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The response of selected genera within the Stichococcaceae to desiccation and saline stress, with respect to their taxonomy

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Betreuerin

Dr. Karin Glaser
University of Rostock
Institute of Biological Sciences
Applied Ecology & Phycology

Gutachter*innen:

Erste Gutachterin
Dr. Karin Glaser
University of Rostock
Institute of Biological Sciences
Applied Ecology & Phycology

Zweiter Gutachter
Mag. Dr., assoc. Prof. Andreas Holzinger
University of Innsbruck
Department of Botany
Research Group Plant Cell Biology

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Abstract

This thesis is about a family of widespread aeroterrestrial green microalgae, the Stichococcaceae. It is at its heart a taxonomic work that has aimed to elucidate the patterns of relationship between the various genera within this family. This began with the rather simple and unremarkable generitype *Stichococcus bacillaris* NÄGELI, from which almost all other extant Stichococcaceae derive their name. The historical and extant problems in working with this group are discussed, with some discussion on possible solutions. This work is thematically separated into the following topics:

First, to supplement traditional molecular and morphological identification of newly described strains within this family, the utility of chemotaxonomic identification with compatible solute expression (osmolytes) was investigated. Sucrose and sorbitol were measured after strains underwent growth in both nonsaline and saline medium, as it is known that salinity can dramatically alter the compatible solute expression profile of algae. The results showed that strains of the novel stichococcaceaen genera have the same qualitative osmolyte production pattern as *Stichococcus bacillaris*.

After quantifying the osmolyte response under saline and standard growth conditions, the next step was to determine how the abiotic stressors of salinity and desiccation differentially affect the taxa. It was found that innate resistance to salinity and desiccation are independent of one another in the Stichococcaceae. Although osmolyte production is tightly correlated to salinity, the mechanisms controlling this seem to be independent of those affecting desiccation tolerance, when not stressed in combination. Halotolerance was more variable between strains than desiccation tolerance.

Third, as an extension to the second theme, the combined effects of salinity and desiccation were studied and measured. The experiments conducted allowed for direct observation of the interaction between salinity, osmolyte production, and desiccation tolerance. *Pseudostichococcus* MOEWUS, 1951 was shown to have higher desiccation and salinity tolerance than other strains of the other stichococcaceaen genera. In summary, this work examines the effects of desiccation and salinity on the Stichococcaceae and connects their ecophysiology and chemotaxonomic profiles to their taxonomy.

Zusammenfassung

Diese Arbeit befasst sich mit einer Familie weit verbreiteter aeroterrestrischer grüner Mikroalgen, die Stichococcaceae. Es handelt sich hierbei um eine taxonomische Arbeit, die versucht, die Verwandtschaft zwischen den verschiedenen Gattungen innerhalb dieser Familie aufzuklären. Die Geschichte der Stichococcaceae beginnt mit dem einfachen und unauffälligen Taxon des Gattungsnamens, *Stichococcus bacillaris* NÄGELI, von der fast alle anderen rezenten Arten der Stichococcaceae ihre Namen haben. Die historischen und bestehenden Probleme bei der Arbeit mit dieser Gruppe werden diskutiert, mitsamt dazugehörigen Lösungsvorschlägen. Diese Arbeit kann in drei Themenbereiche unterteilt werden:

Zuerst wurde, zur Ergänzung der traditionellen Identifizierung neu beschriebener Stämme innerhalb dieser Familie, die Nützlichkeit der chemotaxonomischen Identifizierung mit Osmolyten untersucht. Saccharose und Sorbit wurden gemessen, nachdem die Stämme sowohl in nicht salzhaltigem als auch in salzhaltigem Medium gezüchtet wurden, da bekannt ist, dass der Salzgehalt das Expressionsprofil von Osmolyten in Algen dramatisch verändern kann. Die Ergebnisse zeigten, dass Stämme der neuen Stichococcaceaen Gattungen die gleiche Osmolyten aufweisen wie *Stichococcus bacillaris*.

Als nächstes wurden die Stämme mit abiotischen Faktoren gestresst, um ihre Ökophysiologie mit ihrer Taxonomie zu verbinden. Es wurde festgestellt, dass die natürliche Halotoleranz und die Austrocknungstoleranz bei den Stichococcaceae unabhängig voneinander sind. Obwohl die Produktion von Osmolyten eng mit dem Salzgehalt korreliert, scheinen die Mechanismen, die dies kontrollieren, unabhängig von denen zu sein, die die Austrocknungstoleranz beeinflussen, wenn sie nicht gleichzeitig beansprucht werden. Daraus folgt, dass die Halotoleranz, zwischen den verschiedenen Stämmen, variabler ist als die Austrocknungstoleranz.

Drittens wurden als Erweiterung des zweiten Dissertationsthemas die gemeinsamen Auswirkungen von Salz und Austrocknung untersucht und gemessen. Die durchgeführten Experimente ermöglichten eine direkte Beobachtung der Interaktionen zwischen dem Salzgehalt, der Produktion Osmolyten und der Austrocknungstoleranz. Bei *Pseudostichococcus* MOEWUS, 1951 konnte eine höhere Austrocknungs- und Salztoleranz als bei Stämmen der anderen Stichococcaceae-Gattungen festgestellt werden. Zusammengefasst untersucht diese Arbeit die Auswirkungen von Austrocknung und Salinität auf die Stichococcaceae und verbindet deren Ökophysiologie und taxonomischen Profile mit deren Taxonomie.

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Taxonomy is described sometimes as a science and sometimes as an art, but really it's a battleground.

Bill Bryson

1 Introduction

1.1 An overview of algal taxonomy and diversity

Taxonomy, in its simplest sense, deals in classification. In a more abstract sense, it arbitrates the boundaries between states of existence. This is undoubtedly the case for biology, the most well-known usage scenario for taxonomy, which concerns itself with hierarchically organizing and describing the diversity of life on earth. Good taxonomy is also needed because there is a need to estimate and understand the biodiversity of organisms. For example, global biodiversity is being lost at an unprecedented rate due to human activities ("The Anthropocene Extinction") (Wagler, 2011). It would be impossible to establish protected species and areas if there is not a clear understanding of what species exist and where they naturally occur.

Taxonomy is also history. It shifts, deletes, and creates new boundaries based on previously acquired knowledge. Taxonomists have a reputation for being exacting to a fault, but the nature of their work justifies this. To declare that an organism belongs to a particular group and not another implies that there is enough difference between it and another to draw a distinction. How much difference and in which respects is a topic of rich debate. To complicate matters, it is common and arguably necessary to obscure taxonomic precision for broader understanding, such as by using broad groupings or common names.

The colloquial term "algae" refers to photosynthetic organisms that live in aquatic or moist habitats. It is not surprising that the true biodiversity and taxonomy of "the algae" is highly complex. The popular understanding of what an alga is usually reduced to kelps and seaweeds, pond scum, and, if astutely observed, the green coverings of facades and tree bark.

The precise definition of what comprises an algal *species* depends on the viewpoint of the phycologist (John and Maggs, 1997; Agapow *et al.*, 2004). A physiological scientific definition of an alga may refer to autotrophic organisms that are aquatic or terrestrial, who possess chlorophyll as their primary pigments and may or may not be a "plant." Notable examples of "non-plant" algae are the brown algae (Phaeophyceae), blue-green algae (cyanobacteria, not an alga at all), and the dinoflagellates (Baldauf, 2008). All algae contain chlorophyll *a*, but the proportions and composition of the accessory pigments, such as chlorophyll *b*, chlorophyll *c*, fucoxanthin, and carotenoids differ between the algal lineages (John *et al.*, 2011).

In contrast to this loose definition, there exists scientific consensus on the evolutionary origin of the algae. In brief, the first algal groups evolved roughly 1 to 1.5 billion years ago by primary endosymbiosis of a photosynthetic cyanobacterium by a heterotrophic eukaryote (Yoon *et al.*, 2004; Archibald, 2012). This gave rise to the Glaucophytes and to the green algae/land plants lineages (Viridiplantae or Chloroplastida). The primary endosymbiotic event leading to the evolution of the red algae (Rhodophyta) occurred somewhat later, at around 600 million years ago (Morden *et al.*, 1992; McFadden and van Dooren, 2004; De Clerck, Bogaert and Leliaert, 2012). Together these three lineages comprise the Archaeplastida (Cavalier-Smith, 1998). Secondary endosymbioses of a cell with green or red algal plastids by another heterotrophic eukaryote host resulted in, very broadly, the Chromalveolates, Euglenids, and Chlorarachniophytes. A serial secondary endosymbiosis between a dinoflagellate ancestor and a green algal eukaryote gave rise to the dinoflagellate genus *Lepidodinium* WATANABE, SUDA, INOUYE, SAWAGUCHI & CHIHARA. A further tertiary endosymbiosis from the engulfment of a red algal eukaryote resulted in the complex dinoflagellates. However, the complete evolutionary history of Chromalveolates with red algal plastids is still unresolved (Gentil *et al.*, 2017). These events explain the diversity and often non-monophyletic relationships between extant algae and also why algae contain at at least three separate genomes: the mitochondrial, plastid, main nuclear, and sometimes the "nucleomorph" genome, a residual nuclear genome as the product of secondary endosymbiosis (Moore *et al.*, 2012). The nuclear genome contains the majority of genetic material and protein-coding regions (Kapraun, 2005; Maier *et al.*, 2013). **Fig. 1** summarizes the endosymbioses described above.

As previously mentioned, some algae do not trace their lineage to the Archaeplastida and are, strictly speaking, not "plants". The Chromalveolate supergroup consists of consist of two phyla, the Chromista and Alveolata. To the Chromista belong the Cryptophyceae, Haptophyta, and Heterokontophyta (brown algae and diatoms). The Alveolata house the Dinophyceae (dinoflagellates; often mixotrophic), Apicomplexa, and Ciliophora. The Apicomplexans are notable in that all species are endoparasites, as their plastids have lost the ability to photosynthesize (Bhattacharya, Yoon and Hackett, 2004). The Ciliophora are largely heterotrophic since many have lost plastids, but a minority are mixotrophic (Mcmanus, Schoener and Haberlandt, 2012). Eukaryote-to-eukaryote endosymbiosis events were probably frequent over the course of algal evolution, so photosynthesis may have been nonessential for organisms that retained their heterotrophy from ancestral eukaryotic hosts, thus resulting in algal lineages that

are mixotrophic or even entirely heterotrophic. This can be seen in modern clades as osmotrophy, phagotrophy, or even parasitism in the Apicomplexans and Ciliophora (Oborník, 2019).

In contrast, the Archaeplastidan lineages are largely phototrophic. The Rhodophyta are common in marine environments and are also more dominant than other members of the Archaeplastida, who mainly inhabit freshwater or terrestrial habitats (Baldauf, 2008). This group contains many seaweed genera of economic importance, such as *Porphyra* C. AGARDH and *Palmaria* STACKHOUSE (Kandale *et al.*, 2011), and also the Euglenids. However, in terms of biomass and ecological niche diversity, the Viridiplantae are by far the most successful clade. Green algae and land plants dominate virtually every environment on Earth and comprise ca. 10% of the ocean's biomass (Bar-On and Milo, 2019). Arguably, plants can be said to be "terrestrial algae" because the transition to terrestrial habitats happened long after the establishment of the Chlorophyta; all land plants have evolved from one clade within the Streptophyta (de Vries and Archibald, 2018). The Glaucophytes have a small distribution and have mostly been of interest in clarifying the plastid evolution (Figueroa-Martinez, Jackson and Reyes-Prieto, 2019).

Since the focus of this thesis is on a green algal family, a more detailed introduction of the Chlorophyta, especially of the "core Chlorophytes", is necessary. The core Chlorophytes have attracted the most interest because of their high diversity in terms of ecological habitats, cellularity, and morphology. This group consists of the classes Pedinophyceae, Chlorodendrophyceae, Ulvophyceae, Trebouxiophyceae, and Chlorophyceae. The deep phylogeny and monophyly of the classes Ulvophyceae and Trebouxiophyceae were unclear until recently (Fučíková *et al.*, 2014; Fang *et al.*, 2018). However, recent work based on transcriptome and genome analysis has revealed that the Trebouxiophyceae and Chlorophyceae are monophyletic (Li *et al.*, 2021). Within the Trebouxiophyceae, the core orders are the Chlorellales, Prasiolales, Microthamniales, Trebouxiales, and *Choricystis*/*Botryococcus* clade. Within the Prasiolales, the Prasiolaceae formerly contained taxa now belonging to the Stichococcaceae. Reprising the family name Stichococcaceae (**Section 1.5**) has made clearer which genera belong to the Prasiolaceae, as several phylogenies have not considered *Stichococcus* to truly be part of the Prasiolales (Rindi *et al.*, 2011; Moniz, Guiry and Rindi, 2014; Heesch *et al.*, 2016).

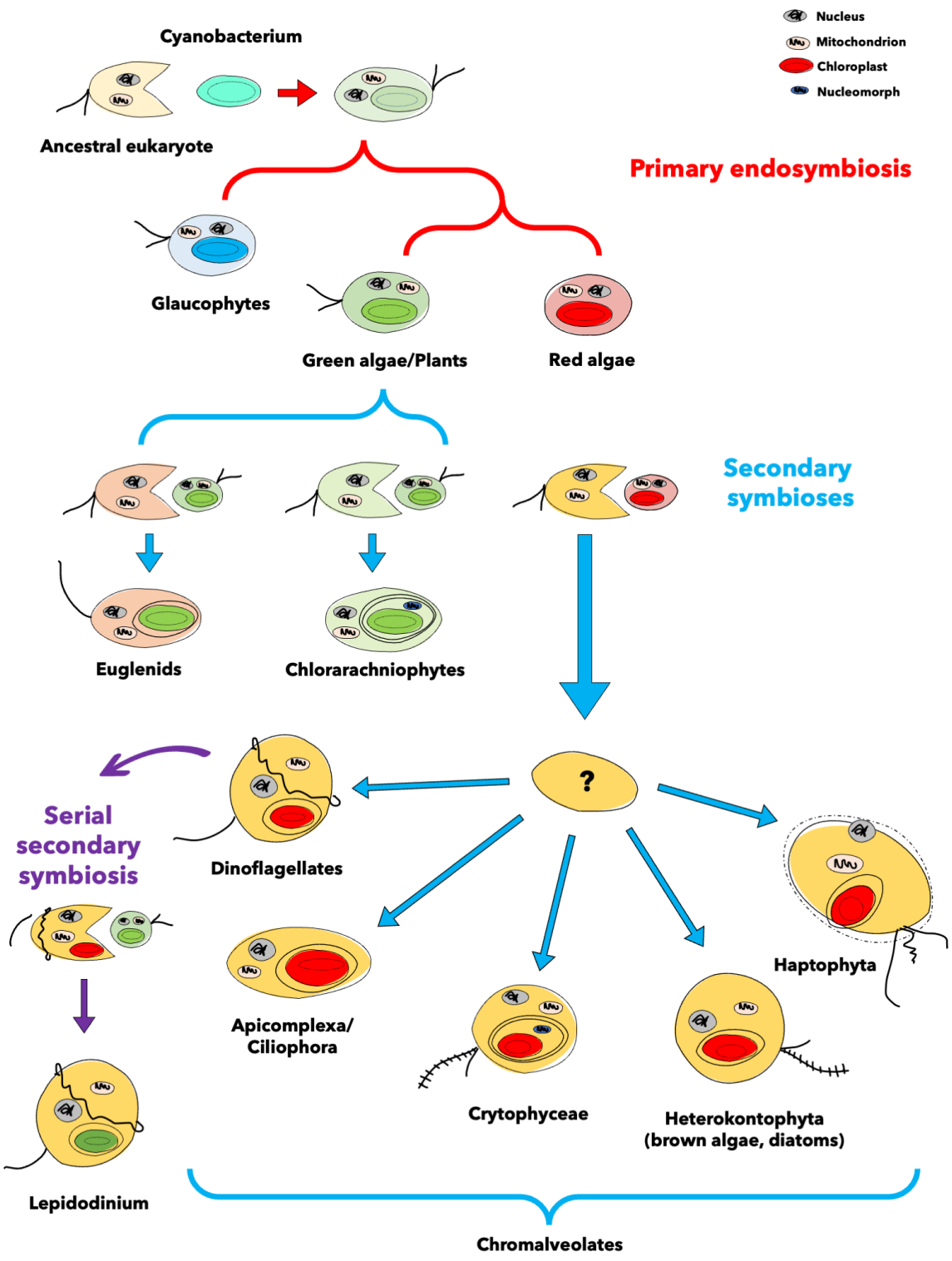

Figure 1 A very simplified schematic of the primary and secondary endosymbiotic events that gave rise to the major algal groups. Arrows indicate gene transfer. Primary endosymbiosis from the engulfment of a cyanobacterium by a eukaryotic host cell resulted in an ancestral "proto-alga" that evolved into the Archaeplastida (Glaucophytes, Chlorophytes, and Rhodophytes). Secondary symbioses of eukaryotes containing green and/or red algal plastids resulted in the Euglenids, Chlorarachniophytes, and Chromalveolates; this additional evolutionary step is still not fully understood. The Chlorarachniophytes and Cryptophyceae possess a nucleomorph within their chloroplasts, a remnant of their ancestral endosymbiotic event. A serial secondary symbiosis of a green alga by a dinoflagellate gave rise to *Lepidodinium*. Further tertiary endosymbiosis (not pictured) resulted in the diverse complex dinoflagellates groups extant today. Adapted from Gentil et al., 2017 and Paini, 2013.

1.2 Algal species concepts

Just as "*What is an alga*?" is difficult to answer, shifting the question to ask then, "*What is a(n algal) species*?" begets a rather roundabout answer. I will define here a species as the smallest evolutionary unit that unifies a set of characteristics that its observers have stipulated, which is free enough to make it suitable for the many species concepts in today; at last count, there were (at most) twenty-seven, most of which are variations of those in **Tab 1**. However, pragmatically speaking phycologists very much need species concepts to do their work, and there have been several utilized in algal taxonomy over the past centuries.

Table 1. A comparison of several, arguably the most successful extant species concepts. Adapted from Wilkins, 2011 and Hörandl, 2022.

Concept	Key points	Use in algal taxonomy
Agamospecies	Speciation by ecological niche adaptation; sexual reproduction does not occur, such as by asexual seed production	Not suitable; more accepted in higher plants
Biospecies	Reproductive isolation; sexual reproduction resulting in fertile offspring	Not suitable, algal sexual reproduction often not observable
Ecospecies	Ecological niche isolation; phenotypically distinct geographic varieties capable of interbreeding within ecospecies	Somewhat popular in botany
Evolutionary species	Evolving lineages; processes that drive speciation determine species; difficulties with hybridization	Rarely used in isolation
Genetic species	Common gene pool, essentially a reproductive species concept	Rarely used in isolation
Morphospecies	Phenotypic variation	Formerly commonly used, still important in species delimitation in the algae
Phylogenetic/polyphasic species concept	Combination of morphospecies, biospecies and evolutionary species	Arguably the most used "concept" today

The oldest species concept in phycology and botany is undoubtedly the morphospecies concept, which has dominated for the past two centuries. In addition, current knowledge of microalgal biodiversity is still based chiefly upon morphological observations through light microscopy, except for organisms with features smaller than the limit of light microscopy, e.g., diatoms, for which electron microscopy is now widely utilized. Although morphological dissimilarity often coincides with species boundaries, cases of phenotypic plasticity, whether innate or environmentally induced (Lürling, 2003; Morabito *et al.*, 2007; Neustupa, St'astny and Hodac, 2008; Verbruggen, 2014), convergent evolution (Potter *et al.*, 1997; Maier *et al.*, 2013; Drobnitch *et al.*, 2015; Yamashita *et al.*, 2021), and cryptic diversity (Beszteri, Ács and Medlin, 2005; Kaczmarska *et al.*, 2014; Koh and Kim, 2018; Amato, Kooistra and Montresor, 2019; Pinseel *et al.*, 2019), overreliance on this aspect leads to an incomplete understanding of taxonomy.

Other species concepts are unsuitable to algal taxonomy in general. For example, the biospecies concept, based on vertical descent, is poorly suited to algal taxonomy because sexual reproduction is enigmatic for some species (Dudgeon *et al.*, 2017), whereas vegetative propagation is extremely common; life and reproductive cycles may be so complex in some algae, such as in the diatoms (Annunziata *et al.*, 2022), that it would be nearly impossible to induce sexual reproduction for observation. Furthermore, successful hybridization between unrelated organisms complicates what comprises a species because it violates the concept of reproductive isolation (Hörandl, 2022).

The advent of molecular sequencing allowed for species delimitation based on genetic distance and helped to solve some of the problems of the morphospecies concept, such that the genetic history of organisms can be inferred through their genetic code. However, phylogenetic inference sometimes presents more problems of its own since it is highly sensitive to the various models, methods and parameters used (Huelsenbeck *et al.*, 2002; Stamatakis, 2006), not all of which give the same result in phylogenetic reconstructions, hence why several methods are often combined and compared (De Bruyn, Martin and Lefeuvre, 2014). The availability of high-quality gene sequences is also another stumbling block, since some organisms are recalcitrant to common sequencing methods and loci (Blaby-Haas and Merchant, 2019). Still, the formation of lineages – gene trees – as postulated through the multispecies coalescent model (MSC) has several caveats (Sukumaran and Knowles, 2017) but remains an important, if not the most important, model in the current understanding of taxonomy (Shazib *et al.*, 2019; Sukumaran, Holder and Knowles, 2021).

Currently, the most widely used species concept is the phylogenetic or species concept (Aldhebiani, 2018), essentially a combination of morphospecies, biospecies, and evolutionary species concepts as needed; this is often what is meant by “polyphasic” or “integrative” methodology. This is tied to evolutionary descent since it is assumed that more closely related organisms will be, in some way, more like each other than distantly related ones – the actual criteria depend entirely on the species concept utilized. In the current algal taxonomy, a morphological description of new species is still non-negotiable. There has been a strong move toward integrating DNA sequence data into the taxonomic delimitation (Henley *et al.*, 2004; Kaštovský *et al.*, 2016; Mikhailyuk *et al.*, 2018; Schultz *et al.*, 2021). Using molecular data to construct phylogenies is essentially creating hypotheses on the evolutionary history of a group of organisms.

1.3 Molecular taxonomic methods and beyond

For classification, molecular data has also provided greater resolution than classifications based purely on morphology alone. For example, the paraphyly of the family Bostrychioideae was suggested from physical structural differences for decades, but molecular data from the 26S SSU rRNA and *rbc*L loci further elucidated the relationships and resulted in the transfer of several *Stictosiphonia* HOOKER F. & HARVEY into *Bostrychia* MONTAGNE (Zuccarello and West, 2006). Sequence data have also given insight into the phylogenic relationships of taxa that were difficult to place based on morphology because of trait instability within and across clades, e.g., *Klebsormidium* P. C. SILVA, MATTOX & W. H. BLACKWELL and *Interfilum* R. CHODAT (Rindi *et al.*, 2011).

DNA sequencing has led to improved species description and their identification in terms of speed via metabarcoding. Metabarcoding extends the work of taxonomy to the practical plane so that short gene sequences (“barcodes”) of high variation from representative organisms act as proxies for entire populations (Le Gall and Saunders, 2010; Zimmermann, Jahn and Gemeinholzer, 2011; Koh and Kim, 2018). The tedious method of having an expert identify algae, particularly the very populous microalgae taxa, can theoretically be sidestepped. This, too, has its costs (Zimmermann *et al.*, 2015), since expert curation is first needed to contextualize barcode reads into taxa placements. There have been large-scale curation efforts, such as the EukRef2 (Del Campo *et al.*, 2018), PhytoREF (Decelle *et al.*, 2015), and PLANiTS (Banchi *et al.*, 2020) annotation collaborations for eukaryotes ribosomal RNA, plastidial 16S rRNA, and plant ITS sequences, respectively. The need for an improved catalog of worldwide biodiversity has grown and has made

metabarcoding a tool for ecological monitoring. The most commonly used loci in green algal research are 18S SSU rRNA, *rbc*L, and ITS-regions, with limited success for the *tuf*A marker (Henley *et al.*, 2004; Caisová, Marin and Melkonian, 2013; Patwardhan, Ray and Roy, 2014). However, some strains are often difficult to sequence due to the presence of large Group I introns in nuclear genomes, which are common in Chlorophyte algae (Haugen, Simon and Bhattacharya, 2005). The introns prevent not only alignment of sequences, but also often lead to failure in PCR amplification in the 18S locus. It is helpful to turn to other tools for taxonomic research.

Chemotaxonomy, or secondary metabolite monitoring, is a branch of applied taxonomy and has been extensively used in pharmacology and biotechnology (Hegnauer, 1986; Frisvad, Andersen and Thrane, 2008; Singh, 2016). Because of the problems inherent to using molecular and morphological methods, chemical characters have been used to explore algal systematics. Chemotaxonomy may also allow hitherto overlooked algae to be studied in more detail if it is shown that they are of commercial value. While this goes against the idealistic ideas in research, “charisma” and utility still drive the valuation of species and should be used as a tool in conservation (Pillon and Chase, 2007; Jarić *et al.*, 2020).

Nevertheless, taxonomy is first and foremost bound to biology, and reducing organisms to mathematical probabilities is problematic. As many phylogenies have shown, using “molecular data” to define taxonomic boundaries may be no less subjective than a cladistic comparison of traits (Leliaert *et al.*, 2014; Leliaert and De Clerck, 2017; Shazib *et al.*, 2019; Willis and Woodhouse, 2020). The amount of variation needed to delineate a species varies on the taxonomic level, specific markers, and target organisms. For example, within the diatoms, a base pair difference of circa 1 percent in the *rbc*L and 18S sequences is sufficient to segregate taxa, and ca. 4-5% for genera (Zimmermann, Jahn and Gemeinholzer, 2011; Abarca *et al.*, 2020). In the green algae, there has also been much research on using secondary ITS-2 structure differences to delimit taxa (Caisová, Marin and Melkonian, 2013; Mikhailyuk *et al.*, 2018). Because the concept of an algal species is subjective and group-specific, peer-reviewed consensus on phylogenetic results is important for taxonomic work.

1.4 Abiotic stressors of dehydration and salinity on microalgae

There are several stressors on dry land for photosynthetic organism, such as high UV radiation, severe temperatures, and in all cases, water balance. On land, solar radiation is not filtered by water, so the danger of photooxidative and UV damage is higher than in aquatic habitats (Holzinger *et al.*, 2014). The risk of desiccation is

always imminent in terrestrial environments; in addition, there are often unpredictable dry-wet cycles (Holzinger and Karsten, 2013; Jung *et al.*, 2020; Samolov *et al.*, 2020). The success of terrestrial algae in diverse ecological niches can be attributed in part to their ability to withstand repeated and extended drying–rewetting cycles (Padisák and Naselli-Flores, 2021).

Photosynthesis inhibition is a primary response to abiotic stress and is observed in both desiccation-tolerant and desiccation-sensitive plants and algae. Decreased photosynthetic activity during drought stress can be due to the limitation of carbon dioxide intake, increased oxidative stress from reactive oxygen species (ROS) (Ben Rejeb, Abdelly and Savouré, 2014) or decreased activity of photosynthetic enzymes. Photosynthetic rates always decrease in response to desiccation, but desiccation-tolerant plants can quickly repair damage to the photosynthetic apparatus, leading to virtually full recovery upon rehydration (Häubner, Schumann and Karsten, 2006; Gray, Lewis and Cardon, 2007; Gasulla *et al.*, 2009; Medwed *et al.*, 2021). Desiccation-tolerant plants and algae are usually homoiochlorophyllous and adapted to shorter drying-rewetting intervals of days and weeks since chlorophyll and chloroplasts are not broken down during desiccation (Tuba and Lichtenthaler, 2011). This contrasts with poikilochlorophyllous organisms, such as resurrection plants, that break down and rebuild their chloroplasts because dry periods could last for months. It would be more efficient to rebuild the entire photosynthetic apparatus instead of conserving them (Charuvi *et al.*, 2019).

Another internal strategy to combat abiotic stress is the production of secondary metabolites. For example, in response to UV radiation, algae can produce protective sunscreen compounds, like mycosporine-like amino acids (MAAs) (Karsten *et al.*, 2005; Oren and Gunde-Cimerman, 2007), pigments (Storme *et al.*, 2015), and carotenoids (Remias and Lütz, 2007). The production of protective secondary metabolites is also common a response to salinity.

Salinity influences a complex network of physiological and biochemical mechanisms associated with microalgal growth and development. Under high salinity stress, algae can alter their metabolism to adapt to a hypersaline environment (Kan *et al.*, 2012). Some produce organic osmolytes such as glycerol, proline, and simple polyols and sugars, which act as “compatible solutes” that replace water to maintain homeostasis in the cell under osmotic stress conditions, such as under dehydration or salinity (Yancey, 2005; Gambichler, Zuccarello and Karsten, 2021). Sugars, in particular, probably stabilize drying cells by directly

interacting with macromolecules and membranes and immobilizing the cytoplasm, resulting in a reversible glass state (Koster, 1991). Osmolytes, particularly sugars and sugar alcohols, have been shown to be helpful in a supportive role for algal chemotaxonomy (Karsten *et al.*, 2007; Gustavs, Görs and Karsten, 2011; Hotter *et al.*, 2018).

In terrestrial ecosystems, salinity stress is tied to water balance because salinity leads to water loss from ion imbalance. While dehydration reduces the amount of water available from the environment, osmotic stress disrupts the normal cellular ion concentrations, so that cells can be surrounded by water yet be subjected to lower water potential; also, high concentrations of Na^+ and Cl^- ions are cytotoxic (Kirst, 1990; Tavakkoli *et al.*, 2011). Marine environments cause mainly osmotic stress since water is plentiful. However, in terrestrial saline environments, the effects of dehydration are exacerbated by salinity. Natural terrestrial saline environments are relatively rare: coastal areas affected by salt spray, saline lakes, and salt marshes. However, anthropogenic effects have led to the salinization of natural habitats, such as through rising sea levels through global climate change. As a result, around 20% of global irrigated land has become salt-affected (Singh, 2021). In Germany, there is a notable example of potash tailing heaps as a byproduct of potassium fertilizer production; they consist almost completely of NaCl and create hypersaline environments around them due to salt dissolution from rain (Sommer, Karsten and Glaser, 2020). This creates an intense selection pressure for halotolerant organisms not normally seen in the natural, unaffected environment, such as halophilic bacteria, algae, and marine plants (Sommer *et al.*, 2020).

It is possible to replicate many abiotic stressors in laboratory settings to study their effects on target organisms. Algae are especially interesting because their metabolism is so sensitive. The metabolic response is quantifiable and in many cases, taxa-specific, such as with osmolyte chemotaxonomy. The toolbox for algal systematics has been expanding for two centuries, and it has become clear that for integrative taxonomy, a broad number of characteristics can – and should! – be used to delineate species boundaries.

1.5. The Stichococcaceae

This dissertation explores the taxonomy of a green microalgal family, the Stichococcaceae. If this seems rather specific, it is because the sheer biodiversity of just “microalgae” alone is sufficient to fill several books (Round, Crawford and Mann, 1990; Baldauf, 2008; Guiry, 2012; Ettl and Gärtner, 2013). Depth, rather

than breadth, is sometimes of the essence when the entirety of biology is to be studied.

The Stichococcaceae is one family within the Chlorophyta, class Trebouxiophyceae, order Prasiolales, well within the Viridiplantae. The name of the family Stichococcaceae was first described by Knut Bohlin (Bohlin, 1901), although this has been apparently overlooked until recently (personal communication, M . Guiry and S. Heesch, July 2021). The Stichococcaceae have previously been referred to simply as part of the "*Prasiola*-clade", sharing the order Prasiolales and family Prasiolaceae, whose members show varying degrees of multicellularity and macro-scale sizing (Karsten *et al.*, 2005; Heesch *et al.*, 2016; Hotter *et al.*, 2018). As cosmopolitan algae, the Stichococcaceae have been found in diverse ecosystems such as facades (Häubner, Schumann and Karsten, 2006; Uher, 2008), tropical rainforest (Neustupa and Škaloud, 2008), low pH habitats (Pollio *et al.*, 1997), as well as in alpine environments and polar habitats (Kvíderová and Lukavský, 2005; Novis, Beer and Vallance, 2008; Syuhada *et al.*, 2022).

The name of the family stems from the genus *Stichococcus*, first described and typified by the taxon *S. bacillaris* by Carl Wilhelm von Nägeli in 1849 (Nägeli, 1849). However, up to very recently, there was no viable holotype material for the strain, so phylogenetic work based on *Stichococcus* was nearly impossible to standardize to a single "known" *S. bacillaris* strain. Furthermore, *Stichococcus* is an old genus that has not escaped the necessity of multiple taxonomic revisions. Since 1849, 49 taxa within *Stichococcus* have been erected, primarily based on morphology (Guiry and Guiry, 2020).

The original diagnosis for the generitype reads:

> "*S. BACILLARIS*, cells 1/900 to 1/800 ligne thick, 1 2/3 to 3 times as long. – Zürich, on moist wooden beams. – The cells are unicellular or linked together by 2, rarely 4 cells. The surface is green and powdery. The membrane is very thin. The chloroplast is evenly colored with chlorophyll, giving the entire cell a green color; the chloroplast can lie to one side of the cell. Colonies have not been observed." (NB: *1 ligne = 2.26 mm*)

The modern description of *Stichococcus bacillaris* has not changed greatly. The venerable algal diagnostic key *Syllabus der Boden-, Luft- und Flechtenalgen* (Ettl and Gärtner, 2013) describes it as single-celled or few-celled, forming uniseriate

filaments that fragment easily (**Fig. 2**). A single parietal laminate chloroplast is present, and a starchy pyrenoid is lacking. No gelatinous sheath is produced. The cells are cylindrical and oblong, sometimes slightly oval.

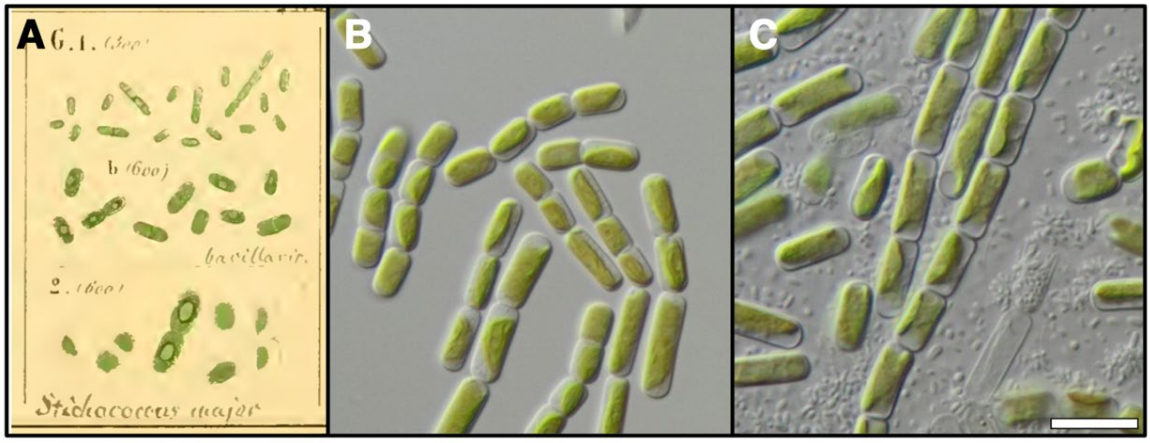

Figure 2 The original description and the holotype of *Stichococcus* from C. Nägeli (A) and two extant strains of *S. bacillaris*: B) CCAP 379/1A and C) CCALA 495. Strain CCAP 397/1B is a sister strain to the epitype, SAG 379-2. *Stichococcus* strains are rod-shaped, have thin cell walls, possess a parietal chloroplast, and can occur singly or in short filaments that are easily disturbed. Scale bar = 10µm.

Because *S. bacillaris* lacks many distinguishing features, such as unique color, cell shape, or chloroplast form, some taxa have been established based on small morphological differences to *S. bacillaris*. One example of such is *S. minor* and *S. major* (Nägeli, 1849), originally described by Nägeli along with *S. bacillaris*. The two taxa were described as respectively smaller and shorter, and longer and larger, than *S. bacillaris*. Upon closer study by Rabenhorst (Rabenhorst, 1868, p. 48), these two strains were subsequently synonymized into *S. bacillaris*. **Fig. 3** gives an overview of the morphology of several strains from the other stichococcaceaen genera.

Taxonomic work with *Stichococcus*-adjacent strains has proven difficult in historical and modern contexts because these strains often have degenerate morphology, such that they are best compared in groups of multiple isolates instead of alone (Neustupa, Elias and Sejnohova, 2007; Hodač *et al.*, 2016). Also, many strains within the Stichococcaceae were notoriously difficult to sequence due to large Group 1 introns of ca. 500 bp in size, which were present in, for example, strains IT-203, ASIB-BS-57, and ASIB-BS-603 *Stichococcus* sp.

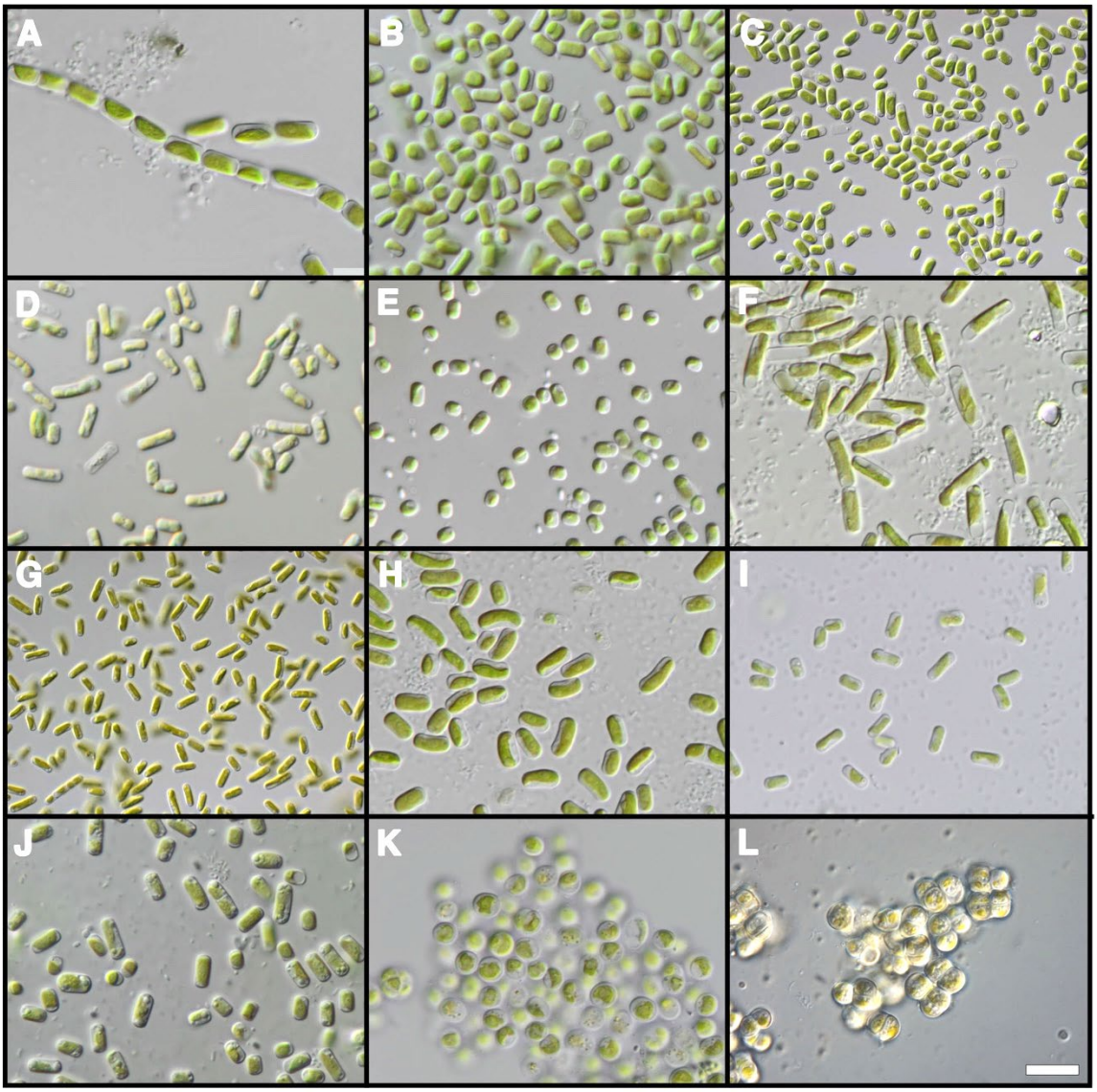

Figure 3. The morphology of selected Stichococcaceae strains. **A – C**: ACOI 95, SAG 56.91, BCAC S41*Stichococcus bacillaris*. **D – E**: ASIB-BS-57, ASIB-Bs-658 *Pseudostichococcus monallantoides*. **F**: LB 1820 *P. undulatus*. **G**: SAG 2481 *Protostichococcus edaphicus*. **H**: J1303 *Deuterostichococcus marinus*. **I**: SAG 2406 *Tritostichococcus solitus*. **J**: J1302 *Tetratostichococcus jenerensis*. **K**: SAG 11.88 *Diplosphaera epiphytica*. **L**: SAG 1.92 *Desmococcus olivaceus*. Scale bar = 10 µm.

The pivotal work of Pröschold & Darienko (Pröschold and Darienko, 2020) is the most current taxonomy of the Stichococcaceae. It lists eight genera, most of which are revisions of taxa formerly known as *Stichococcus*: *Stichococcus* NÄGELI, *Protostichococcus* PRÖSCHOLD & DARIENKO, *Deuterostichococcus* PRÖSCHOLD & DARIENKO, *Tritostichococcus* PRÖSCHOLD & DARIENKO, *Tetratostichococcus* PRÖSCHOLD & DARIENKO, *Pseudostichococcus* MOEWUS, *Desmococcus* F. BRAND, and *Diplosphaera* BIALOSUKNIA. This was done based on morphological and molecular sequence data, focusing heavily on differences in ITS-secondary structure, since, again, introns in the 18S locus made it difficult to get complete and accurate sequences. *Diplosphaera*, *Protostichococcus*, and *Tetratostichococcus*

are monotypic – arguably, *Stichococcus* is also de facto monotypic. Despite extant taxa descriptions for the nine species within *Stichococcus*, the difficulties in differentiating other *Stichococcus sensu stricto* from *S. bacillaris* means that *S. bacillaris* is still the default designation, pending more precise identification, i.e., sequencing. Fortunately, the authors of the study mentioned above were able to designate an epitype (strain SAG 379-2).

I have always found the euryhalinity of *Stichococcus* to be curious since adaptation to salt is common and critical in marine environments, but in terrestrial environments, this need is less acute. Nevertheless, there are notable saline environments that do host algal communities. Some of these communities have been found to contain members of the Stichococcaceae (Sommer *et al.*, 2020, 2021). Ecologically, the Stichococcaceae are ubiquitous and well-adapted to a variety of environments, from polar to tropical regions (Neustupa, St'astny and Hodac, 2008; Hodač *et al.*, 2016; Yoon, Kim and Lee, 2020; Syuhada *et al.*, 2022). Some are well-known as lichen symbionts (Delmail *et al.*, 2013; Candotto Carniel *et al.*, 2015; Hanley *et al.*, 2019) and are also known to be euryhaline and acidotolerant, albeit not *halophilic* (Hayward, 1974; Ahmad and Hellebust, 1984; Hellebust, 1985; Novis, Beer and Vallance, 2008). Thus, they have attracted some attention as biotechnology algae for biodiesel and bioremediation (Iwasaki *et al.*, 1998; Moazami-Goudarzi and Colman, 2011; Olivieri *et al.*, 2011; Makaroglou *et al.*, 2021). I chose to focus on the effects of desiccation and salinity on the Stichococcaceae, as the former is fundamental to life in terrestrial habitats, and the latter shows promise for environmental applications (see **Section 4.4, Chapter 5**).

2 Description of research aims

The work cumulating in this thesis aimed to determine the relationship between the ecophysiology of the Stichococcaceae to their taxonomy. In short, there were three main questions to answer:

Q1. Is it possible to determine taxa using compatible solute expression alone?

Q2. How does the potential for halotolerance affect recovery from desiccation, and are there taxa-specific responses to be observed?

Q3. Is there differential halo- and desiccation tolerance in the Stichococcaceae? If so, which genera distinguish themselves?

To the first: many taxa within Stichococcaceae are challenging to identify morphologically because of their simple morphology, as described in **Section 1.5**. There are few distinguishing features, and a comparison is most straightforward when multiple taxa are available. Of this family are *Pseudostichococcus*, *Diplosphaera*, and *Desmococcus* are the easiest to identify *ad hoc* because they are morphologically distinct from *S. bacillaris*. Although molecular identification is a popular tool, many strains within the Stichococcaceae are notoriously difficult to sequence due to large introns. Using only conserved regions within the 18S/*rbc*L loci mostly does not delimit taxa to a sufficient degree (Pröschold and Darienko, 2020). Large introns in several strains have made it difficult to align the ITS spacer regions. If it is possible to identify taxa quickly and easily via simple compatible solute analysis, it may expedite research in this enigmatic group by allowing for a more precise correlation of results to taxonomic identity.

To this aim, I used High-Performance Liquid Chromatography (HPLC) to quantify the concentrations of sucrose and various sugar alcohols. C^{13}-NMR (nuclear magnetic resonance) analysis was also used to verify the presence of osmolytes. For proline, spectrophotometry was used to determine concentrations from algal biomass. Proline has been correlated with a protective response against various stressors in plants and algae (Khedr *et al.*, 2003; Ben Rejeb, Abdelly and Savouré, 2014; El Moukhtari *et al.*, 2020), and I hoped that quantifying proline may lead to improved knowledge of how halotolerant and halosensitive strains react to salinity. Authentic strains were favored for the studies done here, as they can directly tie taxonomic identity to physiological responses. However, since it was impossible to

find authentic strains for every taxon, I used already established unialgal culture strain instead to ensure genetic homogeneity. Through the very generous contributions of several culture collections and private scientists worldwide, I was able to collect 69 strains to combine with the strains previously established at the University of Rostock that made up the "*Stichococcus*" pool (Appendix, Table1).

In addition to using established unialgal strains from late 2019 to early 2020, I also attempted to epitypify *S. bacillaris* using materials collected from Zürich, the type locality, as no extant *S. bacillaris* strains fully satisfied the ICBN standards for an epitype – i.e., validly published and declared as such at this time.

The second research question expands on the insight won from the first study, since it was clear that salinity affected osmolyte production in *Stichococcus.* Because salinity is analogous to desiccation in affecting water balance, I wanted to determine if the ability to produce high concentrations of osmolytes indicates higher desiccation tolerance. Chlorophyll fluorescence was used as a proxy to determine the stress response under desiccation, measured with a Walz PAM 2500 Chlorophyll Fluorimeter, following many previously done protocols with some minor modifications (Karsten, Herburger and Holzinger, 2016; Medwed *et al.*, 2021; Terlova, Holzinger and Lewis, 2021). A schematic of desiccation chambers is shown in **Fig. 4** below. All fluorescence values were standardized to chlorophyll *a* concentration of the respective strains.

The final research question attempts to contextualize the first and second questions. As cosmopolitan algae, they have been found in diverse ecosystems worldwide. If there is a taxonomic link between abiotic stressors and strain identity, it is reasonable to ask whether the taxa can exploit different ecological niches. A parallel set of desiccation experiment with saline and nonsaline cohorts was set up to see the direct effect of saline stress on recovery ability. For clarity, a schematic of the general setup to establish the saline and nonsaline cohorts is shown in **Fig. 5**.

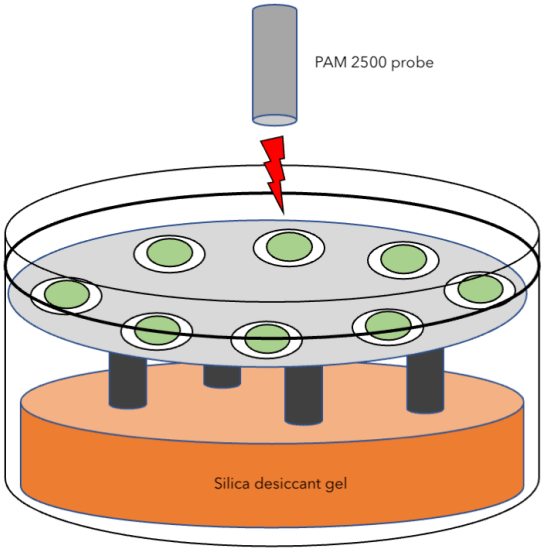

Figure 4 The basic setup of the PAM dehydration and rehydration experiment. Polypropylene chambers were filled with silica gel during the dehydration phase and with the equivalent volume of water in the rehydration phase. The PAM probe was always kept at 7mm from the algal filters to avoid inconsistencies in measuring light conditions. Eight algal filters were used per box, clustered around the rim.

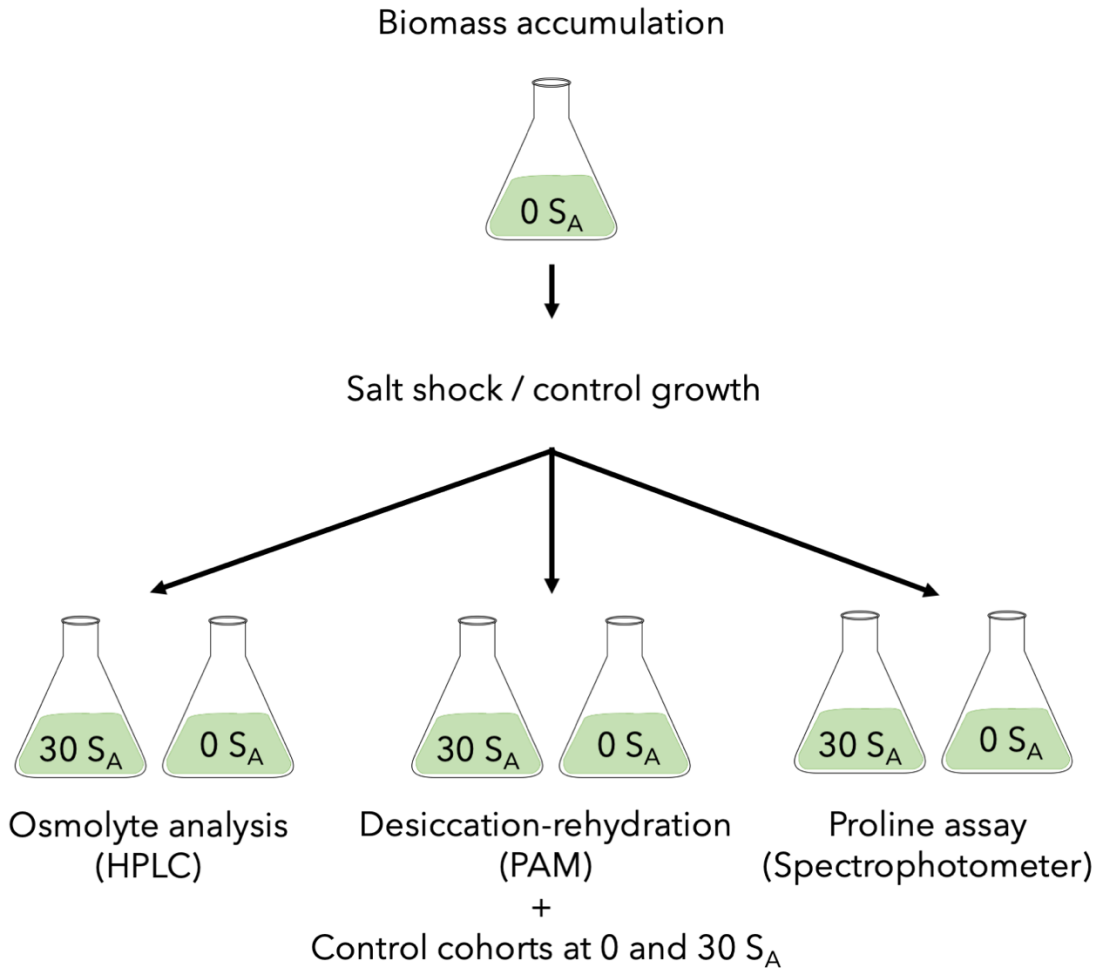

Figure 5 Establishing saline and nonsaline algal cohorts for the work done in the three manuscripts, where the effects of 0 and 30 S_A were to be quantified. Unialgal strains were always used to ensure reproducibility and reduce the risk of contamination. Physical resources limited the number of strains able to be analyzed, hence why there was an emphasis on using representative type strains.

3 Summary of experimental results

The work in the first manuscript characterized the osmolyte expression pattern of selected Stichococcaceae, particularly the new-described genera in 2020 (Pröschold and Darienko, 2020), to determine their relation to *S. bacillaris*. The results showed that all the Stichococcaceae strains studied were characterized by the production of sorbitol and sucrose in high amounts, as is typical of *Prasiola*-clade algae (Gustavs, Görs and Karsten, 2011; Hotter *et al.*, 2018). Furthermore, based on ecophysiological and molecular data, two strains formerly within *Stichococcus* were formally transferred into the genus *Pseudostichococcus* (*P. sequoieti*, *P. undulatus*).

The second manuscript examined the secondary metabolite changes induced by saline stress in a salt-growth challenge. The strains' responses to rapid desiccation and subsequent rehydration were quantified with a PAM fluorimeter. The results showed that although compatible solute production is dramatically affected by salinity, this innate osmotic protection ability is independent of the mechanisms affecting desiccation tolerance when not directly stimulated. Furthermore, halotolerance is more variable than desiccation tolerance within the Stichococcaceae; *Pseudostichococcus* may have the ability to exploit different ecological niches due to their higher halotolerance.
The final manuscript expanded upon the findings of the second manuscript and studied the synergistic effect of salinity on desiccation, focusing on the taxa within *Pseudostichococcus*. The results showed *Pseudostichococcus* strains were able to recover fully after desiccation, with and without salinity stress, unlike the other strains tested. This was correlated with increased proline production under salinity and higher proportion of proline to sorbitol ratio compared to other Stichococcaceae strains. In the other strains, increasing salinity reduced their ability to withstand desiccation. The experiment allowed for a direct correlation between salinity, compatible solute production, and desiccation tolerance.

The main conclusions resulting from the biodiversity and ecophysiology investigations and additional nonpublished research are summarized and discussed in **Chapter 4,** and perspectives for future research are provided.

4 General Discussion

It was possible to significantly contribute to the existing knowledge on the Stichococcaceae, especially since most work has almost exclusively been done on strains identified as *S. bacillaris* (Hayward, 1974; Brown and Hellebust, 1980; Hughes, 2006). The suggested follow-up experiments outlined in the following sections will address some problems that arose during the thesis work.

4.1 The concept of *S. bacillaris*

Before the taxonomic revision of *Stichococcus* into the "-*stichococcus*" genera (Pröschold and Darienko, 2020), one of the primary problems in working with *Stichococcus* was the lack of an actual holotype specimen. While the holotype exists, it is quite literally a drawing from a publication from Carl Nägeli (Nägeli, 1849) (**Fig. 2**) and thus unsuitable for molecular or morphological work. However, the "missing" type strain problem has resulted in many green algal strains with rod-shaped, shortly-filamentous morphology sequestered into *S. bacillaris* – regardless of their actual identity. This problem has been extended into the molecular realm, where gene sequences usually lack accompanying pictures or links to sources with a morphological description altogether.

In an attempt to solve this problem, I researched the history of Nägeli and discovered that he was a Swiss-born botanist that spent many of his productive years as a professor of botany at the University of Zürich (b. 1817, d. 1891) (Hoppe, 1997). Ideally speaking, material isolated from the *locus typicus* that corresponds to the original description of the organism-to-be-epitypified is the strongest candidate for an isotype, defined by the International Code of Botanical Nomenclature, Section 2, Article 9.9 (Turland *et al.*, 2018). *Stichococcus bacillaris* was described in 1849, around the peak of his productive years as a professor. Because the University of Zürich was close to the Old Botanical Garden, I conjectured that he might have spent considerable time there in a professional and personal capacity. *S. bacillaris* has no specific locality description, so it is possible that its original material may have been sampled on short excursions within the botanical garden. Furthermore, the botanical garden has existed in a relatively unchanged state since Nägeli's lifetime when compared to the rest of Zürich.

In July 2019, I acquired, by a generous donation, material sampled from the Old Botanical Garden (**Appendix, Figures 1-5**). After several rounds of isolation by enrichment cultures on standard agar medium (3N Bold's Basal Medium + V, 1.5%

agar), followed by successive streak plating until unialgal cultures that looked similar to *S. bacillaris* were established. However, much of the work done in taxonomy results from serendipity, especially for microscopic organisms that cannot be screened in the field. Unfortunately, luck eluded me in the search for *S. bacillaris* – instead, what was found was *Pseudostichococcus monallantoides* (**Fig. 6**) according to the 18S sequences. Morphologically, the three epitype candidate strains (Z3.1.0, Z3.1.1, Z3.2.0) resembled the *monallantoides* morpheme. These cultures were also used because they were the first to become unialgal.

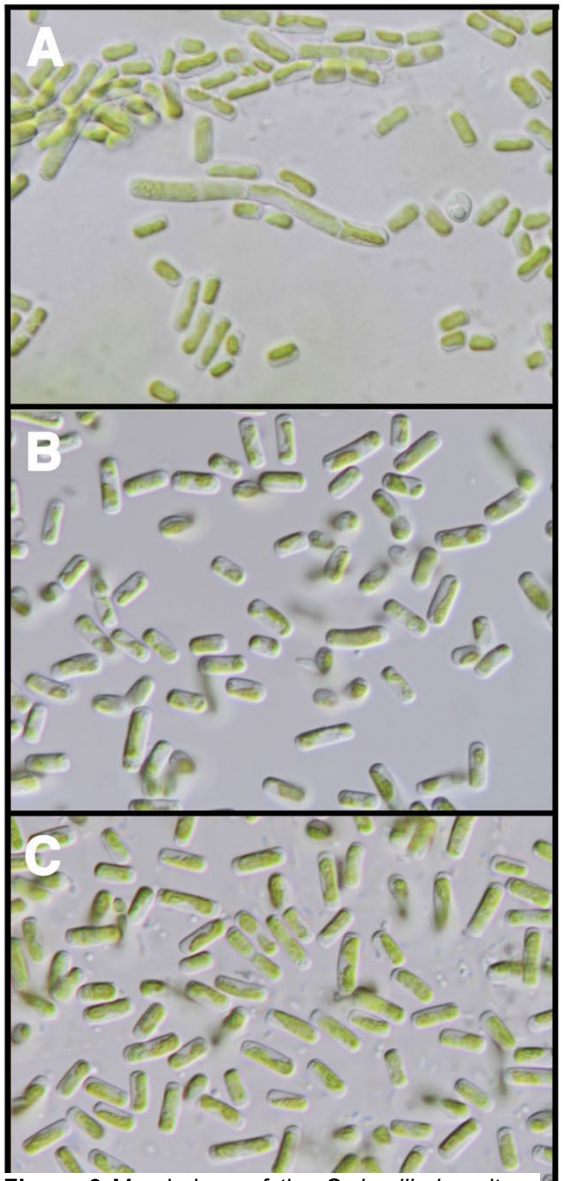

Figure 6 Morphology of the *S. bacillaris* epitype candidate strains Z3.1.0 (A), Z3.1.1 (B), and Z3.2.0 (C), which belonged to *Pseudostichococcus* cf. *monallantoides* after molecular confirmation. Scale bar = 10µm.

Our picture of algal diversity is lacking through the bottleneck of sequencing difficulties and cultivation ability. *S. bacillaris* has been reported in a wide range of environments, from temperate to polar (Häubner, Schumann and Karsten, 2006; Hodač *et al.*, 2016), so the likelihood of it being found in Zürich was quite good. The stress experiments showed that under nonsaline desiccation stress, *Stichococcus* and *Pseudostichococcus* are virtually identical in desiccation and recovery performance. However, it is possible that *S. bacillaris*, which grows well with triple nitrate Bold's Basal Medium (Bischoff, 1963), faces undue competition from other green algae inhabiting quite literally the same ecological niche under enrichment culture conditions. The growth of microalgal species is differentially favored depending on the nutrient composition of culturing medium. Bold's Basal Medium is selective for generalist and fast-growing algae rather than rare or difficult species (Andersen, 2005). For example, *Apatococcus* BRAND is one of the most common aeroterrestrial algae and dominates in biofilms but grows

slowly in culture; as a result, it has been less studied than other taxa (Gustavs *et al.*, 2016; Sanmartín *et al.*, 2020).

I observed that during initial cultivation on 3N BBM on agar with the type material, small algal colonies were visible within one week of enrichment, but they consisted mostly of other common algae, such as *Chloroidium* NADSON and *Scenedesmus* MEYEN, as well as some cyanobacteria. After two to three weeks, the first few *Stichococcus*-like cells were visible, which proved later to be *Pseudostichococcus*. This was also the case when I tried to find *Stichococcus* in soil crusts by enrichment cultivation on agar. Although *Stichococcus* is also ubiquitous and grows well as unialgal cultures in both solid and liquid 3N-BBM, it is possible that it is overtaken by other strains so that *Stichococcus* is harder to isolate from environmental material than expected. One recent study discovered *Stichococcus*-like strains by chance when it survived enrichment media treated with cycloheximide (CHX), used explicitly to eliminate microalgae and fungi in bacterial cultures (Syuhada *et al.*, 2022). A follow-up round of cultivation with CHX should be done to see if I can select for stichococcaceaen strains from environmental samples. For now, the case of the missing isotype from the type locality remains, pending patience and luck, unresolved.

For phylogenetic research purposes, an epitype for *S. bacillaris* has been designated as strain SAG 379-2, deposited at the Culture Collection of Algae at Göttingen University (SAG). This is a German strain, despite there being several Swiss sister strains; it is unclear to me why this was done. Nevertheless, it is now possible to (re)classify rarer taxa of *Stichococcus*, such as *S. mirabilis* LAGERHEIM and *S. lacustris* CHODAT under the new taxonomic schema. As *Stichococcus* is currently monotypic with *S. bacillaris*, with no molecularly confirmed sister taxa, there is plenty to be done in the realm of beta taxonomy.

4.2 The revision of *Pseudostichococcus*

It is somewhat a stereotype in microalgal research that all “green balls” look the same, and this is most certainly a valid saying about *Stichococcus*. Nevertheless, several strains belonging to *Pseudostichococcus* were consistently easier to identify than the other “*Stichococci”* based on their morphology. The cell form is usually longer (> 10µm), squarer, the chloroplasts are often more complex or elongated, the chloroplasts sometimes have a granular appearance, and vacuoles are sometimes visible in older cells (**Fig. 7**). *Pseudostichococcus* was discovered initially as an endosymbiont of “*Enteromorpha”* (*Ulva*) *compressa*, which puzzled its original isolator as to its taxonomic status because it possessed traits of

Stichococcus, *Monallantus* PASCHER, and *Hormidium* (LINDLEY) G. HEYNOLD. After extensive observation following isolation and cultivation in several media, Moewus decided to place the strain into its own genus with an epithet alluding to its enigmatic morphology: *Pseudostichococcus monallantoides*.

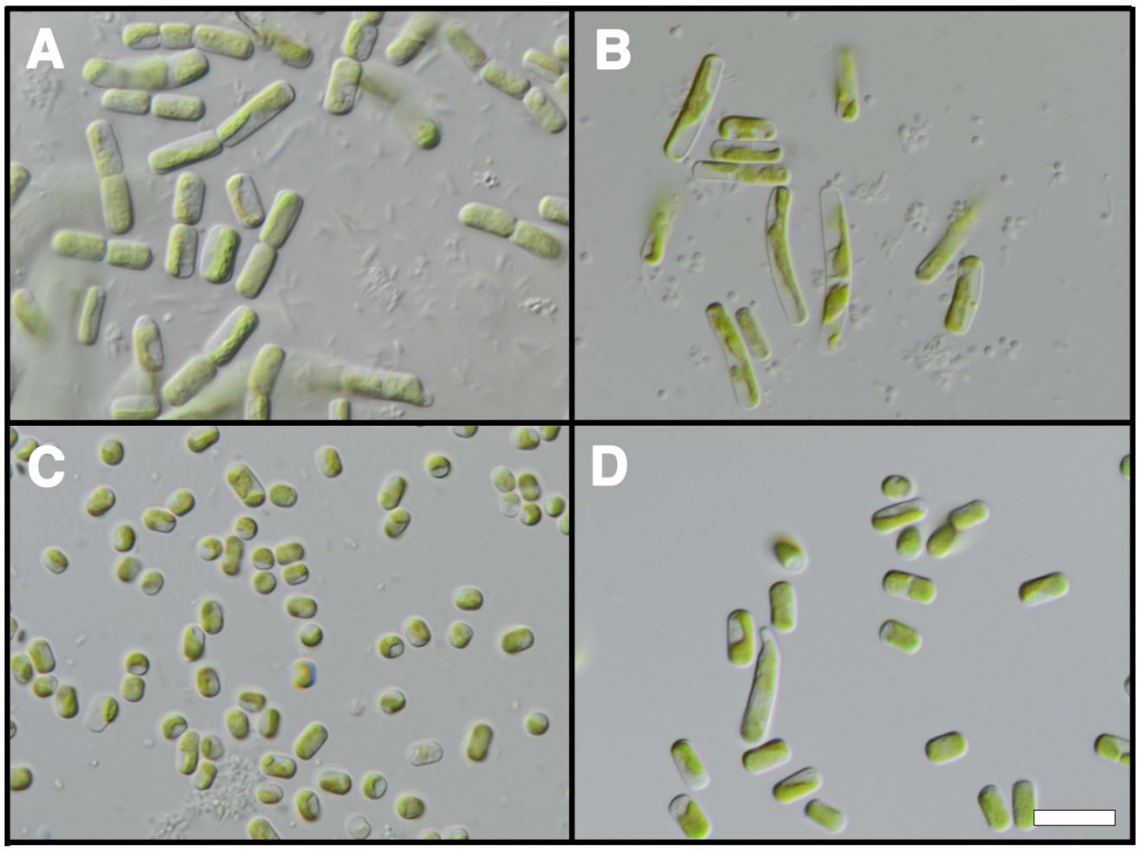

Figure 7 Morphology of strains within the genus *Pseudostichococcus*. A) CALU-1142 *P. undulatus*, B) LB 1820 *P. sequoieti*, C) ASIB-BS-658 *Pseudostichococcus* cf. *undulatus*, D) TT-5-1-K *Pseudostichococcus* cf. *monallantoides*. Scale bar = 10µm.

P. sequoieti is the most easily recognizable taxon from this genus, with expansive, curved cells, large vacuoles taking up ½ to 2/3 of cell space, and bright green parietal chloroplasts that lack a visible pyrenoid body or nucleus. It was precisely this general habitus that indicated very early on that specific "*Stichococcus*" strains may not belong in the genus. In the original description of *S. sequoieti*, this species was compared with several other long *Stichococcus* strains, such as *S. undulatus* VINATZER and *S. mirabilis* for their physical similarity (Arce, 1971). Unfortunately, the strain *S. mirabilis* is no longer extant, so it could not be sequenced. Analysis of *rbc*L and 18S sequences confirmed that the strains *S. sequoieti* and *S. undulatus* from are distinct *S. bacillaris* and belong to *Pseudostichococcus* (**Manuscript 1**).

However, morphology is not always reliable for identification in this genus. Recent work by Sommer et al. 2020 isolated several strains initially identified as

Stichococcus based on morphology but were later shown to be *P. monallantoides*. This was the case for one strain studied in this thesis: ASIB-BS-658 cells are compact (< 5 µm in *l*, *w*) and cylindrical (**Fig. 6c**), somewhat reminiscent of *S. bacillaris* or *Tritostichococcus*. ASIB-BS-658 was originally identified as "*Stichococcus* sp." but consistently segregated with the other strains that now comprise *Pseudostichococcus* – *P. monallantoides*, *P. sequoieti*, and *P. undulatus*. There is a single base pair difference between the other three strains and no difference in the diagnostic V9 Helix 49 region, which does not constitute sufficient support for creating a separate taxon with the tools currently available. Thus, it is highly probable that this genus exhibits cryptic diversity, overlapping with *Stichococcus* and *Tritostichococcus*.

4.3 The effects of salinity and desiccation

Visible changes in cultures and growth rates along a salinity gradient were observed for several strains of the Stichococcaceae family. Their vitality was measured primarily by color, because if the photosynthetic apparatus is undamaged, then cells can recover rapidly (Gray, Lewis and Cardon, 2007; Gasulla et al., 2009; Ritchie and Heemboo, 2021).

The short- and long-term growth data indicated some broad taxonomic trends in this family's ecophysiology (**Q3**). The growth experiments were divided into long-term salinity and short-term shocks. For observing the long-term effects of salinity, strains were subjected to eight weeks of growth in seven media ranging from 0 – 90 S_A to span the range of nonsaline to three times that of natural seawater (**Fig. 8**). Short-term salt shocks were used when high amounts of biomass (ca. 5-10 mg dry weight) were needed within a short timeframe, i.e., for HPLC and proline analysis, as salinity inhibits growth in freshwater algae (Brown and Hellebust, 1980; Fujii *et al.*, 1999); this consisted of raising the salinity of standard 0 S_A medium to 30 S_A (**Fig. 3**).

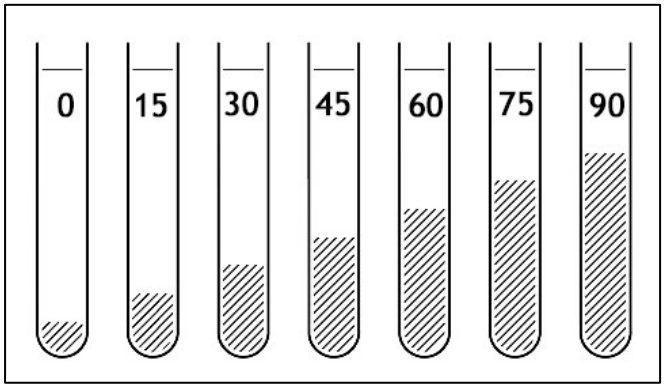

Figure 8 Visual schematic of the salinities in S_A used in the growth experiments. Biomass from each strain was inoculated into a series of seven test tubes to span the salinity range of 0-90 S_A. Filled lines represent relative salinity.

First, salinity was a weak stressor for the Stichococcaceae (**Manuscript 2**). Euryhalinity has long since been reported in

Stichococcus and *Pseudostichococcus.* Although reduced biomass was visible along the salt gradient in all strains, there was good survival of all strains at 30 S_A, with many of the strains investigated showing tolerance up to 60 S_A and some beyond (**Manuscript 2, Fig. 2**). The most obvious physical effects of salinity were bleaching, plasmolysis, and vacuole formation. Unlike in streptophyte algae or other chlorophytes (Chen, Jiang and Wu, 2009; Farghl *et al.*, 2015; Arc *et al.*, 2020), no signs of lipid accumulation were visible. **Fig. 9** illustrates the visible differences between a halotolerant and halosensitive strain, in this case *P. sequoieti* and *D. tetrallantoideus* strains.

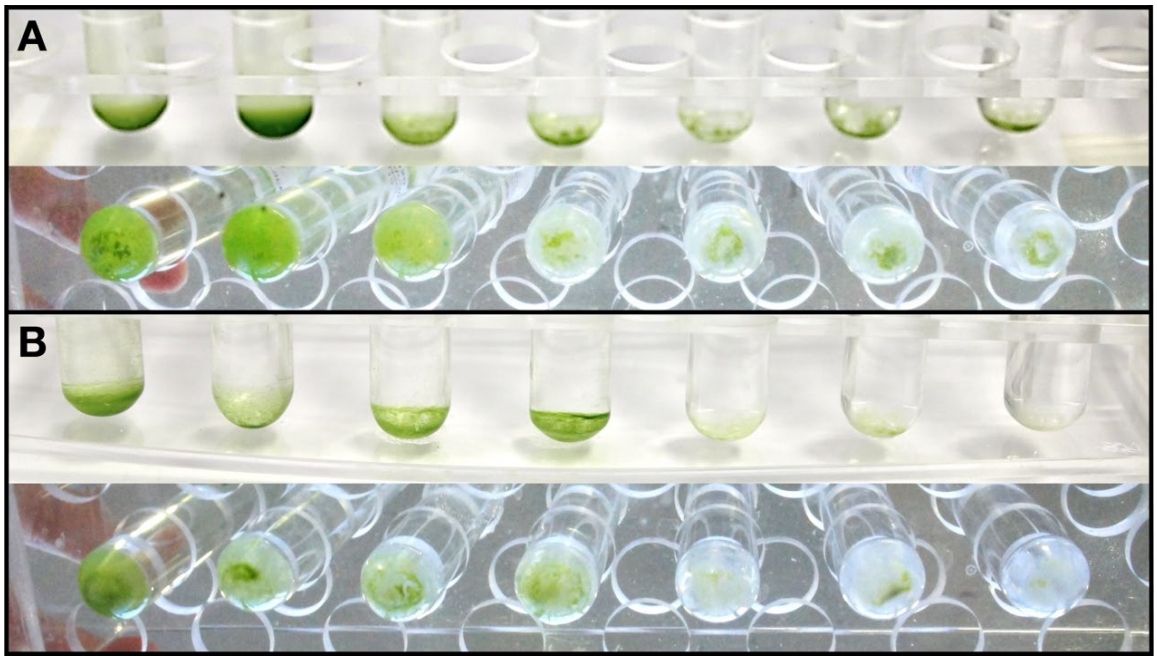

Figure 9 Example of a halotolerant strain (LB 1820, A) and halosensitive strain (ASIB-IB-37, B) at the end of 8 weeks of growth. Tubes correspond to the salinities shown in **Fig. 5** ranging from 0 to 90 SA from left to right. Biomass was markedly reduced at higher salinities. In ASIB-IB-37, cell death (bleaching) was visible starting at 60 S_A, while in LB 1820 growth was retarded but did not halt.

The most halotolerant strains (*Tritostichococcus*, *Pseudostichococcus*) produced proline as their main osmolyte instead of sorbitol and sucrose under salt shock, as depicted in **Fig. 3 (Q3)**. This response is comparable to other euryhaline algae, such as *Chlorella autotrophica* SHIHIRA & R. W. KRAUSS (Ahmad and Hellebust, 1984), *Neochloris oleoabundans* S. CHANATACHAT & BOLD (de Jaeger *et al.*, 2018), and *Picochlorum* W. J. HENLEY & AL. (Foflonker *et al.*, 2016). Proline production is a widespread stress response among plants and algae because it is biologically inexpensive to produce, stimulates or induces stress-protective proteins (Brown and Hellebust, 1978; Khedr *et al.*, 2003; Ben Rejeb, Abdelly and Savouré, 2014), or even quench ROS (Szabados and Savouré, 2010). While the experiments in this study were conducted at a maximum of 30 S_A, previous results from **Manuscript 2** demonstrated that *Pseudostichococcus* and *Tritostichococcus* can grow at

salinities up to 75 S_A. Furthermore, foundational research has recorded *Stichococcus* surviving up to roughly 120 S_A (Hayward, 1974; Brown and Hellebust, 1978). Strains that produce more proline under salinity may be able to better adapt to harsh environmental conditions. However, because halotolerance is complex, increased proline production should not be attributed as the prime protective mechanism. In terms of osmolyte production in response to salinity, there are usually multiple compounds that contribute to maintaining intracellular osmotic balance, such as various sugar alcohols, polysaccharides, amino acids, and glycine betaine (Karsten *et al.*, 2007; Darienko *et al.*, 2010; Kaplan *et al.*, 2012). Furthermore, halotolerance can be genetically regulated via genes coding for active NA^+ pumps or for membrane-stabilizing fatty acids; the 'omics' have begun to provide more insight into halotolerance mechanisms in algae (Gimmler, 2000; Arora *et al.*, 2019; Calhoun *et al.*, 2021). More research is needed on the molecular-level responses to salinity in algae beyond the strains typically favored in biotechnology, such as *Dunaliella* Teodoresco, *Haematococcus* C. Agardh, *Chlorella* Beijerinck*,* and *Nannochloris* Naumann*.*

As desiccation and salinity are both linked to water balance, it was expected that strains with higher halotolerance would also be more desiccation resistant. Halotolerance in the Stichococcaceae was positively correlated with increased osmolyte production and better survival in highly saline media (**Q3**). However, this innate protective mechanism was not correlated with better desiccation tolerance (**Fig. 10; Q2; Manuscript 3**). The desiccation experiments consisted of rapid dehydration (4-6 h) down to circa 10% relative humidity (RH) followed by rapid rehydration to 100% RH for 24 h. The effective quantum yield of photosystem II (Y(II) was measured as a proxy for cell health. Halotolerant strains did not achieve higher end recovery values than halosensitive strains (**Manuscript 3, Fig. 3**). A salt shock reduced the ability of certain strains (*Desmococcus olivaceus*, *Tritostichococcus solitus*, *P. undulatus*) to recover from desiccation. However, these strains already had lower initial Y(II) values at both salinities, which suggests that salinity was not the direct cause for lower recovery. In absolute values, the Stichococcaceae performs similarly in the desiccation experiment without salinity stress to other aeroterrestrial algae, such as *Klebsormidium* (Karsten, Herburger and Holzinger, 2016), *Scenedesmus, Chlorella,* and *Bracteacoccus* TEREG (Gray, Lewis and Cardon, 2007), able to achieve ca. 80-100% of the original Y(II) values.

Because the experimental setup of the desiccation experiment involved exclusively using cells grown in liquid cultures, the sudden transition to a dry environment (fiberglass filters) may have caused additional stress. To increase the extrapolation

potential of the results, a better setup may be to perform desiccation and PAM measurements directly on cultures maintained on agar. First, this eliminates the possible shock of moving between media. Second, perhaps some aeroterrestrial algae will perform more accurately if grown in an aeroterrestrial-like environment versus aquatic, even if most soil protists are adaptable to both liquid and solid media and can grow well in both. Furthermore, extremely rapid desiccation of cells (within 4-6 h) to 10% relative humidity (RH) is unrealistic except in harsh desert environments coupled with solar radiation, such as in the Atacama (Jung *et al.*, 2020). The speed of dehydration influences recovery because slow dehydration results in faster recovery than rapid desiccation (Gray, Lewis and Cardon, 2007; Yuqing *et al.*, 2021). Third, self-shading effects from cells forming a denser layer may increase the length of time that a biofilm is able to survive, compared to their distribution in thin suspensions. A second dehydration experiment using agar-plated algae should be done to compare the effect of slower desiccation to the rapid desiccation done here, which may provide additional insight into the strains' recovery potential.

Finally, the results from osmolyte quantitation showed that this method is of limited value in Stichococcaceae chemotaxonomy at the species level *per se* because there were no differential qualitative responses between the strains (**Q1**; **Manuscripts 1, 2**). All the strains produced proline, sorbitol, and sucrose, and the absolute concentrations increased under salinity. However, this osmolyte profile is one of the only stable physical autapomorphies of the Stichococcaceae and supports their monophyly since distinguishing physical characteristics often overlap in this genus (Pröschold and Darienko, 2020). I aimed to use osmolytes to assist in taxonomic assignments. Strains lacking the Stichococcaceae-specific osmolyte profile may be excluded from the Stichococcaceae when the molecular data is ambiguous. An example of the use of the osmolyte profile to exclude strains from the Stichococcaceae is *Gloeotila contorta* (LEMMERMANN) CHODAT. The first study included a strain named *G. contorta* in the chemotaxonomic analysis. It was assumed that most *Gloeotila* strains, based on morphological and historical similarity to *S. bacillaris*, may belong to the Stichococcaceae. I measured the osmolyte profile of the *contorta* strain and observed a distinct lack of sorbitol, which excluded it from the clade; this was later confirmed after (several rounds) of molecular sequencing.

Previous studies in osmolyte chemotaxonomy have demonstrated similar taxonomic resolution. Several "*Chlorella*" species were reassigned to *Chloroidium* after an integrative analysis, in which the presence of an unusual sugar alcohol

helped to bolster the diagnosis and subsequent taxonomic transfer (Darienko *et al.*, 2010). One project within the Water Framework Directive analyzed the pigments ratios extracted from water samples and was able to correlate them to phytoplankton community composition dynamics; results showed that chemotaxonomy is a promising tool for biomonitoring purposes (Tamm *et al.*, 2015). Thus, the actual utility of chemical analyses may lie in its speed and ease when molecular methods are impractical or fail (**Q1**).

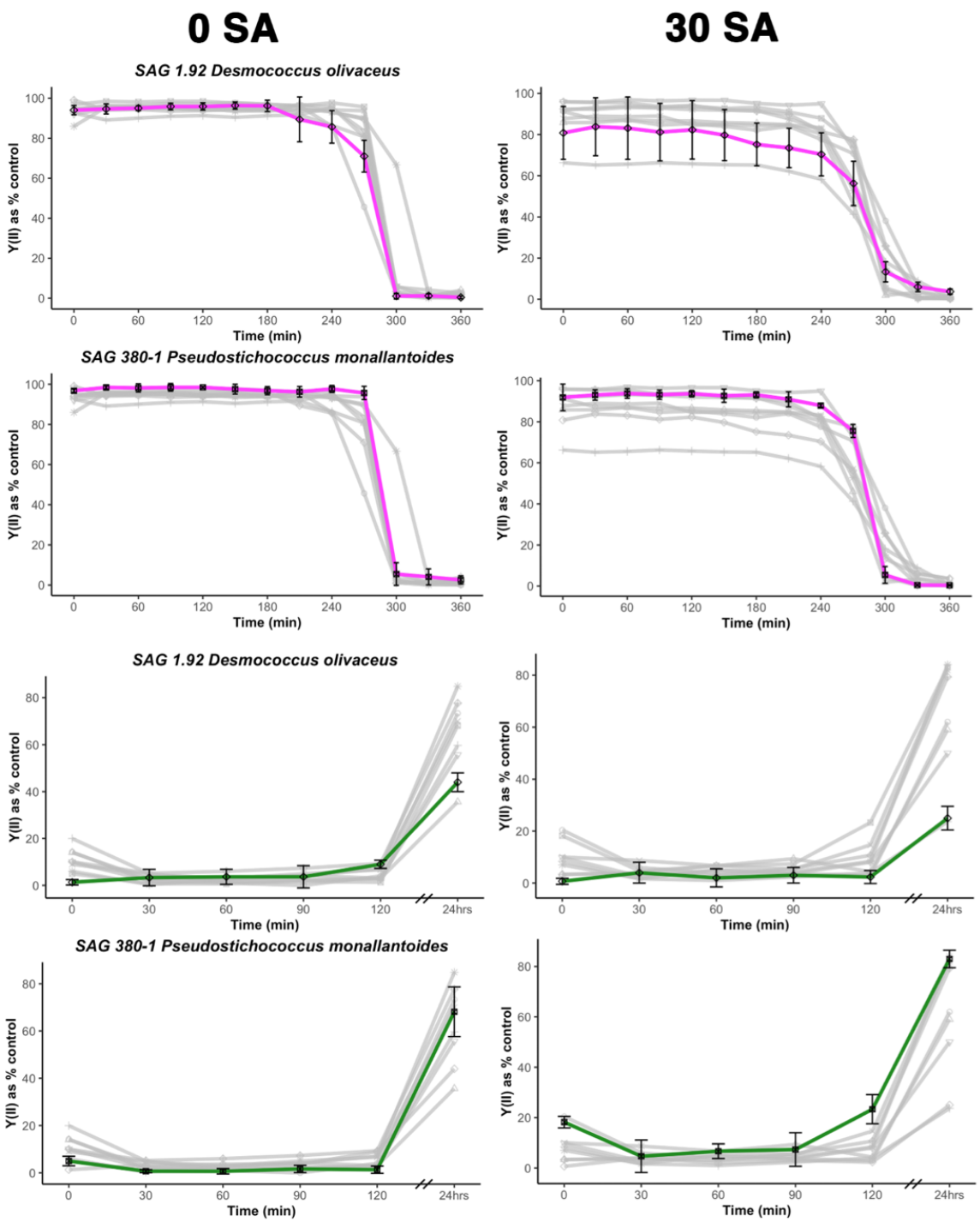

Figure 10 Comparison of Y(II) after desiccation and rehydration in halosensitive (SAG 1.92) and halotolerant (SAG 380-1) strains grown in 0 S_A and 30 S_A media. Magenta lines = average effective quantum yield (Y(II)) per strain during dehydration; green lines = average recovery Y(II); grey lines in the background represent the other strains for comparison. Error bars represent standard deviation (*n* = 3).

5 Conclusion and Outlook

Taxonomy is at once concrete, because of its strict hierarchies, and abstract, because the processes of speciation are ongoing.

As a taxonomist, I believe that there is both beauty and necessity in addressing things by their correct classifications. Just as with any other branch of biological research, being able to precisely attribute the responses of research organisms to a taxonomic entity allows the results to be properly compared and replicated. And as an ecologist, I would like the results of my own taxonomic research to have practical impact.

For example, the strain called *Stichococcus sequoieti* would still retain it innate characters even if it is named *Pseudostichococcus sequoieti*, but this change implies that there is a measurable and adequate difference between the two genera. In the case of the very closely related Stichococcaceae, the differences are subtle but important since to extrapolate responses in a laboratory setting to natural environments. In this work, I have nearly always referred to the Stichococcaceae as one homogenous group throughout this thesis, except for *Pseudostichococcus*. It would be difficult to justify doing otherwise, as the genera are extremely closely related, and genetic delimitation of them has relied on a hodgepodge of methods, such as combing *rbc*L, secondary ITS-2 structures, and biogeographical data (Pröschold and Darienko, 2020). Physiological data helps to tie genetic differences to ecology and allows us to understand where and why strains occur and how they affect the local biomes.

The first task I have at hand is to try to isolate the isotype for S. bacillaris again, as now it seems possible to select for Stichococcaceae in culture and not just *Pseudostichococcus*. Second, I will continue to work on taxa assignment for the 33 "*Stichococcus* sp." strains remaining in the *Stichococcus* pool (**Appendix, Tab. 1**) now that there is a basis for more precise identification. Third, I will try to explore in more detail the cryptic diversity in *Pseudostichococcus*. As with *S. bacillaris*, most strains in this genus are currently assigned to "*P. monallantoides*"; it remains to be seen if *P. monallantoides* is remarkably morphoplastic or underdescribed. A new genotyping method called nanopore sequencing may offer a possible solution to the introns problem that makes working with stichococcaceaen DNA frustrating. Nanopore sequencing is fast, does not rely on PCR amplification, and is inexpensive compared to traditional High Throughput Sequencing (HTS) methods

such as Illumina. The drawback of a rather high error rate (1-6%) compared to ultra-accurate Sanger sequencing; however, this is rapidly being improved upon (Wang *et al.*, 2021). Better genotyping will have direct applied use for the Stichococcaceae in the biotechnology sector, as some strains are easy to maintain in culture, grow in bioreactors, halotolerant and show promise as a bioremediating agent for salt-affected environments as part of young colonizing biological soil crusts (Sommer *et al.*, 2021).

Up to now, the most singularly successful 'alga' in the algal biotechnology sector is a cyanobacterium, *Arthrospira* SITZENBERGER EX GOMONT (spirulina), with *Chlorella* being the only truly famous chlorophyte (Fabris *et al.*, 2020). This sector has become a multibillion-dollar industry designed to exploit the productive capabilities algae, where new growth methods and candidate species are continuously tested. Even when the biotechnological possibilities of are disregarded (**Tab. 2**), there is an astonishing knowledge gap on the Stichococcaceae, and indeed in green microalgae in general.

Table 2. Common general usages of microalgae in the biotechnology sector. Cases where specifically *Stichococcus* strains have been used are underlined.

Domain	Usage	Reference
Fatty acid production	Biodiesel production Biolipid production	Olivieri *et al.*, 2011; Gargano *et al.*, 2016
Pharmacology	Antileukemic agents Vitamin E tocopherols Food and feedstock source	Sivakumar, Jeong and Jackson O. Lay, 2014; Atasever-Arslan *et al.*, 2016; Ścieszka and Klewicka, 2019
Bioremediation	Heavy metal remediation Wastewater treatment Desalination	Bwapwa, Jaiyeola and Chetty, 2017, 2017; Figler *et al.*, 2019; Kaushik and Raza, 2019

Finally, a fourth task that is more like a goal is to study the distribution of this family, or even the Trebouxiophyceae in whole, in biocrusts worldwide. Biocrusts make up to 70% of the plant cover in arid and semiarid biomes and are responsible for ~ 40% of terrestrial biological nitrogen fixation (Weber *et al.*, 2015; Bowker *et al.*, 2018); the Stichococcaceae have been found to be present in biocrusts (Mikhailyuk *et al.*, 2019; Jung *et al.*, 2020; Samolov *et al.*, 2020), but as identification of cultures “by hand” is prone to sequencing and cultivation bias, HTS methods may be the next best way to characterize microbial communities, assuming there is an accurate genetic and taxonomic framework. Currently, large-scale environmental surveys using HTS are mostly being done at taxonomic levels of class, order, even phylum instead of species and genera (Chilton, Neilan and Eldridge, 2018; Palinska, Vogt and Surosz, 2018; Pushkareva *et al.*, 2021; Glaser et al. 2022, in review). Studies on the algal biodiversity using the HTS approach have given a broad picture, but more specific taxonomic knowledge about individual players in microbial communities will allow for a better understanding of ecosystem dynamics. Future researchers may well uncover more secrets by turning to the unassuming green microalgae.

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Appendix

Appendix Figure 1. All material was graciously collected on 19.7.2019, by Prof. Dr. Christoph Scheidegger of the WSL (Eidgenössische Forschungsanstalt für Wald, Schnee und Landschaft), CH-8903 Birmensdorf, Switzerland.

Locality: Switzerland, canton of Zürich, Zürich. Old Botanical Garden at Schanzen. N-exposed slope, patchy stand of park trees. Coordinates, 420 m a.s.l.:

(CH1903 / LV03) 682705 247305
(WGS 84) 47.37139 N, 8.53367 E

Appendix Figure 2. Small piece of rotten wood lying on the ground between tall park trees 5m north of memorial of Johannes Hegetschweiler, 14.12.1789 (Rifferswil) bis 9.9.1839 (Zürich). Collected and marked as “Tube 1”.

Appendix Figure 3. Lying trunks along the border of a visitor's path, 8 m N of memorial of Johannes Hegetschweiler. Collected and marked as "Tube 2".

Appendix Figure 4. Bark of *Taxus baccata*, 17 m E of memorial of Johannes Hegetschweiler. Collected and marked as “Tube 3”.

Appendix Figure 5. Piece of bark of Taxus baccata lying on the visitor's path, 1 m E of memorial of Johannes Hegetschweiler. Collected and marked as "Tube 4".

Appendix 1, Table 1. A listing of the strains investigated over the course of this thesis, with information as available listed. Unfortunately, some strains lacked metadata entirely as to their locality and collector. Although ultimately not all strains were used, all these strains provided insight into the morphological plasticity and ecophysiogical traits of the Stichococcaceae. Blanks indicate unavailable metadata. "≡" and "=" indicate homotypic and heterotypic synonyms, respectively.

STRAIN NO.	STRAIN	STRAIN ORIGIN	COLLECTOR/ISOLATOR
SAG 1.92	*Desmococcus olivaceus**	Vienna, Austria; subaerial	W. Vischer, before 1960
ACOI-1475	*Desmococcus vulgaris*	Coimbra, Igreja de Sta Cruz, Portugal; wall	M.F. Santos, 2002
SAG 10.97	*Deuterostichococcus epilithicus**	Margaraca, Portugal; pond	M.F. Santos, 1991
J 1303	*Deuterostichococcus marinus** ≡ *Stichococcus deasonii*	Dauphin Island, Alabama, USA; soil	T.R. Deason, 1969
ASIB-IB-37	*Deuterostichococcus tetrallantoides* ≡ *Stichococcus allas*	Weißkugel Peak, Ötztal Valley, Austria; soil	H. Reisigl, 1964
ASIB-BS-642	*Diplosphaera chodatii*	Switzerland	
ASIB-S-186	*Diplosphaera chodatii*	Isle of Lavsa, Jugoslavia; soil	K. Scwarz, 1975
SAG 11.88	*Diplosphaera epiphytica**	Waweira Scenic Reserve, New Zealand; lichen phycobiont	E. Tschermak-Woess, 1984
ASIB-BS-217	*Diplosphaera* sp.	Switzerland, Plau dela Rossa, schelliger; in moss cushion with *Hylocomium splendens*	W. Vischer
ASIB-BS-359	*Diplosphaera* sp.	Switzerland	
ASIB-BS-361	*Diplosphaera* sp.	Switzerland	
ASIB-BS-581	*Diplosphaera* sp.	Switzerland	
SAG 41.84	*Gloeotila contorta*	Hagen, Germany; freshwater pond	E. Hegewald, 1983
SAG 2481	*Protostichococcus edaphicus**	Swabian Alb, Germany; forest soil	L. Hodač, 2008
CCALA 496	*Pseudostichococcus monallantoides*	Slovakia, Vysoke Tatry; snowy soil	J. Komarek, 1962
TT-5-1-K	*Pseudostichococcus monallantoides*	Zielitz, Germany; potash tailing heap surroundings	V. Sommer, 2020
ZL-4-1	*Pseudostichococcus monallantoides*	Teutschenthal, Germany; potash tailing heap surroundings	V. Sommer, 2024
SAG 380-1	*Pseudostichococcus monallantoides**	Germany; marine	L. Moewus, 1951
LB 1820	*Pseudostichococcus sequoieti** ≡ *Stichococcus sequoieti*	USA; redwood forest soil	G. Arce, 1971

SY-1-2-P	*Pseudostichococcus* sp.	Shreyahn, Geramny; potash tailing heap surroundings	V. Sommer, 2018
ASIB-BS-658	*Pseudostichococcus* sp. = *Stichococcus* sp.	Blauen-Ettingen, Switzerland	W. Vischer, 1952
CALU-1142	*Pseudostichococcus undulatus** ≡ *Stichococcus undulatus**	Dolomite Mountains, Italy	G. Vinatzer, 1975
CALU-1140	*Stichoccocus bacillaris* var. *minor*	Cherepovets, Russia; soil	E. F. Safonovoy, 1994
ACOI-1477	*Stichococcus bacillaris*	Coimbra, Igreja de Sta Cruz, Portugal; wall	M.F. Santos, 2002
ACOI-95	*Stichococcus bacillaris*	Tocha, Portugal; sphagnum bog soil	M.F. Santos, 1981
BCAC 310	*Stichococcus bacillaris*	Russia	L. Gaysina
CCAP 379/1A	*Stichococcus bacillaris*	Likely Switzerland	W. Vischer, before 1936
J 1301	*Stichococcus bacillaris*	Central Bohemia, Czech Republic; soil	J. Neustupa, 1997
SAG 56.91	*Stichococcus bacillaris*	Czech Republic; freshwater	E.G. Pringsheim, before 1928
SAG 335-8	*Stichococcus bacillaris* = *Gloeotila scopulina*	Sweden; freshwater	V. Czurda, before 1945
CALU-1145	*Stichococcus* cf. *dubius*	Russia	N. V. Velichko, 1998
CCALA 727	*Stichococcus* cf. *minutus*	Svalbard, Norway; soil, subglacial	
CALU-326	*Stichococcus* sp.	St. Petersburg, Russia; soil	L. C. Podgornoy, 1964
ACSSI 273	*Stichococcus* sp.	Russia, Stavropol region; agricultural soil	A.D. Temraleeva, 2018
ACSSI 29	*Stichococcus* sp.	Tula region, Russia; gray forest soil	A.D. Temraleeva, 2019
ACSSI 84	*Stichococcus* sp.	Tula region, Russia; gray forest soil	A.D. Temraleeva, 2014
ASIB-BS-474	*Stichococcus* sp.	Waldenburg, Switzerland; cement wall of house	W. Vischer, 1944
ASIB-BS-57	*Stichococcus* sp.	Botanical Garden, Basel, Switzerland; greenhouse	W. Vischer, 1929
ASIB-BS-603	*Stichococcus* sp.	Brazil	
ASIB-BS-64	*Stichococcus* sp.	Switzerland	
ASIB-BS-670	Stichococcus sp.	Italy	
ASIB-BS-739	*Stichococcus* sp.	Switzerland	
ASIB-BS-794	*Stichococcus* sp.	Switzerland	
ASIB-IB-100	*Stichococcus* sp.	Alps, Tyrol, Austria; soil	H. Reisigl, 1964

ASIB-IB-97	*Stichococcus* sp.	Alps, Tyrol, Austria; soil	H. Reisigl, 1964
BCAC 311	*Stichococcus* sp.	Russia	L. Gaysina
BCAC 313	*Stichococcus* sp.	Russia	L. Gaysina
BCAC KIGI2	*Stichococcus* sp.	Russia	L. Gaysina
IT-058	*Stichococcus* sp.	Bruciarelli, Italy	R. Taddei
IT-102	*Stichococcus* sp.	Bagni die Repole, Italy	R. Taddei
IT-105	*Stichococcus* sp.	Sweden	
IT-106	*Stichococcus* sp.	Italy	
IT-107	*Stichococcus* sp.	(USA) Alaska	
IT-146	*Stichococcus* sp.	Italy	
IT-149	*Stichococcus* sp.	Italy	
IT-155	*Stichococcus* sp.	Italy	
IT-158	*Stichococcus* sp.	Italy	
IT-203	*Stichococcus* sp.	Gessolungo, Italy	R. Taddei
IT-211	*Stichococcus* sp.	Italy	
OD-2-1-W	*Stichococcus* sp.	Oedesse, Germany; potash tailing heap surroundings	V. Sommer, 2018
SWN 282	*Stichococcus* sp.	Vietnam, Ha Long Bay; marine waters	E.S. Gusev, 2014
TT-3-1-L	*Stichococcus* sp.	Teutschenthal, Germany; potash tailing heap surroundings	V. Sommer, 2018
TT-5-1	*Stichococcus* sp.	Teutschenthal, Germany; potash tailing heap surroundings	V. Sommer, 2018
TT-F-2-1-H	*Stichococcus* sp.	Teutschenthal, Germany; potash tailing heap surroundings	V. Sommer, 2018
WT-3-1-H	*Stichococcus* sp.	Wietze, Germany; potash tailing heap surroundings	V. Sommer, 2018
J 1302	*Tetratostichococcus jenerensis** ≡ *Stichococcus jenerensis*	Kampong Kuala Jenera, Kelantan, Malaysia; soil of rainforest tree	J. Neustupa, 2000
CCALA 495	*Tritostichococcus coniocybes* = *Stichococcus chloranthus*	Germany	W. Kruger
SAG 2406	*Tritostichococcus solitus**	Northeim, Germany; karstwater stream rock surface	K. Mohr, 2003

List of Algal Donors

I am deeply, deeply grateful and indebted to the following researchers who very kindly donated various "*Stichococcus*" strains at the very beginning of my PhD journey. Every package was greeted with immense joy, and the strains will live long at the University of Rostock.

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Manuscripts

The following manuscripts comprising this thesis are included in the order of publication:

- Van, A.T., Karsten, U. and Glaser, K., 2021. A chemosystematic investigation of selected *Stichococcus*-like organisms (Trebouxiouophyta). Algae, 36(2), pp.123-135.

- Van, A.T., Sommer, V. and Glaser, K., 2021. The Ecophysiological Performance and Traits of Genera within the *Stichococcus*-like Clade (Trebouxiophyceae) under Matric and Osmotic Stress. Microorganisms, 9(9), p.1816.

- Van, A.T. and Glaser, K., 2022. *Pseudostichococcus* Stands Out from Its Siblings Due to High Salinity and Desiccation Tolerance. Phycology, 2(1), pp.108-119.

Research Article

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A chemosystematic investigation of selected *Stichococcus*-like organisms (Trebouxiophyta)

Anh Tu Van*, Ulf Karsten and Karin Glaser

Department of Applied Ecology and Phycology, Institute of Biological Sciences, University of Rostock, Albert-Einstein-Straße 3, 18059 Rostock, Germany

The taxonomy of green microalgae relies traditionally on morphological traits but has been rapidly changing since the advent of molecular methods. *Stichococcus* Nägeli is a cosmopolitan terrestrial algal genus of the class Trebouxiophyceae that has recently been split into seven lineages, which, along with *Pseudostichococcus*, comprise the *Stichococcus*-like group; there is a need to further characterize these genera, since they are morphologically enigmatic. Here we used organic osmolytes as chemotaxonomic marker to verify the phylogenetic position of *Stichococcus*-like strains and were also able to exclude a strain hitherto identified as *Gloeotila contorta* from this group. *Stichococcus*-like organisms, including those recently revised, were characterized by the production of the polyol sorbitol and the disaccharide sucrose in high amounts, as is typical of *Prasiola*-clade algae. The results demonstrate that organic osmolyte chemotaxonomy can support green algal taxonomic designations as fundamental research.

Key Words: aeroterrestrial algae; chemotaxonomy; osmolytes; *Pseudostichococcus*; sorbitol; *Stichococcus*; sucrose

INTRODUCTION

Aeroterrestrial algae are abundant and ubiquitous microalgae occurring on various substrates like soil, tree barks, or man-made surfaces. However, many algae remain enigmatic in terms of their taxonomic identities and thus true biodiversity (Guiry 2012). One group, the *Stichococcus*-like algae within the order *Prasiolales* (Trebouxiophyceae) are of particular importance in terrestrial habitats, as they are ubiquitous and abundant in terrestrial environments (Rindi 2007, Hodač et al. 2016) inhabiting diverse habitats such as tree bark (Handa et al. 2003, Neustupa and Škaloud 2008, 2010), surfaces of buildings (facades, roof-tiles, etc.) and sculptures (Uher 2008, Gustavs et al. 2011, Hallmann et al. 2013), rocks (Hallmann et al. 2013), soil (Hodač et al. 2016) and biological soilcrusts (Ferrenberg et al. 2017, Glaser et al. 2018, Sommer et al. 2020*a*).

Recent efforts to delineate morphologically simple green microalgae such as *Stichococcus* have relied on molecular phylogeny. However, truly multiphasic approaches using physiological data is hitherto underutilized, with comparatively few studies to date focusing on physiological or chemotaxonomic data to bolster molecular interpretations (Pollio et al. 1997, Neustupa et al. 2007, Darienko et al. 2010, Karsten et al. 2016). Although *Stichococcus bacillaris* is an important alga in bioeconomic applications especially for fatty acids in biofuel production, only limited knowledge exist on closely related *Stichococcus*-like organisms (Olivieri et al. 2011, Mutaf et al. 2019).

One aspect of biochemical traits is of particular inter-

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Received January 21, 2021, **Accepted** June 5, 2021
***Corresponding Author**
E-mail: anh.van@uni-rostock.de
Tel: +49-381-498-6095, Fax: +49-381-498-6072

est: the presence of low molecular weight carbohydrates (LMWC) has both consequences for the cell biology and physiology in terms of osmotic stress tolerance (Roberts 2005, Holzinger and Karsten 2013, Holzinger and Pichrtová 2016) and has been demonstrated to be a stable taxonomic character (Gustavs et al. 2011, Darienko et al. 2015, Hotter et al. 2018). It also provides valuable information on the ecophysiology of the strains, which is usually missing from classical phylogenetic analyses. This approach has been used to analyze carbohydrates as primary metabolites, secondary metabolites, and beyond, chemotaxonomically in both macro- and microalgae. For example, Eggert and Karsten (2010) have shown that the sugars floridoside is accumulated in most Rhodophyta, and digeneaside, a related compound, is instead a marker of specific families within the phylum (Karsten et al. 1999, 2007, Kamiya et al. 2016). The sunscreen and antioxidant compounds mycosporin-like amino acids, abundant in many algal groups (Oren and Gunde-Cimerman 2007), have been used as taxonomic markers in the genera *Bostrychia* (Ceramiales, Rhodophyta) (Orfanoudaki et al. 2020) and *Klebsormidium* (Streptophyta) (Kitzing et al. 2014) as well as in *Prasiola* and *Watanabea* (Trebouxiophyceae) (Karsten et al. 2005).

Prior research in the *Prasiolales* showed that *Stichococcus* have a signature of osmolytes (sorbitol, sucrose, and proline) in response to salt stress (Brown and Hellebust 1978, Hellebust 1985). Hotter et al. (2018) proposed sorbitol as a chemotaxonomic marker for the *Prasiola*-clade within the Trebouxiophyceae, even under non-saline growth conditions. However, other *Stichococcus*-like genera such as *Pseudostichococcus* have not been studied so far. Furthermore, the recent taxonomical revision of the *Stichococcus*-like algae split those into the genera *Stichococcus*, *Protostichococcus*, *Deuterostichococcus*, *Tritostichococcus*, and *Tetratostichococcus* (Pröschold and Darienko 2020). This necessitates further investigation, as these revisions affect organisms that are morphologically diverse at the taxon level, yet very closely related at the genus level.

In this study, a wide variety of *Stichococcus* and *Stichococcus*-like (*sensu* Nägeli; the latter expanded on by Pröschold and Darienko 2020) strains were examined for their ability to synthesize sorbitol, the main osmolyte marker of the *Prasiola*-clade, in order to improve the current knowledge of the physiology of these strains, as they have not yet been extensively characterized. We also investigated *Pseudostichococcus*, which, although distinct from *Stichococcus* and *Stichococcus*-like organisms, is morphologically and genetically similar enough to warrant a more precise characterization. A wide variety of LMWC (e.g., ribitol, arabitol, xylitol) was used as standards, beyond those typical of *Prasiola*-clade algae, as it was unknown what the expression profile of these strains would be. Finally, this method was performed on two strains putatively outside of this group in order to demonstrate its taxonomic utility.

MATERIALS AND METHODS

In total, 26 strains were used for chemotaxonomic analyses (Table 1). Established unialgal strains already deposited at the University of Rostock as well as those from other institutions from around the world were gathered; authentic strains were prioritized when possible. All cultures were maintained at 20–22°C on 1.5% modified Bold's Basal agar (3N BBM + V, Bischoff and Bold 1963, modified by Starr and Zeikus 1993 to have triple nitrate concentration) with a 16 : 8 light : dark photoperiod, with 30 µm photons m^{-2} s^{-1} (Lumilux Cool Daylight L18W/840; OSRAM, Munich, Germany).

Qualitative and quantitative LMWC analysis

Algal suspensions of each strain were grown for four weeks in 150 mL liquid 3N BBM + V medium enriched with additional 10 mL Provasoli's Enrichment Solution per liter, under standard culture conditions outlined above. Medium was refreshed weekly to maintain the algae in the exponential growth phase.

Processing of algal biomass for high-performance liquid chromatography (HPLC) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) analysis was done according to Gustavs et al. (2011). HPLC analysis was undertaken on an Agilent 1260 system (Agilent Technologies, Santa Clara, CA, USA) equipped with a differential refractive index detector; one isocratic method was used to verify LMWC concentration and identity within the algal strain extracts. A Phenomenex REZEX ROA-Organic Acid resin-based column with a Phenomenex Carbo-H+ guard cartridge (Phenomenex, Torrance, CA, USA), was used to separate the solutes. The mobile phase consisted of 5 mM H_2SO_4 at a flow rate of 0.4 mL min^{-1} at 70°C.

For ^{13}C-NMR spectroscopy, dried algal biomass samples were re-dissolved in 0.5 mL D_2O (99.9%). The NMR spectra were recorded with a Bruker spectrometer (^{1}H: 500.13 MHz; ^{13}C: 125.8 MHz, AVANCE 500 Neo spectrometer (Bruker BioSpin, Ettlingen, Germany). Chemical shifts δ are given in ppm relative to the signal for internal

Table 1. The strains used in this study and their culture ID, localities, collector information, and GenBank accession numbers for the 18S small subunit locus, as well as the sequences extracted from GenBank

Strain ID	Species assignment	Locality and habitat	Collector / Isolator	Accession No. (18S)
SAG 41.84	"*Gloeotila contorta*"	Hagen, Germany; freshwater pond	E. Hegewald, 1983	AY422074
SAG 1.92	*Desmococcus olivaceus*[a]	Vienna, Austria; subaerial	W. Vischer, before 1960	KM020049
ACOI-1475	*Desmococcus vulgaris*	Coimbra, Igreja de Sta Cruz, Portugal; wall	M. F. Santos, 2002	-
SAG 10.97	*Deuterostichococcus epilithicus*	Margaraca, Portugal; pond	M. F. Santos, 1991	MT078169
J 1303, SAG 2139[b]	*Deuterostichococcus marinus*[a]	Dauphin Island, Alabama, USA; soil	T. R. Deason, 1969	DQ275460
ACSSI 273	*Stichococcus* sp.	Russia; agricultural soil	A. D. Temraleeva, 2018	-
ACSSI 84	*Stichococcus* sp.	Tula region, Russia; gray forest soil	A. D. Temraleeva, 2014	-
IT-203	*Stichococcus* sp.	Gessolungo, Italy	R. Taddei	-
ASIB-IB-37	"*Stichococcus allas*"	Weißkugel Peak, Ötztal Valley, Austria; soil	H. Reisigl, 1964	-
SAG 11.88	*Diplosphaera epiphytica*[a]	Waweira Scenic Reserve, New Zealand; lichen phycobiont	E. Tschermak-Woess, 1984	AJ416105
SAG 2481	*Protostichococcus edaphicus*[a]	Swabian Alb, Germany; forest soil	L. Hodač, 2008	KP081394
SAG 380-1	*Pseudostichoccocus monallantoides*[a]	Germany; marine	L. Moewus, 1951	KM020066
ASIB-BS-658	*Stichococcus* sp.	Blauen-Ettingen, Switzerland	W. Vischer, 1952	-
LB 1820	*Stichococcus sequoieti*[a]	USA; redwood forest soil	G. Arce, 1971	-
CALU-1142	*Stichococcus undulatus*[a]	Dolomite Mountains, Italy	G. Vinatzer, 1975	-
SAG 56.91	*Stichococcus bacillaris*	Czech Republic; freshwater	E. G. Pringsheim, before 1928	AM412752
CCAP 379/1A	*Stichococcus bacillaris*[a]	Likely Switzerland	W. Vischer, before 1936	FR865746
SAG 335-8	*Stichococcus bacillaris*	Sweden; freshwater	V. Czurda, before 1945	AM412753
ACOI-95	*Stichococcus bacillaris*	Tocha, Portugal; sphagnum bog soil	M. F. Santos, 1981	-
ASIB-BS-57	*Stichococcus* sp.	Botanical Garden, Basel, Switzerland; greenhouse	W. Vischer, 1929	-
SWN 282	*Stichococcus* sp.	Vietnam, Ha Long Bay; marine waters	E. S. Gusev, 2014	-
J 1302, SAG 2138[b]	*Tetratostichococcus jenerensis*[a,b]	Kampong Kuala Jenera, Kelantan, Malaysia; soil of rainforest tree	J. Neustupa, 2000	DQ275461
SAG 2406	*Tritostichococcus solitus*[a]	Northeim, Germany; karstwater stream rock surface	K. Mohr, 2003	MT078171
ACOI-1477	*Stichococcus* sp.	Coimbra, Igreja de Sta Cruz, Portugal; wall	M. F. Santos, 2002	-
SAG 25.92	*Desmococcus endolithicus*[a]			AJ431571.1
ST-2	*Tritostichococcus corticulus*			MT078176.1
SAG 26.83	*Prasiolopsis ramosa*[a]			AY762600.1
Rcon1	*Rosenvingiella constricta*			EF200529.1
SAG 14.91	*Koliella spiculiformis*			AJ306534.1
Prca1	*Prasiococcus calcarius*[a]			EF200527.1
CAUP H 103	*Pseudococcomyxa simplex*			HE586505.1
SAG 43.96	*Prasiola crispa*			AJ416106.1
SAG 211.92	*Watanabea reniformis*[a]			X73991
JK-2015 isolate 6	*Trebouxiophyceae* sp. (*Ekerewekia churiensis*)			KR066789.1

"Species assignment" refers to the most recent taxonomic classification of the available strain or taxon.

[a]Authentic strains.

[b]Equivalent strains from different culture collections.

TMS (δ = 0). The calibration of spectra was carried out externally, using the signals of acetone [5%, δ (^{13}C) = 30.3 ppm] in D_2O. Samples were run in 5-mm diameter tubes at 298 K. For ^{13}C-NMR spectra a sweep width of 30,000 Hz and a number of 10,000 scans were used.

A battery of common compatible solutes served as standards for interpretation of results. One mM solutions of sorbitol, trehalose mannitol, sucrose, glucose, erythritol, glycerol and ribitol were ran and quantified by peak areas. The limit of detection was 0.01 mM and the limit of quantitation was 0.03 mM for the LMWC standards used. Peak areas in the chromatograms were integrated and correlated to a ten-point calibration curve for each substance detected; resulting concentrations are expressed as µmol g^{-1} algal dry weight (DW).

DNA extraction

Culture material was transferred to 2 mL tubes. DNA isolation was done with the NucleoSpin Plant II Mini Kit (Macherey and Nagel, Düren, Germany) or Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Hilden, Germany) following their respective product instructions. DNA concentrations were measured using Qubit Fluorometer (ThermoFisher Scientific Germany, Dreiech, Germany). DNA samples were stored at -18°C until further processing.

Polymerase chain reaction and downstream processing

All polymerase chain reactions (PCRs) amplifying the 18S small subunit (SSU) locus were performed with the basic composition of 12.5 µL MyTaq Mix (Meridian Bioscience, London, UK), 9.5 µL PCR-grade H_2O, 1 µL template DNA, and primers 1 µL at a concentration of 10 pmol $µL^{-1}$ each. The PCR conditions for each primer pair are outlined in Supplementary Table S1. PCR products were visualized on a 1.5% agarose gel and cleaned with SureClean Plus (Bioline, Luckenwalde, Germany) following manufacturer's instructions and sequenced by Eurofins Genomics (Ebersberg, Germany) using the same respective PCR primers. All sequences were submitted to the National Center for Biotechnology Information (NCBI); accession numbers for all sequences can be found in Table 1.

The reads were assembled and edited in PhyDE (Müller et al. 2010), automatically aligned using the MUSCLE algorithm (Edgar 2004) with minor manual proofreading. jModelTest (Posada 2008) was used to find the best model for phylogenetic analysis, the result of which was GTR + G + I (Tavaré 1986). The 18S gene locus was used to obtain phylogenetic trees using the software Geneious v. 8.1.9 (Biomatters Ltd., Auckland, New Zealand) with the add-ons for RAxML (maximum likelihood [ML]) and MrBayes (Bayesian inference [BI]), respectively. Both ML and BI analyses used the GTR + G + I model with 4 rate categories, with the –f a option with 10,000 bootstrap replicates to calculate branch support for the best-scoring tree in ML. This option performs rapid bootstrap analysis and searches for the bestscoring ML tree in one program run. All analyses were conducted under random seed 12,354. The following settings were used for BI: runs with four incrementally heated Metropolis-coupled Monte-Carlo Markov Chains with 5 million generations, burn-in 1,250,000 generations, with a subsampling frequency of 1,000; heated chains = 4, temp. = 0.2; random seed = 11,244. The effective sample size value was >200 and the trace plot indicated convergence. Introns were ignored for the phylogenetic analyses, since they contributed to artificially high differences in the resulting trees. Phylogenetic trees were edited in FigTree v. 1.4.2 (Rambaut 2008). Gene sequences from several authentic strains were used for the phylogenetic analysis (Table 1). The outgroups were *Pseudococcomyxa simplex* and *Watanabea reniformis*.

RESULTS

Molecular phylogeny

The 18S SSU phylogeny recovered is mainly congruent with that of the latest study of *Stichococcus*-like organisms (Pröschold and Darienko 2020, Sommer et al. 2020*b*), except the clade containing SAG 11.88 *Diplosphaera*, CAUP J 1302 *Tetratostichococcus jenerensis*, SAG 2481 *Protostichococcus edaphicus*, and CAUP J 1303 *Deuterostichococcus marinus* (Bayesian posterior probability [B] = 1.00 / ML bootstrap = 54) (Fig. 1). The separation between the non-*Prasiolaceae* and *Stichococcus*-like organisms was well supported (B = 0.99 / ML = 79).

The clades *Stichococcus bacillaris* and *Pseudostichococcus* segregated with high support (B = 1.00 / ML = 100 and B = 1.0 / ML = 99, respectively). However, there was weak support for the segregation of the genera *Deuterostichococcus*, *Tetratostichococcus*, *Protostichococcus*, and *Diplosphaera*. Representative strains from each genus were divided into branches or mixed clades. *Deuterostichococcus* had particularly bad resolution with a

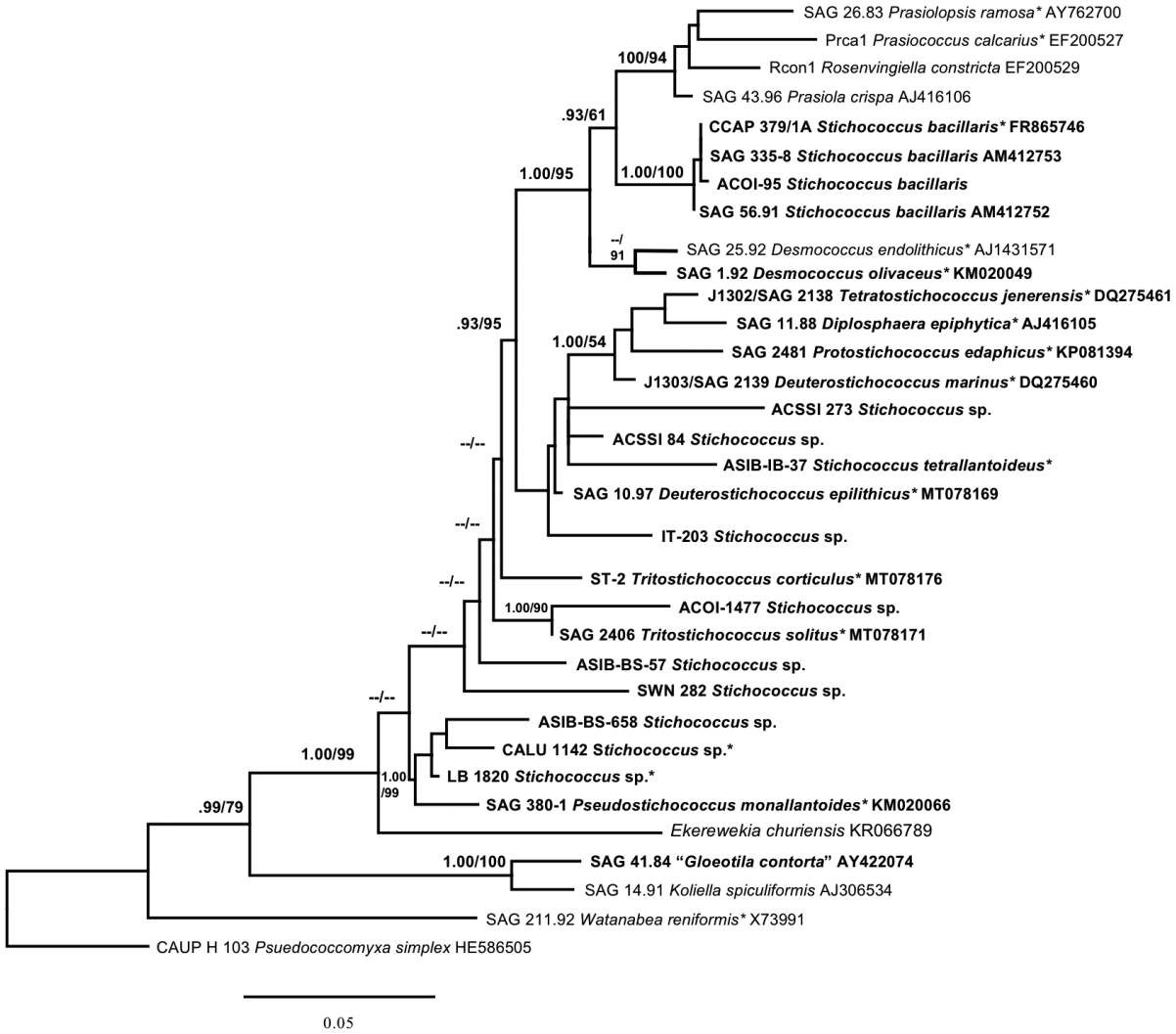

Fig. 1. 18S small subunit gene tree of the dataset strains. Left branch support values = Bayesian inference (BI), right branch support values = maximum likelihood (ML); BI / ML branch support values over 90/50 are displayed. Bold type indicates strains used in this study. The asterisks (*) indicate authentic strains.

low barcoding gap and contradictory support in the two trees, where the patristic distances (*p*-distances) ranged from 1.0–3.0% (BI) and 2.0–9.0% (ML). The clade containing *Pseudostichococcus* segregated with high support (B = 1.00 / ML = 99), apparently with two lineages.

Compatible solute analysis

The qualitative LMWC production pattern of *Stichococcus* and *Stichococcus*-like organisms consist of sorbitol and sucrose (Fig. 2). No other osmolytes were represented in this clade (Fig. 3). NMR analyses verified that the disaccharide of the LMWCs was sucrose and not trehalose, which has a similar retention time in HPLC analysis but differs in molecular structure (Supplementary Fig. S1). There was one strain that had differential osmolyte composition: strain 41.84 *Gloeotila contorta* lacked sorbitol and produced only sucrose, as well as an unidentified compound. Supplementary Table S2 details the absolute concentrations of sorbitol and sucrose measured in each strain.

Osmolyte concentrations varied widely between the *Stichococcus*-like strains, ranging from 28 to ~440 µmol g^{-1} DW for sorbitol and 9 to ~140 µmol g^{-1} DW for sucrose

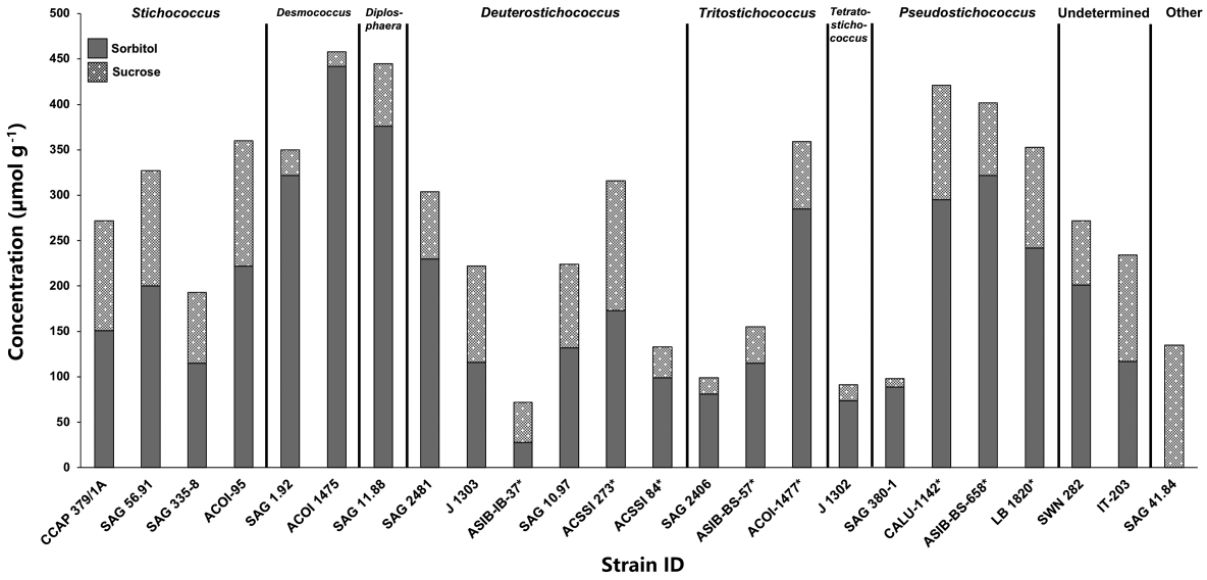

Fig. 2. Proportions of the major osmolytes present in each strain, arranged in the order in which they appear on the phylogenetic tree, separated into their generic assignments (Fig. 1). Unidentified strains did not segregate into a clade in the phylogeny. Concentrations are expressed as µmol g^{-1} dry weight (DW). The vast majority of strains produced more sorbitol than sucrose, though in some strains within *Deuterostichococcus*, such as ASIB-IB-37 *Deuterostichococcus tetrallantoideus*, had an almost 1 : 1 ratio. Strain SAG 41.84 *Gloeotila contorta* does not belong within the *Stichococcus*-like clade of the Prasiolales, as it does not produce sorbitol and falls rather into the *Koliellaceae*. The asterisks (*) indicate strains putatively belonging into the named genus, based on the preliminary phylogeny.

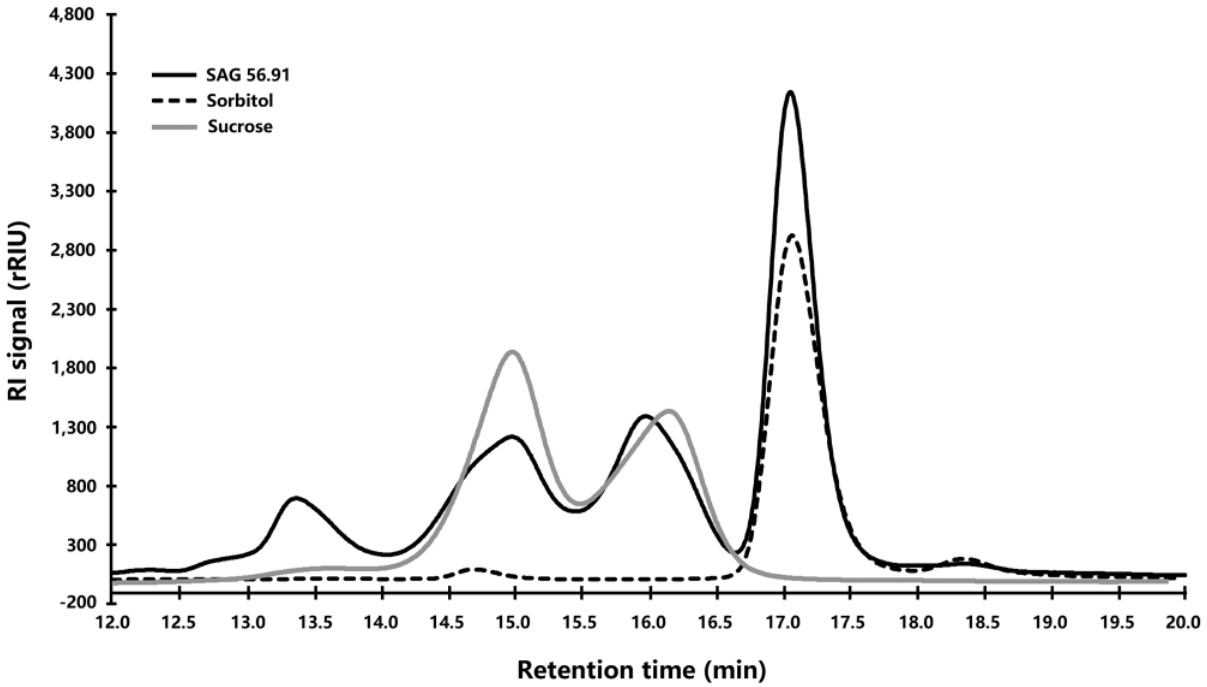

Fig. 3. High-performance liquid chromoatography of cellular extracts of SAG 56.91 *Stichococcus bacillaris*, which also represents the typical osmolyte expression pattern of *Stichococcus*-like strains. Sorbitol shows a peak at 17.1 min, sucrose at 15.1 and 16.3 min. RI signal, refractive index signal; rRIU, relative refractive index units.

(Fig. 2). *Desmococcus* strains SAG 1.92 and ACOI-1475 had the highest concentrations of sorbitol at 322 and 441 µmol g^{-1} DW, respectively; the lowest sorbitol concentration well under 100 µmol g^{-1} DW were detected in the strains ASIB-IB-37 *Deuterostichococcus tetrallantoideus*, CAUP J 1302 *T. jenerensis*, SAG 2406 *Tritostichococcus solitus*, and SAG 380-1 *Pseudostichococcus monallantoides* (Fig. 2). As groups, *Desmococcus*, *Diplosphaera* and the strains putatively belonging to *Pseudostichococcus* had the highest sorbitol production.

The ratio of sorbitol : sucrose varied between the strains and there was little strongly genus-specific ratio correlations to be seen. The *S. bacillaris* group was fairly consistent in this ratio, ranging from 1.2–1.6. The two *Desmococcus* strains had remarkably high ratios of 11.5 : 1 and 27.6 : 1.

DISCUSSION

The strains historically identified as *Stichococcus* have very similar morphology, which, through genetic techniques of recent years, have been shown to have a higher-than-expected diversity. The thorough investigation by Pröschold and Darienko (2020) indicated that the morphologies of *Stichococcus*, *Protostichococcus*, *Deuterostichococcus*, *Tritostichococcus*, *Teratostichococcus*, and *Pseudostichococcus* are, in most cases, both extremely variable within infrageneric taxa yet similar between different genera. Their separation and subsequent description of the genera resulted in there being virtually no traditional autapomorphies, such as differences in cell shape, length : width ratio, chloroplast form, and pyrenoid presence within each genus. The basic descriptive traits of the genera were "cylindrical, hyaline, chloroplast parietal and unlobed, without mucilaginous layer, with or without pyrenoid," with cell dimensions as a main character. However, quantification of the length : width ratio shows that only *Diplosphaera* can be distinguished from the other *Stichococcus*-like genera (Pröschold and Darienko 2020, fig. 14). Only *Diplosphaera* and the cluster-forming *Desmococcus* can be reliably separated from the other *Stichococcus*-like genera on the basis of morphology. It is reasonable to say that for morphological identification purposes from environmental data, most *Stichococcus*-like algae are pseudocryptic. Likewise, the literature dedicated to characterizing these new strains is hitherto scare. The role of chemotaxonomic analysis here was to bolster the existing knowledge on non-morphological traits of these newly described strains, since the morphological descriptions are vague and very little is known about their physiology.

The phylogeny in this study was constructed solely to orient results and not to provide a thorough investigation into the phylogenetic relationships between the taxa. Furthermore, it is oriented to—and with sequences from—the phylogeny from Pröschold and Darienko (2020), as these authors redefined the seven *Stichococcus*-like lineages. Thus, we chose the most widely accepted locus for green algal phylogenetic analysis, the 18S SSU locus and chose to disregard the single Group 2 intron within the locus. Even without using an exhaustive marker array, the overall pattern of clade segregation is consistent with the recently established lineages. Another independent study has also done this in a different context and acquired similar results (Sommer et al. 2020*b*). Several "*Stichococcus* sp." strains tested this phylogenetic hypothesis as well as provided material for the chemosystematic comparison.

The taxon *S. bacillaris* Nägeli 1849 is currently one of the few within *Stichococcus s.l.* confirmed to be of this genus, as many others, such as *S. jenerensis* and *S. allas*, have been transferred to other *Stichococcus*-like clades or genera. In this study's phylogeny, the *S. bacillaris* clade's separation and close relationship to *Desmococcus* parallel the results from the two newest phylogenetic studies. It was not possible to obtain high-quality 18S sequences from strain ACOI-1475 *Desmococcus* and, thus, was subsequently excluded from the phylogeny, but its chemotaxonomy and morphology were consistent with that of the generitype strain SAG 1.92 *Desmococcus olivaceus*. *Protostichococcus*, *Diplosphaera*, and *Tetratostichococcus*, as sorbitol and sucrose producers, align with other members of the *Prasiola*-clade and are genetically distinct from *Stichococcus s.s.* and *Pseudostichococcus*.

Type material for *S. allas* Reisigl 1964, a taxon that now falls within *Deuterostichococcus* after the latest revision, was rediscovered by the authors of the present study and examined chemotaxonomically, with ramifications for the taxonomy of the clade. This strain, ASIB-IB-37, was assumed to be unavailable and hence not directly described or assigned in Pröschold and Darienko (2020), which means that the reclassification of *S. allas* into *Deuterostichococcus allas* was necessarily preliminary, pending type material analysis. In an amendment by the same authors, the taxon *S. allas* was subsequently declared a heterotypic synonym of *Chodatia tetrallantoidea* Kol 1934 (morphologically identical to *S. allas*) and *D. allas* and was then reassigned to *D. tetrallantoideus* (Pröschold et al. 2020). After comparing the genetic sequences of *S.*

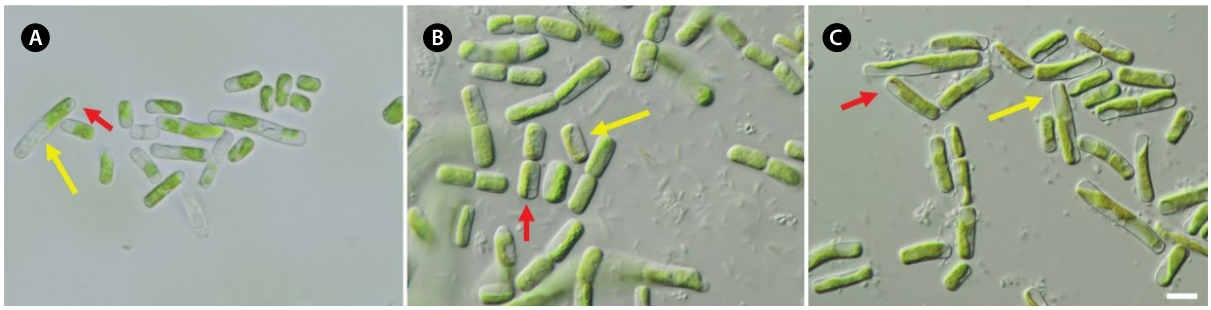

Fig. 4. Morphologies of SAG 380-1 *Pseudostichococcus monallantoides* (A), CALU 1142 *Stichococcus undulatus* (B), and LB 1820 *Stichococcus sequoieti* (C). Yellow arrows emphasize chloroplast form, red arrows the cell habitus. In SAG 380-1 and LB 1820, filaments are rarely seen, while in CALU 1142 short filaments are typical. The chloroplasts cover half the cell length in SAG 380-1, CALU 1142 vertically, and almost the entire cell horizontally in LB 1820. All three strains are characterized by relatively high length : width ratios (although SAG 380-1 has the smallest cells), a cylindrical cell shape with a squared-off appearance at the ends, and frequently large oil and starch droplets in the cells. Scale bar represents: 5 µm. [Colour figure can be viewed at http://www.e-algae.org].

allas and *D. tetrallantoideus* as well as the osmolyte production pattern of strain ASIB-IB-37, it seems that the type strain of "*S. allas*" falls within the near vicinity of *Deuterostichococcus*. Thus, the taxonomic reassignment of strain ASIB-IB-37 be the heterotypic type material of *D. tetrallantoideus* is valid and supports the revision in Pröschold and Darienko (2020).

However, there seems to be insufficient support in the 18S locus to establish *Deuterostichococcus* as a monophyletic clade, especially when including sequences from unidentified strains. In particular, the supports for the branches containing ASIB-IB-37, SAG 10.97, and J 1303 are below the support threshold. A polytomy occurs between the strains ACSSI-84, ASIB-IB-37, and SAG 10.97. Morphological data also does not improve resolution. Of the unidentified strains only ACSSI-84 exhibits a pyrenoid which fits the classical description of *S. deasonii* (*D. marinus*), but this trait no longer supports an autapomorphy (Pröschold and Darienko 2020). To resolve this clade would go beyond the scope of this paper; for now, there is not enough evidence to reassign these three strains into *Deuterostichococcus*.

Assigning strain ACOI-1477 to *Tritostichococcus* is somewhat easier, although still not entirely clear. ACOI-1477 only has five base-pair differences to SAG 2406 (*p*-distance = 1.0% BI, 3.0% ML). Problematic is strain ST-2, which does not segregate with the clade containing the other *Tritostichococcus* strain. Morphologically, ACOI-1477 could fit into either *Tritostichococcus* or *Deuterostichococcus*, as it cylindrical, has a single smooth parietal chloroplast without pyrenoid, and has a length : width ratio of ~2.

In Sommer et al. (2020*b*), one of the few works that have identified *Stichococcus*-like organisms from environmental samples on the basis of molecular data, it was shown that *Pseudostichococcus* consists of two separate clades. Our phylogeny bolsters this finding, as the strains ASIB-BS-658, CALU 1142, and LB 1820 form a sister clade to SAG 380-1 *P. monallantoides* (BI = 1.00, ML = 99). Although this is not definitive, it supports the conclusion that these three strains are more related to *Pseudostichococcus* than to the other *Stichococcus*-like strains. In addition, all these follow the *Stichococcus*-like sorbitol and sucrose production pattern. However, the taxonomic history of these three taxa has uncertainties that we attempt to clarify here.

To examine the validity of this transfer into *Pseudostichococcus* Moewus 1951, it was necessary to analyze the strains CALU 1142 *S. undulatus* and LB 1820 *S. sequoieti*, the authentic strains of their respective taxa (Fig. 4). Unfortunately, there has not yet been a direct comparison of their differences to one another, although the morphological similarity between these two strains has been noticed indirectly in the past by both authorities Vinatzer (1975) and Arce (1971), respectively. *S. undulatus* has a similar size range (*w* 4 µm; *l* 5–30 µm) to *S. sequoieti* (*w* 1.5–3 µm; *l* 5–25 µm), the latter slightly smaller in both dimensions. Vinatzer wrote explicitly in his description of *S. undulatus* that *S. mirabilis* is similar to *S. undulatus*, except that *S. mirabilis* does not form filaments. Furthermore, the illustrations from Vinatzer indicate that *S. undulatus* has a chloroplast that fills the cell length, but only halfway in height. In the original description of *S. sequoieti*, Arce (1971) compared *S. sequoieti* against several other *Stichococcus* strains, one of which was *S. mirabilis* Lagerheim (strain 316) from the Indiana Univer-

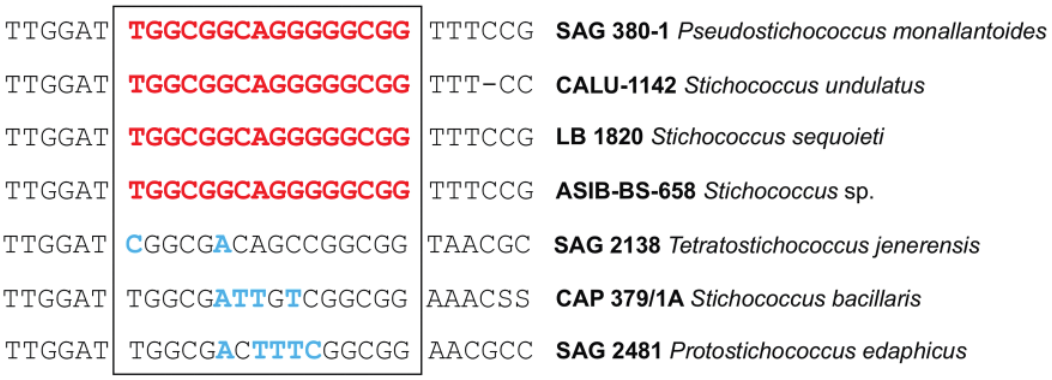

Fig. 5. Nucleotide sequence schematic of the 18S small subunit rDNA V9 region Helix 49 (boxed), diagnostic for the genus *Pseudostichococcus*. For comparison are three authentic strains from other *Stichococcus*-like genera that differ from *Pseudostichococcus*.

sity Culture Collection of Algae (IUCC, now UTEX), with the conclusion that *S. sequoieti* differs from *S. mirabilis* in dimensions (*w* 2.3–3.5 µm; *l* 30 µm) and that chloroplast length in *S. mirabilis* reaches 1/2 to 1/3 cell length whereas that of *S. sequoieti* fills the cell. Starch and lipids are absent in both. Unfortunately, strain UICC 316 is unavailable, as is the only other known strain of *S. mirabilis* (CCAP 379/3), so it was not possible to compare *S. mirabilis* to *S. sequoieti* and *S. undulatus* directly. Here we take *S. undulatus* over *S. mirabilis* as the strain identity and point of comparison to *S. sequoieti* due to type material availability and due to the fact that *S. undulatus* forms short filaments in culture (Fig. 4). The 11/1,760 base pairs difference (*p*-distance = 1.0% BI; 3.0% ML) in the 18S sequence strongly suggests that *S. undulatus* and *S. sequoieti* are distinct taxa; for reference, *S. sequoieti—P. monallantoides p*-distance = 1.0% BI; 2.0% ML; *S. undulatus—P. monallantoides* = 2.0% BI; 5.0% ML. Furthermore, the diagnostic 18S SSU rDNA V9 Helix 49 region (Fig. 5) for *Pseudostichococcus* is identical in all four strains. In this sense, we partially agree with Pröschold and Darienko (2020) that *S. sequoieti* does not belong in *Stichococcus s.s.*; however, it does belong within the *Stichococcus*-like group. In light of the physiological and phylogenetic results, these two strains could be transferred to the hitherto monotypic genus *Pseudostichococcus*. Strain ASIB-BS-658 has the same V9 Helix 49 region, as well as sequence similarity, to the other *Pseudostichococcus* strains and should also be placed into this genus.

Apart from verifying clade membership, we were able to conclude based on the osmolyte composition that SAG 41.84 does not belong to the *Stichococcus*-like organisms due to its conspicuous lack of sorbitol. Our chemotaxonomic results support the conclusions of a previous phylogenetic analysis (Henley et al. 2004), which showed that strain SAG 41.84 does not fit into either the *Picochlorum* / *Nannochloris*-like clade, nor *Stichococcus s.l.*, nor *Gloeotila s.l.* Following the reassignment of many members of the genus *Gloeotila* by Pröschold and Darienko (2020) to *S. bacillaris*, it appears that this strain rather belongs to another family of the order Prasiolales, most likely within the *Koliellaceae*. However, it is not possible to precisely assign this taxon into an existing genus without further taxonomic and nomenclatural work in both *Koliella* and the historical *Gloeotila* clade, barring more precise investigation into the type of the name of the genus, *Gloeotila oscillarina* Kützing. "*Gloeotila contorta*" SAG 41.84 also does not produce osmolytes typical of other closely related groups, such as ribitol or glycerol (*Chloroidium*, *Watanabea*, *Viridiella*, from Darienko et al. 2010), or erythritol (*Apatococcus*, from Gustavs et al. 2011).

The results from the LMWC analysis from this study are in agreement with those of other studies on Treboux-iophyceaen algae (Brown 1977, Brown and Hellebust 1980, Gustavs et al. 2011, Hotter et al. 2018), where it was shown repeatedly that members of the *Prasiola* clade produce sorbitol as an osmolyte. In particular, Brown (1977) and Brown and Hellebust (1978) studied the compatible solute composition of *S. bacillaris* strains directly and showed that sorbitol is the main carbohydrate produced, with proline as an additional osmoprotectant. Our results show that this is a stable (chemo) taxonomic character within the *Stichococcus*-like genera. Sucrose was also present in all *Stichococcus*-like strains, but since it is present in many other green algal families, it is not necessarily taxonomically indicative (Brown and Helle-

bust 1980, Darienko et al. 2015). However, the combination of sorbitol + sucrose may be consistent within the genera studied. A recent work by Medwed et al. (2021) also shows that *Diplosphaera* produces sorbitol + sucrose during growth. These findings support the phylogenetic exclusion of the algal strain SAG 48.41 from the *Stichococcus*-like genera, as it does not produce sorbitol.

Finally, the quantitative data obtained on sorbitol and sucrose production in the *Stichococcus*-like strains provide no phylogenetic resolution. Although there is a slight pattern in the sorbitol : sucrose ratios to be seen (Fig. 2), it is not significant when controlled for genus relationships. Sorbitol is a superior compatible solute compared to sucrose and does not interfere with cellular processes even at high intracellular concentrations (Roberts 2005). The main function of sucrose is, in addition, not that of an osmolyte but rather as a storage compound, as it is the main product of photosynthesis (Everard and Loescher 2016), and hence it only partially contributes to osmotic acclimation. The lower levels of sucrose production in comparison to sorbitol in almost all strains studied support this.

It is unclear why the *Desmococcus* and *Pseudostichococcus*, as well as SAG 11.88 *Diplosphaera epiphytica*, have a higher sorbitol : sucrose ratio than the other *Stichococcus*-like groups. All strains were cultivated under identical conditions, so that the measured concentrations reflect the specific phenotypic biosynthesis. More empirical data, possibly from different cultivation and experimental conditions, would be necessary to determine a relationship between the sorbitol : sucrose ratio and osmotic stress tolerance. This is particularly relevant since the *Stichococcus*-like genera are common aeroterrestrial algae and lichen symbionts (Thüs et al. 2011, Fontaine et al. 2013), and it would be expected that they perform similarly under desiccation.

In conclusion, *Stichococcus*-like algae produce sorbitol and sucrose that serve as compatible solutes to counteract osmotic stress, mainly due to frequent desiccation in the terrestrial habitat. This is a chemotaxonomic marker consistent within the *Prasiola* clade in the Trebouxiophyceae. The novel results show that the newly erected genera formerly in *Stichococcus sensu lato* (*Protostichococcus*, *Deuterostichococcus*, *Tritostichococcus*, *Tetratostichococcus* as well as *Pseudostichococcus*) share this trait and fall phylogenetically into the Prasiolales, although osmolyte analysis alone is insufficient to delineate their generic boundaries. A taxonomically and historically related "*Gloeotila*" strain was reassigned to Trebouxiophyceae *incertae ordinis* based on differential compatible solute production as well as phylogenetic distance. Furthermore, we propose that the taxa *Stichococcus undulatus* and *Stichococcus sequoieti* be reassigned into the hitherto monotypic genus *Pseudostichococcus*.

Summary of proposed taxonomical changes

Because many of the strains in this study are currently known in the respective culture collections as *S. bacillaris* or even solely as *Stichococcus* sp., it will be necessary to reassign them formally into the newly proposed genera of the *Stichococcus*-like clades. Based on the compatible solute analysis, it is possible to confirm or exclude membership from this broad group. Combined with the preliminary 18S phylogeny, this should be sufficient to reassign the following taxa into their new genera. Precise genus-level assignment for the taxa known only generically will require more detailed phylogenetic analysis using the full 18S–28S SSU locus, ideally in combination with plastid markers such as *rbc*L. However, taxa with a valid botanical name are easily transferred, as they serve as their own basionyms and their original descriptions are still valid. The following new combinations are proposed:

***Pseudostichococcus sequoieti* (G. Arce) Van & Glaser, comb. nov.**
Basionym: *Stichococcus sequoieti* G. Arce (1971:25, figs 1-16).
Designated type strain: Strain LB 1820, UTEX Culture Collection of Algae, University of Texas at Austin, Texas, USA.
***Pseudostichococcus undulatus* (Vinatzer) Van & Glaser, comb. nov.**
Basionym: *Stichococcus undulatus* Vinatzer (1975, p. 203, fig. 10).
Designated type strain: CALU 1142 *Stichococcus undulatus*, CALU St. Petersburg Culture Collection, St. Petersburg, Russia.
Sister strain: ASIB-V164.

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Russia), Dr. Tatiana Mikhailyuk (M.K. Kholodny Institute of Botany, Kiev, Ukraine) and Dr. Andreas Holzinger (University of Innsbruck, Innsbruck, Austria). Many thanks in particular to Dr. Rhena Schumann and Dr. Dirk Michalik at the University of Rostock for guidance in HPLC and NMR methodology. Within the framework of the German Research Foundation Special Priority Project: 1991 Taxon-Omics (CrustAlgaeTax-GL909/1-1), we were able to offer additional and critical data on the taxonomic revision of this genus using a chemotaxonomic approach.

CONFLICTS OF INTEREST

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

SUPPLEMENTARY MATERIALS

Supplementary Table S1. A list of primers for nuclear 18 small subunit gene locus and their references (https://www.e-algae.org).

Supplementary Table S2. Absolute concentrations of sorbitol and sucrose measured in cellular extracts of the strains during high-performance liquid chromatography analysis (https://www.e-algae.org).

Supplementary Fig. S1. C^{13}-NMR spectrum of sugar alcohols produced by SAG 56.91 *Stichococcus bacillaris*, overlaid with spectra for sorbitol and sucrose (https://www.e-algae.org).

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Supplementary Materials

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Supplementary Table S1. A list of primers for nuclear 18 small subunit gene locus and their references

Primer name	Primer sequence (5′-3′)	Reference
EAF3	TCGACAATCTGGTTGATCCTGCCAG	Marin et al. (2003)
G800R	CATTACTCCGGTCCTACAGACCAACAGG	Marin et al. (2003)
G500F	GAATGAGTACAATCTAAACCCCTTAAC	Marin et al. (2003)
ITS055R	CTCCTTGGTCCGTGTTTCAAGACGGG	Marin et al. (2003)

Cycling conditions were done according to the references.

Supplementary Table S2. Absolute concentrations of sorbitol and sucrose measured in cellular extracts of the strains during high-performance liquid chromatography analysis

Strain	Species assignment	Sorbitol	Sucrose	Sorbitol : Sucrose ratio
SAG 1.92	*Desmococcus olivaceus*[a]	322	28	11.5 : 1
ACOI 1475	*Desmococcus vulgaris*	442	16	27.6 : 1
J 1303	*Deuterostichococcus marinus*[a]	116	106	1.1 : 1
SAG 10.97	*Deuterostichococcus epilithicus*	132	92	1.4 : 1
ASIB-IB-37	*Deuterostichococcus tetrallantoideus*[a]	28	44	0.6 : 1
SAG 11.88	*Diplosphaera epiphytica*[a]	376	69	5.4 : 1
SAG 41.84	"*Gloeotila contorta*"	0	135	0 : 1
SAG 2481	*Protostichococcus edaphicus*[a]	230	74	3.1 : 1
SAG 380-1	*Pseudostichococcus monallantoides*[a]	89	9	9.9 : 1
ASIB-BS-658	*Stichococcus* sp.	322	80	4 : 1
CALU-1142	*Stichococcus undulatus*[a]	295	126	2.3 : 1
LB 1820	*Stichococcus sequoieti*[a]	242	111	2.2 : 1
SAG 335-8	*Stichococcus bacillaris*	115	78	1.5 : 1
ACOI-95	*Stichococcus bacillaris*	222	138	1.6 : 1
SAG 56.91	*Stichococcus bacillaris*	200	127	1.6 : 1
CCAP 379/1A	*Stichococcus bacillaris*[a]	151	121	1.2 : 1
ACSSI 84	*Stichococcus* sp.	99	34	2.9 : 1
IT-203	*Stichococcus* sp.	117	117	1 : 1
ASIB-BS-57	*Stichococcus* sp.	115	40	2.9 : 1
ACSSI 273	*Stichococcus* sp.	173	143	1.2 : 1
ACOI-1477	*Stichococcus* sp.	285	74	3.9 : 1
SWN 282	*Stichococcus* sp.	201	71	2.8 : 1
J 1302	*Teratostichococcus jenerensis*[a]	74	17	4.4 : 1
SAG 2406	*Tritostichococcus solitus*[a]	81	18	4.5 : 1

"Species assignment" refers to the most recent taxonomic classification of the available strain or taxon. The ratios of sorbitol-to-sucrose are to the far right. Values are expressed in μmol g^{-1} algal dry weight.
[a]Authentic strains.

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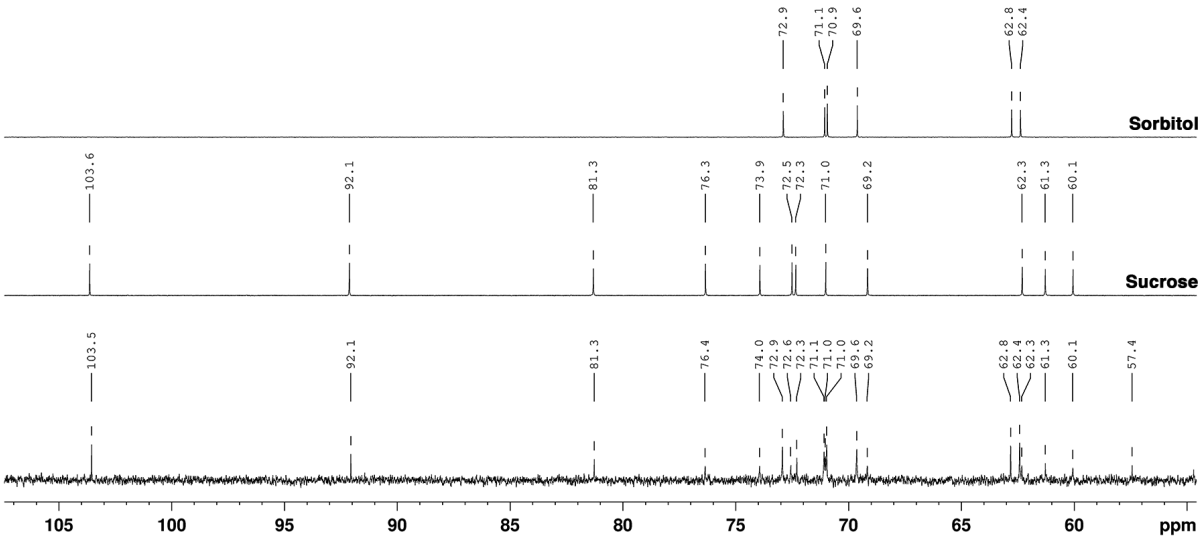

Supplementary Fig. S1. C^{13}-NMR spectrum of sugar alcohols produced by SAG 56.91 *Stichococcus bacillaris*, overlaid with spectra for sorbitol and sucrose. No other sugars or sugar alcohols were present, indicative of a typical *Stichococcus* expression pattern.

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photosystem II (PSII) activity and the production of low-molecular-weight carbohydrates (LMWC) as compatible solutes, also known as osmolytes [1]. LMWC concentrations, particularly sugar alcohols and simple sugars, have been shown to increase under salt stress conditions in various algal groups (Trebouxiophyceae [8]; *Heterococcus* [14]; *Chlorella* [15]; various seaweeds [16]). A recent transcriptomic analysis [17] had shown that under salt and nitrogen stress, the chlorophyte alga *Neochloris oleoabundans* upregulates genes coding for osmoregulation, particularly those for compatible solutes.

Various taxa within the *Stichococcus*-like genera of the Prasiolaceae (Trebouxiophyceae) are ubiquitous and abundant in diverse terrestrial habitats, such as tree bark [18,19], surfaces of buildings [20–22], biocrusts [2], and soil [23]. Due to their simple morphology and resemblance to one another, they have long been functionally grouped into the *Stichococcus* clade. This group currently includes *Stichococcus*, *Protostichococcus*, *Deuterostichococcus*, *Tritostichococcus*, *Tetratostichococcus*, *Desmococcus* as well as *Diplosphaera* [24]. Prior research shows that *Stichococcus bacillaris* can occur, albeit rarely, in marine conditions [25] and is very halotolerant [8,26,27]. Although *Stichococcus* are known to produce sorbitol, sucrose, and proline as osmoprotectants [28,29], there lacks comparative examinations of its response to osmotic as well as matric stress; this is also true for the other genera within the *Stichococcus*-like clade. Here, we aim to characterize the ecophysiological performance of *Stichococcus*-like strains to these pragmatically realized tolerance to salt and desiccation.

2. Materials and Methods

Twelve established unialgal strains from around the world were used (Table 1). All cultures were maintained at 20–22 °C on 1.5% modified Bold's Basal agar (3N BBM+V [30], modified to have triple nitrate concentration [31]) with a 16:8 light: dark photoperiod, with 30 µm photons m^{-2} s^{-1} (Lumilux Cool Daylight L18W/840, OSRAM, Munich, Germany).

Table 1. The strains used in this study and their culture ID, localities, and collector information. Equivalent strains from different culture collections are marked with a "†"; strains marked with "*" are authentic strains. All strains except SAG 1.92 were used in the dehydration and recovery experiment; all strains were used in the salt growth experiment.

Strain ID	Species Assignment	Locality and Habitat	Collector/Isolator
SAG 1.92 *	*Desmococcus olivaceus*	Vienna, Austria; subaerial	W. Vischer, before 1960
J 1303 *, † SAG 2139 *	*Deuterostichococcus marinus*	Dauphin Island, Alabama, USA; soil	T.R. Deason, 1969
ASIB-IB-37 *	*Deuterostichococcus tetrallantoideus*	Weißkugel Peak, Ötztal Valley, Austria; soil	H. Reisigl, 1964
SAG 11.88 *	*Diplosphaera epiphytica*	Waweira Scenic Reserve, New Zealand; lichen phycobiont	E. Tschermak-Woess, 1984
SAG 2481 *	*Protostichococcus edaphicus*	Swabian Alb, Germany; forest soil	L. Hodač, 2008
SAG 380-1 *	*Pseudostichoccocus monallantoides*	Germany; marine	L. Moewus, 1951
LB 1820 *	*Pseudostichococcus sequoieti*	USA; redwood forest soil	G. Arce, 1971
CALU-1142 *	*Pseudostichococcus undulatus*	Dolomite Mountains, Italy	G. Vinatzer, 1975
CCAP 379/1A *	*Stichococcus bacillaris*	Likely Switzerland	W. Vischer, before 1936
J 1302 *, † SAG 2138 *	*Tetratostichococcus jenerensis*	Kampong Kuala Jenera, Kelantan, Malaysia; soil of rainforest tree	J. Neustupa, 2000
SAG 2406 *	*Tritostichococcus solitus*	Northeim, Germany; karstwater stream rock surface	K. Mohr, 2003

2.1. Salt Tolerance

The halotolerance of select strains (Table 1) was determined by exposing them to salinity along a gradient corresponding to natural freshwater, sea water as well as to extreme experimental conditions. These strains were chosen on their ability to produce significant biomass in a short period. A series of nine media was prepared, with salinities ranging from 0 to 90 g NaCl L^{-1} in 15 g L^{-1} increments, corresponding to their values in absolute salinity (S_A). An amount of 30 mL medium was pipetted into standard 150 mm glass test tubes, so that one salinity level corresponded to one test tube. Inoculation

with 1 mL unialgal culture suspensions in the respective salinities followed. The cultures grew for eight weeks; the tubes were photographed weekly and aliquots from the test tubes periodically observed under the microscope for morphological changes. After the experiment, growth was classified by eye using the following categories: healthy, slightly stressed, stressed, mostly dead, and dead.

2.2. Desiccation Tolerance and Recovery Experiments

The experimental setup for desiccation tolerance and recovery largely followed that of Karsten [32] with minor changes to the setup and measurement protocol outlined below. Strains were kept in the exponential growth phase by transfer every three days before experiment began. During the experiment, medium was refreshed daily through decanting and replacement of 25 mL of old medium with fresh, following biomass collection.

For the dehydration experiment, polypropylene desiccation chambers were filled with 100 g freshly activated silica; a perforated metal disc was positioned upon four columns within the chambers. Six Whatman GF/F 25 mm fiberglass filters were distributed along the edge of the metal disk, and 200 µL suspensions of each strain were dropped onto the filters, so that one filter corresponded to one strain. A data logger monitored air humidity and temperature, which were maintained at RH of ~10% and 20–22 °C, respectively. Throughout the duration of the experiment, all polystyrene chambers and algae were kept in a low-light environment (ca. 40 µm photons m^{-2} s^{-1}). The effective quantum yield of PSII (Y(II), ΔF/Fm') of each strain was measured every 30 min until no yield was recorded (420 min) on a PAM 2500 (Walz, Effeltrich, Germany).

The rehydration experiment following the dehydration period was set up in a similar way, except that the silica gel was replaced with 100 mL tap water in a new chamber to create a moist atmosphere (RH ~95%). The filters were moved to the high-humidity chambers and rehydrated with 200 µL of algae growth medium, after which the effective quantum yield was measured every 30 min up to 2 h; a final measurement was done after 24 h of recovery. The strain *Desmococcus olivaceus* SAG 1.92 was not tested due to insufficient accumulation of biomass before experiment begin.

Three technical experimental replicates and two control replicates were taken over a period of one week, where one replicate run took one day. The control replicates were tested at the beginning and end of the experiment, to account for changes in culture vitality. Chl*a* concentrations of the 200 µL biomass from each filter was extracted via ethanolic extraction and quantified spectrophotometrically according to Ritchie [33]. A one-way ANOVA followed by a Tukey's multiple comparison test was performed in R (Version 1.2) implemented in RStudio (RStudio Team 2020) to find the statistical significance of Y(II) means ($\alpha = 0.05$). In both experiments, the average Y(II) values of three experimental replicates per strain were expressed against the two average control values; as both culture and empirical conditions were standardized, it was possible to compare the strain responses to one another directly in an overlaid manner.

2.3. Qualitative and Quantitative Osmolyte Analysis

Algal biomass for high-performance liquid chromatography (HPLC) analysis was cultured in two groups. The first cohort was grown in 150 mL liquid 3N BBM+V medium (as in Section 2) enriched with additional 10 mL Provasoli's enrichment solution [34] per liter, under standard culture conditions outlined in Section 2. The second cohort grew in 150 mL of the same medium at 30 S_A, again under standard culture conditions. Medium was refreshed weekly by decanting 100 mL from cultivation flasks of followed by replacement with 100 mL fresh medium, in order accelerate biomass accumulation. A single replicate was taken per treatment per strain, due to biomass limitations.

Processing of algal biomass for HPLC was done according to a previously published protocol [8]. In brief, HPLC analysis was done on an Agilent 1260 system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a differential refractive index detector. A Phenomenex REZEX ROA-Organic Acid resin-based column with a Phenomenex Carbo-H+

guard cartridge (Phenomenex, Torrance, CA, USA) was used to separate the solutes. The mobile phase consisted of 5 mM H_2SO_4 at a flow rate of 0.4 mL/min at 70 °C.

Solutions comprising 5 mM of sorbitol and sucrose standards were run and quantified by peak areas and retention times. Peak areas in the chromatograms were integrated and correlated to a ten-point calibration curve for each substance detected; resulting concentrations were expressed as μmol g^{-1} algal dry weight (DW). The limit of detection (LoD) was 0.08 mM and 0.09 mM for sorbitol and sucrose, respectively; the limit of quantitation was (LoQ) 0.24 mM and 0.28 mM for sorbitol and sucrose, respectively.

3. Results

3.1. Morphological Changes under Salt Stress

Cells in standard medium without salt showed no signs of osmotic stress over time, whereas cells that grew in saline medium at both concentrations had clear signs of osmotic stress, evidenced by bleaching, formation of vesicles, reduction in biomass, cell size reduction, and thickened cell walls (Figure 1). The severity of osmotic stress was correlated with increasing salinity. Already at 30 S_A, there was visible inhibition of growth in strains *Deuterostichococcus tetrallantoides* ASIB-IB-37, *Stichococcus bacillaris* CCAP 379 1/A, *Tetratostichococcus jenerensis* J1302, and *Desmococcus olivaceus* SAG 1.92. Halotolerant strains *Pseudostichococcus monallantoides* SAG 380-1 (Figure 1E) showed only minor physical changes at the cellular level, where cells became smaller compared to growth in 0 S_A medium.

Figure 2 gives an overview of the vitality of the strains in the tested media at the end of eight weeks of growth in tabular format; Figure 3 show the macro-scale differences between strains under the same conditions. Most cultures at salinities of 30 S_A and above showed markedly reduced biomass, indicative of slowed growth or cell death visible on the macro scale. Growth effectively stopped at 60 S_A for the majority of the strains (nine out of 12). Only three strains—SAG 1.92 *Desmococcus olivaceus*, SAG 380-1 *Pseudostichococcus monallantoides*, and *Tritostichococcus solitus* SAG 2406—survived after extended exposure to 90 S_A. Halotolerant strains, such as SAG 2406 (Figure 3D), maintained more biomass in higher salinities compared to halosensitive strains, such as J1302. There was also a difference in how the biomass was distributed. Certain strains, such as *Deuterostichococcus tetrallantoideus* ASIB-IB-37 (Figure 3C), showed homogenous biomass, whereas others formed a condensed clump under salinity stress (Figure 3A,D). This was not correlated with the morphology of the individual strains, as both filamentous and solitary-form strains could clump or spread, nor with the halotolerance.

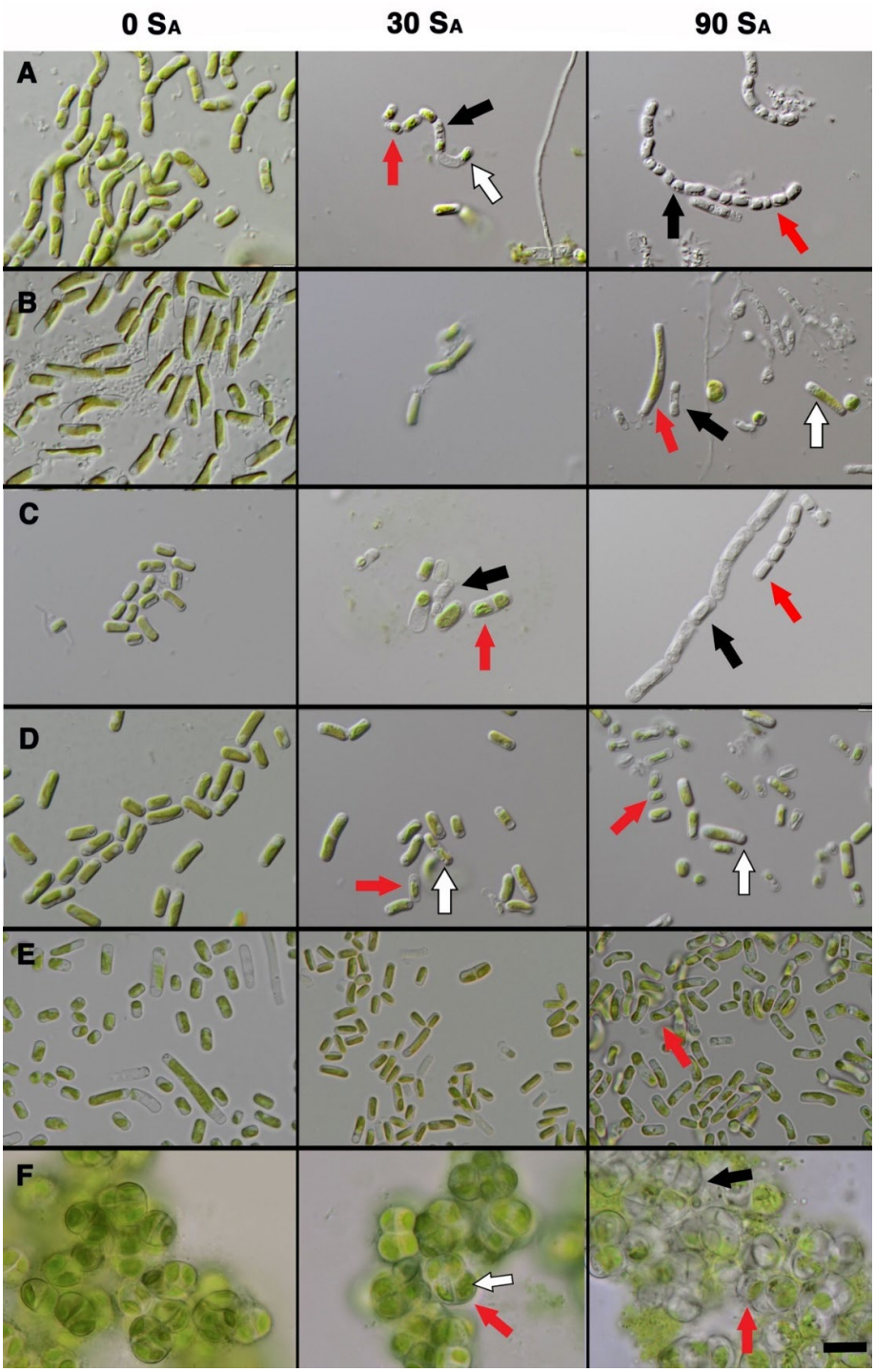

Figure 1. Morphological responses of selected strains in 0, 30, and 90 S_A modified 3N BBM+V media after 8 weeks. Scale bar = 5 µm for all panels. (**A**) *Deuterostichococcus tetrallantoideus* ASIB-IB-37. (**B**) *Pseudostichococcus sequoieti* LB 1820. (**C**) *Stichococcus bacillaris* CCAP 379/1A. (**D**) *Tetratostichococcus jenerensis* J1302. (**E**) *Pseudostichococcus monallantoides* SAG 380-1. (**F**) *Desmococcus olivaceus* SAG 1.92. Red arrows indicate plasmolysis, black arrows chloroplast bleaching, and white arrows the accumulation of storage vacuoles. Most strains in the cohort were still able to thrive at 30 S_A, but in halosensitive strains, there was already some bleaching and cell configuration changes present.

Figure 1. Morphological responses of selected strains in 0, 30, and 90 S_A modified 3N BBM+V media after 8 weeks. Scale bar = 5 µm for all panels. (**A**) *Deuterostichococcus tetrallantoideus* ASIB-IB-37. (**B**) *Pseudostichococcus sequoieti* LB 1820. (**C**) *Stichococcus bacillaris* CCAP 379/1A. (**D**) *Tetratostichococcus jenerensis* J1302. (**E**) *Pseudostichococcus monallantoides* SAG 380-1. (**F**) *Desmococcus olivaceus* SAG 1.92. Red arrows indicate plasmolysis, black arrows chloroplast bleaching, and white arrows the accumulation of storage vacuoles. Most strains in the cohort were still able to thrive at 30 S_A, but in halosensitive strains, there was already some bleaching and cell configuration changes present.

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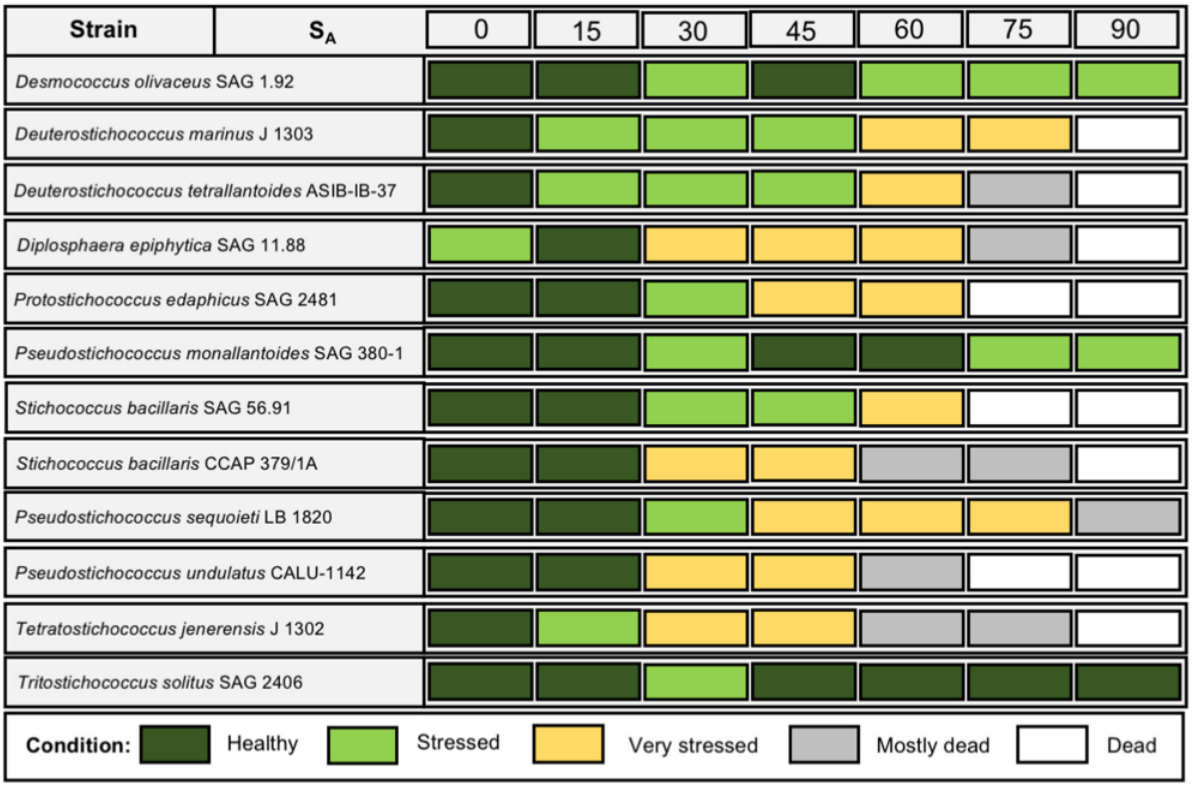

Figure 2. Tabular results of strain survival at 0–90 S_A; individual cultures of strains were evaluated after 8 weeks of growth in the respective media. The condition colors correspond approximately to culture color and health in the test tubes.

3.2. Low-Molecular-Weight Carbohydrate Analysis

Some algae including *Stichococcus* are known to produce osmolytes to withstand desiccation stress. The identification and quantification of these low-molecular-weight carbohydrates was verified through HPLC. Due to extremely slow growth in saline medium, it was not possible to obtain sufficient extract for *Desmococcus olivaceus* SAG 1.92. The major compounds corresponded to sorbitol and sucrose, with retention times at 17.1 min and 15.1/16.3 min, respectively (Figure 4; due to hydrolysis by the acidic eluent, sucrose presented a double peak of fructose and glucose).

The typical osmolyte production pattern of a *Stichococcus*-like strain consisted of sorbitol and sucrose in standard (non-saline) medium (Figure 5). The concentration of sorbitol increased in saline growth in all but four strains (SAG 1.92, *Deuterostichococcus marinus* J1303, SAG 11.88 *Diplosphaera epiphytica*, *Pseudostichococcus* sequoieti LB 1820) (Table S1). Of these four, SAG 11.88 had the largest decrease (176 μmol g^{-1} DW) both in terms of absolute value and percent. The increase in sorbitol after salinity stress of the remaining eight strains varied from ~−85% to 218%, with a median increase of ~29%, corresponding to a median value of 41.5 μmol g^{-1} DW. Strain *Deuterostichococcus tetrallantoides* ASIB-IB-37 showed the highest percent increase in sorbitol (~217%), and strain SAG 380-1 the greatest absolute increase (151 μmol g^{-1} DW).

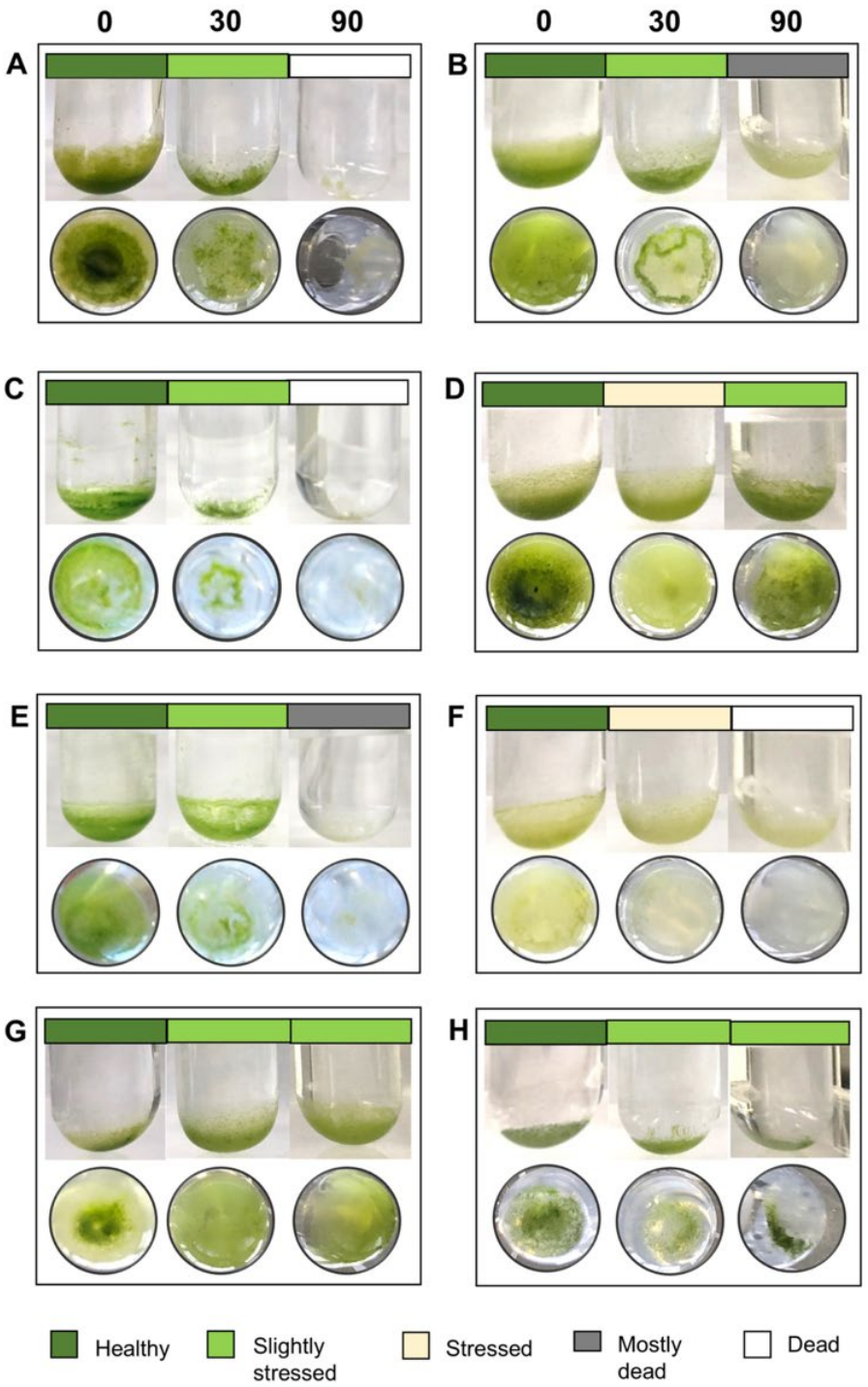

Figure 3. (**A**) SAG 56.91 *Stichococcus bacillaris*; (**B**) *Protostichococcus corpuscleus* SAG 2481; (**C**) *Deuterostichococcus monicus* ASIB-IB-37; (**D**) *Tritostichococcus* SAG 2406; (**E**) *Tetratostichococcus jenensis* J1302; (**F**) *Diplostichococcus epiphyticus* SAG 11.88; (**G**) *Pseudostichococcus monallantoides* SAG 380-1; (**H**) *Desmococcus olivaceus* SAG 1.92. Clumping was observed in (**A**–**C**).

3.2. Low-Molecular-Weight Carbohydrate Analysis

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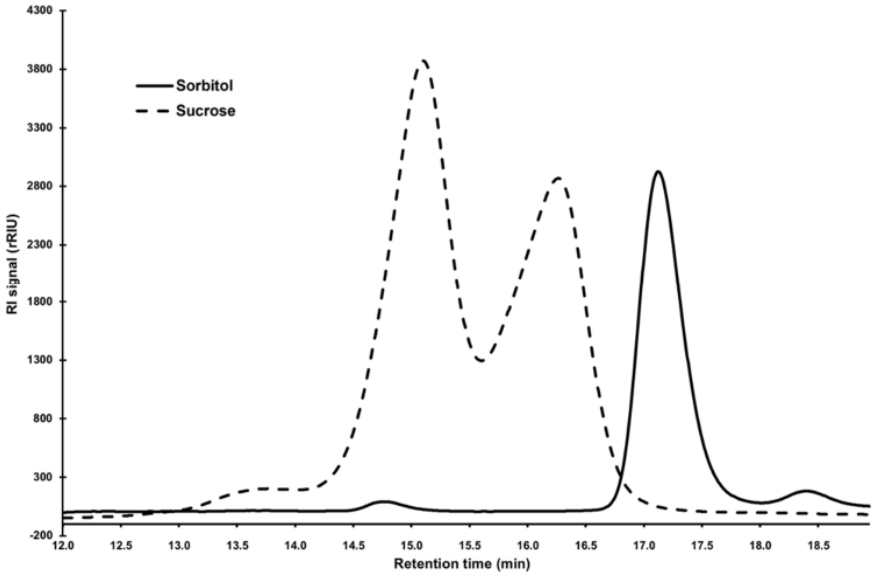

Figure 4. HPLC chromatograms of cellular extracts of pure standards of sorbitol and sucrose, respectively. Sorbitol shows a peak at 17.1 min, sucrose at 15.1 (glucose) and 16.3 min (fructose). RI signal = Refractive Index signal, rRIU = relative Refractive Index Units.

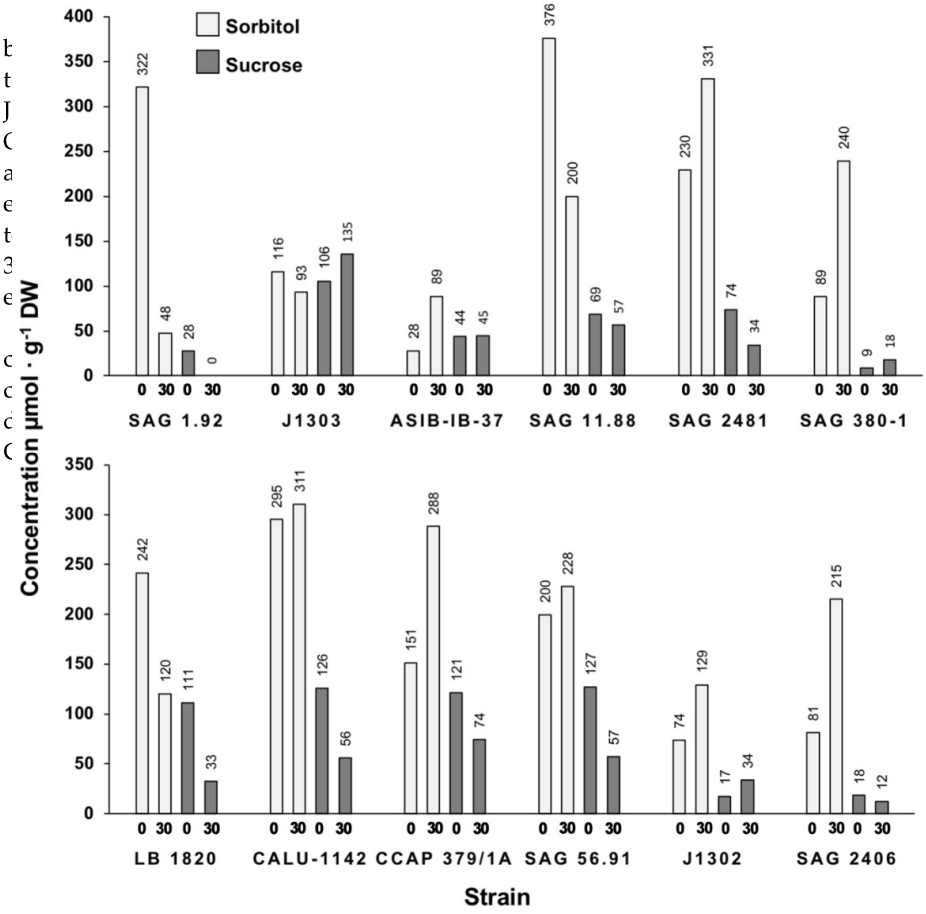

Figure 5. Proportions of the major osmolytes present in each strain during growth at 0 and 30 S_A. Concentrations are expressed as µmol g^{-1} dry weight (DW).

Figure 5. Proportions of the major osmolytes present in each strain during growth at 0 and 30 S_A. Concentrations are expressed as µmol g^{-1} dry weight (DW).

3.3. Dehydration and Rehydration

To follow the desiccation effect on the cultures, the cells were regularly measured with the PAM using the photosynthetic yield Y(II) as an indicator for the vitality of the algae. The initial Y(II) value of ca. 0.6 is typical of healthy algal strains kept under culture conditions and is not indicative of photoinhibition [35]. The strains had very similar de-

of the control value at the end of the 24 h rehydration period. There were no statistically significant differences between the genera *Stichococcus*, *Protostichococcus*, *Deuterostichococcus*, *Tritostichococcus*, *Tetratostichococcus*, and *Pseudostichococcus* representative strains in response (p = 0.283 dehydration; 0.0715 recovery). The highest Y(II) reached by the control was 0.631, on average 0.528, across all control replicates over the duration of 420 min. Table S3 gives final Chl*a* concentrations of the respective filter biomasses.

4. Discussion

The *Stichococcus*-like algae are euryhaline and desiccation tolerant but certainly not adapted to marine or hypersaline environments; reports of *Stichococcus*-like algae in marine environments are rare and, as of currently, taxonomically dubious [25,36,37].

4.1. Growth in Response to Saline Stress

For this study, we defined halotolerant strains as those that were visually vital (indicated by the green appearance of the culture) and continued to grow at 45 S_A and beyond, 1.5 times the average salinity of seawater. Strains that can grow despite saline stress at 30 S_A can be considered moderately halotolerant, since this equals marine conditions, and strains that showed heavy signs of stress and cease growth at 30 S_A were halosensitive. The growth experiment that 90 S_A is the limit of growth for most strains of *Stichococcus*-like algae. Previous work [8] defined a much lower range for good growth of *Stichococcus* sp. (SAG 2060, now *Deuterostichococcus epilithicus*) between 2 and 17 S_A but still measured growth at 65 SA. An earlier study [26] found that growth in the strain *Stichococcus bacillaris* CCAP 379/5 ceased at a salinity of 108 S_A (strain assignment is not yet updated according to recent revision). Pröschold and Darienko [24] cultivated strains from the *Stichococcus*-like group in marine medium (SWES, 30 S_A), and all strains were able to survive, despite salinity-induced morphological changes.

Our results confirm that exposure to 30 S_A and higher diminishes growth capacity (Figures 2 and 3) for four of the tested strains. Three strains, *Desmococcus olivaceus* SAG 1.92, *Pseudostichococcus monallantoides* SAG 380-1, and *Tritostichococcus solitus* SAG 2406, showed even the potential for growth beyond 90 S_A (Figure 2) and are, thus, strongly halotolerant. The four strains *Deuterostichococcus marinus* J1303, *Deuterostichococcus tetrallantoides* ASIB-IB-37, *Pseudostichococcus sequoieti* LB 1820, and *Stichococcus bacillaris* SAG 56.91 are moderately halotolerant and exhibited the beginning of culture collapse at starting at 75 SA. The remaining strains were dead or dying already at 60 S_A. The clearest signs of stress were chloroplast bleaching, cellular conformation change, and the formation starch and fat droplets and plasmolysis (Figure 1). Plasmolysis was visible in both halosensitive and halotolerant strains at 90 S_A and was present in all strains at 30 S_A except for in SAG 380-1, LB 1820, and SAG 2460.

Although *Stichococcus*-like algae are commonly aeroterrestrial and found in non-saline habitats, these taxa have been found in marine-adjacent environments, such as sand dune biocrusts affected by ocean spray [38]. Additionally, in the case of the genus *Pseudostichococcus*, these taxa have even been found in manmade habitats such as the highly saline environments of potash-tailings piles [39], which speaks for its halotolerance. Further, *P. monallantoides* SAG 380-1 is one of the few *Stichococcus*-like strains that was isolated from marine habitat (Table 1). It is possible that the genus *Pseudostichococcus* as a whole is more halotolerant than others within the *Stichococcus*-like clade. The hitherto monotypic genus *Pseudostichococcus* has been revised to also include the strains *Pseudostichococcus sequoieti* LB 1820 and *Pseudostichococcus undulatus* CALU-1142 used in this study, and that the latter strains belong to a second, possibly not halotolerant clade, within *Pseudostichococcus* [40]. Further studies are needed to delineate the ecological traits of *Pseudostichococcus* from those of the other *Stichococcus*-like strains.

4.2. Compatible Solute Production under Osmotic Stress

Here, we quantified the main osmoprotectant compounds of the *Stichococcus*-like group to link the quantitative change to halotolerance. It was expected that there would be a global increase in both sorbitol (main osmolyte) and sucrose concentrations in saline growth versus standard growth.

HPLC analysis showed increased sorbitol production and lowered sucrose production under saline conditions in most of the strains studied (Figure 5). Despite both sorbitol and sucrose being parallel and direct byproducts of photosynthesis [41], which is depressed under abiotic stress [42,43], there were reduced sucrose levels but generally increased sorbitol levels at 30 S_A. Nevertheless, the total concentration of osmolytes increased (Table S1), and the ratio of sorbitol: sucrose changed from approximately 2:1 to 5:1 at 30 S_A. This prioritization points to the critical role of sorbitol in osmotic stress tolerance.

Four strains (*Desmococcus olivaceus* SAG 1.92, *Diplosphaera chodatii* SAG 11.88, *Pseudostichococcus undulatus* CALU-1142, *Stichococcus bacillaris* CCAP 379/1A) showed major decreases in total osmolyte concentrations following growth in 30 S_A compared to 0 S_A (Table S1); only SAG 1.92 was halotolerant. The three remaining strains were halosensitive, and it is possible that saline stress overwhelmed the photosynthesis mechanisms necessary for osmolyte production [42], since there was evidence of bleaching for these three strains at 30 S_A. The persistent growth of SAG 1.92 under extremely high salinities suggests that there may be additional osmoprotection mechanisms at work that circumvent the polyol protection mechanism, such as Na^+/H^+ antiporter gene upregulation, as is the case in cyanobacteria [44], or the presence of Na^+-ATPases [45,46]. In this case, additional protection may come from the production of a very thick mucilage layer, which the other strains lacked. This layer allows cluster formation and reduces the surface area exposed to medium and may contribute to its survival at 90 S_A despite producing very little osmolytes.

The data from this study substantiate the contribution of polyols and sucrose to euryhalinity in aeroterrestrial algae. Polyols acts primarily as an osmolyte due to its high solubility in water and by being biological inert [1], whereas sucrose is a transport and storage molecule [41]; it chiefly protects against desiccation by forming glass structures [47,48]. Based on salt stress data from various studies, polyols seem to contribute more to osmotic stress tolerance compared to mono- or disaccharides. In the stenohaline genus *Klebsormidium*, the main osmolytes are sucrose and raffinose, not polyols, with only very trace amounts of trehalose [49,50], befitting an alga that effectively stop growing at 30 S_A [51]. Fujii [14] and Kremer [52] showed that *Heterococcus* was also euryhaline and produced both mannitol and glucose, whose growth stopped at ~45 S_A. In extremely halotolerant algae, polyols provide insufficient osmotic protection. The alga *Dunaliella salina*, found in extremely saline habitats, produces compatible solutes characteristic of very haloterant algae, such as glycerol and glycine betaine instead of polyols [53,54].

There was no strain-specific relationship between a total osmolyte concentration increase and halotolerance within the *Stichococcus*-like strains. In addition, it was not possible to conclude unequivocally that higher polyol production, either in total or of specific osmolytes, equals higher halotolerance. For example, the most halotolerant strains SAG 2406 and SAG 380-1 had relatively low total osmolyte concentrations, but with a clear sorbitol increase (Table S1). ASIB-IB-37 had lower total osmolyte concentrations than CCAP 379/1A but was more halotolerant in culture (Figures 2 and 5). This nonetheless advances the extensive work previously undertaken in *Stichococcus* [8,15,20,28,29] but indicates the need for more study of the precise saline stress adaptive mechanisms beyond polyol production within the *Stichococcus*-like genera.

4.3. Dehydration and Rehydration

In addition to salinity-induced "physiological drought" [55], dehydration is the second water-related challenge facing aeroterrestrial algae. Since the *Stichococcus*-like clade is not primarily marine, it was assumed that they would be better adapted to desiccation than salt stress.

Dehydration resulting from a lack of water in the environment has different consequences for the cell than osmotic stress, although they are related. Rehydration is usually sufficient to reverse damage to the photosynthetic apparatus, but this is strongly contingent on the extent and length of the desiccation period [56,57]. During the desiccation experiment in this study, algal suspensions were subjected to a long dehydration period (7 h) under very low RH (~10%), comparable to desert-like dryness [57].

Generally, strains that responded faster to desiccation, defined as having a shorter time until zero Y(II), had a lower recovery rate. The high variability of the final Y(II) values suggests that the strains' responses were sensitive to small changes in experimental preparation, of which the most important were biomass density and pipetting technique, since self-shading would protect from desiccation. Individual strain replicates achieved recovery Y(II) of ~90–95%, but this was not associated with one particular run and cannot be attributed to one consistent mechanical error.

All strains reached, per strain average, at least ~40% of control yield value in the recovery experiment, with the exception of CALU-1142 (~30%). This is exceptionally high as none of the *Stichococcus*-like organisms studied were isolated from deserts; all are temperate or tropical aeroterrestrial, and three strains are even aquatic in origin (SAG 380-1, SAG 56.91, SAG 2406). For perspective, true desert algae are expected to be even more desiccation resistant and able to recovery to higher values after weeks of desiccation [57]; desert green algal taxa outperform aquatic taxa in long-term desiccation and recovery, needing on average 900 min to dehydrate fully, compared to the 420 min from this experiment. Karsten [32] demonstrated in a similar experimental setup that *Klebsormidium* strains from African deserts can recover fully after 20–24 h, with little indication of lasting damage to the photosynthetic system. Other studies in Trebouxiophyceaen algae present on tree bark showed that they, as one biofilm unit, are able to survive and recover after being exposed to extended drought periods of up to 80 days [35]. This was reiterated in [58], where a *Diplosphaera chodatii* isolate collected from tree bark reached nearly 100% initial Y(II) after being exposed to water for post-dehydration, albeit after a far shorter desiccation period (45 min at 10% RH).

At 120 min, most strains reached a limit of ~20% initial Y(II) values. It was not until beyond this time that there was an appreciable uptick overall. Medwed [58] and Karsten [32] showed recovery that reached a plateau at ~190 and ~1000 min, respectively, so it is possible that the *Stichococcus*-like strains needed slightly longer to reach peak recovery.

In contrast to the average recovery value of ~40%, a maximum recovery potential of ca. 80–100% of control values was evident in all the strains, even accounting for high variability (Table S2). This was the highest value reached by an individual replicate per strain. Still, because all strains were maintained in liquid medium, it is unclear how important environmental origin is, if strains have been acclimated to liquid medium over a period of several months or even years. Morphology seemed to play a limited role in performance here, as most strains were single-celled and little self-shading was possible. Weakly filamentous strains, such as CCAP 379 1/A and SAG 56.91, are easily fragmented by pipetting and acted equally single-celled. Interestingly, there was little correlation between the Chl*a* content of the cell suspensions (biomass), resistance to desiccation, and subsequent recovery potential (Table S3). The strains with the lowest Chl*a* concentrations (SAG 2481, SAG 406, SAG 380-1) did not show differential responses compared to the strains with higher Chl*a* concentrations. This was also the case with positive outliers, where higher Chl*a* concentration did not result in higher recovery yields.

The data from the salinity stress and desiccation experiments suggest that mechanisms behind ionic and desiccation stress tolerance are independent of one another. Two of the most halotolerant strains (*Tritostichococcus solitus* SAG 2460 and SAG 380-1 *Pseudostichococcus monallantoides*) recovered well after rehydration but not faster than the halosensitive strains. The recovery of SAG 380-1 is comparable to that of CCAP 379/1A *S. bacillaris* and, even, ASIB-IB-37 *Deuterostichococcus marinus*, which had the least total osmolyte production. Despite this, the desiccation tolerance coupled with the ability to survive extended periods

of time in saline environments may contribute to the widely known ecological niche of the *Stichococcus*-like genera.

Nevertheless, the experimental tolerance data collected here are to be interpreted as physiological possibilities, not certainties. Most of the strains used have spent extensive time in storage, sometimes decades, within culture collections; it is possible that genomic changes have occurred, when compared to wild-type specimens [59]. Furthermore, while cryopreservation of stock cultures is recommended to minimize changes, it may also lead to an artificial genotypic selection for cryosurvivability [60]. A cause for optimism is that resistance to genetic changes may vary from alga to alga. *Chlorella vulgaris* strain 211-11b is the oldest isolated algal strain in the world, and no differences between it and its many isolates have been found [61]. This is likely the case within the *Stichococcus*-like strains, since the *Deuterostichococcus* sister strains to J 1303, SAG 2139 (MT078164) and UTEX 1706 (DQ27546) have identical sequences apart from length.

For taxonomists, it is important to refer to the oldest described species for taxonomical revision—in the case of microalgae, it mostly relies on culture collections. As this study was to extend the deep taxonomical revision of *Stichococcus*-like species, we decided to use cultured strains (most of them designated as epitypes) for this experimental setup rather than working with environmental specimens. Further studies with environmental samples of the *Stichococcus*-like strains are needed to determine the effects of saline stress and desiccation tolerance in the ecological context.

5. Conclusions

The patterns observed in the growth experiments under salinity stress, osmolyte analysis, and desiccation stress experiment indicate that the *Stichococcus*-like strains are euryhaline but better adapted to dehydration than osmotic stress. Strains that withstood salt stress did not necessarily recover better in the desiccation experiment—neither was osmolyte concentration indicating the halotolerance. It is unknown if this is true on a genus-wide basis, since most genera had only one representative taxon strain, but the existing results show no phylogenetic correlation in terms of halo- and desiccation tolerance, with the exception of the very halotolerant genus *Pseudostichococcus*. If this is true, then *Stichococcus* and the newly erected genera *Protostichococcus*, *Deuterostichococcus*, *Tritostichococcus*, and *Tetratostichococcus* may occupy a similar niche as aeroterrestrial algae within the Prasiolaceae. This fits their profile as generalists that are able to colonize arid habitats worldwide as well as those exposed to some saline stress, such as in biocrusts on sand dunes [38], deserts [2], and potash piling heaps' surroundings [27,39]. However, the performance of cultures should ultimately be compared to that of environmental samples to provide information on their ecology.

Holzinger [62] used transcriptomics to elucidate the molecular mechanisms behind desiccation tolerance of *Klebsormidium*—it would be useful to see if analogous transcripts exist in *Stichococcus*-like organisms, as this could also ground these terrestrial Chlorophyta along the timeline of terrestrial habitat colonization.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9091816/s1, Table S1: Absolute concentrations of sorbitol, sucrose, and trehalose measured in cellular extracts; Table S2: Average recovery Y(II) at 24 h. Table S3: Chla concentrations of filters used in the dehydration experiment.

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constant exposure and extensive adaptations to high salinities; furthermore, research on plant growth in arid and salinized environments has focused heavily on species that are halophytic or halotolerant, as the precise factors, mechanisms and genetic basis behind salinity tolerance in plants are still not fully understood [12,16,18,19].

Except for studies on the salt and desiccation tolerance of *Dunaliella salina* [20–22], there has been comparatively little research done on this stressor combination in green microalgae, perhaps due to lack of economic pressure. While halotolerant algae have chiefly been used to bioremediate saline waters and salinized soils, crop yield increase is also a growing field in applied phycology [12,23–25]. However, recent work has shown that some algae were able to form young biocrusts on hypersaline potash tailing heaps, with ramifications for salinity bioremediation [26–28]. Among the organisms isolated form salt heaps were *Chloroidium ellipsoideum*, the cyanobacterium *Nostoc*, representatives from *Stichococcus* and *Pseudostichococcus* [27].

The taxonomic history of the genus *Stichococcus* Nägeli, 1849 deserves mentioning here, as it has been frequently revised since its first description [29]. *Pseudostichococcus* L. Moewus 1951, was distinguished from *Stichococcus* Nägeli initially based on morphological and physiological differences [30], and then later via molecular data [31]. Pröschold and Darienko created from *Stichococcus* the genera *Protostichococcus*, *Deuterostichococcus*, *Tritostichococcus* and *Tetratostichococcus* on the basis of molecular and morphological differences [31]. In publications, these *Stichococcus*-adjacent genera have been broadly grouped into the "*Prasiola*-clade", i.e., Prasiolales, or even generally to the Trebouxiophycaeae [31–34]. However, the validly published family name Stichococcaceae has been recently rediscovered and the authority of the family determined to be K. Bohlin 1901 [35] (M. Guiry and S. Heesch, personal communication, July 2020). The members of the Stichococcaceae comprise the "*Stichococcus*-like" genera.

The discovery of *Stichococcus* and *Pseudostichococcus* in a highly saline habitat is unusual, because *Stichococcus* has hitherto been considered euryhaline [36–38]. As with the other *Stichococcus*-like organisms, *Pseudostichococcus* are present in a variety of habitats, such as such as tree bark, surfaces of buildings and outdoor structures, diverse soils and are also components of biocrusts. A previous study indicated that *Pseudostichococcus* may be more halotolerant than previously assumed, as those strains retained vitality despite cultivation at very high salinities [39].

Stichococcus uses the ion exclusion strategy, producing sorbitol and proline as major compatible solutes to withstand osmotic stress [8,37,40]. It was confirmed that members of the seven newly described *Stichococcus*-like genera have the same osmolyte pattern, with the addition of small amounts of sucrose under high salinity. Strains from this clade were highly resistant to strong dehydration and high recovery rates after rehydration, as well as certain strains being halotolerance.

In this study we extend our work done previously [39] and profile osmolyte production and strain vitality under desiccation stress following cultivation in nonsaline and saline media, with a focus on various strains within *Pseudostichococcus*. The combination of stressors is intended to reflect conditions in saline terrestrial habitats, which may guide further efforts in algal bioremediation of, for example, salt heaps or salinized soils. In addition, a comparison between *Pseudostichococcus* and other Stichococcaceae strains expressed as ecophysiological differences will be examined in more detail. When possible, authentic strains were used to maximize taxonomic value, since these are type strains confirmed to be isolated or deposited into a culture collection by the epithet authority.

2. Materials and Methods

Nine established unialgal strains of *Pseudostichococcus* and other Stichococcaceae were used (Table 1). All cultures were maintained at 20–22 °C on 1.5% modified Bold's Basal agar (3N BBM+V, to have triple nitrate concentration) [41], modified by Starr and Zeikus [42] with a 16:8 light:dark photoperiod, with ca. 30 µmol photons m^{-2} s^{-1} (Lumilux Cool Daylight L18W/840, OSRAM, Munich, Germany). For the experiments following, a saline

stock medium was made by adding 90 g NaCl (NaCl, ≥99%, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) to one liter of prepared 3N BBM+V to closely approximate 90 S_A. For simplicity, all media salinities will be expressed in absolute salinity (S_A).

Table 1. The strains used in this study and their culture ID, localities and collector information. Strains marked with "*" are authentic strains.

Strain ID	Species Assignment	Locality and Habitat	Collector/Isolator
SAG 1.92	*Desmococcus olivaceus* *	Vienna, Austria; subaerial	W. Vischer, before 1960
ASIB-IB-37	*Deuterostichococcus tetrallantoides* *	Dauphin Island, Alabama, USA; soil	T.R. Deason, 1969
SAG 380-1	*Pseudostichococcus monallantoides* *	Germany; marine	L. Moewus, 1951
ZL-4-1	*Pseudostichococcus monallantoides*	Teutschenthal, Germany; potash tailing heap surroundings	V. Sommer, 2018
TT-5-1-K	*Pseudostichococcus monallantoides*	Zielitz, Germany; potash tailing heap surroundings	V. Sommer, 2018
LB 1820	*Pseudostichococcus sequoieti* *	USA; redwood forest soil	G. Arce, 1971
CALU-1142	*Pseudostichococcus undulates* *	Dolomite Mountains, Italy	G. Vinatzer, 1975
CCAP 379/1A	*Stichococcus bacillaris* *	Likely Switzerland	W. Vischer, before 1936
SAG 2406	*Tritostichococcus solitus* *	Northeim, Germany; karstwater stream rock surface	K. Mohr, 2003

2.1. Cultivation and Inducing Osmolyte Production

Two cohorts per strain at 0 and 30 S_A were established, for a total of 18 flasks filled with unialgal suspensions. Algal biomass from agar plates was transferred to 300 mL Erlenmeyer flasks filled with 200 mL 0 S_A 3N BBM+V under culture conditions and left to grow for 4 weeks. For the 30 S_A cohort, 100 mL of 90 S_A 3N BBM+V medium was added to flasks to achieve a net salinity of 30 S_A and left to grow for four days; the 0 S_A cohort received an additional 100 mL of 0 S_A 3N BBM+V. Following this, the 300 mL suspensions were used for the experiments described below.

2.2. Sorbitol Quantification

Sorbitol content was determined via HPLC quantification, following a previously published protocol [43]. In summary, ca. 7–10 mg dried algal biomass was extracted at 70 °C in 1 mL 70% ethanol for 4 h, centrifuged, from which 700 µL was removed. The supernatant was removed and dried under vacuum evaporation, then resuspended with 700 µL MiliQ water. The extract was analyzed with an Agilent 1260 system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a differential refractive index detector. Sorbitol standards were prepared from sorbitol (D-sorbitol, BioUltra quality, Sigma-Aldrich Chemie, Schnellorf, Germany) dissolved in MiliQ water to 5 mM and quantified by peak areas and retention times. Peak areas in the chromatograms were integrated and the resulting concentrations were expressed as $\mu mol \cdot g^{-1}$ algal dry weight (DW). The limit of quantitation (LoQ) was 0.24 mM for sorbitol; the limit of detection (LoD) was 0.08 mM for sorbitol.

2.3. Proline Quantification

The proline content of cultures at 0 and 30 S_A was determined spectrophotometrically using ninhydrin, adapted from Bačkor et al. and Lee et al. [44,45]. 5–30 mL algal suspensions were filtered through incinerated Whatman GF/F 25 mm filters and dried at 40 °C overnight. The filter was extracted at 70 °C in 1 mL 1% w/v sulfosalicylic acid for 1 h, then left at 4 °C overnight to allow for maximal proline extraction. The mixture was then centrifuged for 10 min at 13,000× g. 1 mL of ninhydrin reagent (1.25% w/v ninhydrin in 80% glacial acetic acid) was added to 500 µL of the decanted supernatant and heated at 100 °C in a drying oven for 60 min. The reaction was stopped by putting the samples on ice for 10 min. Absorbance was read at 510 nm with a spectrophotometer (Shimadzu UV-2401 PC, Kyoto, Japan).

Absolute amounts of proline were calculated from absorbances using the calibration standard curve. The calibration standard curve (R^2 = 0.9969) for proline covered the concentration range of 0.01–5 mM; dissolved proline (L-proline, ReagentPlus® quality,

=

Sigma-Aldrich Chemie, Schnellorf, Germany) in 1% sulfosalicylic acid was reacted with ninhydrin as with the samples.

Concentrations of proline were standardized to algal dry weight ($\mu mol \cdot g^{-1}$ DW) instead of cell concentration to bypass difficulties in counting filamentous versus unicellular strains, as well as to have a direct comparison with the sorbitol measurements.

2.4. Desiccation and Recovery Experiments

This experimental setup for desiccation tolerance and recovery largely followed that of Karsten et al. [46], with minor changes to the setup and measurement protocol outlined below. Both 0 S_A and 30 S_A cohorts from (2.1) were investigated and three replicates were measured consecutively over three days.

For the dehydration experiment, polypropylene desiccation chambers were filled with 100 g freshly activated silica; a perforated metal disc was positioned upon four columns within the chambers. Eight Whatman GF/F 25 mm fiberglass filters were distributed along the edge of the metal disk and 200 µL suspensions of each strain were dropped onto the filters, so that one filter corresponded to one strain. Relative humidity (RH) was maintained at ~10% and 20–22 °C, respectively. Throughout the duration of the experiment, all polystyrene chambers and algae were kept in a relatively low-light environment (ca. 40 µmol photons m^{-2} s^{-1}), as close to culturing conditions as possible, since it was not possible to perfectly replicate the growing conditions in the experimental setup.

The effective quantum yield of PSII (Y(II), ΔF/Fm') was measured on a PAM 2500 and its accompanying software (Walz, Effeltrich, Germany), with actinic light set at 650 nm (red light) and pulse of 800 ms. The strains were measured every 30 min until no yield was recorded (360 min). The ΔF/Fm' calculation was done within the Walz software using equations previously described [47,48].

For the rehydration experiment immediately following the dehydration period, silica gel was replaced with 100 mL tap water in a new chamber to create a moist atmosphere (RH ~95%). The filters were moved to the high-humidity chambers and rehydrated with 200 µL of algae growth medium, after which the effective quantum yield was measured every 30 min up to 2 h; a final measurement was done after 24 h of recovery.

The two control replicates consisted of algal suspensions at 0 and 30 S_A dropped onto filters as described above. They were held in the desiccation chambers filled with 100 mL water for 360 min, instead of in silica. Measurements were at the beginning and end of the experiment (5 days), to account for fluctuations in culture health under the experimental setup.

2.5. Statistical Analyses

Statistical calculations were performed in R (Version 4.1.2) implemented in R Studio [49]. Mean Y(II)) values of the three replicates per strain and salinity was expressed as a percent against the mean of the respective salinity control values. Absolute Y(II) values were taken directly from the Walz software. Similarly, the mean and standard deviation of sorbitol and proline content were calculated from the three replicates in 2.2 and 2.3. To simplify the proportion calculation, only the mean was used.

A two-way ANOVA was used on the proline measurements to find the statistical variation of means of samples with respect to salinity and strain. A p-value of less than 0.05 was considered significant ($\alpha = 0.05$).

Similarly, a one-way ANOVA followed by a Tukey's multiple comparison test was performed on the samples in the dehydration experiment with respect to salinity. A Pearson correlation analysis was conducted to determine the relationship between salinity and final recovery values in the dehydration experiments.

3. Results

3.1. Changes in Osmolyte Concentrations under Salinity Stress

Nine strains were grown at 0 and 30 S_A and their proline concentration (Figure 1) and proline:sorbitol ratio (Figure 2) was measured using HPLC. A two-way ANOVA yielded a main effect for the salinity, $F(1, 14) = 154.91$, $p < 2 \times 10^{-16}$, such that the average proline amount produced was significantly higher at 30 S_A (M = 425.33 µmol·g^{-1} DW, SD = 356.40) than at 0 S_A (M = 172.26 µmol·g^{-1} DW, SD = 108.46). The main effect of strain was also significant, $F(1, 14) = 27.72$, $p < 2 \times 10^{-16}$. Finally, the interaction effect was significant, $F(1, 14) = 14.85$, $p = 1.45 \times 10^{-14}$, indicating that the variability was due to taxon-specific differences.

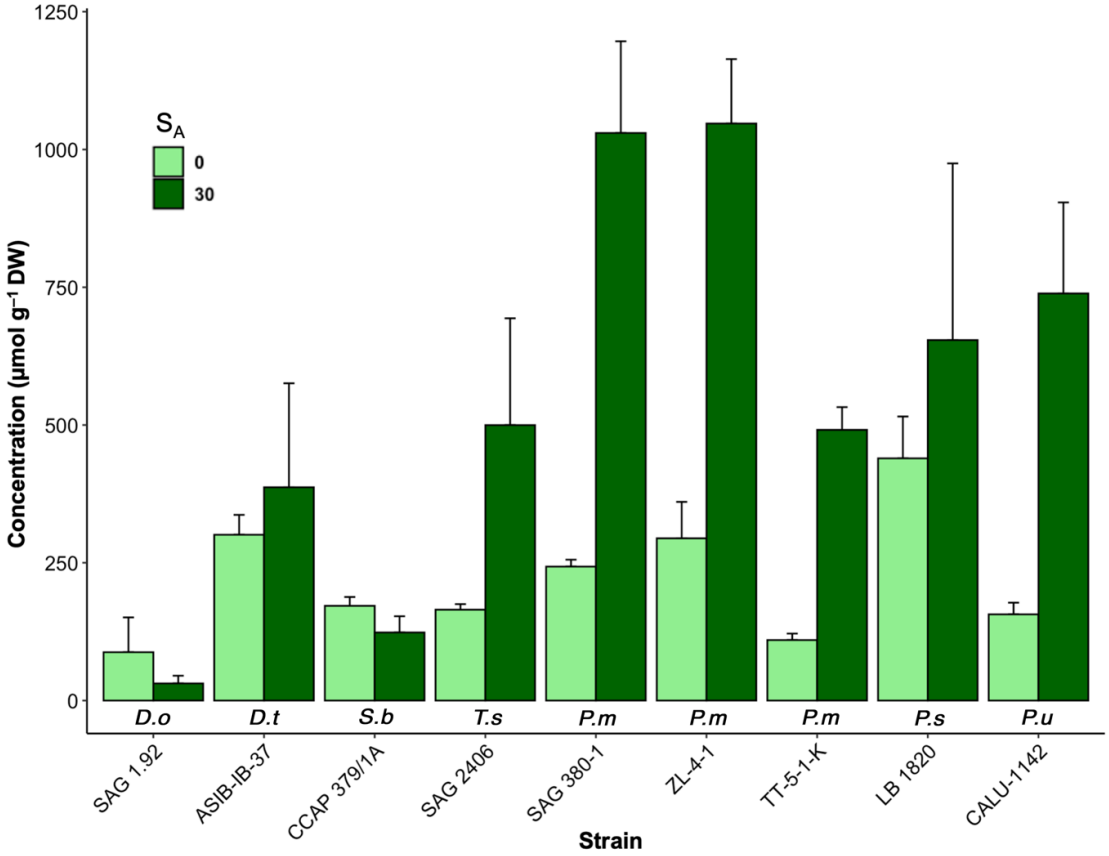

Figure 1. Proline concentrations within the respective strains at growth in 0 and 30 S_A media. Error bars indicate standard deviation ($n = 3$). Differences in the means were significantly correlated to the combined effect of taxon and salinity. Labels beneath the bars are taxon initials and refer to the full names Table 1.

Growth at 30 S_A resulted in increased proline concentrations compared to 0 S_A across all strains except for SAG 1.92 *Desomocccus olivaceus*. The most drastic increases were from *Pseudostichococcus* strains (SAG 380-1, ZL-4-1, TT-5-1-K and CALU-1142), where each strain had a ~3-fold increase in proline compared to 0 S_A medium. A smaller increase in proline concentration (~33%) was observed in *P. sequoieti* LB 1820. Outside of this genus, only SAG 406 *Tritostichocccus solitus* had a similar proline increase. *Pseudostichococcus* strains maintained a relatively steady proportion of proline:sorbitol under both salinities, while *Stichococcus*, *Desmococcus*, *Deuterostichococcus*, and *Tritostichococcus* increased their proportions of sorbitol at 30 S_A.

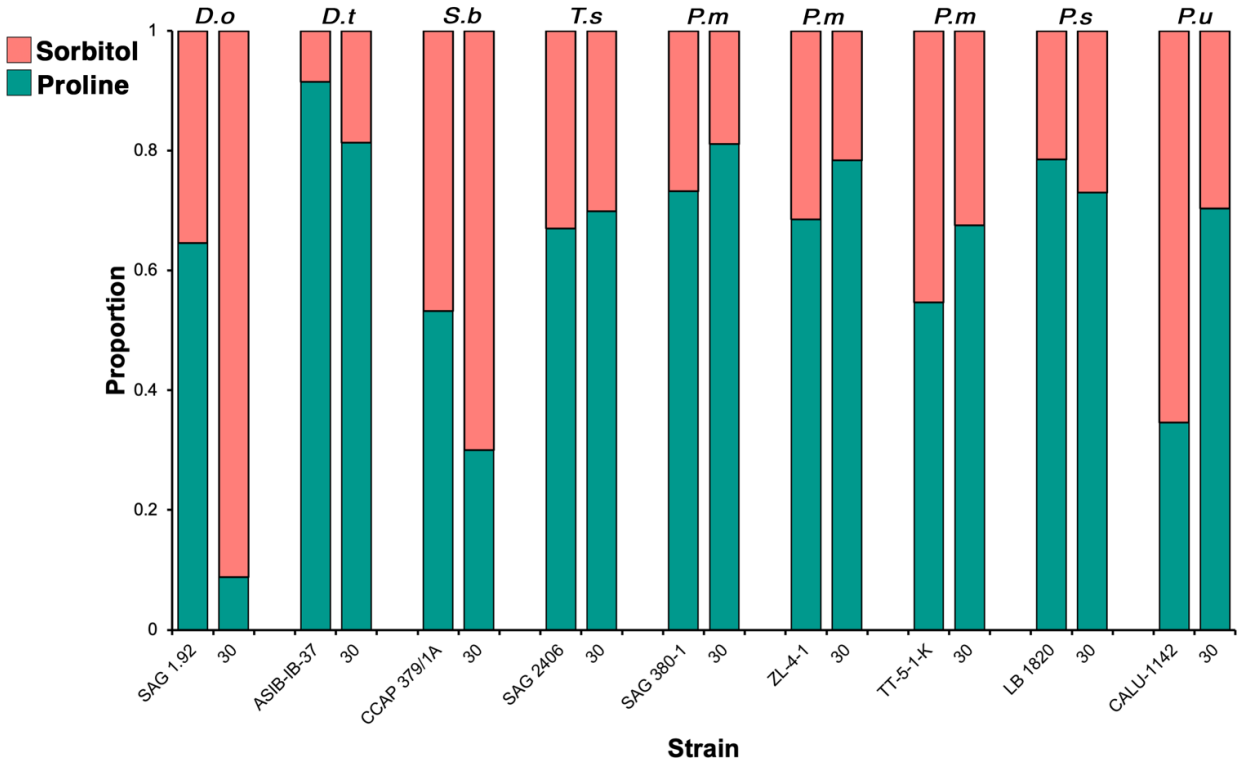

Figure 2. Mean proportions of proline:sorbitol produced in the respective strains at growth in 0 and 30 S_A media. The text labels on the x-axis indicates growth at 0 S_A and "30" indicates the same strain response at 30 S_A. Labels above the bars are taxon initials and refer to the full names Table 1.

3.2. Desiccation and Rehydration

Dehydrating the strains for 6 h at ca. 10% RH resulted in complete inhibition PSII and rehydration led to recovery of all strains after 24 h with varying end values (Figure 3). For reference, the range of starting Y(II) values in the control strains was 0.365–0.660 (average 0.571 $\pm$ 0.078) across all replicates. In the non-control strains, this corresponded to 0.461–0.668 (0.602 $\pm$ 0.061) in the 0 S_A strains and 0.379–0.660 (0.556 $\pm$ 0.068) in the 30 S_A strains. The end phase of desiccation where Y(II) dropped was more gradual and began ca. 30 min earlier in the 30 S_A group compared to the 0 S_A group. The range of end values after recovery phase were narrower for strains grown in 0 S_A (ca. 40–80% of control Y(II)) while the 30 S_A strains ranged from ca. 25–80%, with strains outside the genus *Pseudostichococcus* having lower values up to ca. 60%.

Three strains in the 30 S_A cohort (SAG 1.92, CCAP 379/1A, ASIB-IB-37) consistently had lower average starting Y(II) values (0.483, 0.359, and 0.517, respectively) in the desiccation experiment compared to the other strains. This was not the case at 0 S_A, where the strains had comparably high average starting Y(II) values.

The one-way ANOVA analysis revealed that there was overall no significant difference in average final recovery values between the 0 and 30 S_A cohorts (p = 0.678), and no correlation between final recovery values and salinity ($r = -0.05$). However, when removing *Pseudostichococcus* strains, final recovery Y(II) was somewhat negatively correlated with increased salinity in ($r = -0.518$, $p = 0.00953$). Y(II) activity in most *Pseudostichococcus* strains was virtually fully restored after 24 h following rehydration, except for strain CALU-1142, which had lower final recovery values at both salinities.

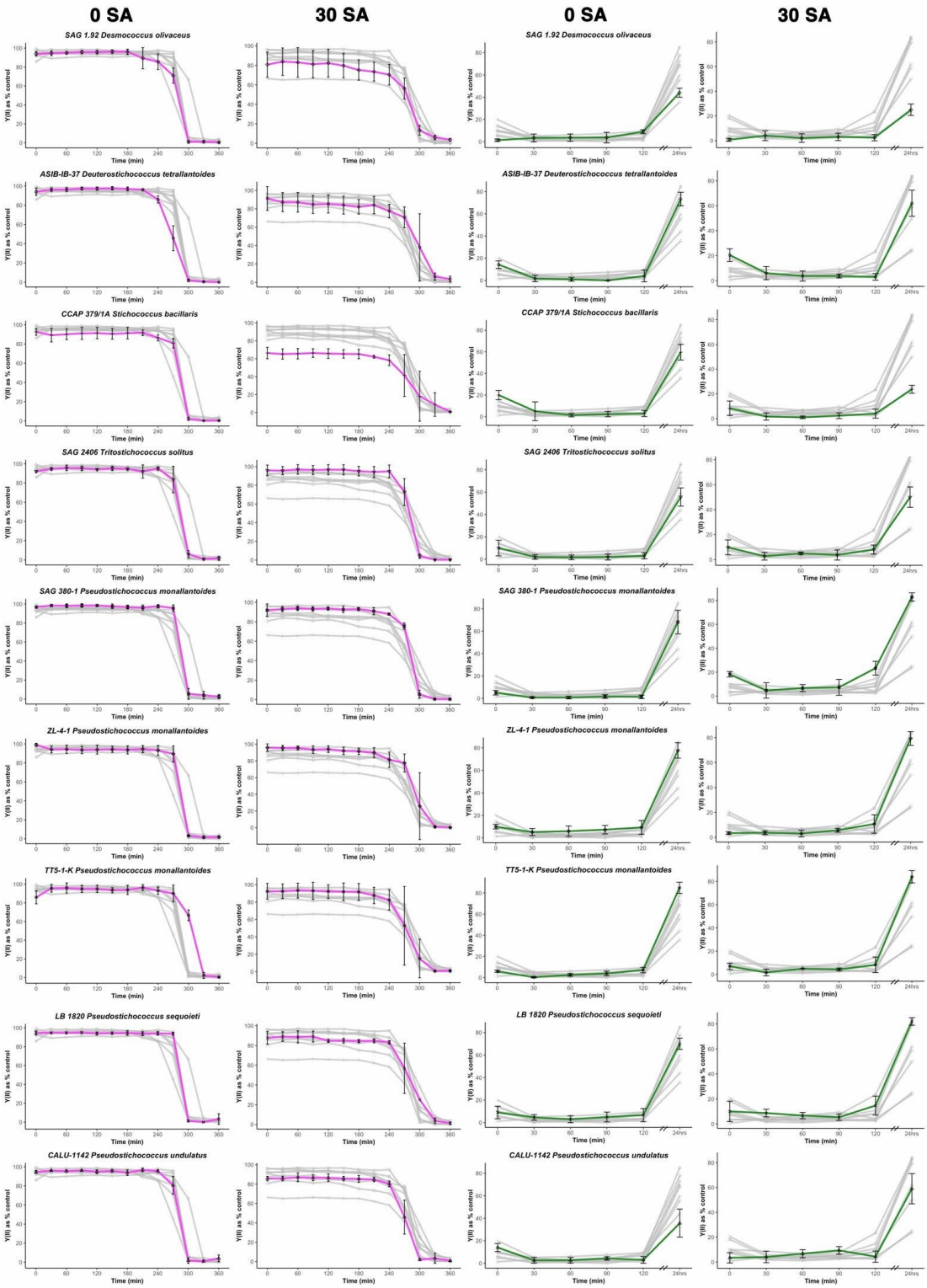

Figure 3. Comparison of Y(II) after desiccation and rehydration in Stichococcaceae strains grown in 0 S_A and 30 S_A media. Each row represents the same strain. Magenta lines show average effective quantum yield (Y(II)) per strain during dehydration, while green lines show the average Y(II) in the recovery phase; grey lines in the background represent the other strains for comparison. Error bars represent standard deviation (n = 3).

4. Discussion

4.1. Effect of Salinity on Proline Production

Osmolyte production is upregulated with increasing salinity and is a protective response to osmotic stress in microalgae. The data on salt-induced physiological changes are scarce for the Stichococcaceae group. This is limited to descriptions of morphological changes induced by salinity [30,31], with more extensive salt stress experiments being done mostly on *Stichococcus bacillaris*, the most known taxon in this group [36,37,50].

Two works from Brown and Hellebust on *S. bacillaris* showed that sucrose, proline, and sorbitol were its major osmolytes, with minor contributions from other amino acids [36,37]. At roughly 30 S_A equivalent (1234 mOsmol/kg), *S. bacillaris* had intracellular concentration of 520 mM for sorbitol, 278 mM for proline, and less than 0.1 mM of sucrose. Unfortunately, as the authors did not use dry weight but rather intracellular concentration, we could not directly compare our values. Nevertheless, our measured proportion of proline:sorbitol for *S. bacillaris* (0.86) at 0 S_A is similar to the 0.97 ratio reported by Brown and Hellebust [37], so it is likely that the results from this study are comparable.

Our recent studies expanded this idea to the Stichococcaceae. In short, sucrose, proline and sorbitol are characteristic osmolytes, independent of salinity [39,40]. Of these three, sucrose was consistently present at lower concentrations than proline or sorbitol. At 30 S_A, sorbitol almost always increased to the range of ca. 48–376 $\mu mol \cdot g^{-1}$ DW; increased sucrose quantities were within the range of 12–135 $\mu mol \cdot g^{-1}$ DW [39]. Here we supplemented this finding with proline quantification, whose contribution is higher than that of both sorbitol and sucrose combined (20–1218 $\mu mol \cdot g^{-1}$ DW), with an average increase of 425.33 $\mu mol \cdot g^{-1}$ DW.

The interaction between salinity and strain accounted for the differential proline response. 30 S_A medium induced higher proline production all strain except for *Desmococcus* and *Stichococcus* strains (Figure 1) but not in the other strains. The major osmolyte for these two strains under salinity seems to be sorbitol and not proline, compared to, *Tritostichococcus* and *Pseudostichococcus*. In these strains, the proportion of proline increases at higher salinity (Figure 2). While sucrose was not directly measured in this experiment, our previous study showed that sucrose generally decreased at 30 S_A in the same strains here [39]. *Deuterostichococcus* had a high proportion of proline at both salinities, but the proportion of proline decreases~10% at 30 S_A. [8,37,43].

Within *Pseudostichococcus*, strains SAG 380-1 and ZL-4-1 *P. monallantoides* produced the highest concentration of proline at 30 S_A. However, as *Pseudostichococcus* was, until recently, monotypic, all *Pseudostichococcus* strains obligatorily belong to *monallantoides* [40]. Furthermore, the strains LB 1820 and CALU-1142 are the only known representatives of *P. sequoieti* and *P. undulatus*, respectively. Molecular verification of the *P. monallantoides* strains in this study will be necessary for clearer taxonomy-based ecophysiological data on osmolyte composition within this genus.

While the experiments in this study were conducted at a maximum of 30 S_A, results from our previous study demonstrated that *Pseudostichococcus* and *Tritostichococcus* can grow at salinities up to 75 S_A [39]. Experimental results recorded the survival of *Stichococcus* of up to 108‰ and 1.6 osmol/kg, roughly equivalent to 10 S_A and 120 S_A, respectively, and that sorbitol and then proline were the main osmolytes, with a minimal contribution from sucrose [36,51]. Proline is energetically "inexpensive" to synthesize, biologically inert and its accumulation is a response to environmental stressors, which includes UV, temperature, drought and salinity [52,53]. Strains that produce more proline under salinity may be able to better adapt to harsh environmental conditions.

Our previous study with these strains grown at 30 S_A show signs of salinity stress, such as enlarged vacuoles and cell conformation changes [39]. Nevertheless, because no growth experiments were performed in this study, the production of proline and other osmolytes cannot be said to directly cause differential fitness in saline conditions within the Stichococcaceae, or to what degree. It is possible that there were strain variation effects for each taxon.

as UV radiation and frost, could significantly change the response to desiccation and osmotic stress. The relative rarity and recent taxonomic status of the genera within the Stichococcaceae means that few empirical results exist on their ecophysiology. More extensive comparison, especially on *Pseudostichococcus*, should be done to determine if they are halotolerance outliers within the *Stichococcus*-like group and if they have biotechnology application potential.

5. Conclusions

At 30 S_A, a plausible natural salinity, most *Pseudostichococcus* strains produce proline as the major osmolyte, in contrast to the other Stichococcaceae. Furthermore, proline contributes to a higher proportion of osmolytes in *Pseudostichococcus* and *Deuterostichococcus* in nonsaline and saline conditions. We showed here that salinity is only slightly correlated with reduced ability to withstand desiccation, hence there are likely other protective factors at work. The results from the desiccation experiment support that the Stichococcaceae are euryhaline, as all the strains could strongly recover from desiccation despite being exposed to salinity.

Author Contributions: Conceptualization, A.T.V. and K.G.; methodology, A.T.V.; software, A.T.V.; validation, A.T.V. and K.G.; formal analysis, A.T.V.; investigation, A.T.V.; data curation, A.T.V.; writing—original draft preparation, A.T.V.; writing—review and editing, A.T.V. and K.G.; visualization, A.T.V.; supervision, K.G.; funding acquisition, K.G. All authors have read and agreed to the published version of the manuscript.

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Declaration of Authenticity

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Rostock, den

Declaration of My Contributions to the Publications

1 A chemosystematic investigation of selected *Stichococcus*-like organisms (Trebouxiouophyta)

This paper was the culmination of about 1.5 year's work on the topic of *Stichococcus* taxonomy and ecophysiology. The idea for the manuscript was provided and encouraged by Prof. Dr. Ulf Karsten and Dr. Karin Glaser. Following this recommendation, experimentation and conceptualization for the writing was mostly done by me. All figures were created by me. The manuscript has been reviewed by all co-authors, who helped revise and provided feedback. I was the final arbiter in terms of which changes were transferred to the final version.

2 The ecophysiological performance and traits of genera within the *Stichococcus*-like clade (Trebouxiophyceae) under matric and osmotic Stress.

The experiments were conceptualized and carried out by me, with experimental troubleshooting and guidance for the phylogenetic part from Dr. Glaser. The writing was mostly done by me. All figures were created by me. The manuscript has been reviewed by all co-authors, who helped revise and provided feedback. I was the final arbiter in terms of which changes were transferred to the final version.

3 *Pseudostichococcus* stands out from its siblings due to high salinity and desiccation tolerance

The experiments were conceptualized and carried out by me, with experimental troubleshooting from Dr. Glaser. The writing was mostly done by me. All figures were created by me. The manuscript has been reviewed by all co-authors, who helped revise and provided feedback. I was the final arbiter in terms of which changes were transferred to the final version.

Conferences and Workshops

- **Taxon-Omics DFG SPP 1991 Annual Meeting.** Montpellier, France. November 11, 2018. **Poster presentation:** “Biological soil crusts as unique microecosystem represent a suitable model system to address taxonomy and cryptic diversity of microalgal key players”.

- **EukRef 2 18S Sequence Database Annotation Workshop.** Roscoff, France. November 4 – November 9, 2018. Workshop to lend expertise to annotate 18S rDNA reference databases for green algae and diatoms.

- **Introduction to automated microscopy and shape analysis of diatoms in facilitating diatom biodiversity estimation.** Bremerhaven, Germany. February 4 – February 8, 2019. Exchange as part of the SPP 1991 Taxon-Omics Project.

- **Advances in Modern Phycology Conference**. Kiev, Ukraine. May 18 – May 19, 2019. **Presentation:** “A taxonomic study of *Stichococcus* Nägeli based on ecophysiological, morphological, and molecular data”.

- **Taxon-Omics DFG SPP 1991 Annual Meeting**. Montpellier, France. June 27 – June 30, 2019. **Poster presentation:** “A taxonomic study of *Stichococcus* Nägeli based on ecophysiological, morphological, and molecular data”.

- **Algal biotechnology techniques and opportunities for the sustainable bioeconomy.** Stuttgart, Germany. November 12 – 13, 2019. Workshop participation.

- **Deutsche Botanische Gesellschaft, 18th Sektion Phykologie Tagung.** Kloster Steinfeld, Germany. March 3 – March 5, 2020.

- **Presentation:** “Loosening the Gordian Knot: the taxonomy of *Stichoccocus* Nägeli”.

Curriculum Vitae

VĂN TÚ ANH

EDUCATION

Dissertation: The response of selected genera within the Stichococcaceae to desiccation and saline stress, with respect to their taxonomy

Faculty of Mathematics and Natural Science

Universität Rostock, Rostock, Germany

August 2018 – May 2022

MSc. Data Science (pending completion)

Eastern University, St. David's, PA, United States of America

June 2021 – May 2022

GPA: 3.95/4.00

MSc. Biology (Biodiversity, Evolution, Ecology)

Freie Universität zu Berlin, Berlin, Germany

October 2015 – May 2018

Thesis: Integrative taxonomy of selected taxa from the genera *Cymbella* C. AGARDH, *Encyonema* KÜTZING and *Encyonopsis* KRAMMER

B.A. Biology

Wesleyan University, Middletown, CT, United States of America

September 2010 – May 2014

GPA: 3.38/4.00

EXPERIENCE

PhD Candidate

Universität Rostock, Rostock, Germany

August 2018 - May 2022

- Identified the effects of abiotic stressors on growth and photosynthesis of biocrust algae
- Performed taxonomic revisions on algae exhibiting semicryptic diversity

Student Researcher

Botanical Garden and Botanic Museum, Berlin, Germany

February 2016 - May 2018

- Assisted in establishing a genetic reference library on diatoms within the German Barcode of Life 2 Project
- Performed phylogenetic reconstruction using Bayesian Inference and Maximum Likelihood
- DNA extraction, PCR, gene sequence analysis, diatom cultivation, SEM microscopy, light microscope