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Encystment of *Alexandrium ostenfeldii* under Controlled Conditions in the Laboratory

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Alexandrium ostenfeldii is a toxic dinoflagellate distributed worldwide. It is one of many species to cause harmful algal blooms that affect other marine creatures and humans. This phenomenon has become more common recently.

The subject of this study was *A. ostenfeldii* originating from the Baltic Sea, focusing on the resting stages of their life cycle that play a very important role. The goal of this study was to get information about what factors trigger the encystment process in the cells and how relevant the sexual reproduction is in this process.

The study was carried out in controlled laboratory settings by monitoring cell growth in different conditions, e.g. in reduced amount of nutrients that are vital for their survival. Three *A. ostenfeldii* clones and cross cultures of these clones were studied to see if sexual reproduction makes any difference. This study was carried out at the Marine Research Centre of Finnish Environment Institute (SYKE).

It was concluded that reduced amount of nutrients was the best way to produce dormant resting cyst. Lowering the temperature produced quantitatively the largest number of cysts but they were 'thin walled temporary cysts'. Nonetheless, further study needs to be done to confirm the dormancy of the resting cysts.

Keywords	*Alexandrium ostenfeldii,* dinoflagellate, harmful algal blooms, encystment, life cycle

Tekijä Otsikko Sivumäärä Aika	Kaisu Jussila *Alexandrium ostenfeldii*n lepoitiöiden muodostuminen valvotuissa laboratorio-olosuhteissa 26 sivua + 1 liite 6.11.2017
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Alexandrium ostenfeldii on maailmanlaajuisesti levinnyt, myrkyllinen panssarisiimalevä. Se on yksi monista lajeista aiheuttaen haitallisia leväkukintoja, jotka vaikuttavat muihin merieliöihin ja ihmisiin. Tämä ilmiö on yleistynyt viime aikoina.

Tutkimuksen aiheena oli Itämerestä peräisin oleva *A. ostenfeldii*, painottuen sen lepovaiheeseen, jolla on erittäin tärkeä rooli sen elinkaaressa. Tämän projektin tavoitteena oli saada tietoa, mitkä tekijät laukaisevat solun muodostamaan lepoitiöitä ja kuinka tärkeä osa seksuaalisella lisääntymisellä on tässä prosessissa.

Tutkimus suoritettiin valvotuissa laboratorio-olosuhteissa, seuraamalla solujen kasvua eri olosuhteissa, kuten vähentämällä niille elintärkeiden ravintoaineiden määrää. Kolmea *A. ostenfeldii* -kloonia ja niiden ristiviljelmiä tutkittiin seksuaalisen lisääntymisen merkitystä. Tämä tutkimus suoritettiin Suomen ympäristökeskuksen (SYKE) Merentutkimuskeskuksessa.

Lopputuloksena päädyttiin, että ravintoaineiden määrän vähentäminen oli paras tapa saavuttaa lepotilassa olevia lepoitiöitä. Lämpötilan alentaminen puolestaan tuotti kvantitatiivisesti eniten lepoitiöitä, mutta ne olivat ohutseinäisiä väliaikaisia lepoitiöitä. Lisätutkimuksia on kuitenkin tehtävä lepoitiöiden laadun selvittämiseksi, eli onko kyseessä varmasti syvässä levossa olevia lepoitiöitä.

Avainsanat	*Alexandrium ostenfeldii,* panssarisiimalevä, haitalliset leväkukinnot, lepoitiöiden muodostus, elinkaari

Contents

Abbreviations

HABs	Harmful Algal Blooms
PSP	Paralytic Shellfish Poisoning
PSTs	Paralytic Shellfish Toxins
psu	Practical Salinity Unit
Spp.	Species (pl.)
STX	Saxitoxin
SYKE	Suomen ympäristökeskus

1 Introduction

This thesis is part of a laboratory study on the life cycle of a toxic alga, *Alexandrium ostenfeldii* (Paulsen) Balech and Tangen. *A. ostenfeldii* is an armored, marine, planktonic dinoflagellate that is a coastal species often found in coastal areas at high latitudes [1] [2]. It causes harmful algal blooms (HABs) and thus produces paralytic shellfish poisoning toxins (PST) [1]. *Alexandrium spp.* is one of the most common genus causing HABs. There are more than 30 known species of *Alexandrium* and at least half of them are toxic or have other way harmful effects. [3]

Alexandrium spp. can reproduce asexually through binary fission, which is the most common way. However, *Alexandrium sp*. can also undergo a sexual cycle. In sexual reproduction, motile mating types fuse, which produces a motile diploid zygote. It is also called a planozygote. The planozygote will swim and then take the form of a cyst. Blooms of *A. ostenfeldii* in shallow coastal waters are induced from resting cysts that form seed banks at the sea floor. Knowledge on the processes leading to cyst formation and germination is thus essential for the prediction of toxic *A. ostenfeldii* blooms. [1]

This graduate study is carried out as a side project for the PhD study of Jacqueline Jerney in the Marine Research Centre of Finnish Environment Institute (SYKE). The goal of this study is to get information about what triggers the encystment process of *A. ostenfeldii*. Experiments are performed to investigate how environmental factors such as temperature and nutrient levels, like phosphorus and nitrogen, will regulate the transitions between the growing cells and resting cysts. Another aspect in the study is to see if the cross cultures will produce more cysts compared to single strain cultures because of sexual reproduction.

In the laboratory part of the study, the respective experiments were set up and it was monitored how cells grow and cysts start to form. Subsamples from cultures were collected and analyzed. There are three different clones of *A. ostenfeldii* that were studied in the cultures. The work included algal culturing, cell counting, quantification of algal growth based on optical measurements, microscopic enumeration of resting cysts, as well as data analysis.

2 Theoretical Background

Alexandrium ostenfeldii is a toxic dinoflagellate and it has wide geographic distribution in temperate waters around the world. Initially it was found from the coast of Iceland [4]. Therefore, it is mostly reported from Europe and North America, the Russian Arctic and the eastern Siberian Seas. *A. ostenfeldii* was for a long time considered to be found only in northern cold-water environments. However, there are now records of *A. ostenfeldii* found from New Zealand, the coasts of Spain and the Mediterranean. This means that *A. ostenfeldii* is widely spread around the world. [5]

Dinoflagellates are algae that are surrounded by a complex covering. This is called the amphiesma and it consists of outer and inner continuous membranes. Between them lies a series of flattened vesicles. Some dinoflagellates possess an armor consisting of cellulose plates in the vesicles called 'thecal plates'. Armored dinoflagellates have two main plate regions which can consist of up to 100 individual plates. The plates are overlapped and they slide apart as the cell increases in size and expands. [6]

Dinoflagellates possess two flagella which differ from one another in appearance and position. Flagellum is a long cluster of protein strand which is used for movement. One of the flagella, the longitudinal flagellum, is in a groove called 'sulcus'. The sulcus begins from the center of one side, to the posterior end of the cell. The longitudinal flagellum beats back and forth causing the dinoflagellate to move to the opposite direction from the flagellum. The other flagellum, the transverse flagellum, is located in the cingulum. The cingulum is in the middle of the cell and goes around it is dividing it into two primary regions. The transverse flagellum makes it possible for the dinoflagellate to turn and also supports forward movement. What is more, together these two flagella cause the dinoflagellate to turn on its axis as it moves through water. [6] [7]

Records in publications from the early 20th century show that *A. ostenfeldii* has been present in the Baltic Sea for a long time. However, it wasn't until 1997 when it was first observed in high concentrations south of Öland. The blooming caused a strong, turquoise bioluminescence which was noticed during night time by local fishermen. A few years later, a similar bloom was reported from the Gulf of Gdansk. Ever since, dense bioluminescent blooms of *A. ostenfeldii* have become a recurring late summer phenomenon at the coasts of Baltic Proper. [5] In recent years, *A. ostenfeldii* has been increasingly observed in shallow waters and bays of the Baltic Sea up to the Archipelago Sea [7].

A. ostenfeldii is a medium sized species. The cell size varies usually between 40-50 µm, although sometimes the cells can be even smaller or larger. The cell length is the same as the maximum width. A light micrograph of *A. ostenfeldii* is shown in figure 1. [8]

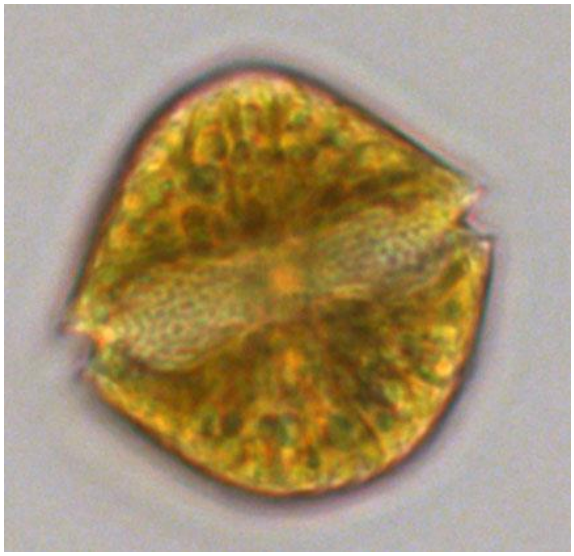

Figure 1. A light micrograph of *A. Ostenfeldii*. [9]

The nucleus is clearly visible in the middle of the cell, as seen in figure 1. The typical golden brown color of dinoflagellate*s* can also be seen. This pigmentation is caused by chlorophylls, the carotenoid beta-carotene and a group of xanthophylls that are unique for dinoflagellates. [10]

2.1 Harmful Algal Blooms

Harmful algal blooms (HABs) occur when populations of algae grow out of control and produce toxic or harmful effects on people and animals who are in contact with them. The toxins can kill fish and make shellfish dangerous to eat. The human illnesses caused directly or indirectly by HABs are rare but can even be fatal. The most famous algae to cause HABs is perhaps *Alexandrium tamarense,* also known to cause ‘Red Tides’ due to its ability to color the water in a reddish-brown tone as a result of really dense blooms. [11]

Only a small portion of algal blooms are toxic. Most blooms are actually beneficial because they are food for different animals in the ocean. However, blooms of some non-toxic species are also categorized as HABs due to their harmful effects on marine ecosystems. When a large number of algae die and decompose, the decaying process con-

sumes oxygen in the water, causing it to become very low in oxygen and resulting harmful or even fatal to animals. In addition, these blooms can also block the light from bottom-dwelling plants. [11] [12] *Alexandrium spp*. is one of the most important HAB species because of its toxicity and its wide distribution in the coastal environments of sub-arctic, temperate and tropical zones [13]. Dense *A. ostenfeldii* blooms during day time in the Baltic Sea can be seen in figure 2.

Figure 2. *Alexandrium ostenfeldii* dense blooms in the Baltic Sea. [9]

HABs occur naturally but occurrence of some blooms has increased because of human activities that disturb the ecosystems, including pollution, increased nutrient loadings, food web alterations, water flow modifications and climate change. The increased blooming has serious negative impacts on the economies and on public health of the affected areas [13]. Algal blooms are monitored and studied worldwide to determine how to detect and forecast the location of the blooms. The purpose is to give advance warnings to the communities and make it possible to plan and deal with the severe environmental and health effects that the blooming causes. [11] [12]

2.1.1 Bioluminescence

Bioluminescence is the ability of an organism to create light. This ability is most common in the deep sea. There are several groups of marine animals that have bioluminescent abilities, e.g. fish, jellyfish, bacteria and microalgae. Organisms use a reaction between

an enzyme and a substrate to create light, but the chemicals used varies depending on the species. It is not well-known how different organisms use bioluminescence, but it might help in finding food, assist in reproductive processes and provide defensive mechanisms. [14] The bioluminescent effect of *A. ostenfeldii* blooming is shown in figure 3.

Figure 3. *Alexandrium ostenfeldii* dense blooms during night in the Baltic Sea. [9]

In the case of dinoflagellates, bioluminescence is a night-time optical phenomenon where cells emit flashes of light induced by pressure on the cell wall. Basically, all bioluminescence in surface waters of marine environments comes from dinoflagellates. This bioluminescence is most likely a defense mechanism against predators. It has been suggested that it alerts their predator's predators, thus altering the marine food cycle in favor of dinoflagellates. *A. ostenfeldii* is considered to be the only bioluminescent dinoflagellate species found in the area of the Åland archipelago. [7]

2.2 Life Cycle of *Alexandrium ostenfeldii*

Alexandrium sp. alternate between asexual and sexual reproduction. The life cycle of *Alexandrium sp*. is shown in figure 4.

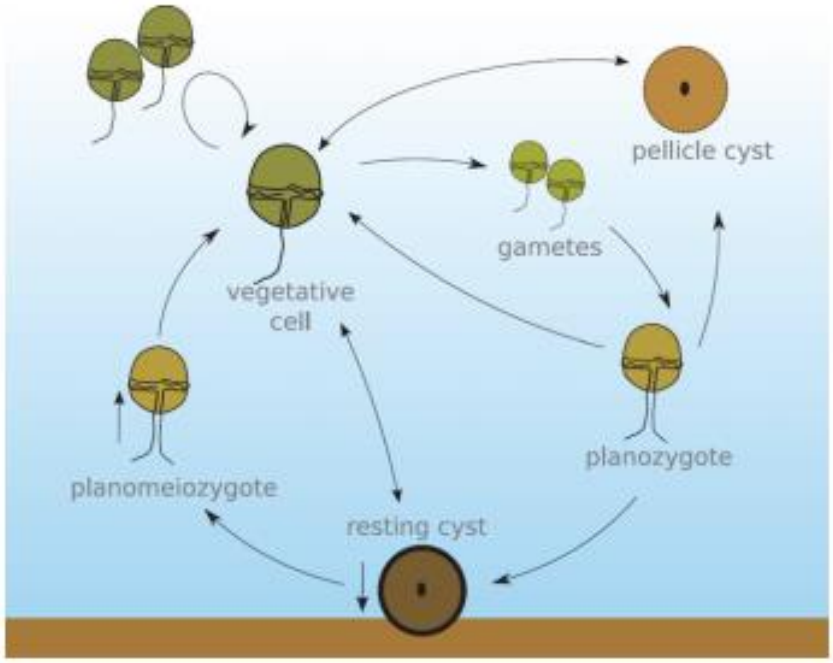

Figure 4. Schematic representation of asexual and sexual life cycle of a dinoflagellate.[15]

During blooming seasons, vegetative cells are rapidly divided through binary fission representing the asexual part of the growth, which is shown on the left-hand side in figure 4. The sexual phase of cycle begins when gametes are formed from vegetative cells. This is illustrated on the right-hand side in figure 4. Two gametes will then fuse together and form swimming zygotes (planozygote). It is characteristic of planozygotes to possess two flagella and a bigger size, compared to vegetative cells. Planozygotes will eventually form dormant resting cyst, called hypnozygotes that sink into the sediment because of their immotile nature and a density higher than water. Nevertheless, planozygotes can survive up to several weeks before falling into the sediment as a resting cyst. [16] [18].

Resting cysts are formed when environmental conditions are unsuitable for growth, e.g. when temperatures fall below a threshold or nutrients are exhausted. Cyst germination is regulated by exogenous or endogenous factors. If the suspension from germination is caused by unfavorable environmental (exogenous) factors, it is called *quiescence*. This can be caused for example by winter conditions, when unsuitable temperature and nutrient conditions prevent germination. If the suspension is caused by active endogenous

inhibition, it is called *dormancy*. The difference between them is that dormant cysts cannot germinate, even under optimal environmental conditions before the maturation is complete. Quiescent cysts, however, are capable of germinating, but are inhibited by environmental factors. The length of the dormancy period ranges extensively, going from a few days up to several months. [16] [17]

Most species can form another resting stage as well, called *a pellicle* cyst, as seen top right in figure 4. This stage is sometimes also called *temporary* cyst. They are formed when vegetative cells come suddenly in contact with unfavorable conditions, such as mechanical shock, a rapid change of salinity or temperature, i.e. when the cells are subjected to stress. Pellicle cysts are non-motile and have a thin single-layered wall. They don't have a mandatory dormancy period: they can quickly germinate into vegetative cells and start to grow when conditions are favorable again. This temporary resting stage allows cells to endure short-term external fluctuations. [5] [16] [20]

Cysts of *A. ostenfeldii* are spherical and relatively large, ranging in size from 35 to 40 µm in diameter. Resting cysts contain a pale granular cytoplasm and usually have a single reddish-brown granule, located in the center of the cyst and present golden-brown pigmentations radiating from the center of the cell towards the periphery. They also contain a well-defined internal belt-like groove. The cell wall is clear and smooth, and it is covered with various amounts of mucus. [18]

2.3 Toxicity of *Alexandrium ostenfeldii*

A. ostenfeldii usually produces paralytic shellfish toxins (PSTs) or spirolides [19]. The best-known PST is saxitoxin (STX). PSTs cause a human neurological disease known as Paralytic Shellfish Poisoning (PSP). It is caused by the ingestion of toxic marine shellfish, another seafood. Symptoms of this disease are: tingling sensation around lips gradually spreading to face and neck, prickly sensation in fingertips and toes, headache, dizziness, nausea, vomiting and diarrhea. In extreme cases, muscular paralysis can occur, resulting in death from respiratory paralysis [20]. PSTs producing species have been found from both cold and warm sea areas. During the blooming season, even large amount of toxins will accumulate and store into the shellfish, without harming or causing them any problems. [1]

The class of macrocyclic imines, known as spirolides, are marine phycotoxins produced by *Alexandrium ostenfeldii*. They contain a cyclic imine group which is only found in a few other marine toxins. Spirolides are often described as fast-acting phycotoxins because they cause neurological symptoms very quickly. Shellfish contaminated with spirolides are a food safety concern and therefore represent also a potential human health concern. [24]

The toxic profiles of *A. ostenfeldii* vary between individual strains but the reasons for this are partly unknown. Salinity of the water might have a role in this because toxin profiles of brackish water (salinity < 30 psu) strains are clearly different compared to strains for oceanic water (salinity > 30 psu). *A. ostenfeldii* strains isolated from the northern Atlantic coast of Canada, USA, Ireland and the Mediterranean produce spirolides, whereas strains isolated from Malaysia and the brackish water Baltic Sea produce PSTs. On the other hand, some strains can also produce both spirolides and PSTs. These include strains from an intermediate salinity, like Danish straits connecting the Baltic Sea to the North Sea, but also from oceanic salinities, like the coast of New Zealand. Other factors like temperature and nutrients have also been suggested to have an effect on the toxic profiles of *A. ostenfeldii*. [19] PSTs concentrations collected from the blooming area from shellfish and small fish exceed the European Commission safety limits of 80 µg 100 g^{-1} [8].

3 Materials and Methods

3.1 Encystment Experiment

Precultures for encystment study grew up to ~10,000 cells/ml. Precultures were grown in 550 ml cell culture flasks filled with 500 ml 6 psu f/2-Si growth medium, see appendix 1. Different *A. ostenfeldii* strains were inoculated from the precultures to reach up starting concentration of ~1,000 cells/ml. There were five different *A. ostenfeldii* clones: B202, B329, B348, B413 and B504 originating from the Åland archipelago in the Baltic Sea. From these five clones, three best growing clones: B202 (A), B348 (B) and B504 (C) were selected for the study.

In the experiment, there were three single clones: A, B and C. There were also three mixes of two clones: AB, AC, BC. Additionally, there was one mix of five clones. These seven different cultures were replicated twice. This is illustrated in figure 5. There were now three parallel sets of cultures in three different conditions. In one treatment, there were 21 samples, with a total number of 63 samples.

Figure 5. Schematic representation of one set of cultures in certain conditions.

One set of 21 samples, as shown in figure 5, was grown under normal growth conditions with 6 psu f/2 -Si growth medium, at 16°C. They were acting as control samples. A second set of 21 samples was grown with 6 psu f/2 -Si growth medium, at 10°C. This was called temperature treatment. Last set of 21 samples was grown using media with nitrate and phosphate reduced to 10% of the f/2 -Si level (f/20 -NO_3 -PO_4), at 16°C. This was called nutrient treatment because the amount of nitrogen and phosphorus were limited. Cultures were grown in 250 ml cell culture flasks. Control and temperature treatment cultures were filled with 200 ml 6 psu f/2 -Si growth medium. Nutrient treatment cultures were filled with 200 ml 6 psu f/20 -NO_3 -PO_4 growth medium.

Later, supplemental experiment was started, where phosphorus and nitrogen were limited separately. One set of 21 samples was grown under normal conditions with 6 psu f/2 -Si growth medium, at 16°C. They were acting as control samples. Second set of 21 samples was grown in media with nitrogen reduced to 10% of the f/2 -Si level (f/20 -NO_3), at 16°C. This was called nitrogen treatment because the amount of nitrogen was limited. Last set of 21 samples was grown with phosphorus reduced to 10% of the f/2 -Si level (f/20 -PO_4), at 16°C. This was called phosphorus treatment because the amount of phosphorus was limited. Cultures were grown in 250 ml cell culture flasks. Control cultures were filled with 200 ml 6 psu f/2 growth medium. Nitrogen treatment cultures were filled with 200 ml 6 psu f/20 -NO_3 growth medium. Phosphorus treatment cultures were filled with 200 ml 6 psu f/20 -PO_4 growth medium.

After six weeks of regular sampling (twice a week a 1.1 ml subsample per culture) for cell and cyst counts, cysts were harvested. First, extra liquid was aspirated leaving about 20 ml media and the cysts on the bottom of the culture flasks. After that, the flasks were roughly shaken to get the cysts that were stuck on the walls of the flasks to detach. In this phase, three replicates from the same treatment were pooled into one flask. Sample was poured into a 50 ml glass bottle and then sonicated for one minute in order to kill the cells. Cysts have the ability to survive this because of their thick cell wall. Following this, the sample was filtered through 30 µm sieving net with filtered sea water and poured into a 50 ml falcon tube. Finally, cyst concentration of the sample was counted and a concentration per mL was calculated.

3.2 Cell Counting

Cultures where gently mixed before taking a subsample. 1.1 ml subsamples were taken regularly from the cultures for counting, two times per week for six weeks. Samples were preserved with a drop of neutral Lugol's iodine solution and stored at +4°C. 1 ml counting chamber (Gridded Sedgewick-Rafter Counting Chamber, 1.0 mL) was used for counting samples. 1 ml of Lugol fixed sample was pipetted into the counting chamber and then the samples were counted under a light microscope (Leica DMI3000B inverted research microscope). Magnification of 100x was used for counting the samples, where all the round cells with a clear halo around them were counted as cysts.

The counting chamber contained a grid consisting of 1,000 squares. A minimum of 400 cells per sample were counted. The number of the squares needed for this was also counted. Finally, the number of cells was divided by the number of squares times 1,000 to get the approximate concentration of cells per milliliter. Subsamples were taken from harvested samples for documentation. 30 pictures were taken from the cysts by microscope to see what type of cysts they were. Finally, cysts sizes were measured to calculate the mean of the size.

4 Results

4.1 Encystment

Cells and cysts were counted through a microscope observation regularly during the experiment. Growth curves based on this data under different conditions are shown in figure 6.

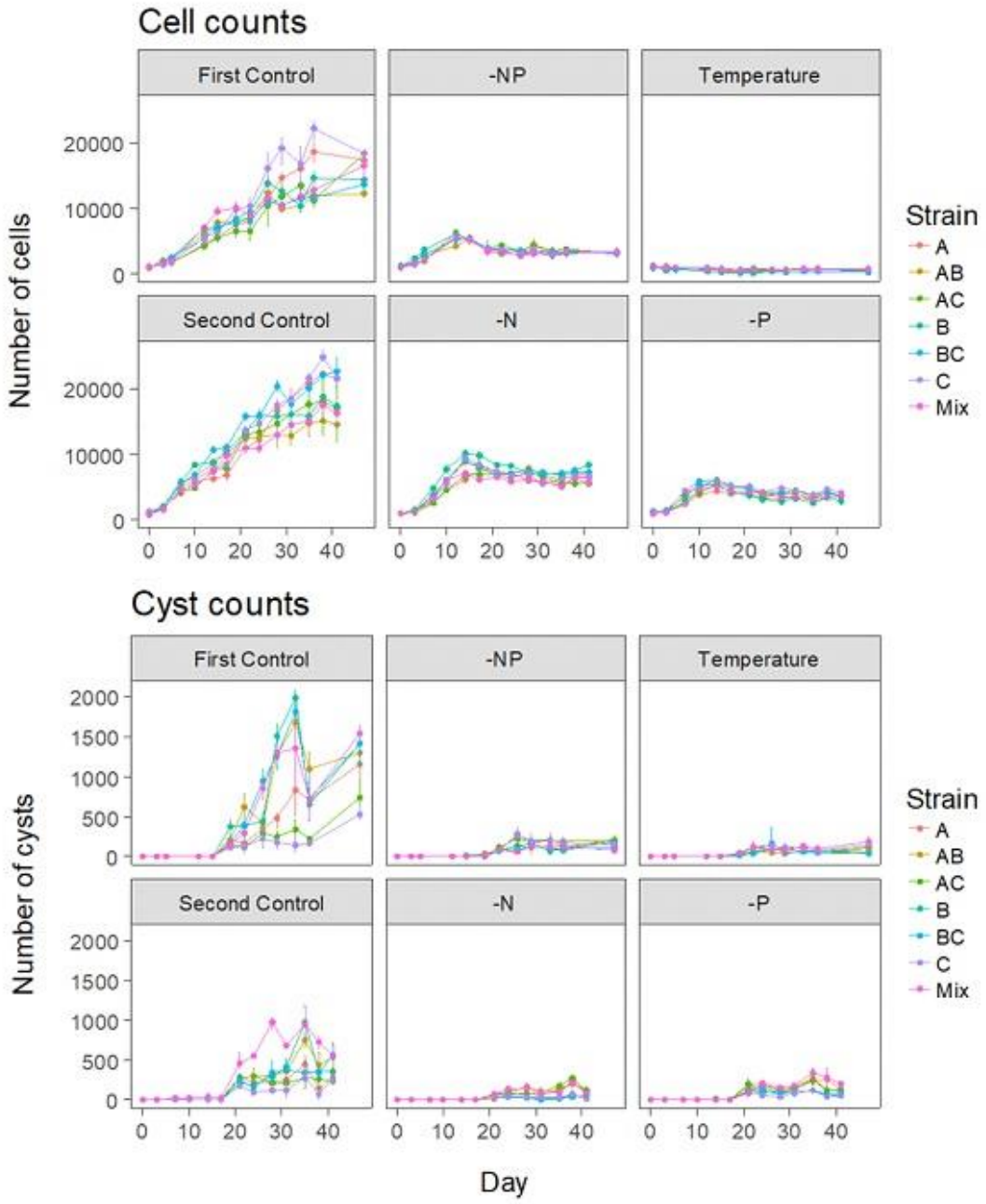

Figure 6. Growth of *Alexandrium ostenfeldii* cells and cysts at different conditions. Error bars represent standard deviations (n = 3).

Alexandrium ostenfeldii grew exponentially in control conditions, as shown in figure 6. Cysts started to form between 16 to 19 days of growing. The dip in the cell growth curve was caused by the formation of cysts. After this, the cells started to grow again due to an unknown reason, which caused a dip in the cyst growth curve, respectively.

In -NP conditions vegetative cells started to grow exponentially like the control, but after 10 days of growing the cell concentration began to decrease. Between days 20 to 40, the cell concentration stayed constant because they reached the stationary phase. Since the conditions were not suitable anymore for the cells to divide because they ran out of nutrients, cysts started to form between 16 to 19 days.

In temperature conditions, cell concentration slightly decreased after the starting point all through to the end. Cysts started to form at the same time as in the other treatments. In the nitrogen treatment cells were growing up to 15 days exponentially, but after that they started to slightly decrease and stabilize, reaching stationary phase to the end of the experiment. In the phosphorus treatment, cells grew exponentially for ~15 days and then decreased and stabilized around the same cell concentration.

Control conditions produced the largest number of cysts, but this was due to the much higher concentration of cells, where a higher growth occurred as a consequence of much more suitable conditions compared to the other treatments. Some strains under control conditions reached up to 20,000 cells whilst others remained at about 10,000 cells. This was due to the fact that some strains grew faster than others. In restricted conditions, the differences were much smaller between the different strains.

More useful information than the absolute number of cysts formed, is the number of cysts formed compared to the number of cells. The cyst to cell ratio along growth period is shown in figure 7.

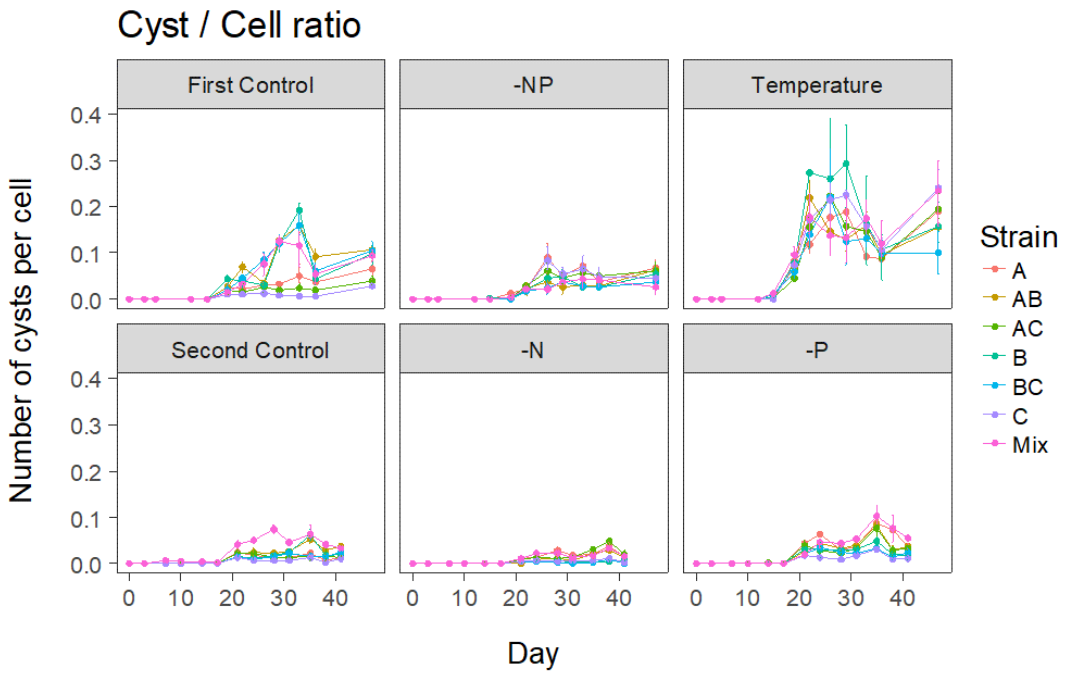

Figure 7. Cyst to cell ratios of *Alexandrium ostenfeldii* variations during growth period. Error bar represent standard deviations (n = 3).

As it can be seen in figure 7, the cyst vs cell ratio varied quite a lot during the growth period. The largest variations took place under temperature conditions. This was presumably caused by the relationship between cell growth and cyst formation, as mentioned before.

The final cyst to cell ratios of different single cultures after growth period under different growing conditions are shown in figure 8. Differences in cyst formation between different conditions are clearly visible in the graphs.

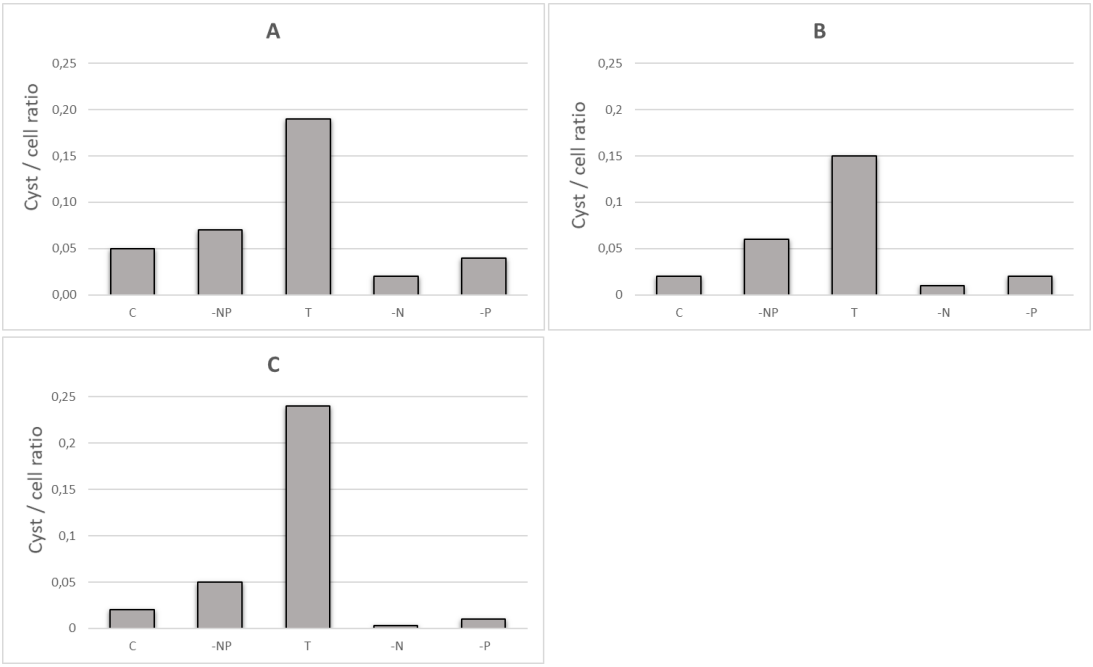

Figure 8. The final cyst to cell ratios of A, B and C clone cultures in different conditions.

The temperature treatment was by far the most productive way to yield cysts with single cultures of A, B and C clones, as seen in figure 8. The C clone produced clearly the most cysts per cell in the temperature treatment, compared to the other clones. The nutrient treatment (-NP) produced the second-best yield with all the clones, but the differences between them were much smaller. The nitrogen treatment produced clearly the least number of cysts per cell with all clones. All in all, the order between different treatments in yield was the same regardless of the clone.

The final cyst to cell ratios of different cross cultures after growth period under different growing conditions are shown in figure 9. In the graphs, differences in cyst formation between different conditions are clearly seen.

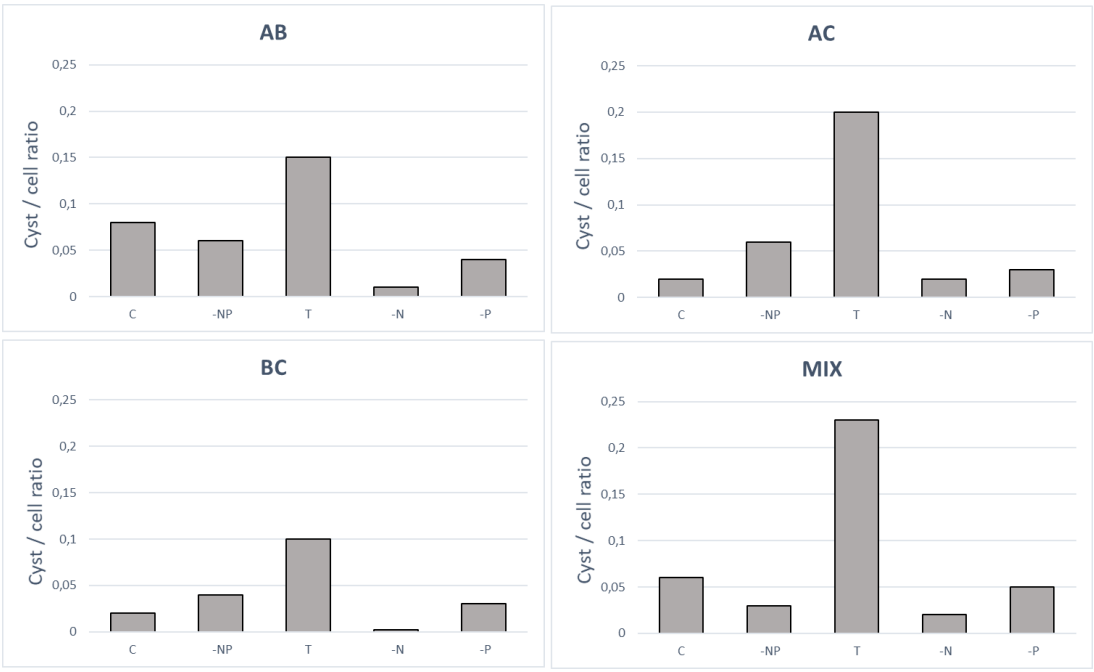

Figure 9. The final cyst to cell ratio of AB, AC, BC and MIX cross cultures under different conditions.

The cross cultures show the same trend as the single cultures. This can be seen in figure 9. The temperature treatment produced the highest yield of cysts, although there were more differences between cultures. With AC and MIX crosses, the yield in temperature treatment is much larger compared to other treatments. Regarding AB and BC crosses, the difference is not as significant. Also, the nutrient treatment did not produce the second-best yield with all the crosses and the order between treatments varied. The results of MIX cross culture are interesting because the nutrient treatment produced less cysts per cell than the phosphorus treatment. This is different from all the other cultures.

It was originally hypothesized that the cross cultures would produce higher cyst yields compared to the single cultures. However, this was not generally the case. In fact, BC cross culture produced the least amount of cysts of all the cultures in almost all conditions. All in all, the yields were quite similar between single and cross cultures.

4.2 Morphology

As mentioned before, there are two different types of cysts, temporary cysts and resting cysts. This caused a need for a morphological analysis to determine which ones the cysts formed in the experiment were. This was determined based on the light micro-

graphs of the cysts. Examples of these light micrographs of cysts formed under different conditions are shown in figures 10-14. In practice, 30 micrographs were taken from each strain under each condition.

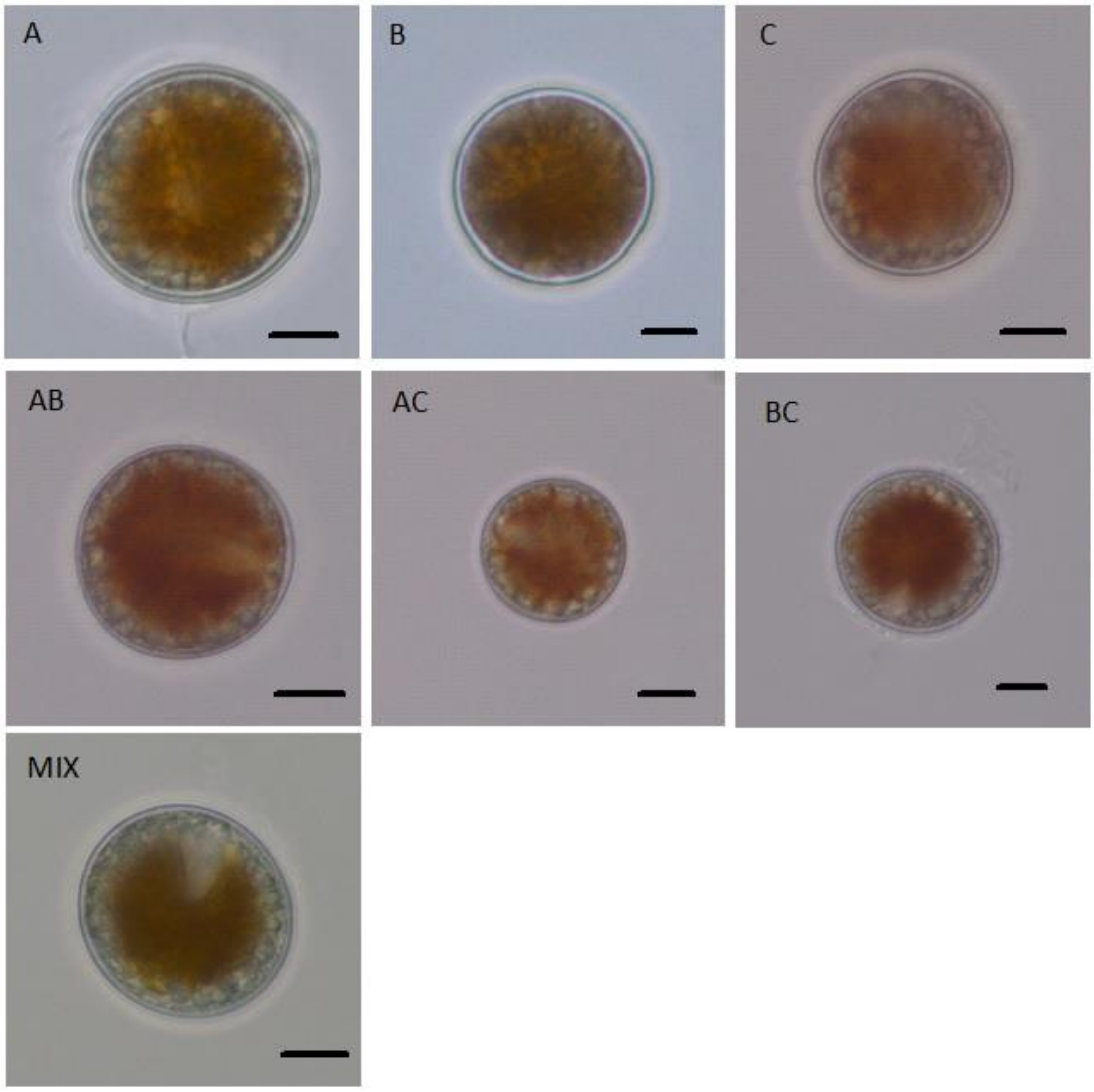

Figure 10. Light micrographs of *A. ostenfeldii* cysts formed under control conditions. Scale bars=10 µm.

In figure 10, light micrographs of cysts of different strains formed under control conditions are shown. The cysts are spherical and they have a clear, smooth and thick circular cell wall, which are characteristic of resting cysts. Yet, the cysts are still heavily pigmented, indicating that they are quiescent resting cysts rather than fully formed dormant resting cysts.

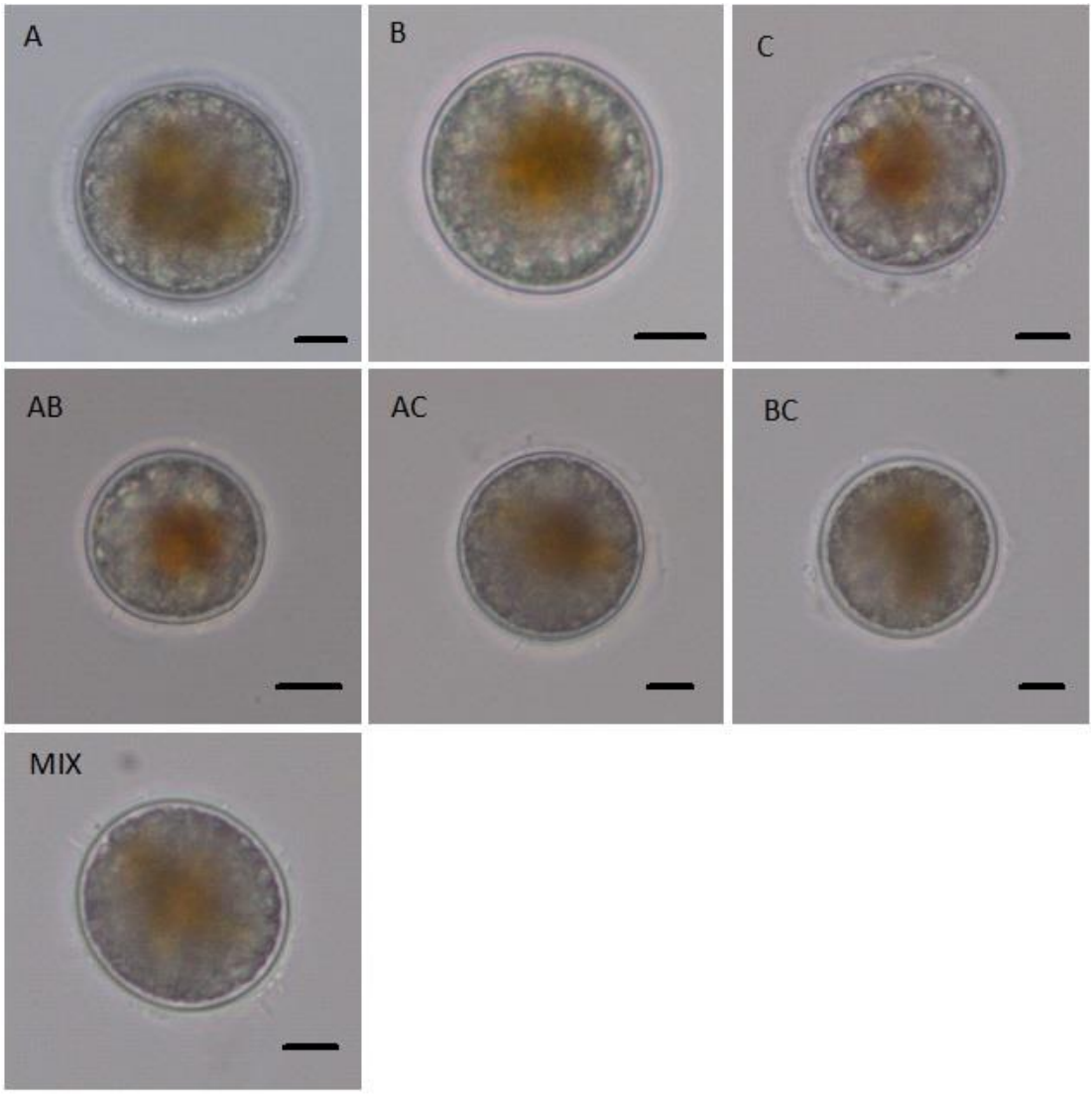

Figure 11. Light micrographs of *A. ostenfeldii* cysts formed under reduced nutrient conditions. Scale bars, 10 µm.

In figure 11, light micrographs of cysts of different strains formed under reduced nutrient conditions (nitrogen and phosphorus) are shown. It seems that these conditions produced cysts that most resemble dormant resting cysts. The cysts are spherical and they have a clear, smooth and thick circular cell wall, surrounded by mucus. They all present a reddish-brown granule in the middle and they have lost pigmentation around them. This is best shown in the micrograph of strain C. Although their characteristics resemble dormant resting cysts, they need to be studied further to confirm the results.

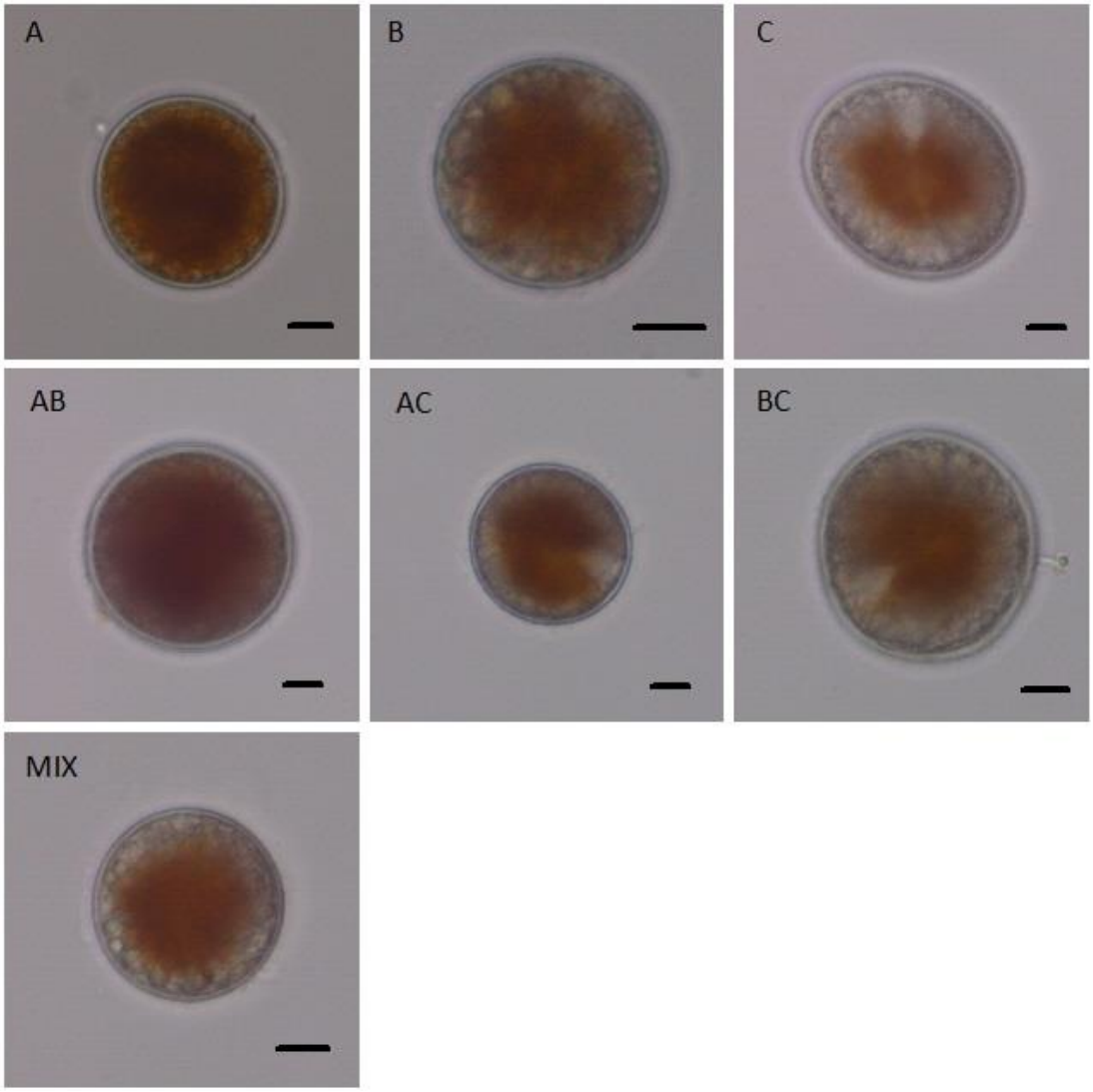

Figure 12. Light micrographs of *A. ostenfeldii* cysts formed under temperature conditions. Scale bars, 10 µm.

In figure 12, light micrographs of cysts of different strains formed under temperature conditions are shown. The cysts are spherical and they have a clear, smooth and thick circular cell wall. The pigmentation is very dark brown in color. Some cysts have begun to lose their pigmentation whilst others have not. They are similar to the cysts formed under control conditions, which indicates they are not resting cysts.

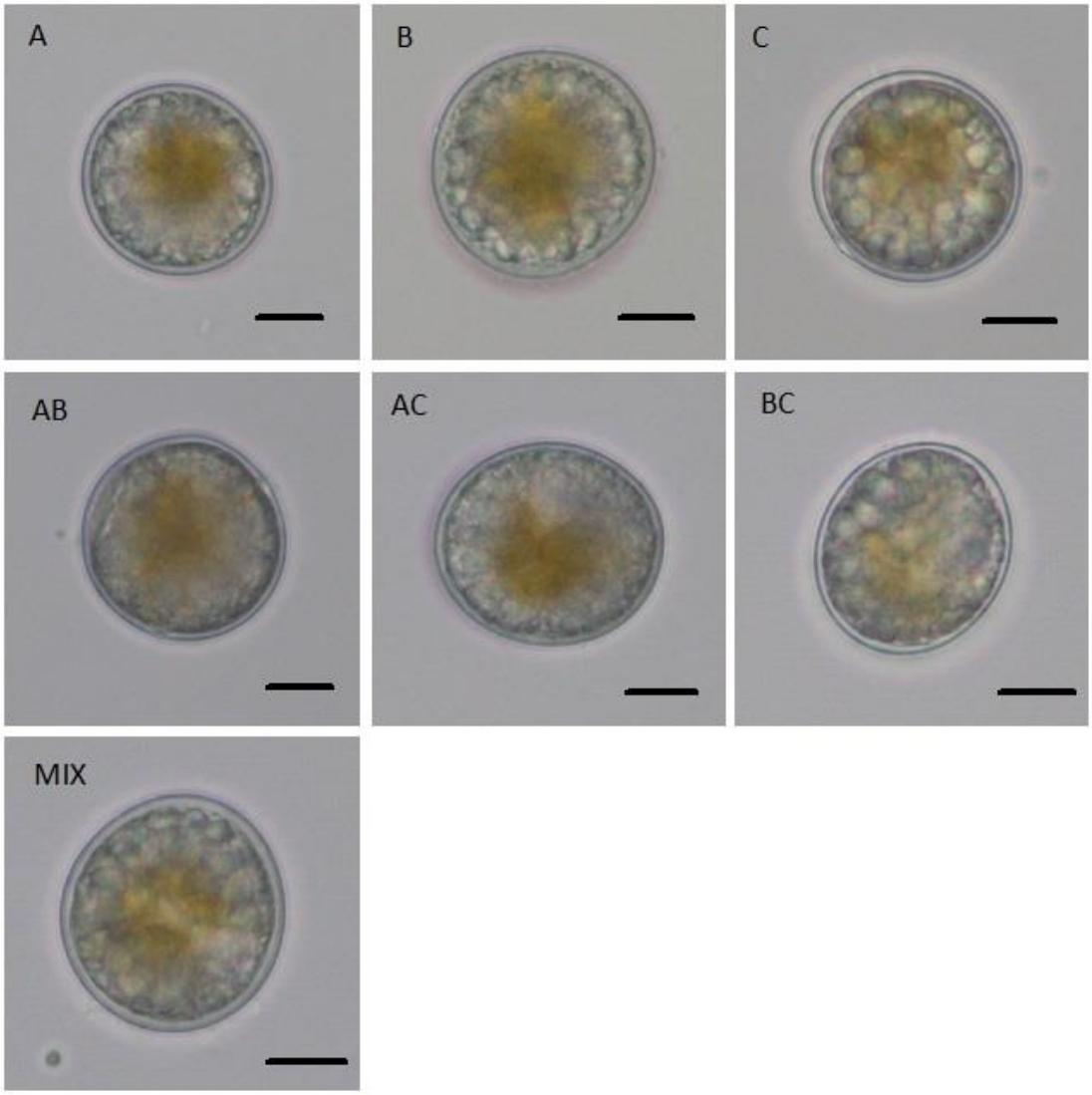

Figure 13. Light micrographs of *A. ostenfeldii* cysts formed under reduced nitrogen conditions. Scale bars=10 µm.

In figure 13, light micrographs of cysts of different strains formed under reduced nitrogen conditions are shown. The cysts are not as spherical as the ones formed under other conditions. They have a clear, smooth and thick circular cell wall, with a yellowish-brown granule in the middle and they have lost pigmentation around them. Because of these characteristics, these cysts resemble dormant resting cysts, but this should be confirmed with further studies.

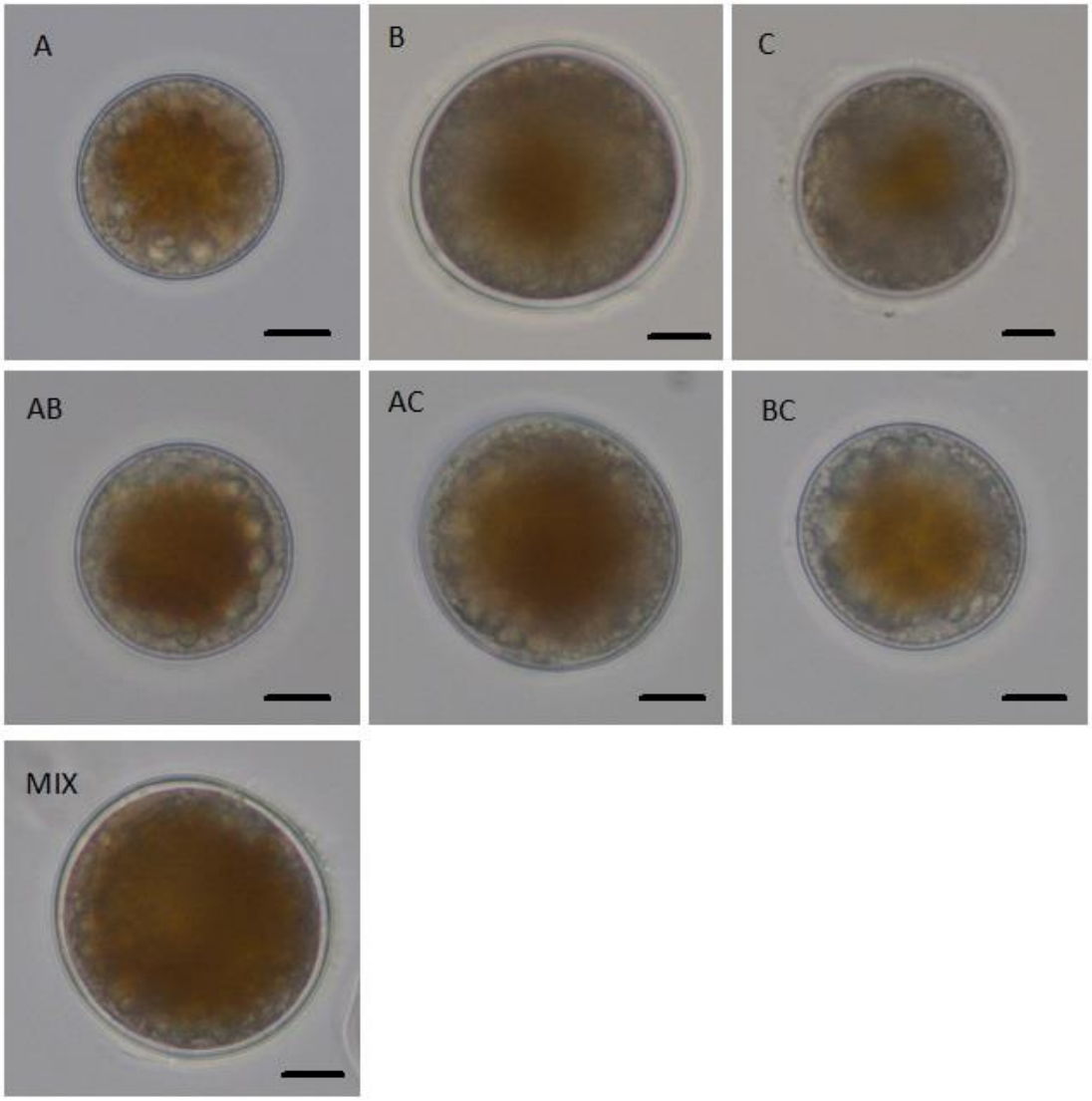

Figure 14. Light micrographs of *A. ostenfeldii* cysts formed in reduced phosphorus conditions. Scale bars=10 µm.

In figure 14, light micrographs of cysts of different strains formed under reduced phosphorus conditions are shown. The cysts are spherical and they have a clear, smooth and thick circular cell wall, with a brown color pigmentation. Interestingly, some cysts have lost some of their pigmentation. These cysts resemble cysts formed under control conditions, which indicates that they are not dormant resting cysts.

5 Discussion

Even though the biggest number of cysts compared to the cell number were formed under temperature conditions, the morphological analysis revealed that they do not look like true resting cysts. In fact, the cysts formed under reduced nutrient conditions resembled dormant resting cysts the most. The cysts formed under reduced nitrogen conditions somewhat resembled dormant resting cysts. However, these conditions produced the least number of cysts compared to the cell number. The cysts formed under control and reduced phosphorus conditions did not resemble dormant resting cysts either. There were no significant morphological differences between different strains under particular conditions.

From the results obtained, it can be concluded that forming cysts under reduced nutrient conditions would be the best approach to produce dormant resting cysts. The yield was decent and the cysts were most likely dormant resting cysts. The nitrogen treatment could be another option, but due to a very poor yield the cysts did not resemble dormant resting cysts as much. In terms of yield alone, the temperature treatment would be the best by far. The cysts did not look like resting cyst, but some had begun to lose their pigmentation. With time, they could have possibly developed into dormant resting cysts.

In nature, encystment is most likely caused by a combination of lowering temperature and decreasing nutrients. This situation occurs in the Baltic Sea during winter, for example. From the results of this experiment, it can be hypothesized that the combination of low temperature and reduced amount of nutrients would produce higher numbers of resting cysts. Regardless, a following study would be needed to confirm if this is indeed the case.

An important factor in this study was its subjectivity, given the fact that the determination of cells and cysts was made visually, with a person interpreting what was observed under the microscope, increasing the margin of error of the results. In fact, one of the difficulties encountered was to determine the difference between cells and cysts during the counting in some cases, especially since the samples were fixed with Lugol and this lead to cell death.

6 Conclusions

The goal of this study was to produce dormant resting cysts of *A. ostenfeldii* and to find what factors trigger the encystment process, assessing if factors including temperature and nutrients play a major role. Another target of this study was to investigate the role of sexual recombination in encystment, with the hypothesis that sexual reproduction would induce the production of more cysts, compared to non-sexual reproduction.

It was concluded that the best way to induce encystment in this experiment was by reducing the nutrients, both nitrogen and phosphorus. These conditions produced dormant resting cysts and the yield was decent. As for sexual reproduction, it did not appear to have a major effect on cyst formation: the cyst yield in these cases was very close to that in single cultures. In fact, in some cases the cyst to cell ratios were even lower.

Finally, it is important to note that the scope of this study did not extend to determining whether the produced resting cysts were actually fully-formed dormant resting cysts. Likewise, the effect of some variations of the different conditions, such as a combination of low temperatures and reduced nutrients, were not studied, which would be interesting to assess since it would provide more information on the subject. Further studies are needed to form a complete picture of all the aspects affecting the encystment process.

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f/2 Medium recipe [21]

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f/2 Medium

(Guillard and Ryther 1962, Guillard 1975)

This is a common and widely used general enriched seawater medium designed for growing coastal marine algae, especially diatoms. The concentration of the original formulation, termed "f Medium" (Guillard and Ryther 1962), has been reduced by half.

To prepare, begin with 950 mL of filtered natural seawater and add the following components. The trace element and vitamin solutions are provided below. Bring the final volume to 1 liter with filtered natural seawater. If the alga to be grown does not require silica, then it is recommended that the silica be omitted because it enhances precipitation. Autoclave.

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
$NaNO_3$	75 g/L dH_2O	1 mL	8.82×10^{-4} M
$NaH_2PO_4\ H_2O$	5 g/L dH_2O	1 mL	3.62×10^{-5} M
$Na_2SiO_3\ 9H_2O$	30 g/L dH_2O	1 mL	1.06×10^{-4} M
trace metal solution	(see recipe below)	1 mL	---
vitamin solution	(see recipe below)	0.5 mL	---

f/2 Trace Metal Solution

To prepare, begin with 950 mL of dH_2O, add the components and bring final volume to 1 liter with dH_2O. Autoclave. Note that the original medium (Guillard and Ryther 1962) used ferric sequestrene; we have substituted $Na_2EDTA \cdot 2H_2O$ and $FeCl_3 \cdot 6\ H_2O$.

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Component	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
$FeCl_3\ 6H_2O$	---	3.15 g	1.17×10^{-5} M
$Na_2EDTA\ 2H_2O$	---	4.36 g	1.17×10^{-5} M
$CuSO_4\ 5H_2O$	9.8 g/L dH_2O	1 mL	3.93×10^{-8} M
$Na_2MoO_4\ 2H_2O$	6.3 g/L dH_2O	1 mL	2.60×10^{-8} M
$ZnSO4\ 7H_2O$	22.0 g/L dH_2O	1 mL	7.65×10^{-8} M
$CoCl_2\ 6H_2O$	10.0 g/L dH_2O	1 mL	4.20×10^{-8} M
$MnCl_2\ 4H_2O$	180.0 g/L dH_2O	1 mL	9.10×10^{-7} M

f/2 Vitamin Solution

First, prepare primary stock solutions. To prepare final vitamin solution, begin with 950 mL of dH_2O, dissolve the thiamine, add the amounts of the primary stocks as indicated in the quantity column below, and bring final volume to 1 liter with dH_2O. At the NCMA we autoclave to sterilize. Store in refrigerator or freezer.

Component	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
thiamine HCl (vit. B_1)	---	200 mg	2.96×10^{-7} M
biotin (vit. H)	0.1 g/L dH_2O	10 mL	2.05×10^{-9} M
cyanocobalamin (vit. B_{12})	1.0 g/L dH_2O	1 mL	3.69×10^{-10} M

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