The Chara plasma membrane system : an ancestral model for plasma membrane transport in plant cells

Zhang, S.

Chapter 2

The culture of *Chara sp.* for research: does and don'ts

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Abstract

The *Chara* algae are popular world-wide as a regulator and indicator for environmental (fresh water system) management, as well as a research subject and model system in different labs for decades. With the rapid development of using *Chara sp.* as a model system to study plant development, cellular biology, hormone and signal systems, etc., there is an urgent requirement for a more consistent and efficient material supply. Thus, we propose and discuss here some efforts of trying to culture *Chara sp.* for a long period in a standard lab condition, including culture settings (e.g. light, temperature) and procedures (planting and harvesting).

Introduction

Chara algae are fresh water, multicellular, green macro-algae closely related to the land plants. They commonly form dense meadows in oligo- and moderately eutrophic waters. In ecological systems, the Charophytes are considered as the "gold standard" in littoral areas, indicating and regulating healthy, clear-water ecosystems (Ibelings et al., 2007; Richter and Gross, 2013; Beilby and Casanova, 2014). The dominance of *Chara* only in clear, non-turbid water system makes them a good indicator for water pollution status (Ibelings et al., 2007; Singh et al., 2013a). With the *Chara* dominance, they can effectively takeup, storage and immobilize the macronutrients especially phosphorus, acting as nutrient sinks and sediment stabilizers to maintain the water clarity (Kufel and Kufel, 2002; Bakker et al., 2010; Blindow et al., 2014). By lowering the nutrient concentration, *Chara* can be used for water management against cyanobacteria, phytoplankton and invasive aquatic plants, helping with the re-oligotrophication of the water body (Ibelings et al., 2007; Hidding et al., 2010; Richter and Gross, 2013). Besides, they are also proposed for phytoremediation applications for decrease of trace metal from the industry (Laffont-Schwob et al., 2015; Poklonov, 2016).

Apart from the great interests of *Chara* in ecology studies, the *Chara sp.* also got special attention in physiological and cell biological studies, especially in the fields of membrane transport, cell motility and electrical signalling, because of their unique internodal-cell geometry (Shimmen et al., 1994; Shimmen and Lucas, 2003; Foissner et al., 2015). Besides an interest in the algae themselves they also provide a unique model system for investigation of especially more general cell physiological processes and their importance as an intermediate in the evolutionary developments from water to land plants (Shimmen et al., 1994; Boot et al., 2012; Zhang and van Duijn, 2014). The major advantages of *Chara* algae as a model system for these types of studies are:

1. *Chara sp.* have giant single cell internodal cells. These cells can reach a length of more than 10 cm with a diameter of around 1mm (Braun et al., 2007; Foissner & Wasteneys, 2014). Most *Chara* species have internodal cells that are surrounded by a cortex of small cortical cells (Fig. 1A). However, certain species of *Chara* have ecorticate (without cortex) large internodal cells (Fig. 1B), such as *Chara australis*, *Chara corallina* and *Chara braunii* (Beilby and Casanova, 2014), which allows for direct observation, measurements and manipulation for cell biological and

physiological studies (Winter et al., 1987; Shimmen et al., 1994). For instance, the cells can be manipulated by centrifugation, perfusion to create different membrane systems for electrical measurements, which set the base and gave a huge boost for the modern plant electrophysiology (Beilby, 2016).

2. *Chara sp.* are closely related to the land plants. As one of the closest ancestor of land plants, *Chara* has evolved a plant-like morphology, with a similar yet simpler morphological and cell composition structure (Casanova 2007). The study of similarities and differences in the metabolism, hormone regulations, cellular processes, makes *Chara* cells a good model system for studies relevant to the land plants, e.g. tropism and polarity growth, wound-healing mechanism, cyto-architecture and development (Braun et al., 2007; Boot et al., 2012; Foissner and Wasteneys, 2012& 2014). Last but not least, the study of *Chara* at the evolutionary perspective, could fill the gap between the Chlorophyta and Streprophta offering a better map at the genomic level, e.g. the mechanism of effective nutrient up-taking and higher salinity tolerant of Charophyceae algae could shed light on the modern agriculture investment (Pedersen et al., 2012; Domozych et al., 2016).

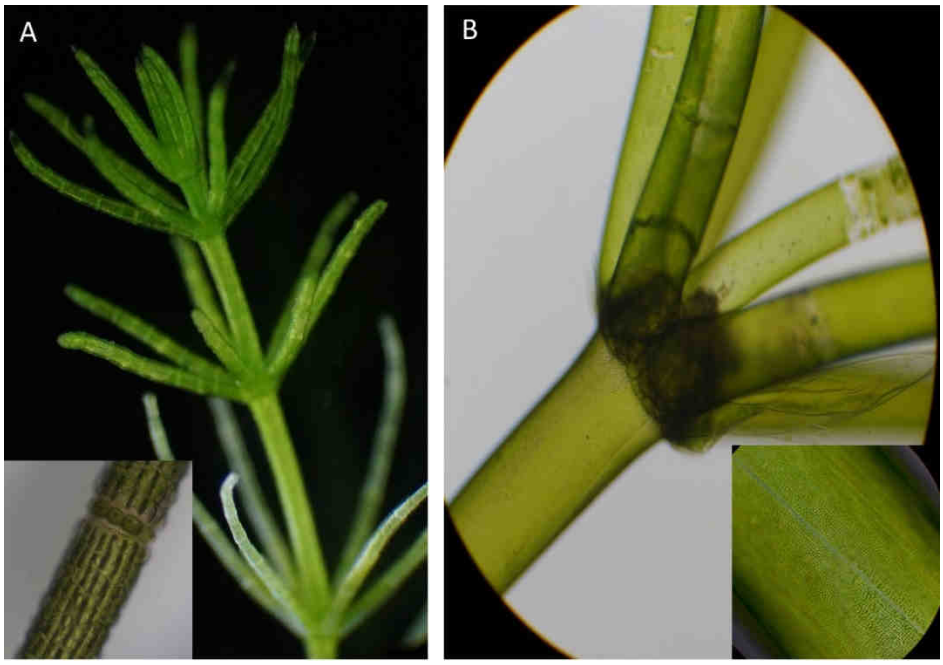

Figure 1. Cortical and ecorticate *Chara* species. (A) *Chara vulgaris* (cortical specie), inserted picture shows the cortical cell layer covering the center internodal cell. (B) *Chara corallina* (ecorticate specie), inserted picture shows the visible chloroplasts layer underneath the cell membrane.

Besides the clear advantages of the *Chara* cells as a model system there are some aspects that put limitations to the use of *Chara* in research. One of the most prominent of these limitations refers to the availability of algae material and (standardized) culture of *Chara*. The difficult aspects in respect to algae material availability can be summarized as:

- Unreliable and unpredictable availability and production in nature. *Chara* is relatively widespread in natural fresh waters, but most of the *Chara* species have an annual life cycle in their wild habitats, which makes the sample collection highly unpredictable and seasonal dependent.
- Absence of information on standard (optimal) growth conditions and growth protocols. The growth and morphology of *Chara* are strongly affected by the environmental conditions. In this respect light conditions (intensity, day-night cycle), water chemistry (nutrients, pH etc.) and soil composition are most prominent in influencing the basic conditions.
- Difficulties and uncertainties in the precise taxonomy and determination of *Chara* species. The methods applied in *Chara* taxonomy are undergoing a continues revolution from rough specimen observing to molecular experimental approaches during the past decades. The genetic distances among "species" in algae is in general larger than in animals or land plants.

The above factors may cause inconsistencies between different independent experiments, due to for instance different culture conditions of different "varieties" resulting in the use of algae with different properties. In addition, the aspects of the taxonomy difficulties may result in confliction or confusing experimental results from labs all over the world using different *Chara* species under identical names.

For starting experiments with *Chara* cells it is easiest to collect the algae material directly from nature and keep them alive in the lab in an aquarium for couple of days or weeks. But a stabilized and standard lab culture for a longer period is still difficult to achieve.

In our research over several years, we have tried to culture the most commonly used *Chara* species in the physiological research in the lab under different conditions. These are *Chara corallina* as the major species and some other species such as *Chara braunii* and *Chara australis*. In these trials, based on culture condition variations and the (scarce, as concerned the description of culture conditions) literature, we were able, after lots of failures, to culture *Chara*

species and keep them in good condition in the artificial environment for more than two years. In this paper we describe to preferred conditions for standardized *Chara sp.* culture, as well as the major pitfalls and conditions to be avoided.

The culturing of *Chara sp.*

In general, *Chara* algae can be cultured in aquaria, water tanks etc. of different sizes. In the culturing the factors of importance to be discussed are:

- Soil structure and composition and soil coverage
- Water quality
- Water volume and level
- Water streaming and mechanical stresses
- Light
- Temperature

Soil and soil coverings

Different soil sources are described in the literature referring to the culture of *Chara*, e.g. pond mud, forest soil, clay, soil & sand mixture (Smith and west, 1969; Weifer and Spanswick, 1978; Klima and Foissner, 2008; Kataev et al., 2012). It is reported that, small granule size substrate (425-710 μm) was shown to benefit the growth of both shoots and rhizoids of *Chara hispida* and *Nitella flexilis* (Andrews et al, 1984a).

Forest soil is among the most popular substrates used for the *Chara* culture (Berecki et al., 1999; Lew, 2015). The advantages of forest soil include that it is easy to collect, and full of nutrients to support the growth of *Chara.* The disadvantages of forest soil are that the composition and structure are undefined and that it not only differs from region to region but also can be very different locally on collection sites and over time. Biological potting and seeding soil without fertilizer can be used as an alternative to forest soil, but also these soils can be very diverse in composition and structure. Therefore, a lot of trial and error goes into testing for the right soil for growing *Chara.* Yet another soil for *Chara* growth in aquaria can be so called aquarium soil with or without nutrients (e.g. Tetra Plant, Complete Substrate). These aquaria soils are better defined, clean and tested for aquarium plant growth.

In relationship to hygiene and cleanliness, forest and potting soil are potentially the biggest source for contaminations. To avoid contaminations, the soil should be at least double autoclaved (Lew, 2015), which greatly reduces the chance of contamination by fungi, bacteria and secondary algae.

We grew *Chara* on many types of soil, but the best results were on either forest soil or (and) aquarium soil. Forest soil was collected from temperate broadleaf and mixed forests (Leidse Hout Park, Leiden, the Netherlands) by removing the top layer (2-5 cm) and collecting the soil underneath to a depth of about10 cm. The forest soil was autoclaved two times before use and aquarium soil was used directly from the package. A layer of less than (about) 3 cm thick forest or aquarium soil was placed on the bottom of the aquarium and covered by 1 cm or more aquarium grit on top. This extra top layer prevents the disturbance of the soil and provides a hard base for the rhizoids to anchor. Both systems can support the healthy growth of *Chara* for a long period (at least two years).

Recommendation: carefully sterilize the soil source collected from the nature to reduce the risk of contamination. Standard commercial aquarium soil and grit for pond plant (e.g. Velda, Tetra Plant) would be a recommended alternative.

Water composition

In natural habitats, *Chara* is often lush in clean and rather hard water (Garcia and Chivas, 2006; Beilby and Casanova, 2014). In literature in many cases artificial pond water (APW) containing 0.1 mM KCl, 0.1 mM $CaCl_2$ and 0.1 mM NaCl (pH about 6.0) was used as a supplement to the nutrients from the soil or used alone for pre-experimental culturing (Smith and West, 1969; Klima and Foissner, 2008). It is recommended to use demineralised (Demi) water instead of tap water. As a standard medium the APW we used, has been proven to be sufficient for the healthy growth of *Chara*. In experiments we tested richer, more nutrients containing media like the Broyer and Barr medium (Lew, 2015) with the same soil conditions. In these experiments, no obvious difference was observed with the growth of *Chara* as compared to growth in APW.

So, we maintained APW as the preferred medium. For a full mature culture, medium was replaced once a month or even less. The old medium was removed by a siphon tube from the bottom of the tank and new medium was added slowly at the other side of tank along the wall to minimize mechanical disturbances (change 1/3-1/2 medium and extra caution with new cultures). The

siphon effect is also helpful in removing the planktonic micro-algae sticking at the bottom or along the tank wall.

Recommendations: 1. low nutrient medium with demi-water; 2. regular (but not too often) exchange of medium to reduce the growth of phytoplankton and cyanobacteria.

Volume and water level

The size of the aquaria and containers used for *Chara* culture are in first instance given by practical considerations. In our experiments two different sets were used for the *Chara* culture. To start new cultures from cuttings (4-6 cells), either by horizontal way or vertical way (see other chapter below), small tanks (24x15x15 cm) were used. 2-3 small tanks were placed into a big tank (60x29x26 cm). The larger tank was covered with Plexi-glass on top to reduce the evaporation. The small tanks were filled to 5 cm (in the beginning) with APW and the big tank was also partly (fully when cultures have grown) filled with APW to create high humidity to reduce the evaporation. Refreshing of the water was done by first removing and then replenishing medium from/to the big tank. The use of this system reduces the mechanical stress due to turbulence and it minimizes the possible secondary algae contamination. Water level could be lower (3-4cm above the top of *Chara* thalli) at the start of a new culture (horizontal way of planting, see below) and fill up gradually during the establishment, until the big tank was almost full.

With large and mature *Chara* cultures, it is possible to transplant them into a big tank with the same substrate and APW medium.

Recommendation: adjust the water level with the growth of *Chara.*

Movement and mechanical stress

In different experiments it was found that turbidity and physical disturbance are rather important negative effectors of the establishment of Charophytes acid-alkaline zones along the internodes, and wave damage was considered one of the top causes of mortality (Casanova and Brock, 1999; Blindow, 1992; Schwarz et al., 2002; van Nes et al., 2002). The *Chara sp.* without cortex (ecorticate) are more fragile to mechanical disturbance than the corticated ones. Apart from the direct damage, turbulence may disperse sediment resulting in a turbid

environment and increased risk of epiphytic and micro-organism contaminants to settle on the *Chara* cells (Kairesalo, 1987).

Recommendation: once the cultures were settled, it is recommended to avoid rough transport of the tanks and be very careful while replacing the medium.

Light and day-night cycle

In the natural habitat, *Chara sp.* are found preferentially in clear, transparent water systems. In these water systems they could be found either in the shallow beds with a few centimetres depth (under shadow) or deeper than 10 meters (Andrews et al., 1984; de Winton et al., 1991; Garcia and Chivas, 2006). This suggests that *Chara sp.* have a good capacity to adjust to and grow under different light conditions. In most studied cases, Charophyte meadows formed the deepest vegetation of the lakes and growth was beyond the depth limit of vascular plants, e.g. *Chara corallina* meadows were found at 10-15 meters and *Chara braunii* at 9 meters under water in New Zealand (de Winton et al.,1991; Schwarz et al., 2002). In addition, the Charophytes tend to form low cover growths in shallow water while high-intense meadows with longer shoots as established below 7 meters depth (de Winton et al., 1991; Asaeda et al. 2007). These all indicate that light intensity has effects on the growth and morphology of *Chara*, and that *Chara sp.* have low light requirements, which has been confirmed by reports that low light intensities of 1-10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ can support *Chara* growth and development (van den Berg, 1999; Bulychev et al., 2013).

Based on these indication in our laboratory cultures, different light intensities have been tested (2-250 $\mu mol \cdot m^{-2} \cdot s^{-1}$). According to our experiences, through different stages of *Chara* culture (planting, recovering, flushing), dimmed light worked fine to support the growth of *Chara*. In particular, dimmed light is highly recommended to start a new *Chara* culture, also since it can effectively suppress the unwanted competition from planktonic algae. To achieve that, the sides of the tanks were covered with black paper to reduce the light from the sides that would reach the bottom part of the tank and a layer of white filter paper was used to cover the top of the tank to create some shade. Though there was no sign of harmful or photo-inhibition effect from a direct strong irradiation once the *Chara* culture reached the lush and dominant condition. To a certain extent, we detected that *Chara* internodal cells showed a darker green

colour under stronger light conditions which confirmed earlier observations (Asaeda et al., 2007).

Different day/night light cycles have been reported among labs, ranging from 12-18 hours for day light (Lucas, 1975; Weifer and Spanswick, 1978; Andrews et al., 1984; Klima and Foissner, 2008; Hidding et al., 2010). 16/8h (light/dark) is applied in our lab.

Recommendation: low radiation in general is in favour of *Chara* dominance over secondary (disturbing) algae.

Temperature

Charophytes can be annual (e.g. *Chara muelleri*) or perennial (e.g. *Chara australis*). With the perennial ones, a wide temperature range for culture can be applied (Casanova and Brock, 1996). Similar to the irradiation level, different temperatures have influence on thalli development and morphology. At lower temperature (5^0- 10^0C), *Chara* thalli tend to show more apical dominance with smaller side branches; while at higher temperature (15^0- 22^0C), they grow faster and develop more and larger side branches (Andrews et al., 1984). Reports also showed that, between 10^0-25^0C, with increasing temperature, the dark respiration rate of *Chara* increases but the highest photosynthetic rates differed among species (Vieira Jr and Necchi Jr, 2003).

Different temperatures (10^0-25^0C) have been used among different research groups for different purpose (Andrews et al., 1984; Mimura and Shimmen, 1994; Klima and Foissner, 2008). For our cultures, we wanted to maintain a high growth rate of *Chara*, and yet supress the growth of secondary plankton algae. So, we avoid the higher temperature of 25^0C, which is more favourable for the quick growth of phytoplankton. Both 18^0C, 22^0C were used and these temperatures can well support the *Chara* growth and healthy oogonia and antheridia were developed during the culture.

Recommendation: lower temperature (18^0- 22^0C) can help to supress the fast-growing secondary algae in favour of *Chara* dominance.

Starting a new culture

To begin a new *Chara* culture, it is very important to start with healthy *Chara* explants. Healthy *Chara* look fresh green and have strong cells which have a high turgor pressure. The physiological status of the *Chara* explants can be easily checked by the presence of a high velocity (50-100 $\mu m \cdot s^{-1}$) of cytoplasmic streaming under a microscope (Kikuyama et al., 1996; Kataev et al., 2012). In general, new cultures are started by using plants from another culture or from nature.

One method to start a new culture is to cut out 4-6 internodal cells, preferably with side branches still on them, from whole thalli. These internodal cells are kept in the similar orientation (all original top parts in the same direction) and are placed (together) horizontally on the soil and subsequently a thin cover of soil is put on top of the cells, leaving the original top part unburied (Lew, 2015). When sufficient explants are available, it is also possible to start a culture by planting complete thalli with rhizoids in a vertical way, in a similar way that plantlets are planted in soil. Big thalli with bright green colour and a high planting density are proven to be an advantage to start a culture. Avoiding mechanical turbidity and dimming the light could encourage the development and anchoring of the rhizoids and support the *Chara* thalli to build up its dominancy.

The density of *Chara* explants being planted is also important for start a new culture. In natural habitat, *Chara* usually grows in meadows/patches, and they form dense vegetation, with high biomass per unit area (Blindow, 1992; van den Berg et al., 1998). Field data also showed that a high early season Charophyte biomass could decrease the probability of algal blooms later in summer (Bakker et al., 2010). And by establishing a proper population, *Chara* can moderate the surrounding environment and cope better with the unfavorable disturbances (Kufel and Ozimek, 1994).

In consistence, in our experience, high density gives better growth both to start a new culture or maintain a stable culture, providing that high density could build up a stronger resistance to non-ideal physical conditions (light/temperature), and a lower chance of epiphytic contamination.

Recommendation: plant *Chara* in clusters (in our experiments the best results were achieved with explants as big as we could obtain).

Diseases/ problems

Competitors /phytoplankton

In natural habitats, *Chara* species are found in lakes that are usually defined as remarkably clear. The dominant position of *Chara* over the phytoplankton is often indicated as strong allelopathic effects of *Chara sp.*, which have been investigated for the past few decades (Berger and Schagerl, 2003 &2004; Gross, 2003; Gross et al., 2007). Some natural compounds were isolated from *Chara sp.* that showed pronounced photosynthesis inhibiting effects (Anthoni at el., 1980; Wium-Andersen et al., 1982; Berger and Schagerl, 2003&2004). However, the allelopathy in situ is still under debate, and the release of allelopathical compounds may also be species specific (Forsberg et al., 1990; Berger and Schagerl, 2004). Likely many more other impactors can be involved in their relationships, yet not well studied (van Donk and van de Bund, 2002). The advantage of an artificial lab culture, under the low irradiation and nutrition level, is that the competition from phytoplankton is rather negligible. Regular replacing the culture medium and wiping clean the side-walls of the tank during the medium replacement could keep this balance well maintained.

Epiphytic growth/ cyanobacteria

The epiphyte community is usually referred to as the mixture of microalgae, bacteria, fungi, inorganic particles and detritus, attaching to the surface of submerged aquatic vegetation (Vis et al., 2006). It has a close negative relationship with the light and CO_2 availability to the growth of macrophyte (Kairesalo, 1987). In the lab culture, epiphytic growth (mainly with cyanobacteria) on *Chara* thalli is usually the top risk to jeopardize the whole culture system (Fig. 2). The dimmed light condition can help with the suppression of phytoplankton growth but not the growth of cyanobacteria, since they have a high capacity of shade-tolerance (Scheffer et al., 1997). Though, cyanobacteria are rather sensitive to temperature and the dominance usually occurs when water temperature is higher than 20^0C (Havens, 2008; Bakker et al., 2010). Thus, the use of a lower temperature, that is still feasible for *Chara* growth and development, like 18^0C or a bit lower is, therefore, in favour to prevent the over growth by cyanobacteria. In general, decreasing the nutrient concentrations can also reduce epiphytic problems to a certain extent. Introducing herbivores (e.g. snails or Daphnids) into the culture was also recommended by other

researchers (Lew, 2015; Bakker et al., 2010) to control epiphytes and secondary algae.

We tried to introduce zebrafish into the culture system, but the result did not turn out well. On the one hand, zebrafish tend to not only eat epiphytic algae but also feed on *Chara* thalli, which lead to some mechanical damage to the *Chara* thalli. On the other hand, the excreta of zebrafish enrich the medium and further encourage the proliferation of cyanobacteria and fungi. The research of fish effects *in situ* also confirmed a poor performance of Charophytes caused by fish exposures (Winton et al., 2002).

In case of some *Chara* thalli gets entangled by the cyanobacteria, immediate removal of the lesions and replacing the medium could reduce the chance of further contamination.

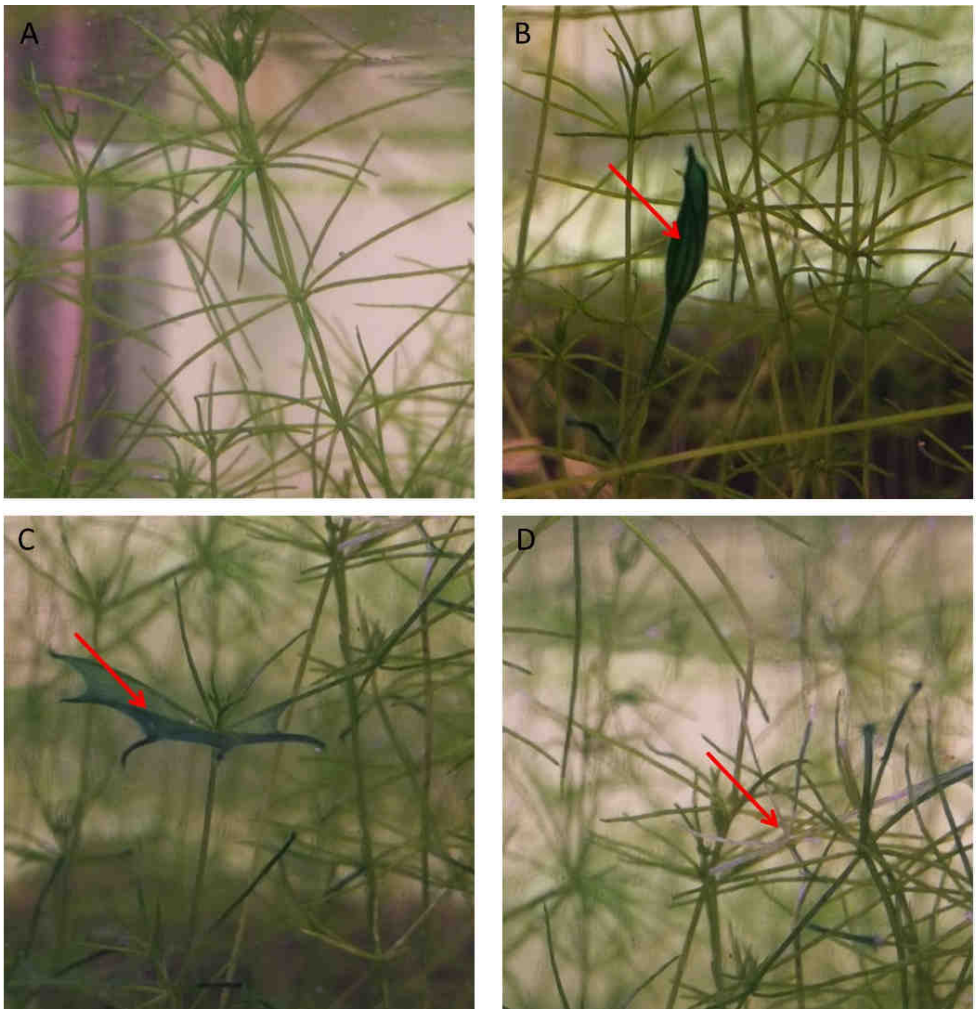

Figure 2. *Chara corallina* lab culture pictures. (A) healthy *Chara corallina* culture. (B&C) contaminated by cyanobacteria (red arrows). (D) transparent dead cells (red arrow).

Hygiene

Even though the *Chara* cultures are non-axenic, it is still important to be aware of the hygienic issues. While handling the *Chara* thalli, harvesting or the exchange of the medium, be sure to wash hands with soap, and remove the soap completely with tap water. Clean the tools (tweezers and scissors) with 70% ethanol and wait till the complete evaporation.

Handling

Shipping

Since the availability of most *Chara* species is (very) limited in most countries, researchers have to rely often on explants of laboratories in other parts of the world. These explants need to be shipped. For the shipment, tubes 2/3 filled with liquid (lake water/ APW), or plastic bags with wet filter paper inside around the explants were two mostly used methods. They both worked fine, though for the latter one it is important to use a box for shipping to avoid any physical pressure during the shipment. The physical condition of the *Chara* thalli before shipment is of great importance and only robust, bright green plant material should be used for shipment. Last but not the least, whole thalli with shoots and rhizomes together can keep the explants in much better conditions than the cuttings.

Harvesting

Referring to the hygiene, harvest the thalli only with clean hands and clean tools (section of Hygiene). Use scissors to cut the internodal cells or tweezers to pull out the whole thalli depending on the requirements. Fresh harvests could be rinsed by demi water and collected in APW solution for further usage.

Conclusions and recommendations

Chara could be an ideal model system because of the giant single cells and because it is assumed that the Characeae are an important link in the development from water to land plants. In the past many interesting results are produced from experiments with Chara.

What is typical for a model system is that experiments could be repeated in every laboratory wherever, provided that a standard protocol has been used.

Unfortunately, in 2017 we are far from having fully developed such a protocol for *Chara* as a model system. We are just on the way. We are still dependent on plant material that has been collected fresh in the field every time again. It is not always possible to find (ecorticate) *Chara* in the neighbourhood of a laboratory because *Chara* doesn't occur (*Chara corallina*, *Chara australis*, *Chara hispida* in the Netherlands) or is very rare (*Chara braunii* in the Netherlands). So, in many cases, we are dependent on the goodwill of colleagues elsewhere. It is absolutely necessary to build up an international network, which we did.

Recommendation: try to get a certain numbr of plants (of one particular species and collected at the same spot) at the disposal. Having reached this result comparison of different culture conditions would be a challenge.

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