The use of allantoin to enhance asexual spore propagation in *Porphyra umbilicalis*

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Photo – Front page: Wild fronds of *Porphyra umbilicalis* on a groyne at Blåvand in the eulittoral zone, February 2016 (picture taken by Lasse Hornbek Nielsen).
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Abstract

Scientific research from USA has investigated and acknowledged the use of *Porphyra umbilicalis* as a maricultural crop, but there is still a lack of scientific research into the reproductive pathways for European populations of *P. umbilicalis* for it to be used in mariculture in Denmark. European populations of *P. umbilicalis* differs from North-west Atlantic population in their reproductive strategies. The European populations primarily reproduce through sexual reproduction, while the North-west Atlantic populations are only known to reproduce through asexual neutralspores. Asexual reproduction allows for a cheaper and less time consuming reproductive pathway in mariculture. The chemical compound allantoin is hypothesised to be able to stimulate asexual reproduction in *P. umbilicalis*.

The effect of allantoin to provoke asexual reproduction was investigated on wild *P. umbilicalis* collected at Blåvand, Denmark. Experiments were performed on fronds collected in late summer and in midwinter. The fronds were treated with three concentrations of allantoin (control (0mM), 1 mM, 10 mM) to test if allantoin would increase asexual spore propagation and increase daily growth of the thallus. It was furthermore investigated if spore propagation and daily growth of the thallus was affected by irradiance level and the size of the piece of experimental thallus used.

Treatment with 10 mM allantoin in the summer population induced asexual spore liberation, and large areas of the experimental thallus changed structure from a robust two-dimensional cell structure to a fragile three-dimensional cell structure. These findings could not be replicated in the winter population. No asexual spore liberation was observed in the experiments on fronds form the winter population. Treatment with 10 mM allantoin was found to increase the daily growth rate of the thallus in the first 15 days of treatment. High irradiance level was found to have a positive effect on the daily growth rate of the thallus over the 30 days of treatment.

The results indicated that allantoin can induce asexual spore formation in *P. umbilicalis* in late summer populations but not in winter populations. Further research is needed primarily to verify the findings and to define the optimal concentration of allantoin for production and liberation of asexual spores, as well as to determine if the liberated asexual spores were neutral spores or blade archeospores.
Resumé

Forskning fra USA har undersøgt og anerkendt *Porphyra umbilicalis* potentiale som en afgrøde til havbrug, men for at kunne overføre den viden til dansk havbrug, er det nødvendigt at få en bedre forståelse af den reproduktive livscyklus hos europæiske populationer af *P. umbilicalis*. Europæiske populationer af *P. umbilicalis* adskiller sig fra beslægtede nordvest-atlantiske populationer ved primært at have seksuel formering, hvor nordvest-atlantiske populationer kun er observeret at formere sig aseksuelt via neutralsporer. Formålet med denne afhandling var at undersøge om den kemiske forbindelse allantoin vil kunne fremprovokere aseksuel formering i danske *P. umbilicalis* populationer.

Allantoins evne til at fremprovokere aseksuel formering blev undersøgt på vildthøstet *P. umbilicalis* indsamlet ved Blåvand, Danmark. Allantoin blev først testet på alger indsamlet i sensommeren, og dernæst testet på alger indsamlet om vinteren. Algebladende blev behandlet med tre allantoin koncentrationer (kontrol (0 mM), 1 mM, 10 mM), og allantoins effekt på produktionen af aseksuelle sporer, samt på algernes vækstrater blev efterfølgende undersøgt. Der blev videre undersøgt, om forskellige lysintensiteter og størrelsen på de eksperimentelle vævsstykker havde effekt på produktion af aseksuelle sporer og vækstrater.

I materiale samlet om sommeren, havde behandling med 10 mM allantoin tilsyneladende en stimulerende effekt på produktion og frigørelse af aseksuelle spore fra vævet, og behandlingen ændrede store områder af thallus fra robust todimensionel cellestruktur til en mere skrøbelig tredimensionel cellestruktur. Der var derimod ingen effekt af behandling med allantoin i vinterpopulationen, og her blev ikke observeret udvikling af aseksuelle sporer overhovedet. Behandling med 10 mM allantoin i vinterpopulationen viste sig at øge den daglige tilvækst af thallus i de første 15 behandlingsdage. Høj lysintensitet viste sig ligeledes at øge den daglige tilvækst henover de 30 behandlingsdage med allantoin.

Resultaterne antyder, at allantoin kan fremprovokere dannelse af aseksuelle sporer i *P. umbilicalis* i sensommeren, men ikke om vinteren. Der er dog behov for yderligere studier til primært at bekræfte resultaterne, dernæst til at bestemme den optimale koncentration af allantoin til produktionen af aseksuelle spore, og til at afgøre om de frigivne aseksuelle sporer var neutralsporer eller blade archeosporer.
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1 Introduction

1.1 *Porphyra* – a commercially important seaweed

The importance of seaweed as a culinarian ingredient differs a lot between different world cultures. Seaweed has been a crucial ingredient in many East Asian countries for centuries, but is of less importance in the European cuisine, with a few exceptions like Wales and Norway where certain seaweed species are included in their traditional cuisine. A range of seaweed species previously grouped in the genus *Porphyra*, are of great commercial importance (Levine and Sahoo, 2010), to a large degree thanks to some of these species being a key ingredient in maki sushi in the form of nori sheets; Nori being the Japanese name for the genus *Pyropia* (former *Porphyra*).

*Porphyra* have traditionally been the genus used to encompass a large group of seaweed species belonging to the order of Bangiales, such as the commercially important Asian species *Pyropia yezoensis* and *Pyropia tenera* (Levine and Sahoo, 2010). A recent revision of the genus by Sutherland et al. (2011) has divided the genus *Porphyra* up into several genera, based on development of local knowledge of Bangiales floras, and by collaborative global analyses based on wide taxon sampling and molecular analyses. The species are now belonging to the genera: *Boreophyllum* gen. nov., *Clymene* gen. nov., *Fusciolium* gen. nov., *Lysithea* gen. nov., *Miuraea* gen. nov., *Pyropia*, and *Wildemania*. The genus of *Porphyra* is now restricted to include five described species and a number of undescribed species. One of the described species is *Porphyra umbilicalis* Kützing 1843, which is the focus of this thesis.

Most papers and books written before 2011 use *Porphyra* as a generalized term, but as this is now misleading, the term “foliose Bangiales” will be used throughout this thesis instead (Green and Neefus, 2015) (Agnes Mols-Mortensen, PhD, Tari Faroe Seaweed, pers. comm.).

At present time, foliose Bangiales is used for a range of different commercial products aimed for the Asian market including sushi, different fast food products and flavour ingredient for soups (Levine and Sahoo, 2010). It is also the principal commercial source of the fluorescent pigment r-phycoerythrin with a market value greater than $12 million per year (Levine and Sahoo, 2010). In an European context, it has been a historical important ingredient in traditional Wallisian and Irish cuisine under the name laverbread (beachfood, 2015) and is sold in many countries as a nutraceutical (Levine and Sahoo, 2010), marketed as being beneficial for health in general due to its high amount of protein, omega-3 fatty acids, vitamin B12 and vitamin A (BIOCEAN, 2016).

The market value of foliose Bangiales is around $1.3 billion (Blouin et al., 2011), and there are cultivated approximately 1.8 million tons (wet weight) in maricultural settings every year (FAO, 2016). Practically the whole industry of foliose Bangiales is based in Asia, with Japan, Korea and China as the most important players. The economic importance of the maricultural industry has driven foliose Bangiales (under the name *Porphyra*) to be one of the most thoroughly described genus of macroalgae, with a huge volume of scientific papers, mainly focused on commercially important Asian species like *Pyropia yezoensis* and *Pyropia tenera*. The only large scale experiments with cultivating foliose Bangiales in mariculture outside of Asia has, to the best of the authors knowledge, been in North America in the 90’s and 00’s (Levine and Sahoo, 2010).

The potential for a Danish market, would most likely be as nori sheets for the large national sushi industry, with sushi restaurants becoming popular in larger Danish cities.

1.2 *Porphyra* in mariculture

Nori (*Pyropia*) has been grown in Japan for centuries and is part of the Japanese cultural history. Up until the 1950’s, the mariculture with nori was not very effective as only the blade phase of the
lifecycle was known and exploited in mariculture. The famous work of Kathleen Drew changed this (Drew, 1954), by linking the blade phase of _Porphyra umbilicalis_ to the shell-boring species _Conchocelis rosea_, showing that _C. rosea_ was the sporophyte phase of _P. umbilicalis_ (Drew, 1954) (Figure 1). This knowledge helped improve nori farming in Japan to such a degree, that Kathleen Drew at present time is celebrated in Japan as “the mother of the sea” each year on the 14th of April.

There has been some interest in establishing a mariculture with foliose Bangiales in Maine, USA. The first attempts with commercially-valuable Asiatic taxon _Pyropia yenzoensis_, imported from Japan, were not successful due to Maine’s coastal environments being suboptimal growing conditions for _P. yenzoensis_ (Yarish et al., 1999). In more recent times, interests have been focused on _P. umbilicalis_ as a candidate for mariculture in Maine, due to _P. umbilicalis_ having several favourable traits for mariculture. _P. umbilicalis_ has high photosynthetic rates, nutrient efficiency and growth rates (Green and Neefus, 2015). The blade phase is present year-round and is the most abundant foliose Bangiales species, spatially and temporally, in Maine (Yarish et al., 1999). A peculiar trait, further in its favour, is that _P. umbilicalis_ is only known to reproduce through neutral spores in Maine (Blouin and Brawley, 2012). In contrast to the complex sexual life-cycle of foliose Bangiales, asexual reproduction has the potential to be a simpler, faster and cheaper reproductive method compared with the established Asian methods of seeding nets with conchospores (Blouin et al., 2011, Levine and Sahoo, 2010). A study from Maine showed that cultivating using neutral spores is a viable reproductive pathway for _P. umbilicalis_ to be used in mariculture (Blouin et al., 2007). Blouin et al. (2007) was successful in establishing neutral spore cultures from harvested _P. umbilicalis_ fronds and in using these neutral spore cultures to seed nets for deployment in maricultural settings.

One large challenge for using _P. umbilicalis_ as a potential new maricultural crop, is the inability of the thallus to stick together. The production of nori sheets is based on granulated thallus from foliose Bangiales naturally sticking together when dried (Levine and Sahoo, 2010). Out of a range of foliose

![Figure 1: The life history of _P. umbilicalis_ showing both the sexual reproductive phase with a conchosporangium and the asexual reproductive phase through neutral spores (Blouin et al., 2011)](image-url)
Bangiales species inherent to France, *P. umbilicalis* showed the worst results when attempted to be made into nori sheets (Tristan Le Goff, team manager at CEVA, pers. comm.).

### 1.3 Reproductive lifecycle of foliose Bangiales

The lifecycle of foliose Bangiales is characterized by two distinct life phases; the blade phase, also known as the foliose phase, and the conchocelis phase. The blade phase is a haploid gametophyte consisting of a single large frond, and is either monoecious or dioecious, which is a species specific trait (Sutherland et al., 2011). The blade phase is the dominating lifecycle-stage of foliose Bangiales and most species live as lithophytes growing in the marine littoral zone. The morphology of the haploid phase is leaf-shaped, with a two-dimensional growth pattern. The second life-stage is a microscopic diploid sporophyte called conchocelis, consisting of thin filaments usually growing through penetrations of the shells of bivalves (Blouin et al., 2011). The conchocelis phase is initiated when non-motile spermatia (male gametes) get in contact with female fronds and fertilize female gametes embedded in the thallus. Spermatia are produced in male fronds as a result of mitosis, where a cell divides into a spore package. The arrangement of spermatia inside the spore package is a species specific trait. The spermatia are released through degradation of the cell wall, and are seen as small spores distinguishable from other types of foliose Bangiales spores by being colourless (Nelson et al., 1999). A fertilized female gamete, called a zygotosporangia, is diploid, and will through mitotic division result in a package of diploid zygotospores that are liberated through degradation of the surrounding cell wall tissue. The liberated zygotospores are non-motile, small, and often have a decentralized nucleus (*P. umbilicalis*, personal observation). When the zygotospores settle they produce long rhizoid-like structures that grow out penetrating the substrate on which the spores have settled, usually shells (Drew, 1954). In vitro, zygotospores can be made to germinate in petri dishes in the absence of a suitable substrate. Laboratory experiments in Portugal with *Porphyra dioica* has developed this technique to such a degree, that petri dish cultures with growing conchocelis phases started reproducing haploid conchospores, which grew to new thallus thereby completing the full life-cycle (Pereira et al., 2004, Pereira et al., 2008). The mature conchocelis produces conchosporangia each of which produce a single diploidic conchospore. The liberated conchospore will germinate into a new haploid thallus after settling on adequate substrate, usually rocks in the littoral zone. Meiosis has been documented to occur when the conchospore germinates, and the four resultant cells, which all have different genetic compositions, form the mature frond through successive divisions (Blouin et al., 2011).

These are the general steps in the lifecycle of foliose Bangiales but there are a range of alternative reproductive pathways with a range of different spore types, thoroughly described by (Nelson et al., 1999). Here follows a short overview of the complete range of spores produced by foliose Bangiales.: 

**Sexual reproduction:**

- Spermatia (also known as antherozoids or β-cells): The male gametes produced by haploid male fronds (*P. umbilicalis* produce 128 spermatia in each package).
- Female gametes: Stay in the thallus of female individuals. Develop into zygotosporangia after fertilisation with spermatia.
- Zygotospores (also known as carpospores): Diploid spores. Produced in zygotosporangia. Develop into conchocelis after being released and having settled on appropriate substrate.
- Conchospores: Diploid spores. Produced in conchosporangia, which are reproductive cells in conchocelis. One conchosporangium develop a single conchospore. Develop into a haploid
blade phase through meiosis after it has been released and has settled on an appropriate substrate (Figure 1).

**Vegetative reproduction:**

- Neutral spores (previously grouped together with agamospores as aplanospores): A single haploid thallus cell undergoes mitotic cleavage into a spore package (*P. umbilicalis* produce 16 neutral spores in each neutral sporangia (Blouin et al., 2011)). Neutral spores will develop into new haploid blades after being released and having settled on appropriate substrate.
- Agamospores: a single haploid thallus cell undergoes mitotic cleavage into a cell package. Agamospores will develop into conchocelis after being released and having settled on appropriate substrate.
- Archeospores (previously known as monospores): A single haploid blade cell developing into a single archeospore. Two types of archeospores exist. Blade archeospores will germinate into a new haploid blade. Conchocelis archeospores will germinate into the conchocelis phase. Blade archeospores and conchocelis archeospores have both previously been described as monospores, which can be confusing, and the term monospores is therefore not recommendable, instead the terms blade archeospores and conchocelis archeospores should be used instead. In the authors experience the term archeospore is often used without defining the type of archeospores, and will in these cases most often describe blade archeospores (Saito et al., 2008).
- Neutral conchospores: Cells in the conchocelis develop into a sporangium releasing a single spore than develop into a new conchocelis.
- Phyllospore: Proposed term by (Nelson et al., 1999) for spores produced by the blade phase where ploidy level, development history and further development is unknown. The term phyllospores thus encompass all of the following spores: male gametes, zygotospores, blade archeospores, conchocelis archeospores, agamospores and neutral spores. Because ploidy level is rarely available and the development history can be difficult and time-consuming work, it is recommended to use the term phyllospores when these conditions are not met.

This all summarizes up in foliose Bangiales having a complex life-cycle, which has been further complicated by different scientific papers giving different names to the same spore types both in in the same and across different foliose Bangiales species. A final consensus has not been met on the naming of foliose Bangiales spores, but the definitions set forth by (Nelson et al., 1999) are commonly accepted and will also be used in this thesis.

1.4 *P. umbilicalis* in Europe and in Denmark

The use of *P. umbilicalis* in European mariculture is somewhat more complex as compared with Maine, in that the European population, based on *P. umbilicalis* fronds collected in England, has been shown to predominantly reproduce sexually (Blouin and Brawley, 2012). The use of *P. umbilicalis* for mariculture in Europe would depend on an investigation of the sexual life-cycle with techniques for cultivation of the conchocelis phase. An alternative strategy would be to improve our understanding of the asexual life-cycle of the European population, to enhance and exploit the asexual spores, or use some combination of the two.

*Porphyra umbilicalis* has been indentified on the west-coast of Jutland, Denmark (pers. obs.). This is somewhat closely placed geographically to the German Island called Helgoland, where Kornmann and
Sahling (1991) listed and described the local *Porphyra* species in 1991, *P. umbilicalis* among them. The genotype of the *P. umbilicalis* populations of Helgoland is believed to be closely linked to the *P. umbilicalis* population found along the west-coast of Denmark. Kornmann and Sahling (1991) observed, that out of 22 collected fronds, 7 developed conchocelis through zygotospores (termed agamospores in the article), and, to their surprise, they found 15 fronds developing into new fronds through asexual spores (termed aplanospores in the article). Kornmann and Sahling (1991) reported the aplanospores to have their origin in unfertilized spore packages containing multiple spores, and to develop into haploid blades. Based on the terminology of Nelson et al. (1999), it can be deduced that the reported spores were neutral spores.

Archeospores are not known in *P. umbilicalis* (Brodie and Irvine, 2003). Denmark lack a complete scientific list for foliose Bangiales species inhabiting the Danish coastline. There is at the moment a process of developing a new Danish algae flora, where foliose Bangiales will be included in a chapter concerning the genus Bangiales (A. Mols-Mortensen, pers. comm.). At present, seven Danish species, that have been verified by molecular identification to belong to this group, can be found in Denmark: *Porphyra linearis*, *P. purpurea*, *P. umbilicalis*, *Pyropia leucosticta*, *P. njordii*, *P. peggicovensis*, and *Wildemania amplissima* (Mols-Mortensen, 2014).

1.5 Asexual reproduction in *P. umbilicalis*

*P. umbilicalis* is not known to have other asexual reproductive pathways than through neutral spores (Brodie and Irvine, 2003, Blouin et al., 2011). The use of *P. umbilicalis* for mariculture has only been researched in Maine, where the seaweed is known to solely reproduce through neutral spores, and as a consequence there does not seem to have been any motivation for researching what triggers or enhance the asexual reproduction in *P. umbilicalis*. In Asia, thorough investigations have been made into which environmental factors influence the asexual reproduction in related Asian species. Investigations into blade archeospore formation have shown, that spore propagation can be enhanced through high irradiance levels (*Pyropia yezoensis* (Li, 1984)), fragmentation of the thallus into smaller tissue sections (*P. yezoensis* (Hafting, 1999b)), low Ca$^{2+}$ concentration in medium (*P. yezoensis* (Takahashi et al., 2010)), high water temperature (*P. yezoensis* (Kitade et al., 1998); *Pyropia pseudolinearis* (Saito et al., 2008)), and treating the thallus with allantoin (*P. yezoensis* (Mizuta et al., 2003); *P. pseudolinearis* (Saito et al., 2008)).

Allantoin is a chemical compound with the formula C$_4$H$_6$N$_4$O$_3$, which is found in several organisms spanning from algae to humans, where it is formed in small quantities through oxidation of uric acid (Shestopalov et al., 2006). Allantoin can work as a nitrogen source and Pineda et al. (1984) has showed that the green microalgae *Chlamydomonas reinhardii*, could grow with allantoin as its sole nitrogen source.

Allantoin has been shown to affect cells in several ways, e.g. modulating activity of the enzyme antioxidant system (Shestopalov et al., 2006), but most interestingly, in the context of this thesis, it is known to mediate cell proliferation (Shestopalov et al., 2006). Allantoin is generally and widely used as a cell proliferant in medical science (Saito et al., 2008) and can be bought in its pure form to be used for skin care (Urtegaarden, 2016).

Allantoin has proven to be a powerful stimulant for enhancing blade archeospore formation in foliose Bangiales. Mizuta et al. (2003) found an increase in formation of *P. yezoensis* blade archeospores with increasing allantoin concentrations. The spore counts increased from ~600 spores without allantoin to ~30,000 spores when the thallus was exposed to 10 mM allantoin. Mizuta et al. (2003) reported that a high number of blade archeospores was embedded in the thallus, and to help spore
liberation, the allantoin treatment was combined with mild homogenization of the thallus after 3 weeks of allantoin treatment. In contrast to P. yezoensis having blade archeospores being a natural part of its life-cycle, Saito et al. (2008) showed that allantoin was able to propagate blade archeospores in Pyropia pseudolinearis, a foliose Bangiales species that would not produce blade archeospores naturally.

To the best of the authors knowledge, this thesis is the first time the effect of allantoin has been tested on P. umbilicalis.

1.6 Aim and hypotheses

The overall aim of this thesis was to investigate the asexual reproduction of a wild Danish Porphyra umbilicalis population collected off the west-coast of Jutland, Denmark. The more specific aims for the study was to test if the fronds of P. umbilicalis would produce asexual spores when exposed to allantoin, and to explore if allantoin had a beneficial or detrimental effect on the daily growth of the thallus of P. umbilicalis.

This thesis tries to answer the hypotheses:

H1: Allantoin can induce asexual spore propagation in Porphyra umbilicalis.
H2: These spores can after liberation be grown into new haploid fronds.
H3: Allantoin can increase the growth rate of the thallus during treatment.

The thesis encompasses two initial pilot studies and two subsequent experiments, through which the hypotheses were tested (table 1).

The first pilot study investigated if a 10 mM allantoin treatment induces asexual reproduction through propagation and liberation of asexual spores in wild P. umbilicalis fronds collected from a late summer population.

The second pilot study investigated if fronds collected from an early winter population would respond differently to a 10 mM allantoin treatment compared with the late summer population from the first pilot study. It further investigated different spore liberation techniques based on the technique used by Blouin et al. (2007) to induce spore liberation through mild shaking.

The first experiment investigated how different concentrations of allantoin, irradiance level and fragment size influenced 1) asexual spore propagation in fronds collected from an early winter population, and 2) the SGR of the thallus during the first 30 days of the experiment.

The second experiment investigated how different concentrations of allantoin influenced 1) asexual spore propagation, and 2) SGR during the first 30 days of the experiment in fronds collected from a midwinter population. This experiment test an alternative spore liberation technique used by Mizuta et al. (2003) and Saito et al. (2008), using mild homogenization to liberate spores.
2 Materials and methods

During this thesis, two pilot studies and two experiments were performed (Table 1).

- **Table 1**: Temporal overview of the experimental setups tested in the thesis. 1.SC, 2.SC and 3.SC are the first, the second and the third spore count. SGR is the specific growth rate of the thallus.

<table>
<thead>
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<th>Date</th>
<th>Replicate (n)</th>
<th>Description</th>
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<td>Experiment 2</td>
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<td></td>
<td>2.SC</td>
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<td>3.SC</td>
<td>02th of May 2016</td>
<td>Test of the effect of allantoin on the survival of cells liberated during homogenization.</td>
<td></td>
</tr>
</tbody>
</table>

2.1 Collection of material

Fresh material of *Porphyra umbilicalis* was collected individually several times during the pilot studies and experiments.

*P. umbilicalis* material was collected at Blåvand strand (UTM: 55.552898407, 8.11977005) in the upper littoral zone on a rocky groyne. *P. umbilicalis* is present year round (Brodie et al., 2008) and was observed at this spot during the whole experimental period from August 2015 until spring 2016.

*Porphyra umbilicalis* is distinguishable from other species of *Porphyra* by not being adherent to paper (Brodie et al., 2008, Brodie and Irvine, 2003), and the collected material was identified as *P. umbilicalis* by placing a frond between two pieces of paper and subjecting the setup to mild pressure for 24 hours. The safest way to identify *Porphyra* species is through DNA barcoding analysis, using the 3´rbcL + 5´rbcL-S marker, which has been found to have excellent species-resolving power within the foliose Bangiales (Mols-Mortensen et al., 2014, Green and Neefus, 2014). This however, was not possible within this study.

*P. umbilicalis* material collected for the two pilot studies and for the first experiment were collected in plastic containers containing sea water from the same area and the material was kept in these containers during the three hours of transport back to the lab. The material was collected during low tides, and large fronds (~ 8 cm in diameter) were favoured over smaller fronds, based on the assumption that the fronds would be more mature and thereby be easier to provoke into asexual reproduction.
Material for the second experiment was collected during low tide were the fronds were between wet and damp. Medium-sized fronds (~ 6 cm in diameter) were chosen for practical reasons (it made it easier to get 15 fronds of identical size), and were loosely wrapped in paper towels and packed in ice for the three hours of transport back to the lab in accordance with the methods used by Blouin (2007). The algae samples were, on return to the lab, placed in a freezer (-20°C) for three days to reduce biofouling of the thallus. A recent study has shown that P. umbilicalis is not harmed by being cooled down to -20°C (Green and Neefus, 2014), and the three days of freezing before start of the experiment should therefore not harm the P. umbilicalis fronds. It was hypothesized that the cooling instead might harm less freeze tolerant species that might be adhered to the thallus as biofoulers, though no sources to support this hypothesis were available.

2.2 Storage conditions for live materials prior to experiments
The P. umbilicalis material for the first pilot study was collected in June, three months prior to the pilot study and was stored in an open 150 litre plastic container, with continuous aeration. The algae material was stored at 10°C under fluorescent light (Phillips Master TL5 HO 39W/840) with an intensity of ~20 µmol photons m² s⁻¹ in a 16:8 L:D regime. Once every second month, the algae material was supplemented with 10 µl l⁻¹ of f/2 medium, which is a 1% dilution of the f/2 medium (Guillard and Ryther, 1962).

The P. umbilicalis material used in the second pilot study and the first experiment were contained in 3 cylindrical containers prior to the experiments. The water was vigorously aerated and 10 µl l⁻¹ of f/2 medium was added twice a month. The algae material was stored at 10°C under fluorescent light (Phillips Master TL5 HO 39W/840) with an intensity of ~100 µmol photons m² s⁻¹ in a 16:8 L:D regime. The algae material was supplemented with 10 µl l⁻¹ of f/2 solution twice a month.

The supplement of 10 µl l⁻¹ of f/2 medium (Guillard and Ryther, 1962) during storage gave the algae 88 µM nitrogen and 4.2 µM phosphor. This is at least a factor two higher than the nutrient concentrations the algae material experienced at Blåvand under natural conditions (Table 2).

The data on nutrient levels at Blåvand is retrieved from ODA (Overfladevandsdatabasen ODA).

2.3 Experimental medium
All water used in pilot studies and experiments was seawater taken from Blåvand collected at the same time as collection of P. umbilicalis material. The seawater was stored in 25 litre plastic containers in a 5°C storage room under dark conditions until use.

| Table 2: The nutrient profile of the f/2 medium used as nutrient supplement, and the nutrient profile of the water at the sampling side when P. umbilicalis was collected. The percentages indicate how much lower the nutrient profile were at the time of sampling compared to the nutrient supplement given in the laboratory. |
|-----------------|-----------------|-----------------|-----------------|
|                  | 1% dilution of f/2 supplement | June 2015 | December 2015 |
| Nitrogen         | 88 µM            | 23 µM         | 26%            |
| Phosphor         | 4.2 µM           | 0.55 µM       | 13%            |
|                  |                  | 43 µM         | 49%            |
|                  |                  | 2.35 µM       | 56%            |
|                  |                  | 32 µM         | 37%            |
|                  |                  | 0.76 µM       | 18%            |

The P. umbilicalis material used in the second experiment was not kept in the laboratory prior to the experiment, but was used immediately after a few days of freezing at -20°C.
To lower the risk of contamination, the seawater used in the two experiments was sterilised by heating it to 75°C three times. The resulting sterilized seawater (SSW) was stored in sealed 5 l glass containers in a 5°C storage room under dark conditions until use.

10 µl l⁻¹ of f/2 medium was added to all SSW used in the two experimental setups, to secure the algae from experiencing nutrient limitation (Table 2).

Diatoms turned out to be a devastating issue in the first experiment. To counteract this issue in the second experiment, all experimental SSW was added a supplement of 0.894 g l⁻¹ GeO² as recommended by Shea and Chopin (2007).

2.4 Measuring growth of thallus

The effect of allantoin on the specific growth rate of the thallus in the first and second experiments was quantified by measuring the area of the thallus before and after the treatment with allantoin.

In the first experiment the total area of the thallus in each replicate was from start 1.9 cm². The area of the thallus upon ending the experiment was quantified by taking pictures with a camera (Nikon DS-Fi 1) in a stereo microscope. The camera was calibrated before use, to a 2 mm ruler for the software used for area measurement. The software program NIS-Elements (ver. 3.22.15) was used for calculating the area of the thallus from the pictures.

In experiment 2 the thallus in each treatment was too large for a stereo microscope. Instead, pictures were taken with a smartphone camera (Fairphone 2) and each picture was calibrated by having a grid with a known size included in the picture. These pictures were analysed, and the areas of the thalli calculated using the software program ImageJ 1.50i.

2.5 Pilot study 1 – Effect of allantoin on asexual spore formation and liberation in *P. umbilicalis*

The first pilot study was started on the 22th of October with algae material collected in June. Six individual plants were chosen and dried in an incubator for 40 min. to damp conditions to increase spore liberation in accordance with Blouin et al. (2007) before being rehydrated in unsterilized seawater in large petri dishes (maxi glass petri dishes). The petri dishes were covered with a lid and were kept without aeration. The seawater was added a nutrient supplement of 10 µl l⁻¹ of f/2 medium at the start of the study, and all replicates was treated with 10 mM allantoin (Urtegaarden, 2016). The petri dishes were stored at 10°C under fluorescent light (Phillips Master TL5 HO 39W/840) with an intensity of ~20 µmol photons m² s⁻¹ in a 16:8 L:D regime.

Over the next six weeks the condition of the thallus and the release of spores to the petri dish was followed visually by use of a stereo microscope, and pictures of spores were taken with a smartphone camera (iPhone 4). A single nylon thread was put into the culture dish to test if any asexual spores that would be liberated would adhere to it. The pilot experiment was discarded after six weeks in favour of second pilot study, partly because the condition of the thallus of all six replicates were continuously worsening, and partly to test if the results could be replicated in a late winter population.

The water was never changed during the six weeks, and the thallus was not given new nutrient supplementation. The salinity in the petri dishes was not monitored.
2.6 Pilot study 2 – Test of different liberation techniques for asexual spores.

The second pilot study was started on the 19th of December with material collected on the 12th of December.

Nine different spore release techniques were tested based on the spore release technique used by Blouin et al. (2007). One replicate of each technique was tested (Table 3). Each replicate consisted of one haploid frond stored in unsterilized seawater in a small plastic petri dish. The seawater was added a nutrient supplement of 10 µl l⁻¹ of f/2 medium, and were treated with 10 mM allantoin (Urtegaarden, 2016) at the start of the pilot study. The replicates were kept for three weeks at 10°C under fluorescent light (Phillips Master TL5 HO 39W/840) with an intensity of ~20 µmol photons m⁻² s⁻¹ in a 16:8 L:D regime.

Four replicates were dried in an incubator for 40 min until damp in accordance with Blouin et al. (2007) and four replicates were kept wet, to test for the potential effect of the incubation period. For spore liberation (Blouin et al., 2007) used an VWR orbital shaker, that was not available for the work of this thesis. The eight replicates were instead treated to one of four different degrees of shaking on an orbital shaker (Fritsch, 220V 50Hz 2.3A 500W) to test which intensity would optimize spore liberation. Two treatments were with mild intensity for either 5 min. or 30 min., one treatment was with high intensity for 5 min. and the last treatment was a control without shaking. The shaking was performed while the seaweed was kept in the experimental petri dishes. The ninth replicate was packed in tin foil and stored for 24 hours at 5°C before being put at high light intensity (100 µmol photons m⁻² s⁻¹) and 20°C for three hours in accordance with the method used by Kitade et al. (1998).

Table 3: Schematic overview of the experimental setup of the second pilot study

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of replicates</th>
<th>Dried in incubator</th>
<th>Kept wet</th>
<th>Shaked</th>
<th>Put in tin foil (5°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>X</td>
<td></td>
<td>Mild (5 min)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>X</td>
<td></td>
<td>Mild (30 min)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>X</td>
<td></td>
<td>High (5 min)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>X</td>
<td></td>
<td>No shaking</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>X</td>
<td></td>
<td>Mild (5 min)</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>1</td>
<td>X</td>
<td></td>
<td>Mild (30 min)</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>1</td>
<td>X</td>
<td></td>
<td>High (5 min)</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>1</td>
<td>X</td>
<td></td>
<td>No shaking</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No results were recorded from this pilot experiment, as the full setup was accidentally ruined before any measurements were made. Based on the available time for the thesis project, a judgement was taken to start experiment 1 instead of repeating the pilot experiment 2.

2.7 Experiment 1 – The effect of thallus size, irradiance level and allantoin on asexual spore formation and liberation, and growth of thallus in *P. umbilicalis*

The *P. umbilicalis* material used was collected in the start of December and had been kept for 1½ month under conditions as previously described. The material had started to degrade to some degree and the fronds had turned from red to brownish-green in colour. The 52 fronds in the best conditions were selected for the experiment. The condition was evaluated based on the colour of the frond, reddish favorized over green/yellow colouring, and the state of the edge of the frond, where some fronds were starting to degrade.
The experimental setup was designed with a total of 18 different treatments, resulting from variations of three different factors: irradiance (~40 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \), ~90 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \), ~160 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \)) (Blouin et al., 2007, Li, 1984), allantoin concentration (0 mM (control), 1 mM, 10 mM) (Mizuta et al., 2003, Saito et al., 2008) and fragment size (small (3 * 0.6 cm\(^2\)), large (1 * 1.9 cm\(^2\))) (Hafting, 1999b). Each treatment had three replicates which resulted in a total of 54 samples. Three groups of each 18 samples, defined by the irradiance, were started with three days’ intervals: The intermediate irradiance intensity was started on the 27\(^{th}\) of January, the high irradiance intensity was started on the 29\(^{th}\) of January, and the low irradiance intensity was started on the 31\(^{th}\) of January.

All irradiance intensities were kept in a 12:12 L:D regime. The light regime was changed to having a longer dark period to simulate the conditions experienced in wild winter populations.

The light source used for all three groups were fluorescent light (Phillips Master TL5 HO 39W/840) and the irradiance intensity was set by adjusting the height of the fluorescent lamps over the experimental setup. Prior to the experiment, the light intensity was measured (Li-cor Li-1000) to map the fluctuation of the irradiance between the individual replicate. The experiment was designed with three separate groups based on specific irradiance, but the fluorescent light source gave very unstable light with a lot of fluctuation: 21-50 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \), 59-112 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \), 77-217 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \).

Two different fragment sizes were tested for an effect of border-to-area ratio. Two cylindrical metal pipes sharpened in one end were used take out the thallus pieces. The small thallus pieces were on third the size of the large thallus pieces, and to get the same area in all replicates three pieces was taken from the same frond for replicates with small thallus pieces. The resulting total area of thallus in all replicates was 1.9 cm\(^2\) (Figure 2).

![Figure 2: Example of the two fragment size tested.](image)

All replicates were kept in large petri dishes (maxi glass petri dishes) with 150mL SSW. The SSW was given nutrient supplement as mentioned earlier, and an additional nutrient addition of 10 \( \mu \text{l} \) of f/2 medium was given after 2 weeks. Weak aeration was applied to all replicates through a silicon tubes ending in the modified tip of a plastic syringe. To compensate for the high evaporation caused by aeration and high irradiance, the replicates were replenished with demineralised water every third day, based on the change in salinity level in the water of the experimental medium. Salinity was controlled by use of a handheld salt refractometer (Atago).

After a four-week period, spore liberation was performed, following a method described in Blouin et al. (2007), on the three groups in three day intervals. The thallus from each replicate was first dried in an incubator for 30 min. until damp. The thallus was then rehydrated in large petri dishes with 150mL new SSW and shaken on an orbital shaker (Fritsch, 220V 50Hz 2.3A 500W) for one hour (20˚C, daylight) at high intensity. The bottom of the petri dishes contained three glass slides (Cover glass,
24x36mm, deltalab) for the liberated spores to settle on and the medium was stirred every 5 minutes, for the liberated spores to distribute evenly during shaking. After one hour the thallus was removed from the petri dish and the petri dish containing the spore solution was stored for the next 3 weeks under the same conditions as before spore liberation.

All spores on the glass slides were counted under a microscope (40x magnification) fitted with an object containing a grid. The grid was calibrated with a 2 mm ruler before spore counting so that the total area of the grid was 1 mm². Five places were randomly chosen on each glass slide, and these five countings were added together to give a single estimate of number of spores per 5 mm² for each glass slide (Figure 3).

Pictures was taken of the thallus 4 weeks into the experiment, after the spore liberation process, and the area of each thallus was measured using ImageJ (ImageJ, 2016). The growth of the thallus during this 30-day period is calculated as the specific growth rate (SGR) (Bruhn et al., 2011) defined as

$$ SGR = \frac{100 \left[ \ln \left( \frac{A_t}{A_0} \right) \right]}{t_t} $$

where \(A_0\) is 1.9 cm² (the area of the thallus at the beginning of the experiment), \(A_t\) is the area of the thallus after \(t_t\), and \(t_t\) is the specific time period of interest, in this case 30 days. SGR gives the average daily growth percentage over the time period. The thallus was discarded after the pictures were taken.

2.8 Experiment 2 – The effect of allantoin on the formation and liberation of asexual spores, and the effect of allantoin on growth of the thallus

The second experiment was carried out between the 6th of March and the 2nd of May. The \(P.\ umbilicalis\) material were collected on the 3rd of March.

In the second experiment, the effect of allantoin on the asexual spore production and liberation, as well as the effect of allantoin on the growth of the thallus, was tested on \(P.\ umbilicalis\) collected in the winter season. Three treatments of allantoin were used: 0 mM (control) (N=5), 1 mM (N=5) and 10 mM (N=5). Whole, intact, seaweed fronds were used, thus using different individuals for each treatment. The fronds were each placed in a glass container with 130 ml of SSW, supplemented with 10 µl l⁻¹ of f/2 medium and 0.894 g l⁻¹ GeO₂. The experiment was conducted at 10°C under LED lamps (Cosna LED 10W E27 A60) with an intensity of 70 ± 20 µmol photons m⁻² s⁻¹ in a 12:12 L/D regime. To hinder evaporation, a glass lid covered each glass containers (crystallizing dish – VWR 95 ml), and replicates were kept without aeration, on the basis that aeration severely enhanced the evaporation, hereby increasing the salinity. Aeration was deemed unnecessary, in regard to the oxygen and carbon dioxide levels in the SSW, because of the high surface to volume ratio. Every fourth day the upper two-thirds of the medium was removed, with care to not removing liberated spores that had settled on the bottom. The replicates were then replenished with new SSW corresponding to the removed volume, that was supplemented with 10 µl l⁻¹ of f/2 medium and 0.894 g L⁻¹ GeO₂.

During the first half of the experimental period (30 days), the allantoin treatment of the fronds was performed. Glass slides (Cover glass, 24x36mm, deltalab) were placed in the bottom of the containers, for released spores to settle on. Three glass slides were placed in each of the 15 containers (n=45), and the released spores that had settled on these slides were counted just prior to homogenization of the thallus on day 30.

After 30 days, asexual spores were liberated from the thallus through homogenization following a procedure described in (Mizuta et al., 2003, Saito et al., 2008). From the thallus of each of the
replicates a 1x2 cm piece was cut with a scalpel. The thallus piece was cut from the edge, so that all samples had 2 cm that originated from the edge of the thallus (Figure 4). Each piece of thallus was homogenized in a hand homogenizer for 1:30 min in 20 ml SSW cooled to 5˚C to decrease the risk of heat produced by the friction of the homogenizer. The dilution was filtered with suction through a nylon mesh (pores size: 100 µM) to remove multicellular pieces of thallus, after which the filter was washed with 80 ml 5˚C SSW. The net volume of the medium in each treatment after homogenization were 100 ml SSW.

The liberated cell solution was kept under similar experimental condition (10˚C, 70 ± 20 µmol photons m² s⁻¹, 12L:12D) as the first part of the experiment, in similar glass containers and with three glass slides (Cover glass, 24x36mm, deltalab) in the bottom for liberated cells to settle on.

In total, three spore counts were performed during the second experiment (Figure 4). The first spore count (1.SC) was of the spores liberated during the first 30 days, and took place just prior to the homogenization of the thallus pieces. Different spore types were not distinguished during this counting, so the spore numbers found, represented the total number of spores liberated, both zygotospores as well as asexual spores. The second spore count (2.SC) and the third spore count (3.SC) were of the free cells on day 11 and day 26 after the homogenization (Figure 4). Distinctions were made between zygotospores and asexual spores, and only the asexual spores were further analysed. The presence of zygotospores in the experiment was a result of some of the fronds used being fertilized female fronds. Because of the fronds not being sampled after gender, any potential results from different allantoin treatments on zygotospore production and liberation would be unreliable.

The 3.SC was assumed to show an identical situation to the 2.SC, based on the absence of new material being introduced and absence of disturbance between the 2.SC and the 3.SC. Any difference between the spore counts are therefore contributed to mortality among the spores.

The three spore counts were carried out after the same procedure described previously for the first experiment.

The second experiment also investigated whether allantoin had an effect on the growth of the thallus of *P. umbilicalis*. Pictures was taken (fairphone 2) before the experiment started, after 15 days, and after 30 days just prior to a thallus piece was taken for homogenization. and the area of each thallus was measured in ImageJ (ImageJ, 2016). The growth of the thallus during these two time periods were calculated as the SGR as described previously the first experiment.

<table>
<thead>
<tr>
<th>Zygotospores</th>
<th>Free cells</th>
<th>Asexual spores</th>
<th>Dead cells/spores</th>
</tr>
</thead>
</table>

Table 4: The cell and spore types encountered during spore counting. The different background colour of the pictures in the table is due to different light adjustments on the microscope at the time of the picture, and is not of importance.
Figure 4: Overview of the experimental setup in the second experiment and of when tests were performed. The cameras symbolize that pictures of thalli were taken, and the microscope symbolizes the three spore counts. 1.SC, 2.SC and 3.SC are the first, the second and the third spore count. The cells liberated through homogenization is symbolized with small red cells in the last two drawings. In reality, the cells were too small to be seen with the naked eye.

Figure 3: Overviews of the spore count design in the second experiment
2.9 Identification of spore types

It has not been possible within the timeframe of this thesis to include methods to investigate ploidy levels of the released spores. Determining ploidy level helps distinguish diploid spores developing into conchocelis from haploid spores developing into the blade phase. It has furthermore not been achievable to do the appropriate analysis of the origin of the asexual spores encountered in the laboratory. Such an analysis encompasses determining the origin of the spores through observations of the spores in the moment of their release, as well as transects of the thallus. Transects can help determining the presence of neutral sporangia, as they can be recognized as packets of four cells in surface view, each dividing to give four cells in transects, making 16 spores in each neutral sporangia (Brodie and Irvine, 2003). The consequence is that the exact definitions of the spores encountered in this work are unknown.

A couple of assumptions have been necessary to make in order to compensate for this lack of knowledge. The further development of different spores is not known to a full degree, but some of the spores started to develop into the conchocelis phase which was evident from their thread-like growth (Table 4) (Drew, 1954). These spores could either be agamospores or zygotospores. They only appeared in a few replicates where they showed in high prevalence, so it can be deduced that they most likely are zygotospores. They are most likely a result of random sampling of fertilized female fronds during the collection of *P. umbilicalis*, which subsequently developed zygotosporangia and released zygotospores in the laboratory. The rest of the spores observed in the laboratory grew in a more blade-like structure (Figure 5 a, c) and are assumed to be haploid spores developing into the blade phase. These spores will be termed as asexual spores. It is unknown if they are neutral spores or blade archeospores. These two types of spores have a high morphological resemblance, and a distinction between them has to be made prior to their liberation from the thallus, where neutral spores derives from spore packages while blade archeospores derives from a differentiation of a vegetative cell (Nelson et al., 1999).

The homogenization in the second experiment fractionized the thallus to liberate sexual spores and asexual spores, along with non-vegetative cells from the thallus. Asexual spores are difficult to distinguish from non-vegetative cells, and thus a cell with three or more nuclei were assumed to be growing, and were defined as an asexual spore.

2.10 Statistics

All statistical tests are performed in Rstudio, Version 0.99.902. The R-package Rcmdr was installed as a help for data import and as a tool for drafting of graphs. The R-package LME4 was installed as the bases for generalised linear mixed models. Graphs and figures were drafted in Rstudio.

2.10.1 Spore counts

The spore counts in the 1.SC in the second experiment were poisson distributed count data. The 1.SC was tested with a generalised linear mixed model (GLMM) assuming poisson distribution, testing if treatment had an effect on the mean number of spores counted on each glass slide. The initial area of the thallus was included as a fixed effect. The three glass slides of each replica were viewed as nested in each container (n=15) and included as a random effect in the model. To get homogeneity of variance, replicates with high amounts of zygotospores were removed from the statistical test. Two replicates from the control group and two replicates from the treatment with 1 mM allantoin, were observed to have a high number of zygotospores, and were removed as outliers.
The spore counts in the 2.SC in the second experiment were normal distributed. The 2.SC was tested with a GLMM with gaussian as family to see if treatments had an effect on the mean number of spores counted on each glass slide. The weight of the 2 cm x 1 cm thallus piece prior to homogenization was included as a fixed effect. The three glass slides (n=45) of each treatment were viewed as nested in each container (n=15) and included as a random effect in the model.

Tests that showed significant differences were further tested by a post hoc pairwise test which compared each treatment pair: control vs. 1 mM allantoin; control vs. 10 mM allantoin; 1 mM vs. 10 mM allantoin. The p-value in the post hoc pairwise test was adjusted by the Bonferroni Correction so that only p-values under 0.0167 was accepted as significant.

A different statistical approach had to be taken for the 3.SC. A comparison between the 3.SC and the 2.SC showed how many spores survived over the time period of 15 days. The 10 mM allantoin treatment had close to a total absence of spores in both the 3.SC and the 2.SC and was thus not included in the test. A permutation test with 10,000 permutations was performed to test if the number of spores surviving over the time period differed between the control and the 1 mM allantoin treatment. For the sake of simplicity, the average percentage of survival was taken for each treatment. This test was performed due to difficulties in estimating if a generalised mix model could be performed on percentage survival.

2.10.2 Area

The SGR of the thallus in experiment 1 was tested with a two-way ANCOVA with the fixed effects: treatment, irradiance, fragment size, and the interaction between light and treatment. The high fluctuation inside each of the three levels of the irradiance variable resulted in irradiance being tested as a continuously variable instead of as a categorical variable. Three replicates with small discs treated with 1 mM allantoin, had almost completely disintegrated thallus at the end of the experiment, and was thus removed as outliers before the test.

The SGR of the thallus in experiment 2 was tested with a two-way ANOVA for two time periods: day 0 to day 15 and day 15 to day 30. The model had a single fixed effect: treatment. Tests that showed treatment having a significant effect were further tested with a Tukey’s range test, testing each treatment pair: control vs. 1 mM allantoin; control vs. 10 mM allantoin; 1 mM vs. 10 mM allantoin. For these derived test the p-value was influenced by the Bonferroni Correction, so that only p-values under 0.0167 was accepted as significant.

2.10.3 Contamination

Contamination of the replicates with filamentous algae was tested for significant differences between treatments in experiment 2 with Fishers exact test of count data.

3 Results

3.1 The pilot studies

The first pilot study showed a fast response to treatment with allantoin. In less than a week large areas of the thallus underwent two types of distinct morphological changes: Large areas changed from the two-dimensional cell structure that is normal for *P. umbilicalis* into a three-dimensional cell structure (Figure 5 a, b) and the three-dimensional area changed colour in what seemed to be the intercellular space to a deeper red colour (Figure 5 b). The new tissue turned more fragile than the surrounding tissue and was easily broken, whereby cells/spores were liberated. The second effect was
a high release of spores from the thallus (Figure 5 d). The pictures in figure 5 was taken a week after the allantoin treatment, and at this time all liberated spores were unicellular (Figure 5 d).

![Figure 5. Pictures of the blade phase of P. umbilicalis taken one week into treatment with 10 mM allantoin in the first pilot study.](image)

The liberated spores were monitored over the following 6 weeks, as they started to grow through cell division. After two weeks the first nuclei divisions were observed (Figure 6 c) in a growth pattern closely resembling asexual spores from *Pyropia yezoensis* (Figure 6 d) (Li et al., 2008). The asexual spores of *P. umbilicalis* (Figure 6 c) and *Pyropia yezoensis* (Figure 6 d) were not identical, but the growth pattern of both were similar in the aspect of undergoing nuclei division before developing rhizoid-like structures in contrast to the growth pattern of zygotospores (Table 4). After four weeks, asexual spores developing into juvenile blades, having developed rhizoid-like structures, were observed (Figure 6 a). The majority of the asexual spores used these rhizoid-like structures to adhere to the petri dish, which they were stored in, but a single cell was observed adhering to a nylon string that were put into the petri dish (Figure 6 b).
3.2 The two experiments

The results for the first and second experiment are described together in the following section. The spore counts in the first experiment did not give any results due to diatoms completely dominating the experimental spore solutions, and the first experiment thus only gave results in regards to growth of the thallus.

3.2.1 Spore counts in experiment 2

The 2.SC showed a significant difference (P<0.01) across treatments of cells and asexual spores being released by homogenization of the thallus (Figure 7). A further analysis showed that the control did not differ significantly from the 1 mM allantoin treatment (P=0.91), but both the control (P<0.01) and 1 mM (P<0.01) differed significantly from the 10 mM allantoin treatment (Figure 7). There were observed almost no living cells in the replicates treated with 10 mM allantoin. There were instead observed high amounts of dead, colourless, cells (Table 4). The dead cells were not counted, as they were difficult to distinguish from the surrounding detritus. Dead cells were observed across treatments, but were most predominant in the 10 mM allantoin treatment.
The 3.SC had 58% less spores than the 2.SC. A test for the effect of treatment (0 mM and 1 mM allantoin) on the percentage of surviving spores did not show any significant effect (P=0.54) (Figure 8).

In the 2.SC (day 11) and the 3.SC (day 26) only a few spores with multiple nuclei were observed (Table 4), and the majority of the liberated spores observed were with a single nucleus.

Only a few of the glass slides in the 1.SC were found to have liberated spores on them, apart from four replicates (12 glass slides) that had high numbers of zygotospores, marked as orange dots in figure 9. The treatments did not differ significantly in number of spores released (P=0.51).
No asexual spores (cells with more than 3 nuclei) were observed in the 1.SC.

3.2.2 Growth of thallus in experiment 1

A statistical test did not show any significant effect of allantoin on the SGR of the thallus ($P=0.10$) (Figure 10), or any effect of fragment size on the SGR of the thallus ($P=0.32$). There was a positive effect of increasing irradiance level on the SGR of the thallus ($P=0.0073$) (Figure 11), but there was no interaction between irradiance and treatment ($P=0.63$).

The thallus of many of the replicates had started disintegrating at the end of the experiment, and most of the thallus had turned into a pale yellow colour.
3.2.3 Growth of thallus in Experiment 2

For the first 15 days of allantoin treatment the average thallus had a SGR of 4.2% per day, and there was a significant difference in SGR between the different treatments \((P=0.0041)\) (Figure 12). The SGR for the specific treatments were on average 3.3% for the control, 3.9% for the 1 mM allantoin treatment and 5.3% for the 10 mM allantoin treatment. The difference was significantly higher for the 10 mM allantoin treatment compared to the control \((P=0.0036)\), though the last showed a strong tendency towards the 10 mM treatment having higher SGR than the 1 mM treatment.

For the following 15 days of allantoin treatment, from day 15 to day 30, no significant difference in SGR between treatments were found \((P=0.54)\). The SGR across replicas was lower for this period than in the period from day 0 to day 15, which can be explained with a beginning deterioration of the thallus across treatments. The best example of this was a replicate (not included in figure 13) from the control group that was smaller on day 30 than on day 15, thus having a negative SGR of -1.2% per day. The degeneration of the edge of the fronds was obvious in all replicas, but from a visual inspection it was clear that the degeneration was larger in the control group than in the allantoin treatments (Figure 14). It was furthermore noted that the thallus across all five replicates in the control group had changed from a brown-red colour to a notable more yellow-green colour, in contrast to the replicates in the 1 mM and 10 mM allantoin treatments, which kept their red colour while turning slightly paler on day 30 (Figure 14). In the homogenization of the thallus, it was noted that the thallus of the control and the 1 mM allantoin treatment were much more fragile and easier fractionized in the hand homogenizer than the thallus of the 10 mM allantoin.
Figure 12: The specific growth rate of the thallus from day 0 to day 15 for the different treatments of allantoin in the second experiment.

Figure 13: The specific growth rate of the thallus from day 15 to day 30 for the different treatments of allantoin in the second experiment. There was no significant difference across treatments (P=0.54). A single outlier from the 0 mM allantoin treatment having a SGR of -1.2% d⁻¹ is not shown.

Figure 14: An example of each treatment’s effect on the thallus. Each replica is at Day 0, Day 15, and Day 30 of the first 30 experimental days of the second experiment.
3.3 Contamination with filamentous algae

In the 3.SC in the second experiment, there was no observation of diatoms, but low contamination with filamentous algae was observed in all replicates. Most replicates were contaminated with only a few single-celled algae, but two replicates were severely more contaminated with high quantities of multicellular filamentous algae (Figure 15). Both of these were in the control group, and the filamentous algae dominated to a degree where it could be seen with the naked eye as a green colouration of the of the glass slides. The growth of the filamentous algae in these two replicates was intense compared with the other 13 replicates, but it was not statistically significant to only observe this intense growth in the two control replicates (P=0.28).

There were also observations of Rotifera in a few replicates.

Figure 15: Pictures taken during the 1.SC. of contamination with filamentous algae from the second experiment.

4 Discussion

4.1 The first pilot study - effect of allantoin on thallus and spore formation

The first pilot study found a clear effect of allantoin on the thallus of the test material. Large areas of the thallus across the six investigated fronds changed from a two-dimensional cell structure into a three-dimensional cell structure (figure 5 a, b) during the first week of allantoin treatment turning the affected areas more fragile. This type of effect has not been reported by the previous studies that have investigating the effect of allantoin on foliose Bangiales (Mizuta et al., 2003, Saito et al., 2008) and is possibly a unique effect for P. umbilicalis.

The change in the thallus was followed by a high number of liberated spores. The origin of these spores was not observed, but are believed to have mainly originated in the three-dimensional cell structure, as these area of the thallus were very fragile and spore release in foliose Bangiales is known to happen through degradation of cell wall (Nelson et al., 1999). These spores were determined as asexual spores as they started to develop into juvenile blades, with multiple nuclei-divisions initially, followed by development of a rhizoid-like structure for adherence to the substrate. It is unknown if the observed asexual spores were neutral spores or blade archeospores. The two types of asexual spores have a similar morphology and the only definite way to distinguish between them is to make transects of the sporangium before liberation, which was not done in this study. Neutral sporangia are arranged
in packages of two by two with four spores in transect (Brodie and Irvine, 2003), whereas single blade archeospores are developed from a single cell.

The liberated asexual spores might have been neutral spores as these are the only type of asexual spores *P. umbilicalis* is known to produce (Brodie and Irvine, 2003). This is especially true for the populations from Main where neutral spores is the only known reproductive pathway (Blouin et al., 2007). However, more likely the asexual spores were blade archeospores, as the liberated spores in the pilot study were approximately the same size as the cells of the thallus from which they were liberated. Blade archeospores are produced as a single product from a sporangium. In contrast, neutral spores are produced in spore packages and blade archeospores are therefore expected to be larger than neutral spores just after liberation. *P. umbilicalis* is not known to produce blade archeospores (Brodie and Irvine, 2003), but Saito et al. (2008) showed that blade archeospore development could be induced by allantoin in *Pyropia pseudolinearis*, a related species that is not known to produce blade archeospores naturally. It thus seems reasonable that allantoin can induce blade archeospore propagation in *P. umbilicalis*.

4.2 Spore counts in the second experiment – the effect of allantoin on spore propagation

No spore counts were obtained from the first experiment due to contamination with diatoms. The second experiments had a notable lack of the asexual spores that were predominant in the first pilot study. There were no observations of asexual spores in the 1.SC and only a small number in the 2.SC and 3.SC. The few cells with more than three nuclei that were observed did not resemble the asexual spores liberated during the first pilot study. The difference between the spores encountered in the first pilot study and the second experiment was, that asexual spores in the pilot study developed rhizoid-like structures, while the multicellular cells in the second experiment had no visible precursors for rhizoid-like structures. The cells in the 2.SC and 3.SC were liberated through homogenization and the multicellular cells observed might not have been asexual spores. The cells could instead be cell clusters embedded in partly fractionized cell wall segments or half developed zygotesporangia containing up to eight spores yet to be liberated (Nelson et al., 1999).

The low numbers of liberated cells in the 10 mM allantoin treatment, compared with the higher numbers released in the control and 1mM treatments, is most likely not a result of allantoin affecting asexual spore propagation. The difference is most likely a result of the thallus of the control and the 1 mM allantoin treatment being more fragile than the thallus of 10 mM allantoin treatment. The resilience of the thallus was not tested, but during homogenization of thallus pieces in the second experiment the thalli from the control group and the 1 mM allantoin group were observed to be more fragile than the thalli from the 10 mM allantoin group. The low numbers of liberated cells in the spore counts of the 10 mM allantoin treatment are most likely also linked to high numbers of dead cells observed in the 10 mM allantoin treatment. Dead cells were observed across treatments, but a quantitative estimate of the dead cells, was not achievable due to the difficulty of differentiating between dead cells and the surrounding detritus. It is unknown why allantoin should have a lethal effect on the survival of newly liberated non-vegetative cells. Comparable studies on different foliose Bangiales species have not observed similar consequence of high concentrations of allantoin (*Pyropia yezoensis*, (Mizuta et al., 2003); *Pyropia pseudolinearis*, (Saito et al., 2008)). In these studies, spores liberated through treatment with 10 mM allantoin were found to grow normally. It should be noted
that the media after homogenization were identical across treatments and that the treatments only differed in the concentration of allantoin supplemented in the 30 days up to homogenization.

The decrease in spores from day 10 to day 26 after homogenization can be contributed to mortality, as well as randomness generated by the fact that although the replicates and glass slides were identical, the spore counts were performed in five new randomly chosen spots. There was no difference in the survival of the spores between the control and the 1 mM allantoin treatment, and thus a treatment with low concentration of allantoin pre-homogenization did not seem to increase mortality of the cells post-homogenization.

There was not observed any effect of the allantoin treatment on the propagation of asexual spore during the first 30 days of the second experiment (1.SC). The results from the first pilot study could thus not be replicated in the winter population.

4.3 Specific growth rate of the thallus in the first and second experiment – the effect of allantoin

The specific growth rate (SGR) of the thallus in the first experiment showed a significant increase in daily growth with increasing irradiance levels going from <50 µmol photons m\(^{-2}\) s\(^{-1}\) to >150 µmol photons m\(^{-2}\) s\(^{-1}\). The increase in SGR with increasing irradiance levels shows that *P. umbilicalis* can tolerate high light intensities, which fits well with their habitat, being exposed to direct sunlight on a daily basis at low tides. Similar results by Kraemer and Yarish (1999) show that *P. umbilicalis* has increased net photosynthetic rate with increased irradiance levels. Kraemer and Yarish (1999) found maximal photosynthetic rates \((P_{\text{max}})\) at \(~100 \mu\text{mol photons m}^2\text{s}^{-1}\), and did not observe any photoinhibition testing irradiance levels up to 280 µmol photons m\(^{-2}\) s\(^{-1}\).

In the second experiment, treatment with 10 mM allantoin showed significantly higher SGR (5.3\% d\(^{-1}\)) than the control (3.3\% d\(^{-1}\)) during the first 15 days. The 10 mM allantoin treatment showed a tendency towards higher SGR compared with the 1 mM allantoin treatment (3.9\% d\(^{-1}\)), but the difference was not significantly higher \((P=0.037)\) as it was influenced by the Bonferroni Correction. Treating the thallus with 1 mM allantoin has previously been shown to influence the SGR of the thallus of *Pyropia yezoensis*, increasing the SGR rate by 1.7 times (Mizuta et al., 2003). Mizuta et al. (2003) also found that the 10 mM allantoin treatment inhibited the SGR of the thallus, resulting in a SGR approximately 60% of the SGR of the control and approximately 15% of the 1 mM allantoin treatment. The detrimental effect of 10 mM allantoin treatment on the SGR of the thallus is explained with allantoin hastening germination of the blade archespores (Mizuta et al., 2003), thereby allocating energy from growth to reproduction.

The results from Mizuta et al. (2003) differ from the results in this thesis, where 10 mM allantoin had the highest SGR of the treatments and where no difference were found between SGR for the control and the 1 mM allantoin treatment. The absence of asexual spores in the second experiment can explain why the SGR of the thallus in the 10 mM allantoin treatment is not lowered by hastening of germination of asexual spores as is the case in Mizuta et al. (2003).

The colour of the thalli in the control turned more green compared with the 1 mM allantoin treatment and the 10 mM allantoin treatment. Hafting (1999a) has shown that starving *Pyropia yezoensis* for nitrogen made the colour of its thallus turn green and decreased the daily growth rate of the thallus. This indicates that the control group could have been starved for nitrogen. Pineda et al. (1984) has shown that allantoin can be used as a nitrogen source in green microalgae, and the higher growth rate in the 10 mM allantoin treatment, as well as the thalli keeping a red-brown colouration,
might thus be contributed to having increased nitrogen concentrations in the media in the form of allantoin.

It would be surprising for the experimental fronds to experiencing nitrogen limitations as two-thirds of the media was changed every fourth day with media having approximately two times higher nitrogen concentrations than the nitrogen concentrations naturally encountered at the collection side at Blåvand. Nitrogen concentrations was not measured in the media, and it is thus unknown if the metabolism of the fronds used up nitrogen faster than it was supplemented.

4.4 Contaminating filamentous green algae – the effect of allantoin

Allantoin might have an effect on filamentous green algae, which can be a contamination hazard for *P. umbilicalis*. Filamentous green algae were present in all replicates in the second experiment, but were only observed showing vigorous growth in two control replicates. The test size was too small for this to be a significant difference between the treatments. The effect of allantoin on contamination species is an interesting subject for further investigations, as it could be helpful in controlling biofouling. It would be a fascinating result if allantoin can be shown to increase the growth of *Porphyra* while simultaneously hinder growth in biofouling species.

4.5 Effect of season on reproductive strategy

The observations of high amounts of asexual spores being liberated in the pilot study could not be replicated in the second experiment, and it is difficult to be certain of the reason behind this observed difference. Most factors in the second experiment were attempted to be as similar as possible to the experimental setup in the first pilot study.

A large difference was the seasonality of the *P. umbilicalis* material. The *P. umbilicalis* material used in the pilot study was from a summer population and they were treated with allantoin in the middle of autumn. In contrast, the *P. umbilicalis* used in the second experiment were collected midwinter. A study on *P. umbilicalis* at Helgoland investigated the reproduction of populations collected in September 1989 (Kornmann and Sahling, 1991), and found both sexual reproduction through zygotospores (termed carpospores in the article) and asexual reproduction through neutral spores (termed aplanospores in the article). It has not been possible to find similar studies on winter populations, and it might thus be possible that the difference in asexual reproduction between the first pilot study and the second experiment is due to the seasonal differences in reproductive strategies in *P. umbilicalis*.

The following factors also differed between the pilot study and the second experiment, but they are believed to be of less or no importance for propagation of asexual spores.

The irradiance level in the pilot study (~20 µmol photons m$^{-2}$ s$^{-1}$), was lower than any of the irradiance levels tested in the experiments, but it has not been possible to find support in the literature for low light conditions being a trigger for asexual reproduction in foliose Bangiales, while Li (1984) have shown that high light conditions enhanced asexual spore propagation.

The material used in the pilot study was, compared to the material used in the second experiment, stored for a longer time before being used, but the three-dimensional growth associated with the release of asexual spores were not observed before it was triggered by treatment with allantoin, and the long storage time are thus not believed to enhance asexual reproduction.

Only the second experiment was frozen before initiation of the experiment, but it is hard to believe that this should hinder asexual reproduction, especially in the light of the first experiment not...
being frozen before use, while still not experiencing the three-dimensional structural change to the thallus.

Seasonality is thus believed to be the crucial difference between the first pilot study and the later experiments, in regard to allantoin's ability to provoke three-dimensional growth followed by liberation of asexual spores.

4.6 Porphyra umbilicalis in mariculture

*Porphyra umbilicalis* has been shown to possess several advantageous traits for mariculture. It grows year round, has high cold tolerance and is very robust against desiccation (Green and Neefus, 2014). It has high photosynthetic rate, is efficient in nutrient uptake and have fast growth rates (Green and Neefus, 2015). In spite of these beneficial traits, *P. umbilicalis* has not been tried for mariculture in Europe, and has only recently been investigated as a mariculture crop in North America, where the formation of spore nets is simplified by the local *P. umbilicalis* populations strictly reproducing by neutral spores. *P. umbilicalis* has not yet been investigated for mariculture in Denmark due to the low number of maricultural project being made in Denmark and Europe, as well as a consequence of the complex life-cycle of European *P. umbilicalis* populations. Sexual reproduction through conchocelis in controlled environment has not been researched sufficiently for efficient use in the production of spore nets. The control of asexual reproduction using allantoin offers a good alternative reproductive strategy, that are both faster and less work intensive (Blouin et al., 2011). The use of asexual spores would also make it easier to integrate the techniques for seeding spore nets developed in Maine, USA, by Blouin et al. (2007).

The use of allantoin in mariculture can be used without concerns to allantoin being a biohazard for the environment or being unhealthy for human consumption, due to allantoin already being present in the environment. It is created in small doses from oxidation of uric acid in many living organisms, including humans (Shestopalov et al., 2006). The small doses potentially used in creating spore nets for foliose Bangiales will be a negligible contribution to the environment. In regard to human exposure, it is already legalised as a component for skin care and can be purchased online (Urtegaarden, 2016).

Allantoin has the secondary effect of increasing daily growth rates of thallus in foliose Bangiales. This has been shown previously by Mizuta et al. (2003) (*Pyropia yezoensis*), who found that 1 mM allantoin treatment stimulated SGR of thallus, and in this thesis were 10 mM allantoin treatment stimulates SGR of thallus (*P. umbilicalis*). It is unclear if the increase in SGR of thallus in this thesis was due to increased nitrogen available as allantoin, or if allantoin enhanced SGR of thallus through other biological processes. Future studies into allantoin and *P. umbilicalis* is needed with excess nitrogen to test the effect of allantoin on SGR of thallus.

The experiments in this thesis into the effect of allantoin on asexual spore propagation and liberation, needs to be repeated on a late summer population, to confirm the effect of allantoin. A few factors need extra attention if the experiments from the thesis were to be repeated. The identification of the species collected would be improved, by making a DNA barcoding analysis, and the identification of the spores liberated needs to be improved with precise transects of sporangia.

Comparable studies in related foliose Bangiales species have shown that several other factors, apart from allantoin, might be able to influence asexual spore propagation. To optimize the asexual reproductive pathway, it is necessary to test if asexual spore propagation can be enhanced from high irradiance levels (*Pyropia yezoensis*, (Li, 1984)), fragmentation size of the experimental thallus (*Pyropia yezoensis*, (Hafting, 1999b)), low Ca\(^{2+}\) concentrations in the medium (*Pyropia yezoensis*, (Takahashi et
al., 2010) and high water temperature (*Pyropia yezoensis*, (Kitade et al., 1998); *Pyropia pseudolinearis*, (Saito et al., 2008)).

Several Danish foliose Bangiales exist in Denmark (Mols-Mortensen, 2014), and it is unknown how these species are affected when treated with allantoin. It is recommended to investigate if allantoin can provoke or enhance asexual reproduction in these related species. *P. umbilicalis* is not suitable for the production of nori sheets, so it would be interesting to investigate alternative Danish foliose Bangiales that are more suitable. But due to several advantageous traits in *P. umbilicalis* (Green and Neefus, 2015) mentioned previously, as well as *P. umbilicalis* having a high protein content (MacArtain et al., 2007), it might still be the most interesting foliose Bangiales species for Danish mariculture. *Porphyra umbilicalis* can be used for other high-quality consumable products than nori-sheets comparable to how foliose Bangiales is used in China in fast-food products and as a flavour component in soups (Levine and Sahoo, 2010).

5 Conclusion

*Porphyra umbilicalis* is a potential new crop for mariculture in Denmark but better understanding of its reproductive lifecycle is needed to optimize the spore-seeding of nets. European *P. umbilicalis* populations are able to reproduce through asexual spores but will usually reproduce through a sexual phase called conchocelis. Spore nets of foliose Bangiales is traditionally seeded with conchospores from the sexual phase, but exploiting the asexual phase of foliose Bangiales can reduce both the time and the cost of producing spore nets (*P. umbilicalis*, (Blouin et al., 2007)). Results from this thesis indicate that allantoin may stimulate asexual spore formation in fronds collected in late summer populations. This effect is shown to be seasonal dependant, since allantoin could not be shown to induce asexual spore propagation in populations collected midwinter. The asexual spore liberation in summer populations is shown to be induced through degradation of the cell wall through changes to the cell structure of the thallus from two-dimensional to three-dimensional. The asexual spores from the summer population was not followed for long enough time, to confirm that they can develop into mature fronds.

Results from this thesis show how allantoin is able to enhance daily growth in *P. umbilicalis* fronds, though it is unclear if this was due to allantoin being a nitrogen source.

Allantoin has been shown to be a powerful stimulant for the production of seed nets by asexual spores for mariculture with *P. umbilicalis*, and thus have the potential to reduce the time and the cost of producing spore nets for Danish mariculture, increasing the feasibility of future experimentation with implementation of *P. umbilicalis* in Danish mariculture. Though further studies are needed on summer populations to confirm the use of allantoin to enhance asexual spore propagations and to test for the optimal concentration of allantoin.
6 Bibliography


LI, L., SAGA, N. & MIKAMI, K. 2008. Phosphatidylinositol 3-kinase activity and asymmetrical accumulation of F-actin are necessary for establishment of cell polarity in the early


MOLS-MORTENSEN, A. 2014. The foliose Bangiales (Rhodophyta) in the northern part of the North Atlantic and the relationship with the North Pacific foliose Bangiales - diversity, distribution, phylogeny and phylogeography.


OVERFLADEVANDSDATABASEN ODA. *oda.dk* [Online]. Available: [https://goo.gl/KrkNWq](https://goo.gl/KrkNWq) [Accessed 29 July 2016].


