its recent discovery, this species has not been recorded in free-living state.

Leptosira A. Borzi. The thallus of the photobiont genus *Leptosira* is composed of cushion-shaped clusters of filaments with short lateral branches (Fig. 15). A parietal chloroplast with or without a pyrenoid (Bakker *et al.* 1997) is located inside spherical cells with a single nucleus. *Leptosira* reproduces asexually by zoospores and aplanospores. However, in the lichenized state, the appearance of the thallus changes dramatically – as a photobiont it forms ellipsoidal or spherical single cells (Tscherma-Woess 1988).

This genus closely resembles the genus *Pleurastrum* (Guiry & Guiry 2022). In the past, several species of *Pleurastrum* were reassigned to *Leptosira* based on examination of morphology and later molecular data (Friedl 1996). Li *et al.* (2021) places this genus close to *Xylochloris* and *Dictyochloropsis*. However, this placement remains uncertain (Sanders & Masumoto 2021).

The genus currently includes seven taxonomically accepted species (Guiry & Guiry 2022), two of which, *L. obovata* and *L. thrombii*, enter lichen symbiotic associations (Tscherma-Woess 1988; Roldán *et al.* 2004). A record of *L. obovata* comes from a cavity in limestone in the Garraf Mountains (Spain), where the species occurred both free-living and as a photobiont of the lichen *Macentina stigonemoides* (Roldán *et al.* 2004).

***Symbiochloris* Škaloud, Friedl, A. Beck & Dal Grande.**

Symbiochloris is characterized by spherical, ellipsoidal, or irregular cell shape with a smooth and thin cell wall (Fig. 16). Young cells have a single parietal chloroplast with lobes. In mature cells the chloroplast is reticulate, the lobes are never arranged in parallel (unlike in the genus *Dictyochloropsis*) and a pyrenoid is absent. Asexual reproduction occurs either by zoospores or by two types of immobile cells, aplanospores and autospores (Škaloud *et al.* 2016). The genus was established to differentiate between two morphologically similar lineages, both including species described under the genus *Dictyochloropsis* (Škaloud *et al.* 2007; Dal Grande *et al.* 2014) and is closely related to the *Watanabeales* (Škaloud *et al.* 2016; Li *et al.* 2021).

Currently, the genus *Symbiochloris* has ten accepted species (Guiry & Guiry 2022) most of which (except of *S. irregularis* and *S. tropica* that are only known as free-living species; Škaloud *et al.* 2016) enter lichen symbiotic associations with a plethora of fungal families (e. g. Peršoh *et al.* 2004; Škaloud *et al.* 2016). Recent sequencing of leaves in tropical forest revealed many *Symbiochloris* sequences (Zhu *et al.* 2018).

In the literature dealing with the diversity of free-living algae, *S. reticulata* was the most frequently observed species. Its occurrence has been recorded on tree bark (beech, ash, maple, alder; Štifterová & Neustupa 2015, 2017), in soil and in moss (Škaloud 2009). Other records of this species come from granite rocks in the Teteriv River Valley, Ukraine, where it formed macroscopic growths (Mikhailyuk 2008) and from the Great Smoky Mountains National Park, USA (Johansen *et al.* 2007; Khaybullina *et al.* 2010).

Another species, *S. symbiontica*, was discovered during environmental sequencing of soil in the Alps (Stewart *et al.* 2021). Morphological confirmations of the same species come from wood and bark of trees of a lowland tropical forest nature reserve, Singapore (Neustupa & Škaloud 2010), granite rocks in the Pivdennyi Bug River valley, Ukraine (Mikhailyuk *et al.* 2003) and soil (Andreyeva 2004, 2005, 2009; Andreyeva & Chaplygina 2006, 2007). Moreover, other species of the genus *Symbiochloris* (*S. ellipsoidea*, *S. gelatinosa*, *S. pauciautosporea*) have been observed in soil of north-eastern Russia (Andreyeva 2005, 2009; Andreyeva & Chaplygina 2006). Additionally, *S. handae* was observed in soil mountain environments of the Alps (Stewart *et al.* 2021).

***Uvulifera* Molinari.** The genus *Uvulifera* (Fig. 17), originally described as *Coccobotrys* by Chodat (1913), possesses a morphology very similar to that of *Apatococcus*. The thallus consists of globular to slightly elongated cells, often forming sarcinoid three-dimensional colonies, and, occasionally short, branched, easily disintegrable filaments. The chloroplast is simple, parietal without a pyrenoid. Reproduction occurs by zoospores or aplanospores (Vischer 1960). The two currently accepted species (*U. mucosa*, *U. verrucariae*; Guiry & Guiry 2022) are closely related to *Xerochlorella* (Mikhailyuk *et al.* 2020). The type species *U. verrucariae* was identified as a photobiont of *Verrucaria nigrescens* based on light microscopy observations (Chodat 1913). Free-living and lichenized cells were observed in limestone cavities (Roldán *et al.* 2004). Moreover, it occurs free-living on buildings (Barberousse

et al. 2006) and in soil (Flechtner *et al.* 2008; Stewart *et al.* 2021).

Class Ulvophyceae

The Ulvophyceae is a species-rich class that exhibits exceptional morphological and cytological diversity. The architecture of the thalli ranges from microscopic flagellated or non-flagellated unicells to large and complex multicellular thalli (filamentous, blade-like or tubular) or siphonous thalli formed by a giant single cell (Leliaert *et al.* 2012). The monophyly of the Ulvophyceae is still uncertain as well as the relationships among the families within the most important orders which include Trentepohliales, Cladophorales, Bryopsidales, Dasycladales, Ulvales and Ulotrichales (Leliaert *et al.* 2012; Škaloud *et al.* 2018, Del Cortona *et al.* 2020; Gulbrandsen *et al.* 2021). Many ulvophycean algae (members of Trentepohliales, Ulvales and Ulotrichales) enter lichen symbiotic associations.

TRENTEPOHLIALES

***Cephaleuros* Kunze ex E. M. Fries.** *Cephaleuros* forms heterotrichous to pseudoparenchymatous orange or red macroscopic discs under the cuticle or epidermal cells of leaves, twigs and fruits of tropical and subtropical woody plants. The filament forming cells are cylindrical to irregular in shape and contain discoid to irregular chloroplasts (Fig. 19). Terminal cells of the filaments often produce gametangia. Sporangiphores and setae develop from terminal cells and emerge through the tissue of the host plant (Suto & Ohtani 2009). Currently, there are 19 taxonomically accepted species and one variety (Guiry & Guiry 2022). However, most *Cephaleuros* species appear to be paraphyletic (Zhu *et al.* 2017). This genus has a negative impact on agriculture as this semi-parasitic alga causes necrosis on leaves (Brooks *et al.* 2015).

Unidentified *Cephaleuros* species have been isolated from a foliicolous lichen *Strigula* (Jiang *et al.* 2020). Detailed observations of the *Strigula* life cycle suggest that lichenized *Cephaleuros* species are also able to live independently, free of the mycobiont. Moreover, lichenization can suppress their reproduction. The mycobiont, on the other hand, will produce pycnidia and perithecia only after the successful colonization of the algal partner (Ward 1884). A genetic study by Zhu *et al.* (2017) proves that many closely-related *Cephaleuros* species exhibit both free-living and lichenized life-styles. In one case, the authors obtained identical strains from two different localities (southern China) and sources: from unidentified foliicolous lichen and from a leaf surface (Zhu *et al.* 2017).

***Phycopeltis* Millardet.** Members of this epiphytic genus typically develop orange circular or irregular discs on the upper leaf surface of vascular plants (Fig. 20). The discs, often varying in shape, are formed by a single layer of laterally appressed dichotomous filaments. Chloroplasts are parietal and lack pyrenoids (Ettl & Gärtner 2013; Škaloud *et al.* 2018). This genus' cell wall contains sporopollenin, a chemically inert biological polymer, and it appears to play a role in plant defences against desiccation and fungal parasites (Good &

Chapman 1978). The life cycle is isomorphic (Ettl & Gärtner 2013; Škaloud *et al.* 2018).

Phycopeltis is non-monophyletic (Zhu *et al.* 2017) and currently includes 25 accepted species (Guiry & Guiry 2022). Its phylogenetic position remains poorly understood (Sanders & Masumoto 2021). Although the genus is widely distributed in tropical forests (Zhu *et al.* 2018), certain records indicate that it also occurs in oceanic Europe (Rindi *et al.* 2004). *Phycopeltis* sp. was isolated from the lichen genus *Tenuitholiascus* (Jiang *et al.* 2020).

Trentepohlia C. Martius. *Trentepohlia* is a widespread genus well-known for its ability to form conspicuous yellowish to red, orange or brown macroscopic growths on bark, rocks, or leaves (especially in tropical habitats). The thallus is composed of uniseriate branched heterotrichous filaments (Fig. 21). The cells are cylindrical to rounded, surrounded usually by a thick cell wall and contain usually many discoidal plastids without pyrenoids. Reproduction occurs by isogametes or zoospores (Ettl & Gärtner 2013). Free-living cells produce a large amount of carotenoid pigments as a protective barrier against UV-light irradiation, unlike lichenized cells that differ not only by the quantities of pigments but also by altered morphology (Honegger 1998).

The genus, which currently harbours 54 species (Guiry & Guiry 2022), is polyphyletic and intermixed with *Printzina* (Rindi *et al.* 2009; Nelsen *et al.* 2011; Zhu *et al.* 2017) and *Phycopeltis* (Zhu *et al.* 2017). *Trentepohlia* includes many lichen photobiont species-level lineages (Nelsen *et al.* 2011; Kosecka *et al.* 2020), among which *T. lagenifera* is so far the only described species (Hametner *et al.* 2014). However, this lineage is a polyphyletic complex of cryptic species (Rindi *et al.* 2009) and, for this reason, it was not considered when searching for articles focused on the diversity of free-living lichen symbionts. Lichenized *Trentepohlia* species do not form a single clade and many closely-related or identical isolates were obtained both from lichen thalli and free-living populations (Nelsen *et al.* 2011; Hametner *et al.* 2014; Zhu *et al.* 2017; Kosecka *et al.* 2020). A free-living strain identical to the photobiont of *Gyalecta jenensis* was shown in Hametner *et al.* (2014). Moreover, Zhu *et al.* (2017) report a free-living *Trentepohlia* strain (SAG 118.80) nested within several identical photobiont strains from China.

ULVALES

Blidingia Kylin. The macroscopic genus *Blidingia* forms cylindrical or compressed, sometimes branched, hollow tubular thalli composed of generally very small cells with lobed chloroplasts (Fig. 22). These tubular structures are attached to the surface by discoidal cushion, which represents the first developmental stage in the asexual cycle and develops after zoospore germination. Sexual reproduction occurs by isogametes. The genus is predominantly marine with two species able to penetrate into freshwater habitats (Škaloud *et al.* 2018) and currently includes seven species (Guiry & Guiry 2022). One of these species (*B. minima*) is involved in an unusual 'borderline' type of association, in which most of the lichen-like organism is formed by the algal partner and the fungus being *Turgidoscolum ulvae*. Thalli of *B. minima*, if inhabited

by *T. ulvae*, appears bigger and much darker. Thus, free-living individuals can be easily recognized (Pérez-Ortega *et al.* 2018).

Blidingia minima is a resilient euryhaline species (Thom 1984). It readily grows in saltmarshes, estuaries as well as intertidal pools (Polderman 1975; Munda 1978; Araújo *et al.* 2006) and was molecularly confirmed from brackish waters of the Baltic Sea (Steinhagen *et al.* 2021). The thalli can be attached to hard surfaces, such as stones, rocks, boulders and wood, or rarely free-floating in sheltered water (Škaloud *et al.* 2018). It is among the first colonizers of artificial substrates (Hruby & Norton 1979). Another genetic study confirms the presence of *B. minima* on the Pacific Coast (Hayden & Waaland 2002). The same strains were used as reference for *T. ulvae* colonized algae in Pérez-Ortega *et al.* (2018). Gallardo *et al.* (1999) report *B. minima* from South Shetlands Islands, Antarctica.

Halofilum Darienko & Pröschold. *Halofilum* is a filamentous genus of mostly coastal marine algae that accommodates species previously assigned to *Dilabifilum*. The short-branched, dense and easily disintegrable filaments consist of vegetative cells containing parietal chloroplasts with pyrenoids (Fig. 23) and reproduce by budding or cell division (Darienکو & Pröschold 2017). The genus currently harbours three species (Guiry & Guiry 2022) including *H. ramosum*, which was isolated from *Wahlenbergiella* and *Hydropunctaria* lichens (Gasulla *et al.* 2019) as well as from stone cracks of coastal rocks in Porth Trecastell, United Kingdom and walls of archaeological remains in Carthage, Tunisia (Darienکو & Hoffmann 2010; Darienko & Pröschold 2017).

Lithotrichon Darienko & Pröschold. Members of this genus, previously accommodated by *Dilabifilum*, form cushion-like thalli composed of heterotrichous filaments when mature (Fig. 24). The cushion-like appearance is caused by the sarcinoid form of the prostrate system. The erect system, formed by short filaments, branches from the edges of the prostrate system. The cells contain parietal chloroplasts with pyrenoids and reproduce by division and zoospore production. These morphological features are, however, not sufficient for successful determination of this genus (Darienکو & Pröschold 2017).

The genus, with two accepted species (Guiry & Guiry 2022), was long believed to be strictly freshwater (Darienکو & Pröschold 2017; Liu *et al.* 2019). *Lithotrichon pulchrum*, for example, inhabits the thallus of freshwater lichens *Verrucaria rheitrophila* (= *Hydropunctaria rheitrophila*; Darienko & Pröschold 2017). Recently, the same algal species was isolated from a marine water sample collected in the Samcheok Harbor, Republic of Korea (Kwon *et al.* 2022).

Paulbroadya Darienko & Pröschold. The thallus of *Paulbroadya* consists of prostrate system, formed from rounded cells or cell-packages and erect system, formed by dense unilateral or bilateral branches (Fig. 25). The morphology is very similar to other taxa previously treated under *Dilabifilum*. These genera can be distinguished only by means of genetic markers (Darienکو & Pröschold 2017).

One of the two described *Paulbroadya* species (Guiry & Guiry 2022), *P. petersii*, was isolated from *Hydropunctaria* and *Verrucaria* species (Thüs et al. 2011; Darienko & Pröschold 2017) but was not, to date, recorded in free-living state.

***Pseudendoclonium* Wille.** *Pseudendoclonium* possesses the typical *Dilabifilum*-like packet-forming to filamentous morphology with cells differentiating into prostrate and erect systems (Fig. 26), which makes accurate determination impossible without molecular methods. In addition, these genera often exhibit considerable phenotypic plasticity (Darienko & Pröschold 2017). The genus with 10 accepted species (Guiry & Guiry 2022) includes free-living (mostly marine, sometimes freshwater, terrestrial or aerophytic; Škaloud et al. 2018) and lichen-symbiotic members. Crustose Verrucariaceae lichens, belonging to the genera *Hydropunctaria*, *Verrucaria* and *Wahlenbergiella* are often associated with *P. commune*, *P. arthropyreniae*, *P. submarinum* and *P. incrustans* (Darienko & Pröschold 2017; Černajová et al. 2022).

Free-living *P. commune* was isolated from coastal rocks, Snake Island, Ukraine and from a concrete block in the tidal zone on Oakland beach, RI, USA (Darienko & Pröschold 2017). A free-living strain closely related to *P. arthropyreniae* was cultivated from material collected from stone surfaces of the Borobudur Temple, Indonesia (Purbani et al. 2020). Environmental sequences of *P. submarinum*, *P. commune* and *P. arthropyreniae* were recovered from the littoral rocks of the North Sea and the Baltic Sea (Schmidtová 2022).

***Rindifilum* Malavasi, Klimešová, Lukešová & Škaloud.** This recently described genus differs from the previously mentioned *Dilabifilum*-like genera by the formation of ovoid to pyriform cells (the prostrate system), later developing into characteristic “hammer-shaped” cells (Fig. 27). It is only the combination of these features, that makes this ulvophycean genus morphologically distinct. *Rindifilum* reproduces by the production of two or four autospores (Malavasi et al. 2022). The type species, *R. verrucariae* (Malavasi & Škaloud 2022) was isolated from the freshwater lichens *Verrucaria scabra* and *Verrucaria margacea* (Malavasi et al. 2022) and to date, has not been observed in free-living state.

***Undulifilum* Škaloud, Černajová & Schiefelbein.** *Undulifilum* forms heterotrichous, brush-like thalli composed of irregularly branched uniseriate filaments, that are typically regularly undulating or curved (Fig. 28). The filaments are built out of long cylindrical cells with a single, parietal chloroplast possessing a pyrenoid. In mature thalli, the terminal cells of the branches are often significantly longer. Reproduction occurs by vegetative division. Other modes of reproduction have not been observed. The only described species *U. symbioticum* lives as a photobiont of Verrucariaceae lichens on seashore rocks (Černajová et al. 2022).

ULOTRICHALES

***Urospora* Areschoug.** This marine alga *Urospora* forms thalli consisting of slender, unbranched, uniseriate filaments

composed of multinucleate cells (Fig. 29), that attach to hard substrates with a multicellular rhizoidal holdfast. The life cycle involves a codium-phase that develops from a zygote. *Urospora* reproduces asexually by four-flagellated zoospores and sexually by biflagellate gametes (Hanic 2005). Interestingly, this macroscopic genus was recently recorded as a Verrucariaceae photobiont (Černajová et al. 2022; Schmidtová 2022). Schmidtová (2022) obtained environmental sequences, identical to *Urospora* sequences from *Verrucaria* lichens, and from littoral rocks in the North Sea and the Baltic Sea.

Class Chlorophyceae

The class Chlorophyceae, one of the three crown branches of green algae, includes morphologically and ecologically diverse organisms that occur mainly in freshwaters. The thallus morphology ranges from unicellular algae (with or without flagella) or simple colonies to branched or unbranched filaments, or even blade-like thalli (Leliaert et al. 2012). The Chlorophyceae are monophyletic and, together with the Ulvophyceae, sister to Trebouxiophyceae (Del Cortona et al. 2020). The class includes six main order-level lineages: Chlamydomonadales, Sphaeropleales, unnamed clade containing *Jenufa*, *Treubaria* and *Golenkinia*, and so-called ‘OCC-lineage’, consisting of Chaetophorales, Chaetopeltidales and Oedogoniales (Lemieux et al. 2015).

Sanders & Masumoto (2021) consider a total of six chlorophycean genera as lichen photobionts, even though they warn that most of them are in need for further revision. In our opinion, most of these genera lack the necessary evidence to be classified as lichen symbiotic algae. For example, Skuja (1943) mentioned a symbiotic relationship between the ascomycete *Pyronema laetissimum* and *Chlamydomonas augustae* (*Chloromonas augustae*; Pröschold et al. 2001). This association, which has been, to our knowledge, only reported once, however, lacks many important characteristics of a typical lichen (Sanders & Masumoto 2021) and the fungus was probably misidentified (Moore & Korf 1963).

Trochiscia was reported as a Verrucariaceae photobiont by Tschermak (1941) and Ahmadjian (1967). Its phylogenetic placement remains uncertain (del Campo et al. 2010; Fučíková et al. 2019) and the identification of this alga is doubtful (Ettl & Gärtner 2013). Genera such as *Chlorsarcinopsis*, *Gloeocystis* and *Radiococcus* are highly polyphyletic, share many morphological features with unrelated genera and can be easily confused for them (Neustupa 2015; Zhang et al. 2018). Yet, all existing records of these algae from lichen thalli are based solely on morphological data (Plessl 1963; Tschermak-Woess 1988; Voytsekhovich et al. 2011). For instance, algae reported as *Chlorsarcinopsis*, symbionts of *Lecidea plana* and *L. lapicida*, could easily be confused for *Trebouxia*, which is known to associate with *Lecidea* species, including *L. lapicida* (Beck 1999; Ruprecht et al. 2012).

SPHAEROPLEALES

***Bracteacoccus* Tereg.** This coccoid aero-terrestrial genus can be characterized by multinucleate, round cells that are solitary or form irregular colonies, multiple distinct plate-like plastids

without pyrenoids and reproducing by zoospores or aplanospores (Fig. 18; Ettl & Gärtner 2013). Fučíková *et al.* (2014) place this genus with its 16 accepted species (Guiry & Guiry 2022) in the Sphaeropleales. Its ability to form lichens (genus *Sulzbacheromyces*) was supported by morphological as well as sequence data (Takeshita *et al.* 2010; Hodgkinson *et al.* 2014; Masumoto 2020). According to Masumoto (2020), *Sulzbacheromyces* isolates of *Bracteacoccus* sp. formed two separate clades that were distinct from free-living taxa.

DIVISION STREPTOPHYTA

The Streptophyta, the second branch of the Viridiplantae, is an immensely important and extraordinarily diversified group of organisms that contains all land plants (Embryophyta) as well as some important groups of algae (Charophyta). The Streptophyta can be divided into four main classes: Zygnematophyceae, Coleochaetophyceae, Charophyceae and Klebsormidiophyceae. Only the latter class harbours one photobiont species. The small classes Chlorokybophyceae, Mesostigmatophyceae and *Spirotaenia* appear to be sister to all streptophytes (Irisarri *et al.* 2021).

Class Klebsormidiophyceae

The class can be characterized by the type of cell division, that occurs by furrowing and the presence of centrosomes with astral microtubules and centrioles, which act as mitotic spindle organizer (Pickett-Heaps 1975). The members of this class form either sarcinoid or unbranched filamentous thalli and inhabit terrestrial and freshwater habitats (Škaloud & Rindi 2013; Mikhailyuk *et al.* 2018b).

Interfilum R. Chodat. *Interfilum* cells are solitary or attached to each other with mucilage forming short filaments. The individual cells are round to ellipsoid, surrounded by a two-layered cell wall, and possess lobed, parietal chloroplasts with pyrenoids (Fig. 30). During division, the daughter cells form within the mother cell wall (Ettl & Gärtner 2013). According to Rindi *et al.* (2011), *Interfilum* is nested within *Klebsormidium*. This aerophytic genus currently harbours three accepted species (Guiry & Guiry 2022). *Interfilum* is the only known lichen-forming lineage of the Streptophyta. *Interfilum massjukiae* and *Interfilum* sp. were reported from *Placynthiella* spp. and *Micarea prasina* as secondary photobionts based on morphological observations. The same algal species were also found to grow epiphytically on the surface of lichen thalli (Voytsekhovich *et al.* 2011). Free-living *I. massjukiae* was observed on the surface of pyroclastic outcrops in Crimea, Ukraine (Mikhailyuk *et al.* 2008).

DISCUSSION

More than 30 green algal genera have been recognized as lichen photobionts. We were able to find evidence, that at least 24 of them were observed in the free-living state. Most of the lichen photobionts are in the class Trebouxiophyceae. This group of photobionts is represented by about 17 known genera (see overview phylogeny in Fig. 31). One of

them has not yet been reported free-living. However, despite much progress, the diversity of this class is still poorly understood. The situation is complicated by the fact that representatives of the class Trebouxiophyceae have limited number of morphological features and show high phenotypic plasticity. Many genera are thus poorly defined and do not form monophyletic groups. Moreover, the majority of the available articles mentioning photobionts of lichens were based only on morphological observations.

Some green algal genera contain both free-living and facultatively lichenised species displaying very similar morphological features. Thus, facultatively lichenized species (occurring in nature in a free-living state) may be confused with strictly free-living relatives (and *vice versa*). For instance, the ubiquitous *Apatococcus lobatus* is morphologically almost indistinguishable from the lichen-forming species *A. fuscideae* (Zahradníková *et al.* 2017). This kind of confusion, however, should not occur in genera that contain facultatively symbiotic representatives only and are morphologically relatively well-defined (such as *Trebouxia* and *Asterochloris*), but even so, all records identified solely based on morphological criteria should be accepted cautiously. Identifying these algae at the species level, but often also at the genus level, is problematic.

Most of the papers on free-living algal diversity included a very small proportion of lichen photobionts (usually only 2–3 species per article) and these algae were usually rare and only rarely dominate. Exceptions were the species *Diplosphaera chodatii*, *Elliptochloris bilobata* and *Chloroidium ellipsoideum*, which were locally very abundant. This may be due to the low competitiveness of photobionts, associated with their relatively slower growth (Elshobary *et al.* 2015), or an inappropriate choice of media that may not suit the needs of photobionts (Ahmadjian 2001), since most of the studies analysed in this review were culture-based.

Despite the claims of some authors, *Trebouxia* and all other lichen-associated algae of the class Trebouxiophyceae seem to be able to live freely, without any connection to the hyphae of fungi. There are many records confirming this (e. g. Bubrick *et al.* 1984; Sanders & Lücking 2002; Hoffmann *et al.* 2007; Roldán & Hernández-Mariné 2009). It is important to note, that the most common photobionts of lichens, *Trebouxia* and *Asterochloris*, have also been shown to be the most commonly found free-living (Tschermak-Woess 1978; Rindi & Guiry 2003; Barberousse *et al.* 2006; Škaloud 2009; Suutari *et al.* 2010; Štifterová & Neustupa 2017; Novakovskaya *et al.* 2020). Lichenized species of the genus *Chloroidium* ranked second, although they are rather rare in lichens. Relatively common photobionts belonging to the widely distributed genus *Coccomyxa*, on the other hand, were rarely mentioned in previous works on free-living algae. The rarest species, with only one record of free occurrence, is *Leptosira obovata*. Moreover, newly described genera and species (*Vulcanochloris*, *Apatococcus fuscideae*) have not yet made it into free-living algal diversity articles.

Free-living photobionts of lichens are able to grow in a wide variety of environments and substrates. Most of the records, however, come from soil and biological soil crusts, probably because these environments have been intensively

studied. Most photobionts do not seem to have a clear preference for substrate type or climatic conditions but rather develop successfully in ecosystems where a cold winter limits survival (Míguez *et al.* 2017). In one case study, Míguez *et al.* (2017) have demonstrated that free-living algae exhibit a similar pattern of responses as their lichenized counterparts under photo-chilling stress.

Soredia, isidia or lichen thallus fragments may, hypothetically, be present in samples together with free-living algae and grow into algal colonies while the fungus disintegrates during cultivation experiments. These algae cannot be considered free-living in the primary sense (they were not found in free-living state in nature). However, many authors have observed free-living cells directly, without prior cultivation. For example, Bubrick *et al.* (1984) found the presence of free-living cells of *Trebouxia* on thin 'skins' of bark scraped from a tree. Tschermak-Woess (1978) confirmed many findings of the same genus free-living on tree bark at many different localities. A remarkable method for observing lichens was used in several of his studies by Sanders. In these, several plastic microscope cover slips were placed among folicolous lichen communities within a neotropical lowland forest (Sanders & Lücking 2002). They were able to observe the undisturbed development of microbial communities and their interactions. Thus, records of the occurrence of free-living cells of *Trebouxia* became an interesting by-products of observations of lichen development (Sanders 2001, 2005; Sanders & Lücking 2002).

Another method of direct observation of free-living algae was proposed by Hoffmann *et al.* (2007). The authors simply attached sterilized coverslips to a sample of forest soil sealed in a petri dish and provided the material with sufficient light. After 4–5 weeks, while observing the dominant organisms of the growths that formed on the slide, they detected free-living cells of *Coccomyxa* cf. *solorinae* and *Elliptochloris bilobata*. Other direct observations of free-living algae identified *Trebouxia arboricola* from rocks and caves (Stoyneva & Gärtner 2009) and the fruiting body of *Fomes fomentarius* (Stoyneva *et al.* 2014). The free-living *Leptosira obovata* was discovered in a cave (Roldán & Hernández-Marín 2009).

Mukhtar *et al.* (1994) attempted to bring further evidence for the possibility of fungus-independent existence of *Trebouxia*. They isolated free-living cells of this alga from the surface of stones of a three-year-old burn site, on which no occurrence of lichens was recorded. Thus, according to the authors, it was not possible that the isolated *Trebouxia* were derived from asexual lichen propagules. However, to study these cells, they used cultivation with BBM medium. This may have resulted in accidental transfer of vegetative propagules, as soredia, to the medium, which although usually falling only a few centimetres from the source (sorals), can be spread by wind over tens of meters (Armstrong 1987) and even tens to hundreds of kilometres (Harmata & Olech 1991).

The question remains whether these algae are capable of long-term survival in nature and are not just cells randomly and temporarily released from lichens, as suggested by Ahmadjian (1988). Sanders (2005) admits that free-living populations of the genus *Trebouxia* could be only transient and lichenization could be a condition for their

longer-term persistence at a given locality. The fact that many of the free-living photobionts found were in close proximity to lichens would argue for this. For example, Novakovskaya *et al.* (2020) collected soil algae in different types of tundra communities in the Northern Urals and two of the total three lichen photobiont species recorded (*Asterochloris excentrica* and *Trebouxia* sp.) came from a site dominated by the lichens *Cladonia rangiferina* and *Flavocetraria nivalis*. Only the species *Elliptochloris bilobata* was also present at two other tundra sites low in lichens.

Novakovskaya *et al.* (2020) suggest that the high abundance of photobionts at a lichen-dominated site may also be because they are species with resistance to the harsh climatic conditions at the site. Unfortunately, it is not clear from this paper whether lichen photobionts were observed directly or in culture and there is a risk that the isolated algae are derived from lichen propagules. The close proximity of potential mycobionts associating with algae of the genus *Trebouxia* is also described by Hallmann *et al.* (2016) in their direct observation of biofilms, and they point out that the cells in question are not necessarily free-living and may originate from lichen propagules.

Thus, it is possible that some species of photobionts are found in small populations in the proximity of lichens (where they do not form close associations to fungal hyphae) that can continually replenish their numbers and are not permanently free-living. Nevertheless, some observations of macroscopic free-living biofilms formed by many lichen symbionts speak against this (Mikhailyuk *et al.* 2003; Mikhailyuk 2008). *Trebouxia* was, for example, found to create macroscopic growth on tree bark comprising only of *Trebouxia* cells (Tschermak-Woess 1978), or mixed with *Apatococcus lobatus* (Rindi & Guiry 2003).

There are still many unanswered and highly intriguing topics surrounding the subject of free-living lichen photobionts. Further long-term studies combining careful microscopic observations and modern molecular methods are needed to gain deeper insights into free-living lichen photobiont communities.

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