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DOI: https://doi.org/10.3897/arphapreprints.e117814

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What we really know about the dormancy, reproduction, and germination of charophytes and the new method of ECO-*IN-VITRO*-CULTIVATION

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Abstract

This paper aims to provide information on previous basic research by charophyte experts mainly in the field of reproduction, dormancy, and germination of oospores. Therefore, published information, the author's PhD thesis and further new results have been combined to summarise the state of knowledge for Characeae of permanent and temporary water bodies. The understanding and integration of evolutionary, systematic, and ecological knowledge enables the successful establishment of laboratory cultures as well as axenic cultures. The combination of ecological field monitoring and species-specific background knowledge led to the new cultivation method of ECO-*IN-VITRO*-CULTIVATION as shown for *Chara hispida*.

This paper is dedicated to two experts of the Characeae research who passed away too early. Tim Steinhardt, the best oospore lab colleague and Andrzej Pukacz with whom I had the talk of transferring field ecology into my *Chara* cultures during my visit in Poznań, when you wanted to teach me the use of all my properties.

Keywords

Charophytes, reproduction, zonobiomes, ecosystem stability

Introduction

Reproduction, one of the most fundamental processes of life is also one of the most critical steps for aquatic non-seeded plants or algae, such as the submerged living, multicellular charophytes, which have evolved by sexual or asexual modes of reproduction. While sexual reproduction depends not only on optimal environmental conditions for the

formation and fusion of male spermatozoids with a female oogonium, asexual reproduction relies on fragmentation and specialised vegetative algae parts such as reserve accumulation roots or nodal cells (Schubert et al. 2016,Holzhausen et al. 2022). The latter one is a short-term mechanism and is usually characterised by a lower level of resistance to environmental factors and a limited survival time. Nevertheless, asexual reproduction is common in charophytes. It has been demonstrated for several species in limnic and brackish habitats (e.g. Steinhardt and Selig 2007, Skurzyński and Bociąg 2011, Holzhausen et al. 2017). Recent studies from North America suggest that vegetative parts of *Nitellopsis obtusa* may also form a perennial reservoir (Pokrzywinski et al. 2020). In contrast, oospores produced by sexual reproduction show a long-term reservoir in aquatic sediments. They are predicted to have a longer and higher survival rate combined with an increased resistance to drought and mechanical stress (Casanova 1994, Rodrigo et al. 2010, Stobbe et al. 2014). However, essential factors such as

1. vital material,
2. dormancy breakage or
3. optimal germination conditions

are required for colonisation after long- and short-distance transport, recolonisation after disturbance and maintenance of populations. But, the knowledge of fundamental molecular processes is limited, the underlying gene regulation and the transcription factors involved are unknown, a reliable differentiation between the processes of dormancy and germination is controversial within the group of charophytes. Because of their applied and evolutionary interest, to maintain long-term cultures without decay of oospore fertilisation and to understand the reproduction and appearance/disappearance of Characeae in aquatic systems, it is particularly important to elucidate the environmental factors and molecular pathways behind these processes.

Thus, this review consists of available and relevant information on

1. environmental influences on gametangia formation and fertilisation,
2. dormancy,
3. germination induction of vegetative and generative units,
4. biomolecular studies and
5. the resulting ECO-*in-vitro* cultivation method based on ecological data on *Chara hispida*.

1. Formation of gametangia and fertilisation

1.1 Sexual reproduction

The fusion of male and female gametangia and the subsequent maturation of the zygote is called sexual reproduction. This mode of reproduction is described for mostly all charophyte species, even if the frequency of gametangia and oospore formation varies as can be seen in the enormous amount of available literature. Both gametangia, the male

antheridia and the female oogonia were produced by the formation of short shoots from the nodal cell of branchlets (Schubert et al. 2016, Schubert et al. In press). Over the last 150 years, various authors published descriptions of cell development and formation of both gametangia. Due to the amount of considered aspects, not all of them can be considered here in detail. More than that, the plethora of techniques for gametagial studies range from simple descriptions over schematic drawings to high-resolution figures of microtome sections. Table 1 summarizes available microtomic/microscopic studies of antheridia, oogonia and rhizoids. The full plethora of studies on internodal cells were excluded (Sundaralingam 1954, Fetzmann 1957, Barton 1965a, Barton 1965b, Nagai and Rebhuhn 1966, Pickett-Heaps 1966, Shen 1967c, Barton 1968, Vouilloud et al. 2015).

1.1.1 Male antheridia

The majority of detailed gametangial studies have dealt with male spermatozoids and have considered the morphology, cell division or phylogenetic relationship of charophyte spermatozoids to bryophytes or Pteridophyta (Belajeff 1894, Mottier 1904, Karling 1926, Karling 1927, Sasaki 1935a, Sasaki 1935b, Satô 1954, Eggmann 1966, Pickett-Heaps 1967a, Pickett-Heaps 1967b). However, almost all comparisons are based on similarities in structure and form. In contrast to numerous evolutionary studies on the genetic regulations of male and female traits in bryophytes or pteridophytes, only a few studies are available for charophytes such as the presence of the MIKC-type MADS box gene in oogonia and antheridia of *Chara globularis* (*CgMADS1*), suggesting the presence of these genes before diversification (Tanabe et al. 2004). These genes are thought to be involved in the control of sex-specific haploid cell differentiation and stress response (Qiu et al. 2023).

The development of the two flagellated spermatozoids is well known within this group of algae (Varley 1834, Thuret 1840). A brief historical overview of investigations is given by Vouilloud et al. (2012). In the 19th century, different authors studied the cell development of all three cell types (scutum, manubrium, capitulum) in detail (e.g., Varley 1834, Braun 1852 , Pickett-Heaps 1967a), a corresponding summary can be found in Schubert et al. 2016, Schubert et al. In press. Intercellular streaming was only observed in the manubrium and capitulum, but never in external scutum cells (Braun 1852). In applied science, antheridia size is part of almost all species descriptions and has been used for species determination e.g., for *Tolypella* species (Holzhausen et al. 2023). However, the developmental stage, environmental conditions, the reproduction mode, and plant position can influence antheridia sizes, which requires standardised and regional considerations necessary (Ernst 1918, Kwiatkowska et al. 1996, Calero and Rodrigo 2022). Fig. 1 shows the typical development of antheridia which changed from green to light yellowish-green to an orange flabelliform dehiscent antheridium, followed by the release of thousands of spermatozoids and the attachment to oogonia. In 1967, Pickett-Heaps suggested a correlation between the orange colour and the accumulation of plastoglobuli as a source of the increasing carotenoid content (Pickett-Heaps 1967a).

In addition to morphology, there are several studies, mainly by Kwiatkowska and co-workers, on the cell-to-cell connection via plasmodesmata and the process of endoreplication in *C. vulgaris* and/or *C. tomentosa* (Kwiatkowska et al. 1990). The type of plasmodesmata (plugged, unplugged) correlates with the developmental stage and differs in the cell types connected and their internal substances (Kwiatkowska and Maszewski 1985, Kwiatkowska et al. 2003, Kwiatkowska 2003). Accordingly, the symplastic linkage between antheridia and thalli is higher in spring and summer than during the winter dormancy (Shepherd and Goodwin 1992). Broken plasmodesmata therefore determine the most mature state of antheridia and should be considered in further studies. Furthermore, autoradiographic studies have shown the circadian translational activity within the different cell types of antheridia (scutum, manubrium, capitulum, spermatozoids), including the protein synthesis activity of ^{3}H-leucine (Kwiatkowska 1991, Kwiatkowska et al. 1995, Kwiatkowska et al. 1999, Kwiatkowska and Papiernik 1999). Studies on DNA methylation were performed by Olszewska et al. (1997)

Cell walls of *C. corallina* antheridia show the presence of cellulose and pectin homogalacturon, whereas arabinogalactan proteins have only been indicated in epidermal shields and anti-xyloglucan in the capitulum (Domozych et al. 2009). The expression of PIN2-like auxin transporters in antheridial filaments was shown by immunofluorescence studies (Zabka et al. 2016). Studies on the effects of gibberellic acid are presented in subsection V, biomolecular studies.

1.1.2 Female oogonia and fertilization

Compared to male antheridia, there are significantly fewer published studies on female unfertilised gametangia, so-called oogonia. While the male gametangium of charophytes (antheridia) is comparable to the antheridia of bryophytes, the two female gametangia differ. Charophyte oogonia are unicellular, ovoid and the fertilisation leads directly to the zygote. In bryophytes, however, archegonia are multicellular and lageniform, and fertilisation leads to sporophyte development, with the formation of spores (Braun 1852). Previous literature has concentrated on descriptions within general cryptogam overviews, on developmental stages of cell divisions by microtome sections and on size variations (e.g. De Bary 1871Covich and Tsukada 1969, Maier 1973, Sawa and Frame 1974, Leitch 1986, John et al. 1990, Leitch 1989, Leitch et al. 1990, Kwiatkowska et al. 1996). The cell development of the female gametangium will not be discussed in detail here. Descriptions can be found in Braun (1852), Sundaralingam (1954), Schubert et al. (2016), Schubert et al. (In press). Microtome studies of female gametangia and included cell compounds are listed in Table 1. Intercellular streaming was observed only in the multicellular basal node, which give rise to the envelope cells, the unicellular internodal cell and the five envelope cells. However, the intercellular streaming within the oogonium could not be observed in detail (Braun 1852).

In 1971, Anton de Bary described the development of the female gametangium up to fertilisation by male spermatozoids (De Bary 1871. Using a Brückean magnifying glass, he was able to observe in detail not only the cell development of the surrounding envelope

cells but also the formation of a tube-like channel, which results from a difference in the lateral diameter of the five narrowed apical parts of the spiral cells to the lateral diameter of the basal parts, which are located at the apex of the oocyte. Masses of spermatozoids rest near the area of the five tube-like channels, fixed by the gelatinous intercellular compound of the five narrowed channels, formed between two apical parts, allowing the intrusion of spermatozoids as a dense bundle formation to move to the intercellular part above the oocyte. In contrast, the intercellular part is only traversed by a narrowed channel, which ends in a widened funnel shape at the apex of the oocyte. A scattered membrane structure allows individual spermatozoids to pass through the apex. The zygote is develops, followed by the formation of a thickened cellulose layer and a thickening and change in the colour of the spiral cells. It is thought, that the absence of this layer is associated with infertility although simultaneously the same amount of amylum is present.

The fusion of male spermatozoids and female oogonia is one of the most fascinating processes in charophyte development. The molecular signalling pathways involved during fertilisation or meiosis are still unknown. The exact time of meiosis is still unknown. For a summary see Schubert et al. 2016, Schubert et al. In press. Five different opposing hypotheses have been proposed for the exact time point of meiosis in charophytes: (I) just before the germination of the oospore (Oelkers 1916), (II) in the early cell stages of the antheridia and oogonia (Tuttle 1924, Tuttle 1926), (III) during the development of the protonema filament (Gonçalves da Cunha 1936, Gonçalves da Cunha 1942) and (IV) immediately after the fertilisation (Shen 1967b, Shen 1967c). Although hypotheses (I) and (IV) are almost identical, both have been listed because of gaps in the descriptions of cell movement and the resting phase before germination. In 1899, Goertz described the observation of a possible delayed fertilisation by the protrusion of starch into the neck of the female gametophyte (V, Goetz 1899). Despite a comparison with *Vaucheria*, no further evidence is found in the literature Oltmanns 1895). The myth of meiosis is not well known, mainly due to complications in microtome sectioning. The high starch content of oogonia and oospores makes the procedure difficult. The establishment of methods for ultrathin sectioning of all developmental stages (from oogonia formation to the released oospore) is one of the most important prerequisites for solving this mysterious process.

Cultivation and field studies have shown that the process of fertilisation may be dependent on environmental conditions such as temperature, light, or salinity. Cultivation or germination conditions, as well as species-specific growth behaviour, should be considered when comparing existing literature. For example, Karling 1924 compared the maturation of oospores under different light conditions. Whereas in greenhouses (high temperature during the day, low temperature at night) the oospores matured, in climatic chambers (high temperature during the day and night) the oogonia remained in an unfertilised state. The same has been proposed for *C. braunii* (Sato et al. 2014, Holzhausen et al. 2022). The same is true for *C. aspera* with respect to light and salinity. The highest rate of gametangiogenesis was found under natural ecological conditions. Greater variation in conditions compared to the natural ecological status resulted in stress responses such as reduced gametangia numbers or cell death (Blindow et al. 2003). In this context, tracer studies on the polarity and concentration of photoassimilates in vacuolar saps (sugar-

phosphates, sucrose, malate, amino acids) of *C. vulgaris*, *C. hispida* and *C. corallina* showed that

1. differences in sap composition differences are caused by seasonal changes and reproduction (MacRobbie 1962, Barr 1965, Winter et al. 1987),
2. apices are sinks in sterile and fertile plants (Schulte et al. 1994),
3. gametangia are sinks during reproductive periods (Schulte et al. 1994),
4. a higher activity of 1,5-bisphosphate carboxylase (RuBPCase) and a fourfold higher ferricyanide-dependent oxygen evolution in branchlets than in internodes (Ding et al. 1991b), and
5. differences between growing and overwintering periods (Shepherd and Goodwin 1992),
6. intercellular transport occurs via plasmodesmata (e.g. Leitch et al. 1990) and
7. the intercellular transport is determined by the rate and concentration of cytoplasmic streaming and a possibly unclear “intrinsic regulatory process” (Ding et al. 1991a).

Assuming that the rate of cytoplasmic streaming depends not only on general seasonal changes but also on the exact environmental conditions, the differences in oospore sizes, as reported by different authors, could probably be explained by the number of cell-cell-connections between branchlets and oogonia and the associated rate of sucrose uptake and starch storage, respectively (Holzhausen 2016). Comprehensive analyses combining phenological and cytological studies, molecular mechanisms and environmental data over several years including the open question of chloride uptake by oogonia could resolve this open question.

1.2 Asexual reproduction

Asexual reproduction by vegetative parts of plants is common in aquatic plants and algae. For reviews about this, see e.g., (Lovett Doust and Lovett Doust 1988, Barrat-Segretain 1996, Barrat-Segretain et al. 1998, Barrat-Segretain and Bornette 2000Cecere et al. 2011, Agrawal 2012). Within the Characeae, vegetative reproduction is a widespread feature in all genera. However, there is one exception, *Chara canescens*, which is the only species that is unable to reproduce vegetatively and is therefore dependent on the formation of oospores for reproduction (Krause 1997). In addition to its unique position, this species can reproduce parthenogenetically or sexually through dioecious populations, and it has been shown that females of parthenogenetic and sexually reproducing populations are genetically distinct (Schaible et al. 2009a, Schaible et al. 2009b, Nowak et al. 2019) and differ in chromosome numbers (Ernst 1917) .

In contrast to generative reproduction, there is a limited amount of literature on asexual reproduction. However, only a few scientists (Braun 1852, Giesenhagen 1897, Migula 1898 Bharatan 1987, Sederias 2003, Skurzyński and Bociąg 2011, Pokrzywinski et al. 2020) have studied this type of propagation in detail for species of the genera *Nitellopsis, Chara*, *Lamprothamnium and Paleonitella*. In most cases, cell division in nodal cells and the emergence of new shoots were mainly visualised by schematic representations, studies

and visualisation with modern microscopy techniques are rare, except for the rhizoids themselves (e.g. Braun et al. 1996, Braun et al. 1999). In 1896, Giesenhagen summarised the differences between bulbils and modified root nodules, based mainly on the work of Migula (Migula 1898) and his own results (Fig. 2A/B). However, Giesenhagen's findings are not entirely consistent with Migula's, as *Chara aspera* root nodules are not unicellular, and Giesenhagen gives a detailed description of their formation and cell division (Giesenhagen 1897). These nodules are cells that store starch as a reserve reservoir (Fig. 2C). However, the different provisioning of individual root nodules, reflected in the different sizes within the complex, has not been fully elucidated. The number of reserve reservoirs is species-specific, with more than four being described as unusual in *Chara aspera*, whereas complexes of three to five or more than 12 have been described for *Lychnothamnus macropogon* (Braun and Nordstedt 1882). Fig. 2C shows a network of reserve reservoirs, with more than four being more common, as described by previous authors. The nearest root node or the apical part of the reservoir cell is assumed to be responsible for the development of new shoots. In his study, Bharathan describes the same type of 'bulbils' for *Chara hornemannii*, *C. aspera*, *L. papulosum* and *L. succinctum*, but in contrast to earlier studies, they are also referred to as bulbils and not as root nodules (Bharatan 1987). Interestingly, the authors' own results have shown that sterilisation and severance of the connecting rhizoid filaments do not lead to the development of new shoots. So far, no signalling pathways are known for these connecting rhizoid filaments. The study by Bonnot et al. (2019) showed that the *Chara braunii* ROOT HAIR DEFECTIVE SIX-LIKE (Stevens and Parsley 2022) genes do not complement the rhizoid or root hair development in mosses and land plants, initiating a completely different function. In contrast to this, the induction of germination by the root/shoot nodule complex is simple and occurs within a few days under laboratory conditions (Giesenhagen 1897, Bharatan 1987, Blindow et al. 2009). In addition to modified root nodes, vegetative reproduction can occur via modified shoot nodal cells. The ability of these cells to produce new shoots after periods of drought, fragmentation or death of apical cells is well documented (Fig. 2D). This ensures the growth of new populations under unfavourable conditions for oospores (Casanova 1994, Casanova 1999, Blindow et al. 2009). Under the ontogenetic assumption that the regeneration of fragmented node cells is based on the formation of side branches or cone-like outgrowths (Giesenhagen 1897) as described for the strawberry-shaped root nodules of *C. baltica*, a limited number of new thalli can be developed by a modified shoot node. These are the same as lateral branches but reduced. Various studies (Braun and Nordstedt 1882, Giesenhagen 1897, Schubert et al. 2016) have been published on the structuring of shoot node cells, including cell division (Fig. 2E-G). Interestingly, different forms of overwintering structures can be observed: star-shaped modified shoot nodes in *N. obtusa* (Fig. 2A/B), strawberry-shaped modified nodal cells in *C. baltica* or cardioid-shaped modified root cells in *C. fragifera*. The existence of star-shaped bulbils in *C. connivens*, as mentioned by Clavaud (Clavaud 1863, Giesenhagen 1897), is not fully confirmed. The phenomenon of reduced, starch-filled bulbils acting as reserves can also be found in other species such as *C. baueri*, *C. foetida*, *C. fragilis*, *C. hispida* or *C. subspinosa*. Bulbils have also been described in *Nitella* species from the southern hemisphere (De Maisonneuve 1859, Casanova 1994, Casanova 1999). However, the formation and gene expression in these vegetative cells is not fully understood, although a correlation with distribution

patterns caused by landmass separation and environmental changes has been hypothesised (Croy 1979). This is supported by barcoding studies that found adaptive mutations of chloroplast and mitochondrial genes in different habitats (Kato et al. 2008, Schneider et al. 2015). The lack of multidimensional approaches, including field, laboratory, microscopic and molecular studies, to address open questions about vegetative reproduction should be addressed in future studies.

1.3 Rhizoids

Charophyte rhizoids function similarly to terrestrial plants, mainly for anchoring but also for absorbing nutrients, which are often associated with microorganisms. The cell development of rhizoids has been studied by different authors. Detailed descriptions of cell development, including cell thickening can be found in the literature (e.g., Braun 1852, Zacharias 1890, Linsbauer 1927, Shen 1967a, Sievers et al. 1991, Braun and Sievers 1993) .

In general, rhizoids consist of two cell walls, a single-layered outer cell wall and a multi-layered inner cell wall. The components pectin and cellulose are described for both. The outer layer of *Tolypella intricata* f. *humilior* also contains mucilage (Fridvalszky 1958). This exudate and polysaccharide- and glycoprotein-rich coating is also known from ecophysiological studies of *Lamprothamnium papulosum*. A correlation between salinity and the thickness of the mucilage secretion was observed (Shepherd et al. 1999). There are no further studies on rhizoid mucilage or oospore-mucilage. Functional characteristics of mucilage on charophyte rhizoids, especially as hydraulic bridges related to nutrient supply and desiccation tolerance, are not known. In land plants and hydroterrestrial algae, mucilage has been shown to act as a barrier to pathogens and harmful metals, to protect root tips and to penetrate the soil (Herburger et al. 2022). In addition to mucilage, vesicles containing $BaSO_4$, which act as gravity-sensing statoliths, are located on the outer cell wall (Hejnowicz and Sievers 1981, Braun and Sievers 1993). The apical part of the rhizoids contains the stationary cytoplasm and the nucleus, whereas the basal part contains the mobile cytoplasm, including the cytoplasmic streaming.

The main focus of research using charophyte rhizoids as model cells is gravitropism. Positive gravitropism, or downward growth, is observed in charophyte rhizoids. The first study was done by Zacharias (Zacharias 1890). He showed that the rhizoid tip cells also grew downwards when the rhizoid position was changed. 100 years later, it was clear that the actin cytoskeleton of *Chara globularis* and *Chara foetida*, which is controlled by actin-binding proteins, plays an important role in the mechanism of gravitropism and in the polarised perception of charophytes (Sievers 1967, Braun and Limbach 2006). A central role in the apical rhizoid zone is supported by immunolocalisation studies of actin polymerisation factor and profilin. Negative gravitropism is described for protonematal cells in contrast to rhizoids.

Further studies on gene expression as described above are not available for charophytes. However, Sandan's studies of *C. braunii* (*C. coronata*) and *Nitella flexilis* showed that root

development was enhanced by IAA-K solution as opposed to pure tap water or Knop's solution (Sandan 1955).

2. Dormancy and germination induction

In both permanent and temporary water bodies, the time between fertilisation and germination is extended by a period of dormancy to increase the possibility of a period of low competition and successful population establishment, although subsequent reproductive capacity is not guaranteed (Casanova and Brock 1990, Vleeshouwers et al. 1995). The adaptive seed trait of a metabollically inert state germination capacity as well as physiological processes such as mobilising resources and activating/deactivating genes leading to morphological changes and subsequently germination are complex and unknown so far. According to Baskin and Baskin (1985), the physiological, morphological, and physical state

1. at the time of maturation and
2. at the time of germination must be considered and combined with environmental habitat conditions at
3. the time of seed state changes and
4. during the maturation and germination process.

However, many classification systems have been established because there is no single definition of dormancy (Bewley and Black 1982). The distinction between developmental time, i.e., primary, and secondary dormancy, is the simplest and most convenient. While primary dormancy is induced during development and maturation (e.g. Crocker 1916, Hilhorst 1995, Hilhorst 1998, Finch-Savage and Leubner-Metzger 2006), secondary dormancy is induced only after release from the mother plant and requires the loss of primary dormancy. Interestingly, although not yet confirmed by molecular studies, the hypothetical physiological dormancy model for terrestrial seeds (Hilhorst 1998) also seems to apply to charophytes. This combined model describes the regulation of secondary dormancy by temperature and the subsequent triggering of germination by the availability of nitrate, light, and gibberellic acids. Fig. 3 shows the modified model of Hilhorst (1998) and implies that dormancy and germination are directly correlated with the amount of phytochrome receptors present. These red (R) and far-red (FR) receptors act as light sensors in several plants/algae (Inoue et al. 2019), which are mainly located in the nucleolus. Several germination studies have identified that germination is triggered by the supply of gibberellic acid (Sederias and Colman 2007, Holzhausen et al. 2022), although evolutionary studies have shown that the gibberellic acid pathway has evolved in mosses (Nishiyama et al. 2018). Further studies are needed to determine whether chemical precursors could explain this phenomenon. In addition, endogenous gibberellic acid has been measured in studies on *Chara tomentosa* and *Chara vulgaris* (Kaźmierczak and Rosiak 2000, Kaźmierczak 2001, Chowdary 2014). Fertilisation was not found to be related to endogenous gibberellic acid content, but to sex. While the concentration in sterile shoots increases towards the apex, the concentration in fertile shoots declines from the apex downwards. In contrast, the levels in male plants exceed those in female plants.

The induction of germination itself is a signal for the end of the resting phase (dormancy) and for the presence of favourable environmental conditions for growth. Sensors are used to provide signals on temperature, light, nutrient availability, and hydration status. Competitive conditions, on the other hand, are undetectable and represent a 'trial and error' approach. Individual factors, although not always fully distinguishable, are considered in the following subsection.

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2.1 Biogeography

The origin of the material (spores/plants/sediments), although neither an abiotic nor a biotic factor, is of crucial importance. This concerns both the biogeographical origin and the occurrence in permanent or temporary biotopes. In addition to morphological differences between regions (Brzozowski and Pełechaty 2020, Holzhausen et al. 2023), there are also programmed physiological differences caused by climates. Most studies with oospores have been carried out on *Nitella* and *Chara* species from permanent water bodies belonging to the zonobiomes III (sub-tropicalal arid climates of sub-deserts and deserts; Pott 2005) and VIII (boreal zone, Table 3). In contrast, most studies on germination use sediments from zonobiome VI, especially from the Baltic Sea (Table 4), while studies on oospore germination are rare.

Studies of *Chara* species from permanent waters in the northern hemisphere are less common. And most studies to date have focused on secondary dormancy, and only a few acquire primary dormancy by leaving the mother plant (Forsberg 1965a, Shen 1966) and on differences in the degree of dormancy (Sederias and Colman 2009), see Table 2. Collectively, these studies have shown that

1. species-specific differences exist due to different temporal and spatial offsets of charophytes (Casanova and Brock 1990, Bonis and Grillas 2002) and
2. freshly extracted oospores have a higher dormancy level than those extracted from sediments, which may be explained by the inhibitory effect of abscisic acid (e.g. Takatori and Imahori 1971, Casanova and Brock 1996),

which is gradually reduced during sediment storage (Sabbatini et al. 1987, Sederias and Colman 2007, Penfield and King 2009). The most effective method for breaking the dormancy of different species of *Nitella* and *Chara* is listed in Table 2**.**

Field studies have shown that charophytes of permanent waters emerge mainly in spring or summer, in their so called “temporal window” (Stross 1989). However, some species prefer autumn or biennial germination periods. The German Charophyte Monograph

“Armleuchteralgen. Die Characeen Deutschlands” (Arbeitsgruppe Characeen Deutschlands) and the forthcoming European Monograph (International Research Group of Charopytes, http://www.sea.ee/irgcharophytes/) provide a good overview of species-specific ecological niches. In contrast to this, the germination of oospores in temporary water bodies is dependent on fluctuations in the water level. Adaptions to these unpredictable variations include short or annual life cycles for *C. braunii*, *C. canescens*, *Tolypella* or *Nitella* species (Casanova and Brock 1990, Brock and Casanova 1991). Although interannual differences cause a high variability of initial environmental conditions on germination and composition of aquatic plant assemblages, simulation studies have shown the effects of the human influence (Rodríguez-Merino et al. 2017). Therefore, it will be necessary to harmonize economic, agricultural and recreational use in order to protect endangered charophyte species as done for T. salina from mediterranean salt marshes (Lambert et al. 2013).

2.2 Temperature

The influence of temperature on oospores has been considered by several authors, both on dormancy breakage (Table 2) and on germination of oospores. The temperatures used for germination approaches with oospores or sediments are summarised in Table 3 and Table 4. In most cases, oospores or sediments were pre-treated for days or weeks using cold temperatures between 4°C and 10°C. In most cases, spores or sediments were pretreated for days or weeks at 4-10 °C to mimic natural vernalisation. Despite conflicting results, the most effective method for breaking dormancy and subsequently inducing germination appears to be cold treatment of oospores followed by irradiation with long-wave light. There are, however, species-specific, and biogeographical differences. For example, the dormancy of freshly extracted oospores (primary dormancy) of *Chara vulgaris* was broken by low temperatures, whereas for the germination of *Nitella furcata* from New Zealand storage at room temperatures is preferred (de Winton et al. 2000). The origin of the samples and the associated climatic conditions appear to be of critical importance. The evaluation of the available data shows that a correlation between conditions for dormancy breakage and zonobiome can be made (Table 2). For example, almost all species in zonobiome V (warm temperate zonobiome) can be broken out of dormancy by pre-treatment at 15°C, whereas species in zonobiome VI require a cold storage phase.

2.3 Light availability

The growth, the preferred reproduction modus and depth distribution of charophytes is determined by the availability of light and the underwater light climate (Barko and Filbin 1983, Canfield et al. 1985, Küster 1997, Middelboe and Markager 1997, Sagert and Schubert 1999), but also by the germination of oospores. However, multiple interactions with ecological factors such as temperature (e.g. van den Berg et al. 1998) or phytoplankton biomass (Brunet et al. 2011) hamper the consideration of all levels of light. Increasing eutrophication, characterised by an increase in nutrient concentration and associated phytoplankton growth, can also rapidly terminate the existence of charophytes. A number of studies have shown that the reduced availability of light, caused by a high

density of phytoplankton, can be a decisive factor in the decline of charophytes (Blindow 1992, Arthaud et al. 2012). In addition, light acts in a variety of ways. Photon flux density, spectral composition or the duration of light exposure (periodicity) can all have important signalling effects. For seeds, studies have also shown that the genetic basis, the position in the sediment, the absorptive capacity of the coat and the state of dormancy play a crucial role (Grime 1979, Grime et al. 1981, Pons 2000). Results are partly contradictory despite the large number of studies on light-induced oospore germination. While several authors have attributed a positive effect to the intensity and quality of the light, others have not observed any effect of these factors on the germination of oospores (Shen 1966, de Winton et al. 2004). This inconsistency may be due to biogeography, species specificity, the type of sample material used (oospores versus sediment sample, fresh oospores versus oospores from diaspore banks) and the synergy thereof. The results of existing literature are summarized Table 3 and Table 4**,** divided into experiments with sediments or only oospores separated from sediments. For example, Shen (1966) did not observe any effect of light on the germination of *C. contraria* oospores, whereas Sabbatini et al. 1987 identified this factor as the breaking of dormancy. In this case, however, it is difficult to derive a biogeographical response, because Shen does not specify the origin of the oospores of *C. contraria*. Sabbatini et al. 1987 uses oospores from zonobiom III, which come from the dry areas of the temperate zone, characterised by strong annual rainfall variations. These inter-annual rainfall variations, combined with high summer temperatures, initiate the positive trigger for light-induced germination. Similar contradictions could be found for *C. globularis*. Proctor 1967 described a positive effect of light, while de Winton et al. (2004) and Holzhausen et al. (2017) could not confirm this. Furthermore, de Winton et al. (2000) demonstrated the germination capacity of *C. australis* and *C. globularis* under dark conditions (< 0.5µmol photons/(s*m^2)). However, there are differences in methodology, particularly in the origin which may explain the wide range of response behavior (Table 3). In any case, such inconsistencies highlight the need for further basic research on charophyte dormancy and germination to develop reliable, species-specific protocols, including habitat and geographical differences (permanent waters vs. temporary waters, zonobiomes) for continuous cultivation allowing the full life cycle.

2.4 Nutrients and phytohormons

The chemical and physical properties of the substrate and medium, including invertebrates and microbes determine the success or failure of charophyte germination and growth, in addition to the main cues of temperature and light. This includes aspects such as:

- **oospore sterilization agent and concentration** (Forsberg 1965c, Sederias and Colman 2007, Sederias and Colman 2009, Holzhausen 2019, Holzhausen et al. 2022
- **inorganic media** (Forsberg 1965a, Forsberg 1965b, Forsberg 1965c, Proctor 1967, Andrews et al. 1984, Wüstenberg et al. 2011)

- **content of organic material** (Buljan 1949, Gumiński 1983, Smart and Barko 1984, Kalin and Smith 2007, Pörs and Steinberg 2012, Holzhausen 2016, Holzhausen et al. 2017, Holzhausen et al. 2022)
- **cyanobacteria / phytoplankton** (Casanova et al. 1998, Rojo et al. 2013a, Rojo et al. 2013b, Fukushima and Arai 2015, Pełechata et al. 2016)
- **nutrient concentration** , e.g., phosphorus, sulfides or nitrates (Reid et al. 2000, Sederias and Colman 2009)
- **pH value** (Shen 1966, Kim and Mun 1997, Quanter 2020)
- **phytohormones** (Sederias and Colman 2007, Tarakhovskaya et al. 2007, Holzhausen et al. 2022)
- **salt concentration** (Winter and Kirst 1990, Winter et al. 1996, Shepherd et al. 1999)
- **substrate density, structure, and water content** (Boedeltje et al. 2002, Matheson et al. 2005, Porter 2007)
- **antibiotics** (Christian 2004)
- activity of **benthic invertebrates** (Kuczewski 1906, Fukuhara and Sakamoto 1987, Kotta et al. 2004, Hansen et al. 2011
- **epiphytic associated microorganisms** (Hempel et al. 2008, Kataržytė et al. 2017, Rodrigo et al. 2021)
- beneficial **growth promoting substances and phytopathogens** (Wajih and Sinha 1980, Ghazala et al. 2004, Lusweti and Pili 2021)

Interestingly, studies have shown that pure inorganic medium inhibits oospore germination (Imahori and Iwasa 1965). This was strongly supported by a high percentage of germination experiments (see Table 3). In most cases, the addition of organic compounds/ organic material was critical to induce charophyte germination. However, this also increased the risk of unwanted contamination. However, there is a lack of studies on the interaction of germination-promoting micro-organisms. For terrestrial plant seeds and macroalgae, promoting seed germination and growth development was confirmed by producing phytohormones providing water and/or minerals, nitrogen fixation or pathogen defence (Tsavkelova et al. 2007, Spoerner et al. 2012, Wichard 2015). For Characeae, information on associated bacteria is rare (Hempel et al. 2008, Kataržytė et al. 2017), and information on soil microbes, especially in the rhizosphere of *Chara* meadows, is completely lacking.

For most of the above aspects there is a consensus in the currently available literature. Discrepancies and partly contradictory results exist only for the use and influence of sterilising agents. These range from combined alcohol and calcium hypochlorite to sodium hypochlorite and hydrogen peroxide. The same applies to the concentration (1%-30%) of substance used. While some authors have not been able to detect any effect on the germination of oospores, others have reported an inhibition of germination after the use of high concentrations of hydrogen peroxide (Forsberg 1965c, Holzhausen et al. 2022).

2.5 Desiccation/ redox potential

Seeds stored in sediments show long-term viability, whereas seeds stored in moist conditions may lose viability over time (Villiers 1974). Changes in desiccation/redox potential manipulation have also been observed to have species-specific treatment responses Kalin and Smith 2007. Desiccation of *Nitella cristata* var. *ambigua* oospores increases germination, whereas no effect was observed for *Nitella sonderi* (Casanova and Brock 1996). Dried oospores of *C. canescens*, *C. contraria*, *C. evoluta*, *C. hydropitys*, *C. globularis*, *C. rusbyana*, *C. sejuncta* and *C. zeylanica* germinated after freezing (-20°C), cold (3°C) and warm temperatures (24°C) over a period of up to four years in studies by Proctor (1967). A high percentage of germination of *C. canescens* after six years of wet storage (room temperature, dark) has been shown in own experiments.

Furthermore, a decisive effect of desiccation and salt concentration on germination has been shown in studies of the germination capacity of *Tolypella salina* oospores from different French salt marsh sediments (unpublished data). A significant increase in the number of seedlings after desiccation was observed when comparing germination rates of wet and pre-dried sediments. These results suggest that targeted temporary desiccation and reduction of digested sludge could allow for a continuous recolonisation of *Tolypella salina* in France.

2.6 Seed size and burial depth

In addition to physiological triggers, morphological and geological factors have influence on oospore germination. Only a small number of studies on this subject can be found in the literature. The seed size of oospores and spores allows the availability of energy reserves in the form of starch grains as a resource for the growth of the transparent seedling part (Venable and Brown 1988, Casanova and Brock 1990, Casanova and Brock 1996). The relationship between burial depth and ability to germinate was investigated by Dugdale et al. 2001 using New Zealand oospores. Their results of highest germination rates up to 50 mm were confirmed by most germination studies of sediment samples. Interestingly, they found a relationship between seed size and burial depth: Heavier *C. globularis* oospores germinated from deeper sediment zones than lighter *Nitella* oospores. Those oospores from deeper sediment layers can be considered as a potential internal oospore bank as shown by restauration studies of so-called “ghost-ponds” (Alderton et al. 2017, Sayer et al. 2023). Mediation by soil invertebrates is only known for the seeds of e.g., *Zostera marina* (Blackburn and Orth 2013) but not for charophytes although Kuczewski (1906) described the supply of the water snail *Limax paludosa* for clear water cultivation vessels.

3. Growth and *in-vitro* cultivation

Charophytes have been cultivated for more than 100 years. In most cases, charophytes were cultivated using laboratory-based or greenhouse-based techniques. Problematically,

the epiphytic microorganisms grow independently of oxygen or the duration of cultivation. Only a few authors (Kuczewski 1906, Karling 1924) have attempted to overcome these problems by adding grazers such as snails or daphnia. A drastically reduced selection of cultivation experiments and conditions can be found in Table 5. Due to a lack of information on cultivation and propagation methods, studies involving short-term experiments on photosynthesis are not listed. Nevertheless, they may provide information on light intensities applicable to cultivation methods. In recent decades, aquaculture and outdoor mesocosms have been additionally used to grow and reproduce algal material, e.g., for (electro-) physiological studies or regeneration of aquatic systems (e.g. Tazawa et al. 1979, Tazawa et al. 1987, de Winton et al. 2000, Beilby et al. 2006, Rodrigo et al. 2010, Holzhausen 2019, Blindow et al. 2021). In contrast to constant indoor cultivation conditions, outdoor experiments or greenhouse cultivation require the documentation of environmental conditions to determine influencing factors such as growth, expression of morphological traits or gametangia production. In addition to the extreme growth of microorganisms in culture vessels, the development and maturation of gametangia is often suppressed completely or decreases over time. According to the existing literature, this does not only depend on the periodicity or preference for vegetative growth, but also occurs with repeated reactivation of the material, as can be seen in Karling (1924). The influence of various abiotic factors such as temperature, light regime or media composition including amino acids and vitamins has been the subject of a large number of studies over the last 100 years, but only a few have considered the development of gametangia during experiments. Effects of amino acids and vitamins, added individually or in mixtures, on the growth of protonemata and adult thalli of *Chara zeylanica* were studied by Imahori and Iwasa (1965). Here they identified casein hydrolysate and polypeptone as effective for promoting growth in protonemata and adult plants, whereas yeast extract and individual amino acids were only marginally effective. In particular, the growth of adult plants was promoted by the vitamins and phytohormones cobalamin, nicotinamide, GA and kinetin (6-furfurylamino-purine). However, thiamine (B1) and pyridoxal (B6) only promoted the growth of the protonemata. Further studies were carried out by Libbert and Jahnke (1965) who found an antagonistic effect of indoleacetic acid/auxin (IAA) and antiauxin (PCIB) in *Chara vulgaris* (*Chara foetida*), *Chara hispida* and *Chara subspinosa* (*Chara rudis*).

4. Biomolecular studies

The number of biomolecular studies on the Characeae is lower than on other plant organisms. However, over the last 100 years, various cell wall studies, phytohormone studies and cytological studies have been carried out.

In addition to the cell walls of antheridia, the **chemical composition** of charophytes has also been studied in internodal cell walls of various species, mainly to elucidate the ion exchange in the cell walls. Overall, charophytes contain the same proteins, pectates, lignin, hemicellulose, and cellulose as land plants (Anderson and King 1961a, Anderson and King 1961b, Anderson and King 1961c, Anderson and King 1961d, Foissner et al. 1996, Proseus and Boyer 2005, Proseus and Boyer 2006, Proseus and Boyer 2012). Only the

percentages of these differ from those of land plants, e.g., the values for lignin in roots/ rhizoids. The highest proportions of uronic acid anhydrides and proteins were found in dried cells of *C. australis*, *C. foetida* and *N. translucens* (5-50% of ash). Sugars such as glucose, uronic acid, galactose, arabinose, xylose, mannose and rhamnose were detected in descending order of abundance. Recent studies have shown that *Nitellopsis obtusa*, *Chara aspera* and *Chara subspinosa* lacks hydroxyproline and arabinogalactan proteins, which are common in land plants (Pfeifer et al. 2022, Pfeifer et al. 2023).

Over the last 30 years, Foissner and co-workers have published a vast amount of **cytological and molecular** work on *C. australis*, *C. braunii* and *Nitella* internodal cells. This includes wound healing, exocytosis (Foissner et al. 1996), nuclei fragmentation (Foissner and Wasteneys 2000), the detection of sterol-rich domains (Klima and Foissner 2008), the formation of lipid droplets near the endoplasmatic reticulum (Foissner 2009), the involvement of charasomes in pH banding (Schmolzer et al. 2011, Foissner et al. 2015, Sommer et al. 2015, Eremin et al. 2019) and the inhibition of vesicle transport by Bredfield A (Bulychev and Foissner 2020). The majority of these studies have been carried out using FM labelling. In addition, molecular and biochemical work was done on vesicular transport and plasma membrane repair by identifying a CaARA6-like protein with GTPase activity and a CaVAMP72 protein (Hoepflinger et al. 2013, Hoepflinger et al. 2014), as well as studies on OH- transporters including the identification of the Slc4-like gene CaSLOT (Quade et al. 2022). Genomic data and protocols available for *C. braunii, C. australis* and *C. corallina* have accelerated the ongoing molecular work on charophytes using *in-vitro* cultures (Tsutsui et al. 1987,Nakanishi et al. 1999, Nishiyama et al. 2018, Bonnot et al. 2019, Phipps et al. 2021, Haraguchi et al. 2022, Quade et al. 2022, Heß et al. 2023).

Phytohormone signalling is known to be essential for seed maturation, dormancy, germination, and senescence. In land plants and bryophytes, the roles of abscisic acid (ABA), gibberellic acid (GA_3) and auxin are well studied. Their associated enzymes are key players in plant signalling processes, germination and stress response (von Schwartzenberg 2006, Anterola et al. 2009, Thelander et al. 2018). Within Characeae, little is known about the detailed gene regulatory network and the proteins involved in transporting phytohormones and performing specific functions. In the past centuries, the focus on physiological studies has dominated this research, which e.g., demonstrated the auxin accumulation in charophytes. Here the first studies on auxin in charophytes were carried out by the group of Libbert and co-workers. The synthesis of IAA via a tryptophan-independent pathway was suggested by cut-off studies of apices (Libbert and Jahnke 1965). The presence of polar auxin transport (PAT) in internodal cells and membrane-associated PIN2-like proteins in male antheridia during proliferation but not during spermiogenesis is confirmed by immunofluorescence labelling studies in *C. corallina* and *C. vulgaris* (Boot et al. 2012). Interestingly, PIN2-like protein expression and auxin accumulation were found to be associated with callose and plasmodesmata connectivity between cells, suggesting that these functional features evolved long before land plants. The highest expression and accumulation were found in capitula cells. In addition, the use of exogenous IAA had been shown to (i) shorten the proliferative period while PCIB reduces mitotic activity (Godlewski 1980, Zabka et al. 2016) and (ii) ensure the presence of

rhizoids during the development of gametangia (Sievers and Schröter 1971) However, recent phylogenetic analyses based on genomic data could confirm proteins involved in phytohormone biosynthesis and signalling (Feng et al. 2023).

Within the charophytes, hormone extraction was performed for C. braunii and C. australis including salicylic acid (SA), ABA, jasmonic acid (JA), indole-3-acetic acid (IAA), jasmonate-isoleucine conjugate (JA-Ile), indole-3-acetyl-aspartate (IAA-Asp), strigolactones and the JA precursor cis-(+)-12-oxo-phytodienoic acid (cis-OPDA) using LC-MS/MS methods and deuterium-labelled standards (e.g., Delaux et al. 2012, Hackenberg and Pandey 2014, Beilby et al. 2015, Waters et al. 2017, Schmidt 2021). After detecting low levels of ABA in *C. foetida* cells (Tietz et al. 1989), Hackenberg and Pandey identified the associated G-proteins (Hackenberg and Pandey 2014). Like land plants, they could potentially be involved in phytohormone signalling pathways, as suggested by the findings of a synchronous seasonal and circadian change in ABA, serotonin and melatonin concentrations (Beilby et al. 2015). Furthermore, melatonin findings in *C. australis* have been suggested to be protective against reactive oxygen species. This compound is known for the freshwater and brackish water species *C. tomentosa* with its reddish tips (Beilby 2016). Detailed interdisciplinary studies to elucidate this evolutionary feature, which has not yet been found in Zygnematophyceae or Coleochaetophyceae, are essential to elucidate its function, molecular signalling pathway and microorganism-associated association (non-sulfur bacteria and cyanobacteria). Evidence for the presence of the serine-threonine phosphatase (PP2C), which acts as an ABA signalling sensor (Ma et al. 2009) has been identified in the Chara genome (Nishiyama et al. 2018). Gibberellin-like substances have been ascertained in extracts of *C. braunii* (*C. coronata*) by paper chromatography (Murakami 1966). Additionally, different studies have shown a concentration-depending promoting effect of exogenous GA_3 concerning male gametangia development, germination initiation or RNA and protein biosynthesis in charophytes (Godlewski and Kwiatkowska 1980, Sederias and Colman 2007, Holzhausen et al. 2022). Concentrations of 10^{-7} and 10^{-8} M GA_3 shorten the duration of mitotic divisions in antheridial filaments, increases the number of spermatids per filament by about 200 and increases the lengths of spermatoid cells (Godlewski and Kwiatkowska 1980). Interestingly, Godlewski found a stimulating effect of GA_3 on the incorporation of 8-^{14}C adenine into DNA and RNA in dependence of the cell cycle stage of antheridia as well as an increased capability of the antibiotics actinomycin D binding that indicates at least an indirect participation at the transcription level. The chemical compound responsible for the gibberellin activity could not be identified so far and needs to investigate in further studies. One possible explanation for land-plant-like reaction of exogenous gibberellins in charophytes could be the presence of uniport-transporters with activities for different nutrients and simultaneously phytohormone conjugates. Those transporters are well known for plants, especially within the nitrate/peptide transporter family (NRT/PTR) or the SWEET sugar transporters. Recently, in *Arabidopsis thaliana*, the uniporter SWEET13 (AtSWEET13) was identified to transport sugars and gibberellin. Orthologs of this AtSWEET13 proteins can be found in different streptophytic algae genomes.

5. ECO-*in-vitro*-CULTIVATION

The intensive study of the charophytes shows that there are various studies on reproduction, germination and dormancy that can be used on an ad hoc basis and as fundamental resource. The understanding and integration of evolutionary, systematic, and ecological knowledge enables the successful establishment of laboratory cultures as well as axenic cultures (unpublished).

Further own studies have shown that this integration of e.g., monitored field data is essential to allow dioecious species from permanent water bodies to complete the life cycle under laboratory conditions. Monitored light and temperature profiles from mesocosms at the University Rostock, as shown in Fig. 4, were used to identify temperature and light ranges in which the physiological processes of dormancy breakage and germination initiation (Fig. 4A), gametangia development (Fig. 4B) and maturation (Fig. 4C) take place. The transfer of these natural conditions to *in-vitro* cultures of different dioecious species successfully induces the formation of gametangia upon maturation of the oospores. This method of ECO-*in-vitro*-cultivation is particularly useful for species that are poor gametangiogenic *in-vitro*, such as dioecious species. These reproduce mainly vegetatively in culture, which greatly reduces the formation of gametangia. In addition, the analysis of the solids used, such as organic additives, but also the analysis of the liquid medium, provides information about the chemical components and their content (Holzhausen et al. 2022, unpublished data). These are currently used to optimise the inorganic medium.

This type of cultivation allows for a complete reproductive cycle and the establishment of a variety of *in-vitro* species for future molecular work, so that in addition to the rapidly reproducing freshwater species *C. braunii* further freshwater species or salt-tolerant species such as *C. canescens* or *C. tomentosa* can be used to identify e.g., reproduction-related gene networks at different reproduction states.

Acknowledgements

10 years of enthusiasm for charophyte research would not have been possible without the financial support of the universities Rostock, Marburg and Halle/Wittenberg and the associated chairholders, H. Schubert, M. Quint and S. Rensing. My greatest thanks go to Arne Schoor, who gave up a part of his position at the University of Rostock and made it available to me. Furthermore, the author is particularly grateful to the support by K. von Schwartzenberg and E. Woelken (University Hamburg) and the funding of the federal states of Mecklenburg-Western Pomerania (“Landesgraduiertenförderung – MV” and the “Professorinnenprogramm II”), Schleswig-Holstein (V. Wetzky, U. Hamann) and Brandenburg (R. Mauersberger, A. Hussner and K. Vasters; E+E Project CHARA-SEEN). The professors of the Institute of Biosciences at the University of Rostock are to be thanked for the provision of equipment, chemicals, mesocosm space and laboratories. These are R. Schröder (sterile bank, equipment), M. Hagemann (climatic chamber), F.-O.

Lehmann (room for the climatic chamber), U. Karsten (agar). A. Schoor, S. Thümecke, A. Siegesmund and P. Nowak are to be thanked for their professional support and their well-being on this path. Thanks to M.T. Casanova for the workshop in spermatozoid isolation at the National History Museum London.

Conflicts of interest

The authors have declared that no competing interests exist.

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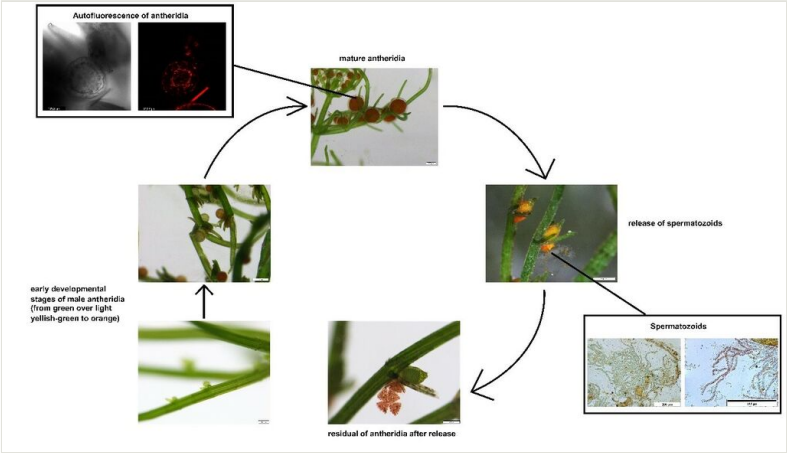

Figure 1.

Life cycle of charophyte antheridia. First developmental stages characterised by the green (A) and yellow-greenish colour (B) of antheridia. (C-D) fully mature antheridia (C) including their autofluorescence in detail (D). (E-F) release of spermatozoids from open antheridia (E) and a detailed view of extracted spermatozoids (F). (G) empty and fanned shield cells of male antheridia after the release of spermatozoids.

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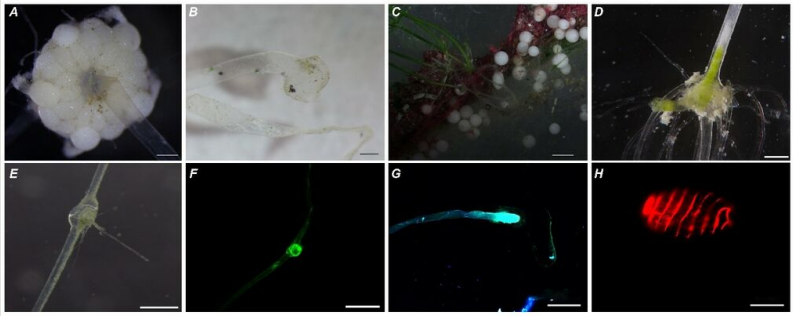

Figure 2.

Reproductive units and rhizoid cells of charophytes. *Nitellopsis obtusa*. (A) fully developed star-shaped bulbil, scale 1mm. (B) Cell at the beginning of bulbil development, scale 200µm. (C) root nodules with associated microorganisms from *Chara aspera*, scale 1mm. (D) vegetative reproduction of *Chara filiformis* by nodule cells, scale 500µm. (E) characteristic S-shaped cleavage plane of rhizoid cells (*) of *Chara vulgaris* with additional cells for secondary rhizoids (arrows), scale 1mm. (F) root cell of *Chara braunii* S276, stained with DAPI, scale 200µm. (G) root tip of *C. braunii* S276, stained with DAPi, 200µm. (H) oospore of *C. braunii* S276, stained with PI, scale 200µm.

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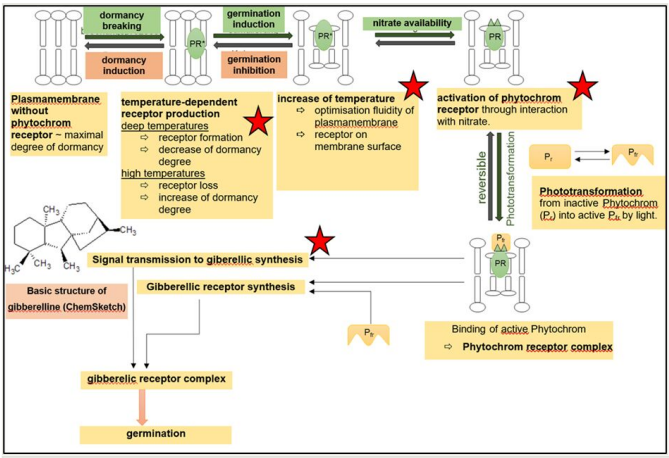

Figure 3.

Modification of the hypothetical physiological dormancy model of Hilhorst (1998). Red stars indicate available studies for charophyte oospores. Gibberellin-like substances or precursors of gibberellin have not yet been identified.

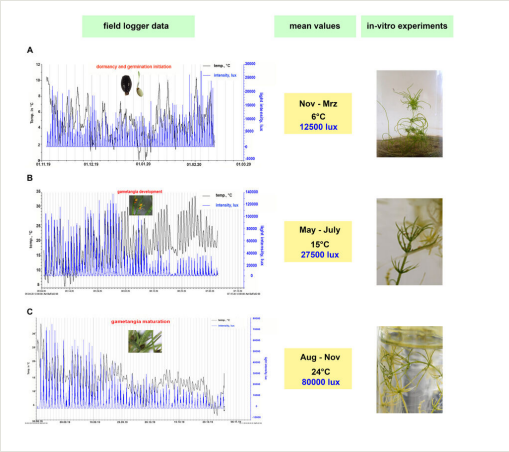

Figure 4.

Details of ECO-*in-vitro*-cultivation of *Chara hispida*. Field data are monitored for the physiological processes of dormancy and germination initiation (A), gametangia development (B) and gametangia maturation (C). The resulted mean day values were adopted successfully to *in-vitro* cultures of *Chara hispida* and initiate gametangia development and maturation.

Table 1.

List of cytological studies on gametangia, rhizoids and vegetative thallus material of charophytes. The respective object and cell type, methods, fixative, embedding, staining and washing solutions, and identified organelles are recorded.

reference	*o* bject	method	fixative
Johow (1881)	***C. foetida* (*C. vulgaris*)** branchlet gametangia rhizoids nodal cells	-	- picric acid
Zacharias (1885), Zacharias (1888), Zacharias (1890)	***C. foetida* (*C. vulgaris*)** male and female gametangia rhizoids	- microscopy	-
Overton (1890)	***N. syncarpa*** ***C. hispida*** female gametangium, branchlets and internodal cells	- staining - microscopy - reaction against conc. acids	-
Kaiser (1896)	***C. foetida* (*C. vulgaris*)** ***C. hispida*** ***C. crinita* (*C. canescens*)** ***N. syncarpa*** ***N. flexilis*** apical cell node cell branchlets oogonia/antheridia cortex cells	- sectioning (3-5 µm)	- 1% sublimate solutio - picric acid - Flemming's solution - Herrmann's solution
Goetz (1899)	***Nitella flexilis*** ***Nitella opaca*** ***Chara foetida* (*C. vulgaris*)** internodal cells oogonia/oospores	- sectioning - oogonia 10µm sections - zygotes 20µm sections	- Rath's mixture - Zenker's mixture
Mottier (1904)	***C. fragilis* (*C. globularis*)** spermatozoids	- sectioning (3-5µm)	- chrome-osmic-acetic
Oelkers (1916)	***C. fragilis* (*C. globularis*)** ***C. foetida* (*C. vulgaris*)** ***N. syncarpa*** generative/vegetative tissues	- sectioning (30µm) - degradation of sugars	- vegetative: chromium acid - generative: alcohol c xylol resp. chloroform
Riker (1921)	***C. fragilis* (*C. globularis*)** ***C. verrucosa* (*C. virgata*)**	- sectioning (5 – 7µm)	- Flemming's solution - Merkel's solution - Benda's solution
Karling (1926)	***C. coronata* (*C. braunii*)** ***C. fragilis* (*C. globularis*)** ***N. gracilis*** antheridia/oogonia apical cells nodal cells	- microscopy	- Flemming's solution - Merkel's solution

Linsbauer (1927)	***C. rudis (C. subspinosa)*** ***C. foetida (C. vulgaris)*** ***C. fragilis (C. globularis)*** rhizoids	- microscopy	- 1% chromic acid
Schmuckler (1927)	***C. contraria* var. *hispidula*** oogonia	- microscopy	-
Walther (1929)	***N. syncarpa*** ***N. hyalina*** ***N. batrachosperma*** oogonia/antheridia oospores node cells branchlets internodal cells	- sectioning (6-16 µm)	- Flemming's solution - osmic acid - xylol - ethanol
Sasaki (1935a), Sasaki (1935b)	***N.* sp.** ***C.* sp.** spermatozoids	-	- 2% osmic acid
Sundaralingam (1954)	***C. zeylanica***	- sectioning (5-10µm)	- Nawaschin's fluid
Satô (1954)	***C. braunii*** spermatozoids	- electron microscopy - phase contrast microscopy	- 2% osmic acid soluti formalin
Barton (1965a), Barton (1965b)	***C. vulgaris*** mature lateral cells	- sectioning	- 4% - 6% glutaraldeh (addition of phosphate potassium permangana
Hollenstein (1966)	***C. sp.*** spermatozoids	- X-ray diffraction	-

Shen (1967a), Shen (1967c)	***C. contraria*** ***C. zeylanica*** older internodes rhizoids protonema leaves shoot apices	- sectioning (25µm) - smear - whole plant part	- 10% neutral-buffered hydrolysis in 1N hydroc - Permount - Navashin's solution - 10% neutral-buffered
Shen (1967b)	*Chara zeylanica* sperms	- Feulgen spectral absorption	- erythrocytes containi
Pickett-Heaps (1967a), Pickett-Heaps (1967b), Pickett-Heaps (1968)	***C. australis*** ***C. fibrosa*** ***C.* sp.** ***N.* sp.** antheridia oogonia	- sectioning	- 6 % glutaraldehyde v phosphate buffer and c chloride - post-fixation with ver buffered 1% osmium te
Sawa and Frame (1974)	***T. nidifica*** plant apices with oogonia	- sectioning (1-5µm)	-
Leitch (1986), Leitch (1989), Leitch et al. (1990)	***C. delicatula* (*C. aspera*)** ***C. hispida*** ***L. papulosum*** oogonia	- sectioning - (80-100nm; >200nm)	- paraformaldehyde ar hydroxide - sodium cacodylate a glutaraldehyde - osmium tetroxide in s cacodylate
Moestrup (1970)	***C. corallina*** spermatozoids	- sectioning - formvar/carbon-coated grids for electron microscopy	- 2% osmium tetroxide phosphate buffer - glutaraldehyde
Stabenau et al. (2003)	***C. fragilis* (*C. globularis*)** homogenates	- sectioning	- 3.5% glutaraldehyde phosphate buffer - 1.5% osmium tetroxi
Hodick (1993)	***C. fragilis* (*C. globularis*)** protonema from nodes	- microscopy	-

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Sievers and Schröter (1971), Braun and Sievers (1993), Buchen et al. (1993), Braun (1996a), Braun (1996b), Braun and Wasteneys (1998)	***C. globularis*** ***N. pseudoflabellata*** protonema rhizoids	- immuno-fluorescence labelling - sectioning (2-3µm) - slow-rotating-centrifuge-microscope	- 3% paraformaldehyc - 1% paraformaldehyc glutaraldehyde in MSB - 3% glutaraldehyde ir - sodium cacodylate, 1 mM $CaCl_2$, 0.8% $K_3Fe($ - agar

Table 2.

List of existing references for **oospore dormancy breakage** including the habit (permanent/ temporary) and zonobiome after Pott (2005).

species	habit	zonobiome	most effective method to break dormancy (storage conditions)
Chara aculeolata	p	VIII	+4°C for about 2 months
Chara aspera	p	VIII	+4°C for about 2 months
Chara australis	p p	V V	wet under semi-natural conditions
Chara braunii	p	I/V	wet storage 22°C
Chara canescens	p/t p p/t	VI VI VI	Dry oospores, low temperatures 12weeks at 10°C in the dark dessication at 5°C
Chara contraria	p/t p p	N/A III* VII	drying of oospores wet oospores at cold temperatures (3°C) fresh oospores from mother plant
Chara corallina	p	V	15°C and darkness for up to 4 days
Chara globularis	p p	III* V	dry oospores at cold temperatures (3°C) 15°C and darkness for up to 4 days
Chara hispida	t	VI	drying of oospores, cold treatments of 1-3months at 4°C
	p	VIII	+4°C for about 2 months
Chara muelleri	p/t	V	dry storage in either dark or ambient light conditions
Chara papillosa	p	VI	dessication at 20°C
Chara rusbyana	p	III*	dry oospores at cold temperatures (3°C)
Chara vulgaris	p p p p t	VIII VIII VIII VIII VI	36days at 18-22°C wet storage 60days at 4°C and red light dark at 4°C drying of oospores, cold treatments of 1-3months at 4°C
Chara zeylanica	p p/t p	VIII N/A III*	4°C for about 2 months cold temperatures (5-7°C) for 10 days wet oospores at cold temperatures (3°C)
Lychnothamnus barbatus	p	VI	dessication at 5°C
Nitella cristata var. *ambigua*	p/t	V	cold treatment (4-5°C)
Nitella furcata	p	VI	4°C and darkness
	p	V	15°C and darkness for up to 4 days
Nitella flexilis	p	VIII	247days sediment storage at 18-22°C, decline of redox conditions in medium
Nitella sonderi/ Nitella subtilissima	p/t	V	drying of oospores

* sample sites are not given for all strains of the collection

Table 3.

List of references for **oospore germination approaches** including light and temperature conditions. Listed are the species name, the usual habit (occurrence in temporary/permanent water bodies), the zonobiome (in correspondence to the published material), experimental temperature(s) in °C (T), the light cycle (LC, intensity in µmol photon/(s*m^2) and light:dark cycle in hours, method of germination approach and reference. Missing information is marked with -.

* Oospores in Proctor (1962) were obtained from the faeces of waterfowls. Only *Chara braunii* and *Chara zeylanica* are present at the study site in Texas. The origin of the oospores is therefore unknown.

** Imahori and Iwasa (1965) mentioned the use of material from Proctor's experiments (oospores from Texas).

species	habit	zonobiome	T	LC
C. aculeolata	p	VIII	20-25	N/A | N/A
C. aspera	p	IV VI VIII VI VIII	21-29 10-16 20-25 20 room temp.	298 ± 20 | 14:10 300 | 12:12 and 16:8 ~40 | continuous 95 | 16:8 N/A | natural light
C. australis	p/t p	V V	14.4 – 27.8 14-16	N/A | N/A 0.1-147 | 14:10
C. braunii	p/t	I/V/VI III III* III**	22 25 (50) 24 25	< 30 | 16:8 N/A | N/A N/A | continuous ~ 10 | 12:12
C. canescens	p/t	IV III*	21-29 24	206 ± 26 | 14:10 N/A | continuous
C. contraria	p/t	N/A III III* III** VII	22 25 24 25 23	~ 50 | 12:12 N/A | N/A N/A | continuous ~10 | 12:12 90-100 | 16:8
C. cf. *contraria*	p	VI	20	400 | 12:12
C. corralina	p/t	III**	25	~10 | 12:12
C. delicatula	p	III	20	0-10 | continuous bright light, continu dim light, dark
C. globularis	p	V III III* VIII III** V	14-16 25 24 20-25 25 N/A	0.1-147 | 14:10 N/A | N/A N/A | continuous light ~40 | continuous ~10 | 12:12 N/A | 6% ambient light
C. gymnopitys	p	V III**	- 25	N/A | continuous ~10 | 12:12
C. hispida	p	IV VIII	21-29 20-25	298 ± 20 | 14:10 ~ 40 | continuous
C. hornemannia	p	III*	24	N/A | continuous light
C. hydropitys	p	III**	25	~10 | 12:12
C. intermedia	p/t	VI	10	N/A | dark conditions
C. muelleri	t	V	14.4 – 27.8	N/A | N/A
C. sejuncta	p	III	20-25	~40 | continuous

C. vulgaris	p	VIII III VIII VIII VIII	20-25 20-30 20 22	~40 | continuous N/A | dim light N/A | 12:12 (wet/dry) N/A | natural daylight, north-directed window
C. zeylanica	p/t	N/A III III VIII III** III	22 25 (5-37) 24~40 20-25 25 10-12 (night), 18-20 (day	~50 | 12:12 N/A | N/A N/A | continuous light N/A | continuous ~10 | 12:12 50 | outdoor
N. cristata var. *ambigua*	p/t	V	14.4 – 27.8	N/A | N/A
N. flexilis	p	VIII	20 (wet/dry)	N/A | 12:12
N. furcata subsp. *megacarpa*	p	VI	18	monochromatic light | 4, followed by darkness and 18 or broad-band light
N. hyalina	p	IV	21-29	298 ± 20 | 14:10
N. macrocarpa	p	III**	25	~10 | 12:12 |

Table 4.

List of references for **sediment germination approaches.** *Listed are the species name, the usual habit (occurrence in temporary/permanent water bodies), the zonobiome (in correspondence to the published material), experimental temperature(s) in °C (T), the light cycle (LC, intensity in µmol photon/(s*m^2) and light:dark cycle in hours, method of germination approach and reference.* Missing information *is* marked *with* -.

species	habit	zonobiome	T	LC
C. australis *C. globularis*	p	V	14-16	0.1-147 | 14:10
C. australis *C. muelleri* *N. sonderi* *N. stuartii* *N. subtillissima* *N. tasmanica*	p/t	V	14.4 - 27.8	treatments: (I) wet winter (II) dry winter (III) dry summer germination in glass houses
C. baltica / var. *liljebladii* *C. contraria* *C. globularis* *C. canescens* *N. obtusa*	p	VI	15	15-130 | 16:8
C. canescens	p	VI	15	~2 | 12:12
C. canescens	t	V	N/A	N/A | N/A
C. canescens *C. contraria* *C. vulgaris* *T. nidifica*	p	VI	15	100 ± 20 | 12:12
C. contraria *C. globularis* *C.* sp. *N. obtusa*	p	VI	15	100 ± 20 | 12:12
C. connivens *C. aspera* *T. nidifica* *L. papulosum*	p	VI	15	80-90 | 12:12
C. filiformis	p	VI	15	15-20 | 16:8
C. corallina *C. fibrosa* *C. globularis* *N. hookeri / cristata* *N. leptostachys* *N. pseudoflabellata*	p	V	N/A	N/A | natural light cycle
C. fibrosa *C. zeylanica*	p	II	N/A	N/A | direct sunlight
C. hispida *C. vulgaris*	p	IV	N/A 20	N/A | natural light cycle N/A | natural light cycle 45 | 12:12
C. spp.	p	V	20	5 | 14:10
L. macropogon	t	V	N/A	N/A | partial sunlight with 15% sl light fibreglass screen mes treatment
L. sp aff. *macropogon*	t	V	N/A	N/A

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L. papulosum *N. flexilis/opaca*	p	VI	15	30-130 | 16:8
L. barbatus	p	VI	15	30-40 | 16:8
N. micklei *N. parooensis*	t	II	N/A	N/A
N. mucronata	p	VIII	20	N/A | daylight, south-directed win
N. mucronata *T. glomerata*	p	VI	15	110-130 | 16:8
N. sp. *C.* sp.	p	IV	N/A	N/A | greenhouse
T. nidifica/salina	(p)/t	VI	15-20 salt treatment	110-130 | 16:8
C. contraria *C. globularis* *C. hispida*	p	VI	15-18	110-130 | 16:8
C. aspera *C. contraria* *C. globularis* *C.* sp.	p	VI	15-18	110-130 | 16:8

Table 5.

List of references for ***in-vitro*** **cultivation approaches.** Listed are the species name, the cultivation conditions, the observation as well as the respective reference.

species	cultivation conditions	observations
C. canescens		oospore length differences by temperature and li substrate and culture medium
C. corallina	Soil-water medium, windowsill with natural light/dark cycle	Aneuploidy and polyploidy in charophytes; cytogen species
C. corallina	Aquarium with deionized water, fluorescent lamps (L:D =14:10), soil / sterilised forest soil	anion channels, *in-vivo* caspase 3-like proteinase activity
C. corralina	tap water with soil extract and rotten leaves, plastic buckets, 25 ± 1 °C, fluorescent lamps with 50-60 µmol/m^2 s^{-1}), L:D = 15:9	intracellular transport of photoassimilates
C. foetida	dark half-dark full daylight	loss or abnormally cortication in limited light conditions
C. fragilis	windowsill (all cardinal directions) / greenhouse / electric illumination	gametangia development in dependence of collection d and continuous illumination
C. fragilis/ *C. foetida*	Glass aquarium 4L; sludge, garden soil, quartz sand or dolomite sand as substrate and/or floating cultures; destilled water, spring water or mineral media (Detmer, Crone, Beyerinck, Artari, Pringsheim and Benecké); addition of "Purissimum" and "Pro Analysi" salt.	modified mineral medium of Crohn; bicarbonate/C experiment; effects of chemical compounds on charoph cultivation
C. fragilis/ *C. foetida/* *C. contraria*	Munich tap water, thalli w/wo rhizoids	Effect of $CuSO_4$, alkaloids, strychnine, nicotine and caffe on the morphology of gametangia
C. fragilis/ *C. foetida/ C.coronata/* *N. mucronata*	Vessels with slufge, garden soil, clay, sand or quatz; sunlight, shade and deeper shade	effects on different soils and light conditions on charoph cultivation
Chara hispida	windowsill	loss of cortication
Chara strigosa	cultivation in sodium chloride solution under full or reduced light conditions	high light conditions + full light leads to reduced length grow
Chara vulgaris	buffered lake water (HEPES); 22°C, 14:10	15W lumilux tubes sterile plants: 10hlight:14h dark
Chara vulgaris	Forsberg mineral medium, Flora white lamps (6.2W*m^{-2}), L:D = 14:10 L:D = 24:0 L:D = 1:23	continuous illumination: exceed mitotic activity (new node shortens internodal cells, increased rhizoid formation, initi antheridia, reduced oogonia formation; prolonged darkness: halves the mitotic activity, prolo internodal cells, blocking of rhizoid formation, reduction antheridia and oogonia formation
Nitella flexilis	outdoor conditions	high temperature and sunlight influenced oospore producti