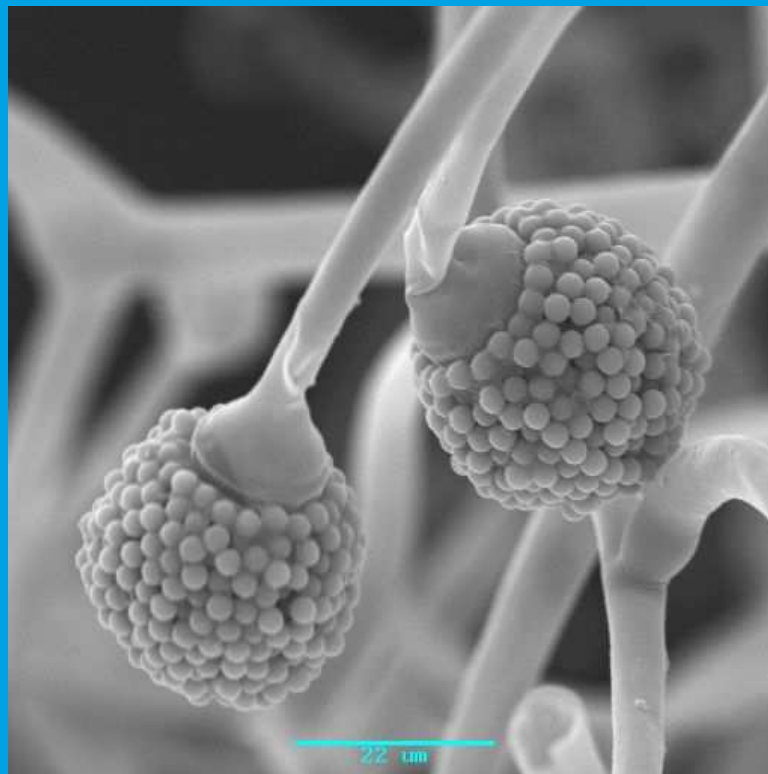


# MICROALGAL BIOPROSPECTING

SPORE SWELLING AND GERMINATION AS A BIOASSAY FOR THE RAPID  
SCREENING OF CRUDE ALGAL EXTRACTS FOR ANTIFUNGAL ACTIVITY

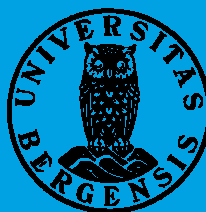


By

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Thesis for the degree of *Cand. scient.* in algal physiology

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## IMAGINATION

There is a dish to hold the sea,  
A brazier to contain the sun,  
A compass for the galaxy,  
A voice to wake the dead and done!

That minister of ministers,  
Imagination, gathers up  
The undiscovered Universe,  
Like jewels in a jasper cup.

Its flame can mingle north and south;  
Its accent with the thunder strive;  
The ruddy sentence of its mouth  
Can make the ancient dead alive.

The mart of power, the fount of will,  
The form and mould of every star,  
The source and bound of good and ill,  
The key of all the things that are,

Imagination, new and strange  
In every age, can turn the year;  
Can shift the poles and lightly change  
The mood of men, the world's career.

By John Davidson (1857-1909)

## ABSTRACT

Microalgae and cyanobacteria has through the last couple of decades been increasingly recognized as a valuable source of a variety of bioactive compounds. Many species have been investigated, but these represent mostly strains from tropic or subtropical areas. Norway has, with its long coastline, access to both temperate and polar waters, and thus a unique opportunity to prospect for marine organisms that might possess novel cold-water adapted properties.

Screening for bioactivity is commonly performed *in vivo* in a bioassay purposefully designed for revealing a defined bioactivity (e.g. fungicide or antibacterial activity). This allows the testing of many crude extracts. The search for new drugs against fungal infections is a major challenge to current research in mycotic diseases. In this Thesis a new method (bioassay) targeting spore swelling and germination to assess antifungal susceptibility is developed and evaluated. Traditionally, antifungal activity has been investigated using disk diffusion assays or micro-well plates. Inhibition is measured as a function of radial growth, inhibition zone or turbidity. Construction of a bioassay composed of germinating fungal spores bears the prospect of being a more rapid method, allowing more extracts to be screened within a shorter time frame. It also can be used to reveal antifungal action at an early state in the prospecting by the mode of its action on the spores. A strain of *Absidia glauca* Hagem served as model organism. A Beckman Coulter Multiziser<sup>tm</sup> 3 particle analyser was applied for the determination of bioactivity and investigation of the sporangiospores. Effects of crude extracts from different microalgae were investigated as changes in volumes of, or as a decreased germination rate rather than percentage of germinating spores. 21 microalgae (15 different marine Chlorophytes, 5 selected Arctic and Antarctic terrestrial Cyanobacteria, and 1 additional Chlorophyte isolate of unknown origin) were cultivated for the production of biomass, harvested and extracted (hydrophilic and lipophilic extracts), and screened for antifungal activity. Among these a strain of *Phormidium sp.* (NIVA 209) proved to be an interesting candidate for fungicide effects. Several hydrophilic extracts improved germination rates. The method was concluded to be a suitable contender for the detection of bioactivities. These results supports that suppression of spore swelling provides early indication of preservative potential and the type of swelling curve produced indicates the mechanism of fungistasis.



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Svein Atle Uldahl

Bergen, February 26, 2006.



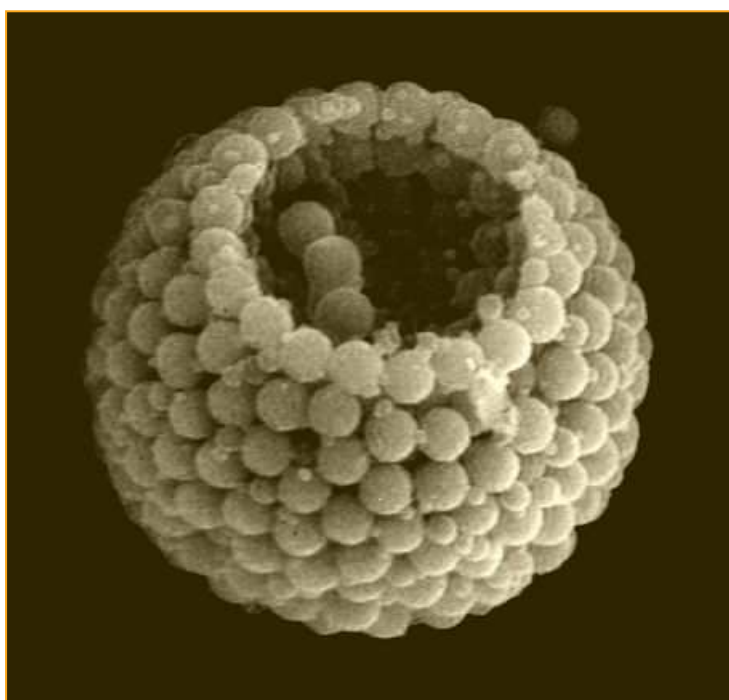
## ABBREVIATIONS

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CFU	Colony-forming unite (CFU / ml)
DMSO	Dimethyl Sulfoxide
DW	Dry weight
ESZ	Electrical Sensing Zone method
H	Hydrophilic extracts
L	Lipophilic extracts
MEX	Malt extract medium
MIC	Minimum inhibitory concentration
NCCLS	National Committee for Clinical Laboratory Standards
O.D	Optical densities
PBR	Photobioreactor
PGRs	Plant growth regulators
SEM	Scanning Electron Microscope
SS-rDNA	Small-subunit rDNA

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**KEYWORDS:** MICROALGAE – CYANOBACTERIA – BIOASSAY – BIOACTIVE – FUNGICIDE – BIOPROSPECTING – SCREENING METHOD - ALGAL EXTRACT – *ABSIDIA GLAUCA* – COULTER COUNTER - ESZ



# 1 INTRODUCTION

## 1.1 *Bioprospecting*

“Bioprospecting is the investigation of nature for exploitable and valuable genetic and biochemical resources (*def. by Author*).”

In the following section bioprospecting is briefly reviewed, aimed to introduce and to elucidate why prospecting microalgae and cyanobacteria is a promising field worth spending time and effort investigating. It is also a preface to screening methods and the different factors that have to be considered searching for bioactivity. This is background information necessary in the development and evaluation of a new screening method or assay.

### 1.1.1 **The search for exploitable bioactive compounds**

Humans have throughout history always depended upon the exploitation of nature. With the increasing sophistication of our societies, new ways of using natural resources have been discovered and developed. This have in turn led to an improvement of human nutrition, health and wealth fare. Nature has thus been the source for medicines, nutrition supplements and other utilizations long before the technological age. Examples are numerous, from the traditional folk medicine based on different herbs to the colouring of textiles. Today, many of the processes that lay behind natural biological systems are understood, and thanks to new technology more available for use. Biodiversity has also been revealed as a new resource for the benefit of human civilization. Human societies face great global challenges in the future, in regard of nutrition, environment (e.g. pollution) and especially in medicine and health; problems that needs to be solved. We are in a constant battle with microorganisms that develop resistance to our drugs, and with new and older diseases. The requirement for new commercial products on the international markets is increasing, and is a strong economic motivation. Therefore, the attention on to the potential of nature as a source of novel, bioactive compounds is growing, both in pharmaceutical industries and in aqua and agricultures worldwide. Exploitable biology goes well beyond drugs: novel crops protection

agents, biocatalysts, and biomaterials are among the many important industrial targets. Among the armamentarium of new biocatalysts are the so-called extremozymes, such as thermozyms.

Bioprospecting has commonly been associated with higher plants and the rain forests. The reason for this is that plants have been more easily available, and most of the drug development until now has evidently profoundly relied on ethnobotanical knowledge (Knutsen, 1997). Microorganisms have never had the same position in human history, and therefore lead information is not available to the same extent. Nevertheless there are some examples. Cyanobacteria have been used as a source of nutrition (*Spirulina sp.* / *Arthrospira sp.*) in both South America (Peru) and in Africa. There also exist documented use of fungus, actinomycetes and algae (also marine) from China for about 4700 years ago. The macroalgae *Porphyra (Nori)* has been used directly as food. Many of the pharmaceuticals and antibiotic components in use today are the result of systematic screening of terrestrial organisms like higher plants and earth microbes. Aspirin<sup>®</sup> and penicillin<sup>®</sup> are among these. Of the nearly 13 500 known natural occurring antibiotics, 5500 is produced by actinomycetes, 3300 higher plants, and of these about 90 is in actual use (Borowitzka, 1995 (a)). Antibiotics remain the largest marked of natural derived drugs, but the reported discovery of microbial metabolites with non-antibiotic activities has increased progressively over the past 30 years and now exceeds that of antibiotic compounds (Hill, 1998). A classic example of Bioprospecting, is the discovery of cyclosporine A (cyclic peptide) isolated from a fungus (*Tolypocladium inflatum*, Deuteromycete) found in an earth sample from Hardangervidda (Norway, 1969). This compound possesses an immune suppressing effect on patients, and has revolutionized surgery and organ transplantation (Stähelin, 1996). Synthetically produced substances have been the basis for the developing and screening of new pharmaceuticals. But lately there has been increasingly focused on natural products as sources for new bioactive compounds among international researchers and in the pharmaceutical industry. One prerequisite to natural-product discovery that remains paramount is the range and novelty of molecular diversity. Bioprospecting offer an opportunity to make use of and learn from the solutions already existing in nature, instead of only depending upon the development of synthetic molecular compounds. Newly discovered bioactive products do not usually become drugs per se, but may enter a chemical transformation program in which the bioactivity and pharmacodynamic properties are modified to suit particular therapeutic needs. Nature has evolved numerous biochemical solutions to many different problems, and has a superior ability to fabricate stereospecific compounds with very specific bioactivities (Knutsen, 1997; Metting & Pyne,

1986). Therefore, it is advantageous to derive new bio-chemical inventions from this source, when these are more likely to be “well tested”, biologically functional and degradable. The discovery of artemisin (artemesinin or Qinghaosu) illustrates this. Through the investigation of traditional Chinese folk medicine, this novel and highly unstable molecule was extracted from a Chinese herb (*Artemisia annua L.*, “sweet wormwood”) and has shown to be highly effective against the malaria parasite (*plasmodium sp.*), which it rapidly kill. The parasite is thus left with no or few chances for adaptation and resistance, a major problem with preceding synthetic malaria drugs developed.

Marine organisms have drawn increasingly attention in the last two decades, as promising sources of bioactive substances. Oceans cover more than 70% of the earth’s surface, and this marine environment represents more than 95% of the biosphere. It is estimated that the marine biological diversity is higher than in tropical rain forests, so in regard of bioprospecting, it is in many ways considered as the “new rain forest”. It is commonly believed that life originated in the world’s oceans. This consequently means that marine life forms have had a longer evolutionary path to adapt to their environments, than the terrestrial ones. Subsequently one can assume that they must have developed diverse mechanisms for survival, which would involve chemical defence-systems together with other biochemical properties. The marine environment has already proved to contain a much larger and diverse variety of habitats than what was assumed to exist only for less than two decades ago. This strongly suggests that it represent an immense and largely untapped reservoir of biological and genetic diversity. There are several features that make the marine environment unique and can provide clues for the detection of novel natural products. A sedentary lifestyle is common among marine organisms. Sessile organisms have to defend themselves with chemical means of protection. These compounds might be incorporated into their tissue or released into the water. Such secondary metabolites can be used in niche protection to detect, paralyse, confuse or to keep predators away (e.g. a bad taste). They may also be used to warn others of the same species that a danger is present or in competition for territory (signalling). Such strategies are abundant among marine organisms. Metabolites dissolved into the water are rapidly diluted and, therefore, need to be highly potent to gain the wanted effect. This is one of the reasons that such compounds from the sea is recognized to be highly adapted and with very specific bioactivity, that might be useful in the search for new drugs candidates for the marked (Haefner, 2003). Many marine organisms form symbiotic associations (e.g. light organs of fish), and the mechanisms behind are not always understood. Many species encounter extreme environments, like heat (generated by tectonic activity), high pressure or variations of high

and low salinity. Species from arctic and antarctic waters live under extreme light and temperature, and might possess unique adaptations to these conditions. Species living in such low temperatures also have a slower metabolism, and this might suggest that they produce fewer metabolites, which has to be even more highly bioactive. Shrimp alkaline phosphatase (SAP) isolated from the melting water of shrimps by Biotech Pharmacon ASA (Tromsø, Norway) is an example of cold-water adaptation. This enzyme is heat-inactivated, and is applied in preparation of PCR-generated DNA sequencing.

Bioprospecting is a time consuming process, where new products and markets have to be identified, and a compound that covers commercial demands and social needs have to be discovered. Prospecting for exploitable qualities in nature is a systematic work for the discovery of new molecules and biochemical principles. One difficulty scientists are facing is the problem of cultivating the organisms in the laboratory. Some substances are too difficult and expensive to synthesize in quantity (Yasumoto & Satake, 1998), or their molecular composition or activity is unknown. This challenge can be solved by increasing the understanding of life history, ecological place and function, and needs for survival and growth of these organism. Definitive characterization of organisms (i.e. in microbiology) is a crucial act in the search for natural products, and the ability to dereplicate strains avoids duplication of efforts (i.e. the ability to prevent isolations of identical species or strains of microorganisms and the rediscovery of identical natural products). Moreover, it is important to discriminate strains at the infraspecific level (see below). Once a biotechnological target has been identified, two questions follow. First, what might be the best-producing organisms to investigate? Second, what screening procedure should be used in order to elicit the desired activity or property? Bioassay screening methods are used to detect a specific biological activity that may lead to development of a new therapeutic drug or industrial product. There are mainly two approaches in use, biochemical (molecular) and cellular (cell-based) assays. The term “bioactive molecule” is an expression commonly used including substances that may affect life processes at low concentrations (beneficial or harmfully). A considerable effort has been and is expended in the development of screening assays, particularly as a response to the need to evaluate large numbers of samples in high-throughput screens and the expectation that many new targets will be identified in the wake of genome sequencing projects (Bull *et al.*, 2000). There is a strong view that biopharmaceutin leads are more likely to be detected in cell function assays (*in vivo*) than in the *in vitro* assays (Hertzberg, 1993).

### 1.1.2 Biotechnology, bioinformatics and biodiversity

Biotechnology is based on the search for and discovery of exploitable biology. This field has undergone dramatic changes in the last decades as a consequence of scientific and technological advances. Biotechnology has, thanks to genetic analysing methods, also revealed a biodiversity much larger than ever believed in the past.

Biotechnology starts with the assembly of appropriate biologically material for the screening after a desired attribute. It then moves through the selection of the best option among a few positive hits for further testing and purification, before culminating in the final development of a commercial product or process. The impact of biotechnology to date has been most pronounced in the pharmaceutical sector. It has become an important tool in modern human medicine, nutrition and increasingly in the industry as industrial processing (e.g. enzymes). It is a radical innovation that generates new industries, and its versatility is so great that existing industries that have previously not used biological systems are now exploring such options. Since its resource is biology, an understanding of ecology as means for revealing new opportunities of discovery is necessary. This applies to the field of taxonomies as well. Biotechnology is presently undergoing what can be described as a paradigm shift in exploitable biology (Bull *et al.*, 2000), due to the profound change in search and discovery tactics. This paradigm is a consequence of the shift in what is referred to as traditional biology (i.e. specimen collection, system observation and laboratory experimentation) to the bioinformatics (i.e. data collection and storage, and the retrieval and integration of the database in order to generate knowledge), which has undergone a revolution in the last decade. Formation of large international databases where information of genomes, protein sequences, biodiversity, metabolic pathways and other information is stored makes it possible to search for relevant knowledge and conduct only essential experiments based upon this information. By mapping the genome of organisms, it is possible to create medicines from functional genomics (i.e. defining transcriptome and proteome status of a cell, tissue or organism under given conditions). Genomes of several organisms are today mapped and intensively used in scientific research. The use of polyphasic taxonomy is another way of optimising the search and discovery of new bioactive compounds. This approach helps distinguishing among known species and to recognize new ones. Detection of new, novel and rare species is made easier due to the availability of classifications based on genotypic and phenotypic data. This approach will also be valuable in the challenges facing systematic and

the need of establishing well-defined taxa, a stable nomenclature and improved identification procedures. Rapid and unambiguous characterizations of large numbers of isolates are requirements in screening for natural products or biocatalytic activities of industrial interest. Also the ability to exclude previously screened organisms and to discriminate between microorganisms at the infraspecies level (i.e. examine the genetic diversity within a defined species) is important in this context. Different methods are available for this purpose. Some molecular techniques lack the ability to distinguish between strains below the species level or between members of recently diverged specie (molecular fingerprinting and *ssrDNA* sequencing), while others that have this resolving power are time consuming. Curie point pyrolysis mass spectrometry (PyMS) has been shown to be of value in rapidly grouping microorganisms isolated from environmental samples into clusters and to recognize phenotypic differences between strains of the same species.

Biological diversity is the foundation of biotechnology and all bioprospective research. Estimates of microbial diversity and physiology have risen the last decades as a consequence of the advances in biotechnology, and have obviously been underestimated in the past. Especially in the field of marine biology there has been revealed unsuspected levels of diversity, much as a result of the discovery of new extreme and till now totally unknown habitats. The abyssal and hadal oceans (depths below 2.000 and 6.000, respectively) were regarded as biological desserts, but this view has changed especially concerning microbial diversity. The recent discoveries of additional deep-sea environments (sub-sea floor sediments, cold fluid seeps, brine lakes, carbonate mounds, mud volcanoes, hydrocarbon seeps, and gas hydrates) open up new opportunities for bioprospecting. The discovery of two marine ecosystems independent of solar energy within a decade has radically changed the perspective of where and how life might be found to exist. Microbial diversity proves to represent a large an untapped resource. Knowledge of microbial diversity in terms of species richness, local and global distribution, and ecosystem function remains very incomplete. Similar morphologic features can often mask the microbial diversity, and thus make it difficult to discriminate between species of different genotypic and phenotypic composition. New biotechnological methods investigate and discriminate species at the genetic level, and have contributed to a new understanding of microbial diversity. Another problem encountered when new species are collected and isolated, is that often only a fraction of the species present in a given community or location can be cultured. Therefore many or most species are lost long before they reach the laboratory and can be investigated. This might be a consequence from the use of wrong cultivation medium or wrong physiological conditions. In the



laboratory, culture conditions are often made optimal, and this might only favor opportunistic species that will grow rapidly and dominate the culture. Therefore, these conditions will not represent the actual composition of species in the ocean. A possible approach to this problem might be to use oligotrophic cultures, where conditions are not optimal, but instead close to a minimum of survival. This may favor growth of species that otherwise would have been outnumbered by fast growing and opportunistic species. Detection and cultivation methods are crucial in isolating organisms from highly oligotrophic, extreme and high-pressure (e.g. barophily, require pressure *in situ* for growth) habitats. This indicates that chemostate or dilution to extinction culture procedures be used. Another factor that can influence growth is the phenomenon referred to as “quorum sensing” (or the pheromone theory). Some species may lay dormant until the right signal molecules are present (growth suppressed until abundance of specimen is high). Signal molecules involved in this communication are called virulence factors, and have been documented in some bacteria. If this is also the case for algae, it might explain the difficulties of cultivating some species. Other growth factors might also be present in the water for microorganisms to initiate growth. Better understanding and knowledge of the geographic distribution (biogeography) of microorganisms will assist in determining the extent of microbial diversity, identifying threatened taxa and the ecological function of an organism. It will also assist the search and discovery for exploitable biological novelty. Whether microorganisms are endemic or cosmopolitans is also important in preservation of the gene pool. Because of their direct value as a major resource for biotechnology development, the conservation of microbial gene pools is a crucial issue. *Ex situ* collections is and will continue to be essential for ensuring that a source of living cells are available for research and industrial purposes. Some organisms isolated from environmental samples cannot always be found again, or if they are, they might lack the desired properties exhibited by the earlier strains. But it is not feasible to maintain an adequate representation of all known species of microorganisms and cell lines in *ex situ* collections. A future requirement may well be the provision of the DNA rather than the organisms themselves.

As a consequence of the fast development of biotechnology and bioinformatics, the possibilities of undiscovered novelty are much higher than ever before. Life is found virtually on every place of this world, and the diversity and physiological adaptations that lie behind are most likely to be highly valuable for biotechnology in the future. Life has been found blossoming in hostile places, and has changed the boundaries of life as it was thought of and defined for only a few years ago. Today, much of the biotechnological research is on the molecular level, and many biomolecules are discovered, mapped and eventually utilised

commercially. Biotechnology is one of the key technologies of the 20<sup>th</sup> century, that have changed and influenced industries, global problems, and that bring great expectations for the future. Genomics and new technology can promote the search for new natural products by increasing the understanding of factors that regulate growth and gene expression and microbial diversity, complementing the way to drug development. Biotechnology is in principle driven by economic demands. The paradigm shift in biotechnology search strategies has hence opened new opportunities, and new technology together with the realization of the recourse biodiversity represent, makes bioprospecting a very great present interest.

## ***1.2 Screening microalgae and cyanobacteria for bioactivity***

### **1.2.1 Screening history and methods**

For more than 50 years microalgae and cyanobacteria has been exploited for the production of various substances. Algae have been used for agricultural and aqua cultural purposes. They are especially valuable due to their high content of proteins and polyunsaturated fatty acids (PUFA). Microalgae are an important and essential live food for hatchery rearing of bivalve molluscs and penaeid shrimps as well as the culturing of several zooplanktons. Algae have been used for soil conditioning and for the control of soil erosion. Both macro and microalgae are used as fertilizers (e.g. nitrogen fixers in rice fields). Cleaning of wastewater and sewer is another area of use already launched in the 1950's, and become in the 1960's, together with bacteria, considered used as organic recyclers in space exploration (extraterrestrial life-support systems) due to these qualities. Use of microalgae as sources for energy (e.g. hydrogen) has also been investigated. Microalgal biotechnology has been extensively investigated, and areas of potential uses, production systems (culturing, harvesting and processing), commercial products and industries has been considered, evaluated and developed. Today, microalgal biotechnology is widely recognized as a source of great potential in several articles and reviews (Borowitzka, 1986; Borowitzka, 1995 (a); Burja *et al.*, 2001; De La Noue, 1988; Metting & Pyne, 1986; Moore, 1989; Namikoshi & Rinehart, 1996; Pulz, 2001 (a); Pulz & Gross, 2004; Pulz, 2001 (b)). Estimates of algal diversities range from about 40,000 to a number as high as 400,000 species believed to exist

at present. It is likely to believe that the true number is between 200,000 and 400,000 species based on estimates of microbial diversities (Norton, 1996) They inhabit nearly all of the ecosystems of the world, from deserts to polar seas.

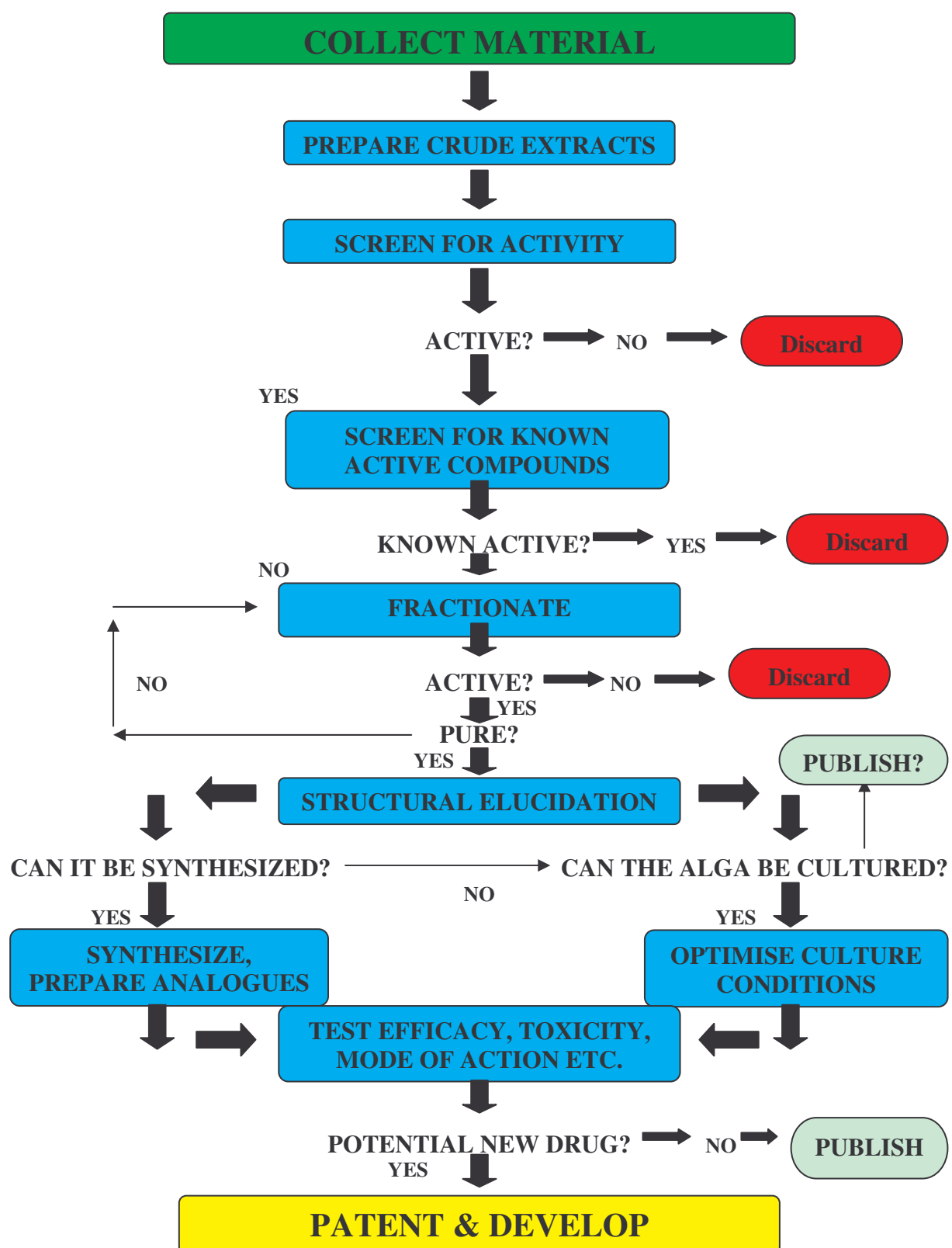
Large-scale systematic screening of marine organisms for bioactive has been mostly concerned with sponges and macroalgae. However, more recently there has been a shift in this focus towards marine microorganisms (Borowitzka, 1995 (b)). The systematic examination of algae for biologically valuable substances, especially antibiotics, began in the 1950's. These studies were concerned mainly with *in vitro* studies of the substances. But in the 1970's focus was shifted to emphasise *in vivo* examination of activities using an extensive range of screens at the Roche Research Institute of Marine Pharmacology (RRIMP) in Sydney. It was also RRIMP that pioneered the screening of crude extracts rather than pure compounds, and the use of bioactivity in the screens to direct the isolation and identification of the active compound. Most of this work focussed on macrophytes, and the microalgae were begun to be studied widely first in the 1980's (Borowitzka, 1995 (a)). An increasingly amount of effort has been put into the field of algal research, especially in the two latest decades. Applied algology has developed rapidly over the last 40 years, especially in Germany, United States, Japan and Israel. As a result, several bioactive substances have been found and isolated. Despite this, none have yet reached the drug store (Knutsen, 1997). It has been suggested that one of the reasons for this might be that methods for algal research and the phycological lexicon is not broadly familiar to industrial microbiologists (Metting & Pyne, 1986). Of the many thousand of species of microalgae and cyanobacteria, only a few (30-40) have been considered for mass-cultivation or are presently of real commercial importance. Researchers and scientists have traditionally been more concerned about marine microalgae and cyanobacteria than in freshwater or terrestrial species in their investigations. This research has also focused more on tropical or sub tropical species than on temperate and polar species. Cyanobacteria have also been given much more attention than eukaryotic microalgae. Therefore, cold waters surrounding Norway and Svalbard might turn out to be an immense resource for bioprospective research, as species here will most likely have developed their own unique bioactive substances as an adaptation to their environment, that differ from those encountered in tropical regions. Many species have already been isolated and characterized from these waters, but the majority of them are today not to be found in any culture-collection (Knutsen, 1996). In Norway, professor Gjert Knutsen recognised early in the 1990's that Bioprospecting would become a valuable tool in the search for novel bioactive compounds. Today, he has developed a set of methods for the collection, production and screening of

microalgae and cyanobacteria together with his co-operatives and students (PhD and master-students). He initiated in 1994, with the support of the Norwegian Research Council, the project "Bioactive substances from marine cyanobacteria and microalgae". Main objective was to acquire through experiments a set of well-tested methods necessary for the exploitation of the resources of these microorganisms. Today, several of these projects have gained progress. A culture collection of more than 400 isolated cyanobacteria and microalgae exists on the Institute of Biology (UIB), and screens of these and testing of bioactivity has yielded promising results. Screening is accomplished through the cooperative work of other scientists.

Screening for bioactive components demands methods that optimise the overall work and that give a high yield in a short amount of time to the lowest cost. Which method that is most suitable, depends on the desired bioactivity screened for, and the species involved. Methods have to be further tested, adjusted and developed for optimization, so that the best system to detect wanted properties in an efficient and economic way is established. In the preliminary screen, small or medium scale laboratory cultures are to be preferred for biomass production. Isolation, growth conditions and the extraction of biomass are steps that can be optimised for a more effective screen. Since many of the bioactive components produced by microalgae and cyanobacteria might be secondary metabolites, stressing of cultures can trigger the production of these. In the exponential growth phase, most of the metabolic apparatus in the cell are concerned with growth only, so harvesting cells in the stationary phase might also be an alternative as secondary metabolites might be synthesised at the end of the primary growth phase and into the stationary phase. Stress can be achieved by exposing algae for high light irradiances or UV- light. Altering of the nutrition available for growth, like removing nitrogen or phosphorus, is also an alternative. High or low temperature or salinity is another option. If bioactivity is revealed, isolation and purification of compounds follows for further testing, and finally for structural elucidation. If cultures of algae then are to be cultivated for the production of a known substance, the production has to be optimized. This is achieved by increasing the growth rate (optimum values for light, CO<sub>2</sub>, nutrition and temperature), and to investigate in which way the algae can be grown to produce high quantities of the desired substance. Again, the solution might be to stress the algae in some manner, as some secondary metabolites are produced as a response to such factors in the environment, and not under optimal conditions for growth. Selection of cells with higher production qualities, or genetic engineering might also be considered. There are a number of different factors that can influence the results of a screen. Isolation of organisms is to some extent season dependent. Specimen composition and their physiology (e.g. the production of metabolites) can vary

throughout the year. A sample of a species during a spring bloom does not necessarily give a representative isolate of the organism. One problem with cultures is that they tend to be highly monotonous genetic as they often are cultivated from the line of one cell and its descendants. The geographical location of a search is also of great importance. Different species appear on different locations to any given time, and the representation in a given location is dependent on factors as competition, light regime and nutrition. Another problem outlined in section 1.1.2, is that many species are difficult or cannot be cultivated in laboratorial conditions. Figure 1.1 displays a detailed flow diagram for the screening process, from the isolation of an organism to the final marketing of a finished product. From an industrial perspective, the goal of microbial biotechnology is to profit from the commercial developing of products for the market. This has to be taken into consideration when time is spent on research, and the succession relies on the identification of a desirable metabolite and microalgae that produce and accumulate the desired metabolite. Then a large-scale production process has to be established, and a market for the product must be identified and evaluated (Olaizola, 2003). Microalgae have an extremely high productivity compared to higher plants. This, together with the prospects of growing algae around the year using the right production system, give algae an advantage in biomass production and economic yielding. Photobioreactors (PBR) and open cultivation systems (ponds / channels) are different strategies for this purpose (Pulz, 2001 (b)). PBRs are to prefer for the cultivation of biomass for the use in biotechnology to produce valuable substances and for special applications, whereas open systems are predominating in mass cultivation because of cost considerations, and are more convenient for the production of e.g. food additives ( $\beta$ -carotene from the extreme halophile *Dunaliella salina*, biomass from *Spirulina sp.* and *Chlorella sp.*). Open systems rely on natural light for illumination, which make them seasonal and / or geographical dependent. But this in turn is cheaper than artificial illumination. Closed and semi-closed PBRs can be located outdoors, and supplement with artificial light when needed. These seem to be the more promising field for high-value products in particular, as culture conditions and growth parameters (i.e. temperature, nutrition, turbulence, pH, salinity,  $\text{CO}_2$  /  $\text{O}_2$  levels, and light intensities) can be controlled. Cultures can also be grown axenic if necessary. Such photobioreactors can obtain high biomass densities, and water and nutrition can be recycled. Microalgae can thus be grown around the year on a continuous basis and be cultured on marginal lands in arid regions of the world, utilizing waters unsuitable for conventional agriculture. For the industrial process, a fast growing alga is required. Alternatively, if the algae containing the wanted substance have growth rates too low for an

## SCREENING FOR BIOACTIVE MOLECULES FROM MICROALGAE



**Fig. 1.1.** Flow diagram illustrating the different steps followed in the search for bioactive molecules from microalgae (Borowitzka, M.A.)

economically warrantable production, the substance can be tempted isolated, whereas it can either be synthesised if possible, or serve as a model structure. After culturing, recovery of the biomass and further processing to purify the metabolite from the biomass is necessary. Biomass can be harvested by centrifugation, filtration, flocculation, or in some cases by gravity sedimentation. These processes may be preceded by a flocculation step. When a compound is identified, and can be produced in large quantities, the marketability and profitability of the product has to be evaluated. It is not enough to assume that a product that is available will be a success (an “if we make it consumers will buy it” attitude). Market research and marketing resources are an important factor in the success of a commercial product.

### **1.2.2 Bioactivity in algae**

Microalgae display a diversity of primary and secondary metabolites, and release several of these substances to their environment actively or passively living or dead after decomposition and lysis. Apart from common growth factors and nutrients, other bioactivities are of especially great concern, namely those that affects other organisms positive or negative as antibiotics, algicides, toxins, pharmaceutically active compounds and plant growth regulators (PGRs).

A great number of bioactivities or bioactive components from microalgae have been reported. Several of these possess antibiotic properties, and many with a novel structure have been isolated and characterized. Biomolecules with other bioactivity have also been detected, and many of these have the potential of being utilized in pharmaceuticals or in agriculture, as tools in scientific research, or as models in the development of new medicines. Most of these bioactive substances have been isolated from cyanobacteria as these have been more extensively investigated, but microalgae are increasingly being reported as good candidates. Both endocellular substances (e.g. osmoregulators like glycerol, sorbitol, and mannitol) and exocellular substances (mainly polysaccharides, hydrocarbon or polyacrylates) can be obtained from microalgae and cyanobacteria. The diversity of exo-polysaccharides is impressive and undoubtedly represents considerable potential for the food industry. Polysaccharides from both cyanobacteria and microalgae have been tested, and show promising results expressing immuno-stimulating, antineoplastic or cytostatic effects (Pulz, 1993). A demonstration of the ability of microalgae and cyanobacteria to produce highly



effective bioactive compounds is phycotoxins. Many prokaryotic (especially *Anabaena*, *Microcystis* and *Aphanizomenon*) and eukaryotic microalgae can be toxic, and create problems for fisheries and water quality deterioration. The same properties making some species or genera harmful might be qualities that give them possibilities for their economic utilization (Skulberg, 2000). Detrimental properties of algae have gained more attention than other activities. Phycotoxins include an array of cyclic peptides, alkaloids, polyethers and glycolipids. Some show cytotoxic activities, and are investigated for potential use as anti-cancer drugs. Most known metabolites isolated from marine eukaryotic microalgae are in fact toxic (Daranas *et al.*, 2001). Striking is hepatotoxins (microcystins, cyclic heptapeptides) and neurotoxins, the two types of toxins characterized from toxic cyanobacteria. Peptide hepatotoxins (most often occurring) are interesting due to the potential use in cancer research. They can be employed as useful tools in studies of cellular regulation processes. Especially many microcystins have been characterized (obtained from *Microcystis*, *Anabaena*, *Nostoc* and *Oscillatoria*), and structurally related toxic nodularins (cyclic pentapeptides) have also been isolated (*Nodularia*). Among the neurotoxins isolated are anatoxins and homoanatoxins, and also saxitoxins produced by certain marine dinoflagellates. Some neurotoxins among the cyanotoxins have the potential of being applied in medical research as they exert their effects on specific ion channels in nerve and muscles membranes (e.g. Homoanatoxin-a). A diverse group of components isolated from different cyanophytes that are non-toxic cyclic depsipeptides (and a few linear peptides), has been described to possess serine protease inhibition activities. Some other proteinase inhibitors and inhibitors of other enzymes have also been described. Others have been demonstrated to be cytotoxic (cytostatic) or antineoplastic (e.g. dolastatin 13). Some still have an unknown biological activity. Cyclic peptides and depsipeptides are common bioactive and structural compounds. Others are linear peptides, guanidines, phosphonates, purines and macrolides (Namikoshi & Rinehart, 1996). Some cyanobacteria and a few microalgae have been tested for anti-virulence activity. Despite limiting results, these appear encouraging. Activity against *Herpes simplex* virus type 2 and “respiratory syncytial virus” have been detected. Antiviral and antineoplastic activity has been investigated in approximately 600 and 1000 strains respectively (Patterson *et al.*, 1991; Patterson *et al.*, 1993) in a period from 1987 to 1993. It was found that certain taxonomic groups are more likely to display antiviral activity (*Chroococcales*), in the contrast with results of the screening for anti cancer compounds, which identified different taxonomic groups (*Scytonemataceae* and *Stigonemataceae*) as prolific producers of cytotoxic agents. In an attempt to identify likely sources of bioactive cyanophytes, the substrate material from

which the strains were collected in the field was characterized. It was then found that epilithic (i.e. attached to rocks) and epidaphic (i.e. on the surface of soil) algae proved to be the most likely to display bioactivity, while marine strains (independent of substrate) tended to be least active. No correlation was found between growth rates and the bioactivity in the strains investigated. Amphidinolides with promising therapeutic potential from a group of more than 20 macrolides with cytotoxic properties has been isolated from the dinoflagellate *Amphidinium* sp. (Ishibashi & Kobayashi, 1997; Kobayashi *et al.*, 1994; Tsuda *et al.*, 2000). Several antimicrobial compounds have been detected and isolated, dominated by antibacterial substances. Fatty acids, glycolipids, acrylic acid, bromophenols, terpenoids, carbohydrates, N-glycosides, peptides, polysaccharides and others are among these (Borowitzka, M.A. (a) 1995), several detected using *in vitro* assays, and might therefore not have *in vivo* applications. Antibacterial activity was reported to be present especially in the more lipophilic extracts, and only inhibited the growth of Gram-positive bacteria (Mundt *et al.*, 2001). Algae possess many other interesting bioactivities. These include pigments, vitamins, carotenoids, and fatty acids among others. These not only have potential medical or agricultural uses, but might also be implemented into nutrition and processing to name two important fields. Potential economic valuable unsaturated fatty acids like  $\alpha$ -linolenic acid and arachidonic acid are abundant among algae. Phycocolloids like agar and alginate, and other polysaccharides like carrageenans, are economically the most important products from algae. Due to their phototrophic nature, which involves the exposure to high oxygen and radical stress, they are an important source of antioxidants (e.g. carotenoids, lipids, enzymes, polyphenols and vitamins such as C and E). They also possess potential economic important pigments, like astaxanthin (*Haematococcus*) and  $\beta$ -carotene (*Dunaliella*). Screens from many invertebrates show in some cases striking similarity to bioactivity found in cyanobacteria, structures are also often very similar to those. Symbiotic relations or associations between invertebrates and cyanobacteria are common, and it is quite possible that bioactivity from sponges and tunicates originate from metabolites produced by their cyanobacterial partners. Similarities from other bioactive compounds to those of cyanobacteria, like the one detected in the Indian sea hare *Dolabella auricularia* (Namikoshi & Rinehart, 1996), is so striking that it is believed that it might originate from cyanobacteria in the diet of this mollusc. This might also be the case in other samples, including microalgae, where the detected bioactivity is produced from a substance belonging to an associate fungus or bacteria. As a part of the diet of the organism were the compound was first isolated from, the compound has accumulated or been incorporated into the organism, and is thus detected in screens (Suenaga, 2004).

### **1.3 Aims of present research**

Aims of the present research was to use volume changes during spore swelling and germination, applying the Coulter Counter particle sizing system, as a bioassay system to determine quantitatively fungicidal effects from extracts of cyanobacteria and microalgae. A considerable part of this Thesis was the development and testing of the method. Consequently, much of the “material and methods” part has to be modified and adapted progressively to the results during research. Thus, “material and methods” can also be viewed as the final recipe for the practise of the method. A procedure for the method will contain several elements that need to be clarified, and then tested before the method can be viewed as complete and evaluated as successful or disclaimed as unsuited for the purpose of screening extracts or measuring volumetric properties of the spores. Aims of this Thesis can be summarized in the following five main steps:

#### **1. Preliminary research.**

- 1.1. Growth and harvesting of spores from the fungal model organism selected.
- 1.2. Construction and testing of experimental set-ups and bioassay.
- 1.3. Fungal spore behaviour - investigation of the process of swelling and germination.
- 1.4. Optimising of the viability and germination condition of the fungal spores.

#### **2. Growth, harvesting and extraction of algal biomass.**

#### **3. Standardization of method**

Standardization will involve inoculum sizes and assay conditions, spore harvesting (age, method, and storage), sample preparations and standardization with known fungicides.

#### **4. Screening for bioactivity**

Selected microalgae and cyanobacteria are finally tested for bioactivity in the refined bioassay. At this step, the method will be more or less completely developed.

#### **5. Discussion and evaluation of the bioassay as a method for screening.**

If time allows, and a positive hit is encountered, growth alterations will be conducted on the algal specimen that contain the bioactive compound for investigations on growth conditions on the bioactivity, and for optimization of the compound. Further examination of the potential positive hits after screening for isolation and structural analyses of the bioactive compound is neither in the aim nor within reach of this work.

## 2 THEORY

### 2.1 The Coulter Principle

Wallace Henry Coulter (1913-1998) developed and patented in the late 1940's a new technique to simultaneously count and size particles that were homogeneously suspended in a conducting liquid. The principle of using electronic impedance to count and size microscopic particles suspended in a fluid was invented, and has since then been referred to as the Coulter principle. This method was originally designed and implemented to facilitate blood cell analyses. However, it did not take long before this method was found to have a wide range of applications in both research and in the industry. It increases the sample size, thereby



Figure 2.1

reducing errors, and at the same time decreases the analysing time compared to microscopic methods. The Coulter Principle, also known as the Electrical Sensing Zone (ESZ) method, is a method based upon an electrical current flowing between two electrodes in a conducting liquid. Separating these two there is a cylindrical opening, called the aperture (or "Sensing Zone") (figure 2.1). The magnitude of this current is low, typically about 1.0 mA, but the resistance or "pinch" created by the aperture creates a considerable current density within the aperture. An amplifier converts the fluctuations in resistance into a voltage pulse large enough to be measured accurately. As the suspension is forced to pass through the aperture, any particles following it will displace its own volume of conducting liquid, and thus create a momentarily increase in the impedance. This will create a pulse in the electric current, which can be measured by the system. **"The Coulter Principle states that the amplitude of this pulse is directly proportional to the volume of the particle that produced it."**

These pulses can therefore further be scaled into units of volume, which create a size spectrum. In addition, if a metering apparatus is used to draw a known volume of suspension through the aperture, the concentration of particles per unit of volume can also be measured. The diameter of the particle can be derived when the volume is known, to give a linear measure. Particle surface area can also be calculated. However, in medical and biological research, traditionally it is preferred to give the results in units of volume.

## **2.2 Model organism – *Absidia glauca***

The following is a general description of *Absidia glauca*, the fungal model organism that was engaged in this research project. The specimen was chosen due to its rapid growth and high spore production. It is also a well-known experimental organism.

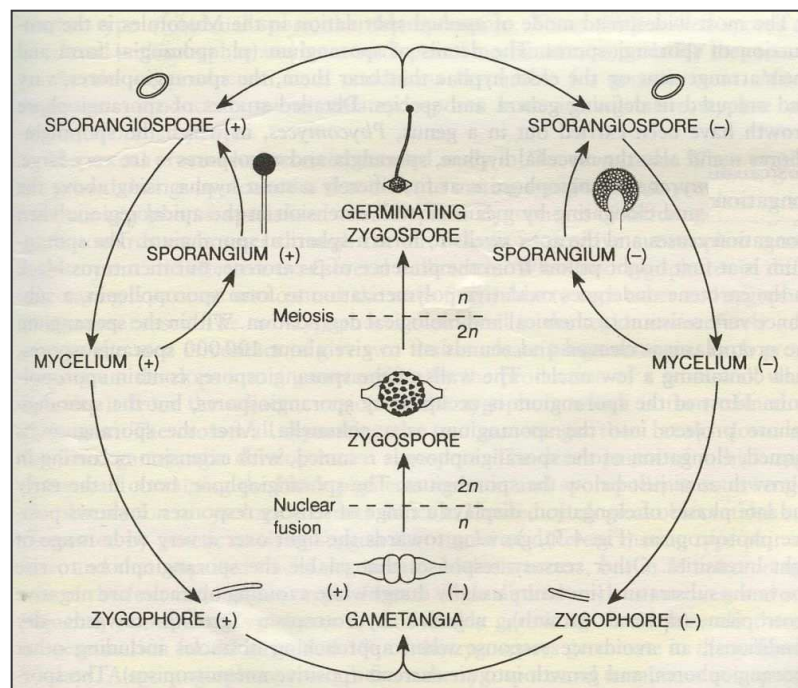
### **2.2.1 *Absidia Glauca* – taxonomic classification (systematic)**

In the kingdom of Fungi, two divisions are recognized, the Myxomycota (the slime moulds, a phylogenetically diverse group with amoeba as the trophic (feeding) phase, and also in some classes, plasmodia) and the Eumycota (heterotrophic eukaryotes with a trophic phase that is typically mycelial but sometimes unicellular, as in yeasts). Fungi are a large group with about 250,000 species, of which more than 300 species have been reported to be potentially pathogenic to humans (Gupte *et al.*, 2002). *Absidia glauca* (Norwegian; *Gråmugg*) belongs to the division Eumycota, subdivision Zygomycotina (alternatively phylum zygomycota in some literature); class Zygomycetes in the order Mucorales (Carlile, 1994a). Subdivision Zygomycotina is characterized by zygospores, and consist of two classes; Zygomycetes and the Trichomycetes (obligate parasites of arthropods). The class Zygomycetes is a diverse group of five orders of terrestrial fungi characterized by sexual reproduction by fusion of gametangia, the production of a resting sexual spore (zygospore), and asexual reproduction by non-motile spores. *Absidia glauca* further belongs to the order *Mucorales*. This group is a widespread and abundant one, and is characterized by a saprotrophic lifestyle feeding on dead or decaying organic matter, and have coenocytic hyphae (aseptate, protoplast not separated by cell walls) with many nuclei and few septa. Other species of this order includes *Mucor sp.*, *Rhizopus sp.* and *Phycomyces blakesleeanus* to name a few.

### **2.2.2 Growth and lifecycle**

Growth is by rapid extension of aerial hyphae called stolons. When these reach a suitable substratum, more slender hyphae, rhizoids (i.e. filamentous outgrowth from prothallus that

function as a root), develop and penetrate the substratum. The most common mode of asexual sporulation in the Mucorales is the production of sporangiospores (figure 2.3).



**Figure 2.3:** Lifecycle of the Zygomyces. Scanned image from “The Fungi” (Carlile, 1994a).

A sporangiospore is produced in the sporangium, where the protoplasm is cleaved and rounds off to give rise to many thousands of spores, each containing a few nuclei. The spore contains sporopollenin (an alcohol very resistant to chemical and biological degradation, derived from oxidative polymerisation of  $\beta$ -carotene) in its wall that makes it durable. The sporangium itself rests on the erect hyphae that bears it, the sporangiophore, which projects into the sporangium as a columella (i.e. sterile structure within a fruiting body in fungi). The sporangiophore displays a range of sensory responses in the phase of elongation. The sexual process involves the formation of zygophores, when two colonies of different mating type (designated + and -, respectively, if self sterile like *A. glauca*) grow vegetative in close proximity of each other. These aerial hyphae fuse their cell walls when they come into contact with each other, and then swell to form two multinucleate progametangia. Each progametangium develops into a gametangium, by the production of a cross wall, leaving the rest of the zygophore as the suspensor. The cross wall then breaks down between the two gametangia. They fuse and develop into a zygospore, which is a thick-walled sexual spore in zygomycete fungi, and which later on will undergo meiosis and still later germinate under favourable conditions.



### 2.2.3 The sporangiospore

Released from the sporangium, the spore will initiate germination as soon as it is exposed to suitable conditions. If not, it will be dormant with low metabolic activity. The germination is a three-step process (dEnfert, 1997). **1. Activation;** requires environmental factors that act as triggers. These vary among species, but water, oxygen and CO<sub>2</sub> are universally required and in addition low molecular weight nutrients (sugars, amino acids) and inorganic salts. **2. Isotropic growth;** the first morphological change where spores undergo a period of swelling (the term spherical growth may be used instead of swelling) and wall growth. This process begins with a passive uptake of water, followed by numerous metabolic activities including respiration and RNA and protein synthesis (i.e. increase of biomass). The passive phase is simply driven by osmosis. When first initiated, the process will continue as long as conditions will allow. A new inner wall is synthesized beneath the original spore wall throughout this second step, and the cell wall of the germ tube is continuous with this new wall. **3. Polarized growth;** after some time, cell wall deposition becomes polarized, resulting in the formation of a germ tube. The old outer wall weakens through the process, and after a few hours, the germ-tube breaks through the old spore wall. Several germ-tubes may be produced. The germ-tubes grow and branch, and give rise to the mycelium, thus defining the limit between spore germination and vegetative growth. The precise mechanisms that control these three steps remain poorly understood. Trehalose ( $\alpha$ -D-glucopyranosyl- $\alpha$ -D-glucopyranoside) mobilization occurs very rapidly upon induction of spore germination controlled by sensing of the extracellular carbon sources, concomitant with accumulation of glycerol (and thus a dramatic increase in intracellular osmotic pressure). The actin cytoskeleton appears to play a major role in establishing polarized growth, and for the germ tube elongation. Striking features of the spore is the cell wall (multi-layered although thinner-walled due to rapid germination), its low water content and chemical composition that often differ from the hyphae of the same specimen. The germination usually requires the same temperature as for growth, but not necessarily. Light has also been demonstrated to affect germination for some species.



### **2.3 Experimental organisms – Cyanobacteria and Microalgae**

Microalgae comprise a large and diverse group of photosynthetic organisms. They widely differ in morphology, physiology and site of habitation, and are thus much a heterogeneous group despite being unicellular / filamentous and photosynthetic. They are all photoautotrophs (CO<sub>2</sub> as the primary source of carbon), characterized by the presence of two photosystems (PSII and PSI) and the use of H<sub>2</sub>O as the photoreductant in photosynthesis, releasing O<sub>2</sub>. In addition they require nitrogen and phosphorus, some minerals and trace elements, depending on the specific species. Some species are not obligate phototrophs, but can live facultative, or in some cases even as heterotrophs through phagocytosis. They are the most important primary producers, and also the most important extant CO<sub>2</sub> consumers in the world. They are the basis of the food chain of the oceans, and inhabit nearly all ecosystems of the world. Many species are generalists and will tolerate a great range of environmental conditions, and symbiotic associations are common. The term microalgae is used for all of the microscopic, unicellular or filamentous algae, including the prokaryotic photosynthetic cyanobacteria (in traditional “botanic classification” they were described as blue green algae). Eukaryotic algae are divided into 14 different groups (Margulis, 1990). Cyanobacteria comprise a single taxonomic and phylogenetic branch (phylum) within the domain *Bacteria* (Eubacteria), most closely related to the Gram-positive bacteria (Castenholz, 2001). A phylogenetic relationship among the cyanobacteria based on 16 ss-rDNA sequences divides the Phylum into 16 internal related clusters. About 2,000 species in 150 genera are recognized (Pulz, 2001 (b)). They share the basic cellular features of other bacteria, and in addition they possess unique and diagnostic characteristics as a group. The cell wall is of a Gram-negative type, and often with a considerable thick peptidoglycan layer. Numerous species possess an “envelope” outside the outer membrane (sheath, glycocalyx or capsule) and some possess specialized cells (e.g. heterocysts, akinetes, hormogonia and termini). For additional information on physiology, morphology and systematic of Microalgae and Cyanobacteria, please look into the following referred literature (Becker, 1994; Castenholz, 2001; Garrity, 2001; Richmond, 1986; Rippka *et al.*, 1979; van den Hoek, 1995).

## 3 MATERIAL AND METHODS

### 3.1 Fungus

#### 3.1.1 The fungal isolate

A strain of *Absidia glauca* Hagem from the culture collection at the Department of Microbiology, University of Bergen, was used in this research. This strain was originally isolated and described by Hagem (1907) from soil in western Norway. At present, professor Finn Langvad keeps it as stock. The strain consist of to mating types (designated + and –, see section 2.2.2). Both mating types were kept under the same conditions. Only the – mating type was applied in this research as model organism, although the + strain was briefly investigated as a candidate.

#### 3.1.2 Maintenance of stock cultures

Stocks were stored suspended in 4 ml sterile distilled water in cryo-tubes kept in a refrigerator at 4°C. They were prepared by stamping agar plates containing growing mycelia with a hollow cork borer, producing circular bits from the agar plate whit a diameter of about 0.5 cm. Two or three of these where then transferred to the cryo-tubes, where they would be kept until needed. Stock cultures could be kept for approximately one year or more, but new ones were prepared every third or fourth month.

#### 3.1.3 Growth

*A. glauca* was grown on malt extract agar medium (MEX) (appendix I, table A.1). Spores were germinated in liquid MEX medium (appendix I, table A.2) filtrated as described in section 3.6. *Absidia glauca* was cultivated on agar dishes (Petri plates) in an incubator (Fermacs incubator) at  $22.0 \pm 0.5$  °C for 14 days, the time selected for optimal spore

production and maturation (see results). All dishes were incubated upside-down to avoid moisture to evaporate from the medium and accumulate on the inside of the lid. New plate-cultures were prepared from these cultures whenever needed, strictly using sterile techniques. Inoculums were kept at a relative constant level mainly by streaking the graft-needle containing some mature (sporulated) filament (hyphae) in a small cross in the middle of the agar dishes. Like this new cultures would raise from spores and small fragments of hyphae, the inoculums being invisible to the naked eye. This to assure that re-cultivating would have more or less the same inocula for all cultures. Cultivation was logged using a scheme to keep the different cultures apart, so that several cultures could be grown simultaneously. This made scheduling and planning of future tests, re-cultivation and harvesting easy and structured, and any changes in morphology, growth or growth conditions were monitored.

### **3.1.4 Spore harvesting**

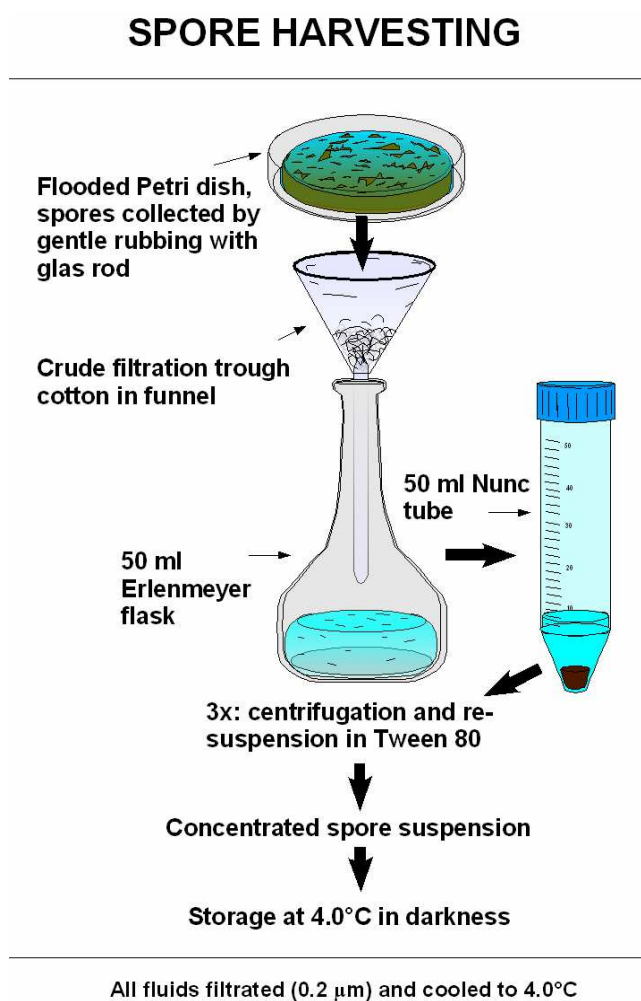
Spores were harvested after the culture had been incubated for 14 days. This age was chosen to allow the culture to be mature and fully sporulated, and for the reproducibility of experiments. First flooding the Petri dish with 10 ml sterile distilled water (4°C) and then rubbing the culture gently with a sterile bent glass rod released the spores. The subsequent spore suspension was roughly filtered through washed and sterilized cotton (2 x 30 min. at 750 W in microwave oven, when moist) positioned in a funnel into a 50 ml Erlenmeyer flask. This procedure was repeated once, and the cotton finally washed with 5 ml sterile distilled water (figure 3.1).

### **3.1.5 Preparation of spore suspensions**

All spore suspensions were transferred directly to 50 ml Nunc tubes after harvesting, and centrifuged for 5 minutes at 1000 rpm at 4 °C (Beckman J2-HS Centrifuge, USA). After centrifugation, the supernatant was removed and the pellet re-suspended in 10 ml sterile distilled water containing 0.1% (v/v) Tween 80, using a vortex shaker for 30 sec (MS1 Minishaker, IKA<sup>®</sup> Works, INC) to suspend it completely. Then it was centrifuged once more, to wash the spores free of debris. This process was repeated a third time (so that spores were washed twice), and finally re-suspended in 5 ml sterile distilled water containing 0.1% (v/v)

Tween 80 and shaken for another 30 sec. before storage (figure 3.1). This produced a high-density suspension of more than  $2.0 \cdot 10^8$  spores / ml per Petri dish. All fluids used to harvest and suspend spores, were prepared from distilled water that had been filtered according to section 3.6, and cooled to 4°C before use to preserve and keep the spores from initiating the swelling process.

**Figure 3.1:** Flow diagram illustrating the steps in spore harvesting and preparation.



### 3.1.6 Storage of spores

Before storage (or before experimental use), the concentration of the suspension was determined and adjusted to  $1.0 \cdot 10^8$  spores / ml, serving as starting point for all subsequent work. Suspensions were stored in refrigerator at 4°C in darkness until needed, for max. 7 days after harvest (see results). All subsequent handling of the suspension, like preparation for screening, was initiated by shaking the tube for at least 30 sec., followed by sonication for another 5 minutes to dissolve the pellet (performed twice in this order). This was done to homogenize and separate spores. The spore suspension was shaken for min. 30 sec. before any use during experiments to assure that spores did not cling or sediment, and was at all times before introduction to the MEX medium kept on ice to stay cooled down. All unnecessary disturbances of the suspension were avoided.

## 3.2 Algae

### 3.2.1 Algal isolates

A total number of 21 different selected microalgae (5 cyanobacteria, 15 greenalgae and one additional Chlorophyte isolated during this work), were cultivated for biomass-production, extracted and screened for bioactivity. The greenalgae were first employed, and thus served in the improvement of the method (extraction and screening) mutually with the model organism *Absidia glauca*. All cyanobacteria were screened after the method was satisfactorily developed, tested and considered complete, as they were considered as the main algal species in this work.

#### Cyanobacteria strains

Five clones received from the culture collection at the Norwegian Institute for Water Research (NIVA) was applied in this work. Species and place of origin and isolation are listed in table 3.1. These strains are all terrestrial, but were kept and cultured in a freshwater based Z8 medium (see below).

**TABLE 3.1**  
Cyanobacteria strains.

Cyanobacteria species	Strain	Place of isolation
<i>Merismopedia angularis</i> Thompson	NIVA-CYA 199	Soil (cryoturbation), collected from Sørneset, Spitsbergen - 06.07.1984.
<i>Nostoc</i> sp.	NIVA-CYA 192	“Diorite gravel in edge of melting snow”, Stabben, Jutulssessen, Dronning Maud Land, Antarctic - 20.01.1985.
<i>Phormidium</i> sp.	NIVA-CYA 181	“Sand ground, weak influence of Snøpetrell”, Stabben, Jutulssessen, Dronning Maud Land, Antarctic - 20.01.1985.
<i>Phormidium</i> sp.	NIVA-CYA 207	Soil sample from Ny Ålesund, Spitsbergen - 01.08.1985.
<i>Phormidium</i> sp.	NIVA-CYA 209	Soil sample from Bjørnhamna, Spitsbergen - 01.08.1985.

## Greenalgae strains

Fifteen different strains from the culture collection originating from the Bioprospective activity at the algal group (Institute of Biology, University of Bergen), was applied in this work. Species and place of isolation are listed in table 3.2 (species designated by number). Currently none of these strains have yet been classified. They are isolated by Kjersti Hansen from different marine locations in the south and west coast of Norway.

**TABLE 3.2**  
**Greenalgal strains.**

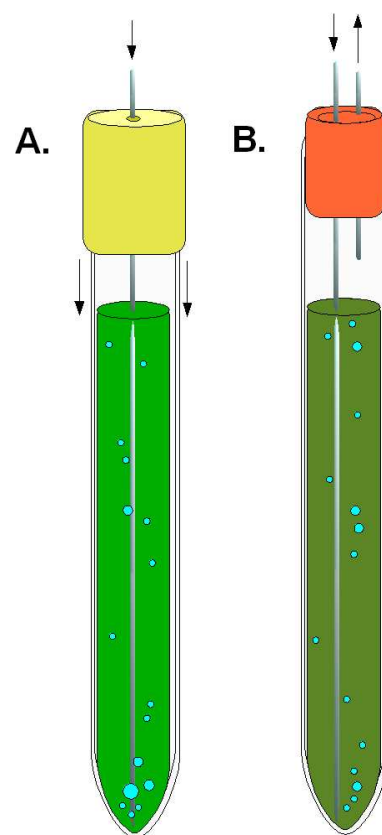
<b>Greenalgae species (not classified)</b>	<b>Strain</b>	<b>Place of isolation</b>
Marine greenalgae	G 8	Store Lungegårdsvannet, bro v/ Florida (4 Bf)
Marine greenalgae	G 9	Langesund (5.5 Bf)
Marine greenalgae	G 13	Vold, indre (Skien) (10 Bf)
Marine greenalgae	G 17	Masfjorden (25 Cf)
Marine greenalgae	G 18	Langesund (5.5 Df)
Marine greenalgae	G 20	Masfjorden (25 Df)
Marine greenalgae	G 23	Vold, ytre (Skien) (9 Ef)
Marine greenalgae	G 44	Store Lungegårdsvannet, Ford i Straumen (3 Af)
Marine greenalgae	G 45	Nordnespynten (1 Åf)
Marine greenalgae	G 46	Nordnespynten (1 Af)
Marine greenalgae	G 48	Vold, indre (Skien) (10 f)
Marine greenalgae	G 52	Nordnespynten (1 Ef)
Marine greenalgae	G 61	Store Lungegårdsvannet, Ford i Straumen (3Hf)
Marine greenalgae	G 63	Store Lungegårdsvannet, Ford i Straumen (3Cf)
Marine greenalgae	G 73	Store Lungegårdsvannet, bro v/ Florida (4Wf(2))

### 3.2.2 Maintenance of stock cultures

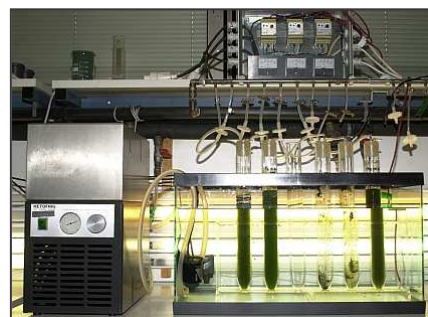
Stocks were kept in 50 ml Erlenmeyer flasks closed with aluminium foil at room temperature ( $22.0 \pm 2.5$  °C) and artificial room and seasonal window illumination (approximately  $37.0 \mu\text{mol m}^{-2} \cdot \text{s}^{-1}$ ). Cyanobacteria stocks were in addition stored in an illuminated refrigerator at  $17.0 \pm 2.0$ °C in a 12:12 light: dark cycle at  $45.0 \mu\text{mol m}^{-2} \cdot \text{s}^{-1}$  quanta. All stock-cultures were regularly re-cultivated every third to fourth month.

### 3.2.3 Growth of algal biomass

Biomass production was accomplished in non-axenic semi-closed growth systems consisting of 250 ml slim glass-tubes (3.5 cm inner diameter) covered with plastic lids. Aeration and thus turbulence supplied through a hollow glass rod extending through the lid onto the bottom of the tubes (figure 3.2.A), creating a slightly higher pressure inside compared to the outside. This provided protection against any potential contaminants. All greenalgae strains and stocks were cultivated on a standard *Chlamydomonas* medium added 24 g/l NaCl (Kuhl and Lorenzen, 1964) (appendix 2) at room temperature ( $22.0 \pm 2.5$  °C). Cyanobacteria strains and stocks were cultivated on standard freshwater-based Z8 medium (Staub 1961; modified by Kotai 1972, NIVA 1976) (appendix 3). Growth system assembling that of the Chlorophytes initially, but modified into a closed system after experiencing serious contamination problems (see results). Sealing tubes from the outside environment with a rubber stopper solved this (figure 3.3.B). Although grown in a closed system, no attempt to remove all bacteria from the medium was done, and growth was hence still considered non-axenic. Growth temperature fixed at  $20 \pm 0.5$  °C (for reproducibility of screens). This was achieved by keeping the glass tubes suspended in a 60 L aquarium filled with water from an ion exchanger filtrated through a filter with 5.0 µm pore size (Seradest S 650). Water was circulated into a cooler (Hetofrig, Heto – Birkerød) using an aquarium pump. A thermostat connected to the cooler monitored the temperature within the aquarium, and started cooling when temperature rose above 20.0°C. A second water pump assured further circulation inside the aquarium itself to avoid establishment of a thermocline (figure 3.3).



**Figure 3.2:** The two principal growth tube arrangement applied for **A.** Chlorophyceae strains (“open system”) and **B.** Cyanophyceae strains (“closed system”). Arrows indicate the flow of air through the system.



**Figure 3.3:** Growth system for algal biomass.



### 3.2.4 Illumination

Illumination was achieved with white fluorescence light tubes (Philips TLD 58W/84o, Holland / OSRAM L 58W/840, Lumilux cool white, Germany) mounted in front of the cultures, and the intensity regulated by the number (1-5) or the distance between the light and the cultures. All the greenalgae were grown under the same light regime at  $182.63 \mu\text{mol photons}\cdot\text{m}^2\cdot\text{sec}^{-1}$  (equals three fluorescence light tubes at a distance of 30.0 cm). Cultivation of cyanophytes demanded some precautions due to their poor physiological state when received. Extremely low growth rates and high light sensitivity, made it necessary to adapt them through an increasing illumination regime to higher radiation to obtain higher growth rates (see results). This was achieved by first adapting re-cultivated stock cultures to room temperature and room illumination, both slightly higher than what they experienced during storage in the refrigerator (section 3.2.2). Stocks kept like this were used for preparation of inoculums when needed. Cyanobacteria were first grown at  $33.21 \mu\text{mol photons}\cdot\text{m}^2\cdot\text{sec}^{-1}$  (cultivation step 1 - adaptation and initiation of growth phase) using light-filters mounted in front of the tubes (standard filters printed onto transparent plastic sheets). Illumination was kept constant at this value until growth occurred and achieved some densities. Then filters were removed (cultivation step 2 – exponential growth phase, occurred from 1 till four weeks after inoculation), and the cyanophytes were grown at  $102.95 \mu\text{mol photons}\cdot\text{m}^2\cdot\text{sec}^{-1}$  (equals 1 light tube at a distance of 30.0 cm) until harvested. Light intensities were measured frequently to keep them constant using a Biospherical Instruments Inc. (California) QLS-100 radiation metre, with a spherical detector (calibrated). Scalar irradiance was here measured as  $\text{quanta cm}^{-2}\cdot\text{sec}^{-1}$ , and then calculated to  $\mu\text{mol quanta m}^{-2} \text{sec}^{-1}$  ( $1 \mu\text{E m}^{-2}\cdot\text{sec}^{-1} = 6.022\cdot 10^{13} \text{ quanta cm}^{-2}\cdot\text{sec}^{-1} \leftrightarrow \text{quanta cm}^{-2}\cdot\text{sec}^{-1} / 6.022\cdot 10^{13} = \mu\text{mol quanta m}^{-2}\text{sec}^{-1}$ ). Light was measured on the inside of the growth tubes, calibrated in water.

### 3.2.5 Aeration

Cultures were aerated with humidified air containing 1% CO<sub>2</sub>-gas. This assured that the media at all times were saturated with inorganic carbon for photosynthesis. CO<sub>2</sub> enrichment was monitored daily (Qubit Systems Inc.). This is an important factor for the optimisation of growth condition. Aeration also created turbulence in the culture, assuring that cells were

homogenously distributed throughout the medium / cultivation tube, and did not easily sediment on the bottom of the tubes. Before entering the cultivation tube, air was filtered (Gelman Sciences bacterial air vent, 4210, pore size 0.3  $\mu\text{m}$ ), and then led into a hollow glass rod (stuffed with cotton at the entrance for further filtration) extending through the lid to the bottom of the tube (figure 3.2.A).

### 3.2.6 Harvesting

Cultures were allowed to grow till they were considered to have reached the end of the exponential phase / start of the stationary phase before harvested (i.e. when densities of cultures were highest possible). This was determined only by morphological means by following the cultures visually and in light microscope on a daily / weekly basis. Growth was monitored only by this method, as this was merely cultivation for biomass production and not experiments considering growth. Biomass densities of both cyanobacteria and green algal cultures were measured using dry-weight (section 3.7) when harvested, to establish the productivity of the cultures.

Biomass was harvested by centrifugation using a Beckman swing out centrifuge for 400 ml beakers (Beckman J2-HS Centrifuge, USA). Depending on the species, 3000-4000 RPM for 10 minutes (Chlorophytes) and 6000-9000 RPM for 20 minutes (Cyanophytes) were necessary for sedimentation. Generally, the lowest speed was tried at first, and then the speed was increased if necessary. After centrifugation, pellets were washed by re-suspension in distilled water and repeating of the centrifugation process at the same speed. Bacteria still present on the pellets were further re-moved by cleaning off this layer (separated from the algal layer as a distinct red, orange or yellow colorization). Dissolving the bacterial layer by gently adding distilled water directly onto the pellets, and then removing it with a Pasteur pipette solved this. Re-suspending marine algae in distilled water might cause severe damage to the cells by decreasing the osmotic pressure around the cells and consequently make the cells go into lysis. This could lead to the loss of valuable compounds from the cells. Therefore some cells were removed from the pellet of each Chlorophyte and suspended in distilled water in reagent-tubes for approximately 30 minutes. They were then observed in the microscope and checked for any visible damages to the cells. All of the cells tolerated this short exposure, allowing them to be centrifuged and washed free of remaining salts.

### 3.3 Extraction of algal biomass

All 16 microalgae and the 5 cyanobacteria strains were cultivated to obtain biomass for the rapid screening of candidates (see section 3.3.1 for isolates).

#### 3.3.1 Extraction methods

Different extraction methods are to be found in the literature. The method used by Patterson and his research team (Patterson *et al.*, 1991) is the one that were used as a model for this work. The choice of using this method is also based on previous experience, as it has been utilized in previous bioprospective work at the algal group at the department of Biology (UiB) (Hatfield, 2004), and will therefore ease the opportunity to compare different results produced by other work than here. Some adjustments were necessary to adjust the method to this thesis (see below).

Algal biomass was cultivated and harvested as previously described (section 3.2.3 and 3.3.6). After harvesting, the biomass was freeze-dried (lyophilised) for approximately 20 hours (overnight), the biomass weighted (Mettler Toledo AG 64 microbalance) and then prepared for bioassay by the extraction of hydrophilic and lipophilic extracts completed according to Patterson:

<b>H:</b>	Hydrophilic extracts.	3:7 Ethanol: water
<b>L:</b>	Lipophilic extracts.	1:1 Dichloromethane: 2-propanol

Extracts were mixed at a ratio of 40 ml solvent per gram algal biomass. This equal 2.0 ml solvent per 50 mg dries algal biomass. All extracts were kept at temperatures below 25°C. They where also kept in darkness throughout the extraction-process to avoid any light damage to the material. All handling of dry biomass and extracts were performed in an outlet with ventilation to avoid inhalation of any volatiles from the dry biomass (because of the uncertainties of what the biomass might contain) or residues from the organic solvent. A few modifications were done when preparing the final extracts of the cyanophytes. A detailed description of extraction follows:

### 3.3.2 Hydrophilic extracts

50 mg freeze-dried biomass was weighted and blended with 2 ml (3:7) ethanol: Milli-Q ultra pure water (Millipore Co., USA) in a micro tube (2 ml, polyethylene). The tube was further sonicated (Ultrasonic cleaner, Branson 200) for 10 minutes, and placed for extraction overnight (20 hours) at 4°C. The following day the extract was separated from the cells by centrifugation at 13 000 RPM (mini Spin, Eppendorf) for 10 minutes. The hydrophilic extract was removed, freeze-dried (contained in glass containers to avoid cross-contamination, figure 3.5) and re-suspended in 0.4 ml Milli-Q ultra pure water and stored at  $-30.6 \pm 0.1^\circ\text{C}$  until further use. H-extracts obtained from Cyanophytes were concentrated to twice the strength of those prepared from greenalgae, by re-suspending two tubes prepared according to the procedure above in 0.2 ml Milli-Q water, before mixed to a final volume of 0.4 ml.

**Figure 3.4:** H-extracts and lyophilising of these.



### 3.3.3 Lipophilic extracts

The remaining biomass was first freeze-dried to remove remaining moist, then blended with the lipophilic solvent (2 ml (1:1) dichloromethane: 2-propanol (isopropanol)). The micro tube had been tested in advance and proved to resist both the solvent and the centrifugation without any obvious damage to the polymer it was made of. The tube with the lipophilic extract was sonicated for 10 minutes, and placed for extraction overnight (20 hours) at 4°C. The next day the extract was separated by centrifugation at 13 000 RPM for 10 minutes and the lipophilic extract removed and reduced to dryness by gently blowing N<sub>2</sub>-gas at the surface of the solvent. This was accomplished by placing the extracts under a sterile 5 ml Pasteur pipette (blocked with cotton at the top) mounted to a rack and connected to a N<sub>2</sub> bottle through silicon. The dry biomass was then re-suspended in 0.4 ml ethanol (Chlorophytes) or 0.2 ml DMSO (Cyanophytes) and stored at  $-30.6 \pm 0.1^\circ\text{C}$  until needed. Lipophilic extracts were kept at the same concentration in the bioassay due to problems of dissolving any higher concentrations, but by re-suspending in half the volume of DMSO (compared to ethanol), the same amount of extract could be used in the bioassay in a higher concentration of extract to solvent ratio.

### **3.4 Microscopic observation**

#### **3.4.1 Light microscopy**

Cultures and experiments were at all times monitored through microscopic observations. A Leika light microscope was applied here (Leika DM-RBE, Leika GmbH Germany). Digital pictures were taken through the microscope with a Nikon camera (Nikon D1, Japan) (also used for all other photographs in this thesis). A measuring ocular calibrated to the different magnifications (10x, 40x and 100x magnification oculars) was used to calculate cell and spore sizes. All microscopic observations were performed frequently to monitor growth of the different algal species, and to check that cultures were free of any contaminants. This was not necessary for the growth of *A. glauca* strains, when any contaminants on the agar dishes would be clearly visible as distinct colonies. Microscopic observations were however crucial in studies of changes in morphology of fungal spores compared to measures in the Coulter Counter. This also provided useful and necessary information about the strains. Microscopic observations were thus an important tool during all experimental performances.

#### **3.4.2 Electron microscopy**

Structures of vegetative mycelia, sporangia and most important the spores of *A. glauca* were investigated with a Scanning Electron Microscope (SEM). The main objective was to study the surface, size and shape of the sporangiospores, hard to observe in light microscope. They also tended to become semitransparent with diffuse edges in the light microscope, which contributed to the difficulty of determining the nature of their surface. SEM has the advantage over light microscopic images due to its higher resolution. The five different cyanobacteria species were also investigated with SEM, to gain more information about their surface morphology and species dependent details that could contribute to a more accurate taxonomic description. The different species were prepared according to the procedure of critical point drying described by professor Finn Langvad (Langvad, 2001) in his electron microscope course manual (basically after Cole & Samson: Patterns of Development in Conidial Fungi. Pitman, London, 1979). This procedure was also used for the preparation of cyanobacteria,

but with a few modifications. Since preparations for SEM is designed to observe superficial structures only, damages inside the cells due to fixatives are not that important, as they would be for Transmission Electron Microscopic (TEM) investigations. Therefore, the procedure can be performed quicker. A more detailed description of the preparation of the different material is outlined in the following:

#### **Procedure for fixation and critical point drying of biological material for SEM:**

1. Fixation in 6% glutaraldehyde in cacodylate buffer + 1% OsO<sub>4</sub> in cacodylate buffer 1:1 kept in ice-bath or refrigerator at 4°C for min. 3 hours.
2. Washing in buffer 10x (cacodylate buffer – 3.2 g Sodium cacodylate per 100 ml distilled water (= 0.1 M), adjusted to pH 7.2 with 0.1 N HCl (8.3 ml per 100 ml buffer)). The syringe was replaced with a new one at this step to avoid any residues of the glutaraldehyde solution. Remains could lead to deposits of Osmium in the next step, which could slightly ruin the preservatives.
3. Post fixation in 1% OsO<sub>4</sub> in cacodylate buffer for min. 2 hours.
4. Washing in buffer 10x, after the replacement of the syringe used in step 3 with a new.
5. Washing in distilled H<sub>2</sub>O, 5x.
6. Dehydration in ethanol. First 50%, then 75%, 96% and 2x times in 100%, 15 minutes for each solution. Material was kept on 100% ethanol until next step was carried out.
7. Critical point drying.
8. Mounting of material onto aluminium specimen stubs. Material was attached with carbon sticker (leads electricity).
9. Coating of the specimen stub with gold-palladium in a Sputter coater (Polaron SC 502, Fisions instrument).

Specimen stubs are at this stage ready for microscopic observations in SEM. They were kept sheltered for dust and mechanical damages in a box. Specimens were observed in a JEOL scanning electron microscope (JSM 6400 Scanning Microscope, Japan).

#### **Preparation of *A. glauca* and Cyanobacteria for scanning electron microscopy:**

Five sterilized polycarbonate membrane filters (pore size 0.2 µm, 25 mm diameter) were placed onto an agar MEX dish. Each of these were then inoculated in the middle and incubated under standard cultivation conditions for 14 days. A second dish was prepared with

three filters placed onto the agar in a straight line, inoculated at one end of this creating a growth gradient. A third dish was inoculated in the middle without any filters, and proved to be the dish that displayed the best growth patterns for further preparations and observations in SEM. Preparations of *A. glauca* spores were performed by filtration of fixatives through a syringe assembly (figure 3.6). Polycarbonate filters were stamped to the desired size with a device special made for this purpose, and further placed inside a filtering unit. This unit containing the filters was then attached to a syringe (5 ml) at one end and closed with a needle penetrated into a rubber cork at the other. Fixatives could then be drawn through the assembly after spores first had been filtered onto the filters. When fixatives had passed through, the filter was left suspended in the respective solutions as demanded for fixation. Then the next fixative could be introduced after washing in buffer. This assured that the material was kept on the filters, and that they were always kept in the fixatives and did not dry out. Filters were not removed before the step of critical point drying. For all preparations of Cyanobacteria, the same procedure as for *A. glauca* spores was applied. Filters had cultures filtered onto them, or filaments were directly removed and placed onto them. The material was further prepared for SEM without any delay to keep the material fresh.



**Figure 3.5:** Syringe assemblage for SEM preparations.

### 3.5 Spore counting and sizing

#### 3.5.1 Beckman Coulter Multizer<sup>tm</sup> 3

For investigations and all measurements (i.e. counting and sizing) of fungal spores, and for the screening of all extracts, a BECKMAN COULTER Multizer<sup>tm</sup> 3 was applied. It is the most versatile particle size analyzer available, and provides size and volume distributions in number, volume, surface area and mass, with an overall range of 0.4 – 1200  $\mu\text{m}$ . Calibration was performed frequently. Between periods of experiments, the Coulter Counter was cleaned with 2.0 % bleach. The aperture was also cleaned with a brush to make sure no growth or bio



film attached. A saline electrolyte of 9 % NaCl (w/v) dissolved in distilled water (filtrated according to section 3.6) was used. Electrolyte solutions were stored at room temperature. For all handling and the analyses of samples, the software program developed for and installed in the companion computer associated and connected to the BECKMAN COULTER Multisizer<sup>tm</sup> 3 was applied. Background noise was measured separately, stored and subtracted from measures at start of each experiment (i.e. for all subsequent measures). All measures were performed in a window covering approximately 2.0-30.0  $\mu\text{m}$  (displayed as diameter to allow real-time microscopic comparison).



**Figure 3.6:** Beckman Coulter Multisizer<sup>tm</sup> 3.

### 3.5.2 Haemocytometer

In addition to the sizing and measurements of spores in the Multisizer, spore numbers and size distributions were determined using a counting chamber (haemocytometer, Thoma). This was done both to verify Multisizer measures and for purposes where it no longer could be applied. The chamber depth was 0.02 mm and the grid composed a square divided into 4 x 4 main squares ( $0.2 \times 0.2 \text{ mm} = 0.04 \text{ mm}^2$ ), that each was further subdivided into 4 x 4 smaller squares ( $0.05 \times 0.05 \text{ mm} = 0.0025 \text{ mm}^2$ ). All spores were counted using the main squares ( $8.0 \times 10^{-4} \text{ mm}^3$ ), as their size best fits the size dimension of these. Three lines, dividing each of them from each other, bordered the main squares. Counting a minimum of 5 squares was regarded in most cases as sufficient to obtain statistically representative material. As a rule, more than 300 spores were counted and only two of the four edges (above and to the right) were counted when spores were not situated within the square, but touched the boundary of the area. A sample of approximately  $1.0 \times 10^6 - 1.0 \times 10^8$  spores / ml was inserted under the cover glass and distributed evenly by the capillarity force of water. Adding 200  $\mu\text{l}$  in one channel did this. As far as possible, dead spores were separated and excluded by observation, and not included in the count.

### **3.6 Filtration of suspensions and liquids**

All spore suspensions and liquids (e.g. electrolyte, distilled water and medium) were filtered when required according to the following procedure: Filtration through a layer consisting of a glass micro fibre filter (Whatman GF/C, pore size 1.2  $\mu\text{m}$ , diameter 47 mm) overlaying a membrane filter (polycarbonate, pore size 0.2  $\mu\text{m}$ , diameter 47 mm). This was done to remove any debris in the solution that could interfere with Coulter Counter measurement. Thus the level of counting noise was reduced to a minimum. Filtration of MEX and DW (see below) were done without the membrane filter due to large amounts of particles. All filtrations were performed using a water jet pump (pressure ca. 0.8 Bar).

### **3.7 Dry weight**

Dry weight (DW) was used to determine the biomass of alga and spores. Algal cells and spores were collected on washed, dried (70°C for 24 hours, then kept in a desiccator containing a moisture absorber until needed) and pre-weighted (Mettler Toledo MT5 microbalance) filters by suction filtration (section 3.6). Samples were then washed to avoid errors originating from absorbed salts on the cell surface and salts present in intercellular water. A common technique employed is to wash marine alga with an isotonic solution of ammonium formate (0.5 M used here). This salt keeps the washing agent isotonic to the growth medium, preventing cell damage caused by osmotic pressure and loss of biomass due to the lysis of cells. Ammonium formate has a boiling temperature at 180°C, and sublimates easily when kept in a heater at a temperature above 60°C for 24 hours. This method is confirmed to be satisfactory for dry weight determination by a test comparing different washing agents (Zhu & Lee, 1997). DW of cyanobacteria and spores were washed with distilled water only (isotonic to the freshwater based Z8 medium and the MEX medium). For each culture 5 (all algae and some spore samples) or 3 parallels were run to diminish errors. Each parallel was washed with 5 ml 0.5 M ammonium formate or distilled water twice. Filters were then dried at 70°C for another 24 hours, and cooled down in the desiccator for minimum 2 hours (until stable weight at room temperature) before weight was measured. Biomass (mg/ml) was estimated as the difference of weight between filters before and after filtration, divided on the volume filtrated.

### **3.8 Preliminary research and tests**

A set of preliminary tests were performed initially to assess the assembly of the bioassay and the cultivation systems for the used organisms. This involved harvesting spores and determination of spore behaviours (i.e. swelling, germination and how to approach these experimentally in a bioassay) before the construction of the experimental set-up was developed. Primary, it had to be assured that spores could be grown, harvested and examined in the Multisizer. Second, a bioassay for screening had to be further developed and constructed from this information, and all parameters (e.g. analytically, densities, volumes, incubation conditions) had to be determined and established. Then it had to be established a proper method for investigating and assessing antifungal activity (i.e. inhibition of swelling and / or germination). Elements investigated were as following;

- Observations on growth and morphology of *A. glauca* (especially sporulation).
- Determination of age and method to harvest and store spores (section 3.1.4).
- Investigations and determination of swelling and germination in the Coulter and microscope. Preliminary approach to determine and assess bioassay targets.
- Final development of bioassay.
- Standardizing swelling and germination, and against known fungicides.

Further, a method for growth, harvesting, extraction and screening of algal biomass had to be established (section 3.2.6 and chapter 3.3). All of the following elements described in this chapter are the methodology that was developed from these preliminary tests.

### **3.9 Bioassay**

Construction of the bioassay was performed on the basis of preliminary research. It serves as the template used for all subsequent work with spore suspensions for tests on spore behaviours and screening. All experiments were carried out under the same standardized incubation conditions. *Absidia glauca* spore cultures were germinated in 50 ml Erlenmeyer flasks submerged in a temperature-controlled water bath (GFL type 1083, Gesellschaft für Labortechnik m.b.H. & Co.). Cultures were kept at a constant temperature of  $27.0 \pm 0.5^\circ\text{C}$ , and circulation was achieved by automatic shaking at a moderate speed. Main ingredient was growth medium (MEX), total volume depending on the experiment, either 4.0 or 4.25 ml.

Spore suspensions were prepared as described (section 3.1.5 and 3.1.6), and composed 0.5 ml of a total culture volume of 5 ml. This gave a further  $10^{-1}$  dilution of the suspension (approximately  $1.0 \times 10^8$  spores / ml), and the inoculum concentration became thus approximately  $1.0 \times 10^7$  spores / ml. The remaining 0.5 / 0.25 ml consisted either of a fungicide component, algal extract or any of the other tested substances. All diluting of these to the desired concentration was done with Milli Q water. All controls of each experiment had 0.5 ml of Millie Q water added instead of extract or other substances. This was also performed in general tests on spore behaviour, to make growth conditions equal in all experiments. Cultures were sealed with aluminium foil to avoid evaporation, and for general precautions. Sterile techniques were used. When constant temperature was reached, adding the spores to the Erlenmeyer flasks prepared with all experimental elements started the experiment. Before measures in the Coulter, samples were prepared by placing the whole culture in a sonicating bath (Ultrasonic cleaner, Branson 200) for 5 minutes before the sample (100  $\mu$ m diluted in 10 or 20 ml of electrolyte) was transferred into a beaker. Additional blending was performed by using a glass rod to stir the solution thoroughly to further homogenize right before measurements. This last step would dilute the suspension and after some mixing also provide further anti-clumping of the spores. For each volume measurement in the Coulter, three parallels were measured, and averages stored as the final result. Tests were terminated by adding 5 ml ethanol into the Erlenmeyer flask. Degree of inhibition was measured with the following formula (expressed as percentage of inhibition):

$$(1) \quad \text{Inhibition (\%)} = \frac{\text{Control } (\Delta \text{ vol.}) - \text{Treated } (\Delta \text{ vol.})}{\text{Control } (\Delta \text{ vol.})} \times 100$$

Controls were always averages of controls run simultaneously. Treated means cultures treated with extracts, known fungicides, DMSO or ethanol. Average difference in volume at start subtracted from the increase of volume after six hours, comprise delta volume ( $\Delta$  vol.).

### ***3.10 Standardization of spore swelling and germination***

Experiments performed to investigate spore swelling and germination were done in the described bioassay, and served foremost to standardize and set parameters to prepare the

assay for screening algal extracts. If necessary adjustments were revealed, these were performed (discussed in chapter 5), and the bioassay described in section 3.9 is the refined bioassay developed for this method. A clear definition on germination was needed. The most common definition in use is to consider a spore germinated if the length of this germ tube reaches one-half of the largest dimension of the spore.

***Definition;*** *A spore was defined germinated when the germination tube was half the diameter of the spore.*

### **3.10.1 Standardized swelling and germination**

Standardization of swelling and germination of spores were performed on newly harvested and untreated spores, i.e. without centrifugation, washing and re-suspending in Tween-80. This was achieved by directly transferring the harvested spores without delay or treatment to the bioassay, only suspended in filtered and autoclaved distilled water. Cultures consisted of 4.5 ml MEX medium and 0.5 ml spore suspension. Measurements were performed immediately after their addition to the medium, and three parallel cultures were run simultaneously. Samples were taken every 30 min. from each culture for 6 hours. It was considered important to investigate spores immediately upon their suspension, to investigate the immediate response. As the process of harvesting and transfers to the bioassay, before measured in the Coulter comprised about 10 – 15 min. suspended in water, a second test (“absolute zero”) was performed by measuring them at the very instant of harvest (washing spores from mycelium in a funnel trough cotton directly to a beaker). Temperature optimum for the swelling and germination of spores was not investigated due to the lack of a proper method and time, but was based on results from the same strain by Frøyen (Frøyen, 1975).

### **3.10.2 Storage of spores**

The viability of spores was tested during 14 days of storage. Two suspensions were kept, one used for incubation of cultures, the other being a back up. Spores were kept in a refrigerator at 4.0°C in darkness during the whole experiment, and were only removed for sampling. All handling and preparation of spores as previously described. 4.5 ml MEX was

added 0.5 ml suspension and incubated for 24 hours under standard bioassay conditions. Adding 100  $\mu$ l formaldehyde terminated growth immediately. The method and procedure followed for the fixative is one described by Jahn Thronsen (Thronsen, 1978). The agent is a 20 per cent aqueous solution of formaldehyde (made from a solution of diluted paraformaldehyde) neutralized with hexamethylenetramine (100 g to 1 litre of the 20 % solution). This gives a neutral to weakly alkaline solution. 2 ml of the 20 % formaldehyde solution was added to 100 ml of sample (100  $\mu$ l to 5 ml cultures), and the final concentration of the agent thus became 0.4 per cent. This served to eliminate all further growth. It was assumed that only total biomass was conserved, and changes in spore volumes might occur. This would affect volumetric properties when spore samples were measured in the Coulter, leading to wrong inhibition estimates if this preservative were used in the screening of algal extracts. The effect of formaldehyde on the volumes of spores after time of addition was therefore investigated. For each culture, investigations of spore size in the Coulter were performed to detect changes in volume at start and when germinating. Even though cultures were sealed, some loss of water by evaporation was expected. Tests with distilled water showed that about 100-200  $\mu$ l could be expected to evaporate after 24 hours, but as the spore number would remain the same, so also the concentration of nutrients, it was assumed that this would not affect growth conditions. Three parallels were run for each culture, and growth of the spore suspension was measured at day 1, 2, 3, 6, 8, 11 and 14. Dry weights (according to section 3.7) of spores were also measured (three parallels), and was subtracted from the growth measures to give actual growth. The suspension was also investigated in the light microscope to reveal any morphological changes. To obtain DW measurements, germinated spores in the growth medium had to be collected at a given time, set to 24 hours to allow some growth after germination, due to the expected low biomass accumulation before germination. Growth of mycelium is a linear process, and as long as all growth factors were kept constant, the thesis was that biomass at 24 hours should reflect the amount of germinated spores, and also the time of germination if a delay would occur.

### **3.10.3 Effects of spore concentrations in the bioassay**

Preliminary tests showed that a high spore density was to prefer over lower ones due to better measures in the Coulter. To examine to what extent the concentration would have any effects on the viability of the spores, 9 different spore concentrations were used.

Concentrations chosen were within the desired range giving optimal measurements in the Coulter instrument. Further tests would be performed from these results if needed. At start of the experiment, nine 50 ml Erlenmeyer flasks were prepared with 4.5 ml MEX medium and placed in the water bath. All handling of spore suspensions and incubation conditions were as previously described. DW of spores (5 parallels) was measured directly from the suspension. The spore concentration gradient was logarithmic and had the following concentrations;  $5.0 \times 10^7$ ,  $2.5 \times 10^7$ ,  $1.25 \times 10^7$ ,  $6.25 \times 10^6$ ,  $3.125 \times 10^6$ ,  $1.56 \times 10^6$ ,  $7.81 \times 10^5$ ,  $3.9 \times 10^5$  and  $1.95 \times 10^5$  spores / ml. Three parallels were run for each concentration. All cultures were started simultaneously, incubated for 24 hours before growth was stopped with paraformaldehyde. DW measures were performed as previously described. Results were expressed as the weight of un-swelled spores subtracted from the total amount of dry biomass in the culture, which gave total growth in 24 hours. This weight further divided on the concentration of spores in each culture gave an estimate on growth. With a test growth period of 24 hours one could expect that the medium would become growth limiting, especially at the higher spore concentrations. Germination after 6 hours was measured in the Coulter. Experimental aim of this section and the previous (section 3.10.2) served also as a combined effort to assess DW as a usable tool for the method.

### **3.11 Standardization with known fungicides**

To be able to interpret and express screening results, and to investigate inhibiting properties on swelling spores, two known fungicides were applied to standardize antifungal susceptibility. The agents used were the potassium salt of **sorbic acid** ( $C_6H_8O_2$  /  $C_6H_7KO_2$ ) (Fluka Chemika 85520, Sorbic acid, Potassium salt, Assay >99%) and the sodium salt of **benzoic acid** (sodium benzoate) ( $C_6H_5COOH$  /  $NaC_6H_5COO$ ) (Norsk medisinaldepot, Nariri Bonzoas, Natriumbenzoat). Sorbic acid is a polyunsaturated fatty acid, and is a fungicide widely used in the food industry as a food preservative. It reacts with potassium and calcium to form their salts. It is a tasteless acid naturally found in the fruit of the mountain ash (genus *Sorbus*), widely used to maintain microbial stability in foods and beverages. Benzoic acid is the simplest aromatic carboxylic acid containing a carboxylic group bonded directly to a benzene ring. It is slightly soluble in water, and is then weakly acidic. For preservative applications in foods, drugs and cosmetics, it is converted into its salts and esters. Sodium



benzoate is much more soluble in water than is Benzoic acid, and is widely used as one of the principle weak acid antimicrobial preservatives in the food and beverage industry (concentrations limited usually not exceeding 0.1 %), used to preserve especially acid food (converts to benzoic acid in acidic solutions, normally used in environments below pH 3.6), but also cosmetics, deodorants and toothpaste. It occurs naturally in many plants and resins, and is also detected in animals. Benzoic acid possesses antimicrobial properties due to its interference with energy metabolism (discussion). A 1.0 % stock solution (w/v) of each of the two agents was prepared in a standard liquid MEX medium. These solutions were the basic for further dilutions and were kept cooled in refrigerator at 4 °C until needed. Preliminary investigations establish the gradient used to standardize inhibition. Controls without any agents, but with H<sub>2</sub>O were always run simultaneously in each experiment. Covering the range from no inhibition to full inhibition completed standardization. Total culture volume was 5.0 ml, whereas 4.0 ml was MEX medium, 0.5 ml was spore suspension and the remaining 0.5 ml fungicide solution. Inhibition was measured after 2, 4, 6 and 24 hours.

### ***3.12 Extracting solvent control assays***

A set of control assays were performed to accomplish a standardisation of the screening results of algal extracts. This was done to assess effects on the spores of the solvent used in extracts, to make sure that none of the ingredients used for extraction or preparation of the bioassays were affecting spores. **Ethanol** (C<sub>2</sub>H<sub>5</sub>OH, 98% Arcus AS) was used as solvent in the lipophilic extracts of greenalgae. **DMSO** (Dimethyl Sulfoxide, C<sub>2</sub>H<sub>6</sub>SO) (SIGMA min. 99.9 %) was assessed used in lipophilic extracts of the Cyanophytes, as it was searched to find an alternative solvent for ethanol. To investigate the effect on germinating spores, each solvent was diluted to 7 different concentrations with Milli-Q water. The bioassay was composed of a total culture volume of 5.0 ml, of which MEX constituted 4.0 ml (not 4.25 ml as used for other tests, due to solvent concentrations above 5.0 %) and spore suspension 0.5 ml. All growth conditions and preparations were as previously described for the bioassay. Measurements were performed at the start of the experiment (time 0) and after 2, 4 and 6 hours. Additional measurements were performed after 24 hours of incubation to assess the stability of inhibition. Time series had an interval of 10 minutes between the start of each culture. The ethanol and DMSO gradients were; 0.1%, 0.5%, 1.0%, 2.5%, 5.0%, 7.5% and

10.0%. Each solvent concentration was added with Milli-Q water in a total of 0.5 ml to the bioassay. The experiment was started by adding the spores. **Acetone** and **Methanol** was briefly investigated as candidates besides DMSO to replace ethanol. These were tested only in two concentrations (1.0 % and 5.0 %) at time 0, 6 and 24 hours.

### **3.13 Screening for bioactivity**

All screenings were performed according to the following composition based on the results presented in section 4. Total culture volume was 5 ml, of which the different basic ingredients constituted a fixed volume and were mixed together in the following ratio:

- 4.25 ml MEX medium.
- 0.25 ml algal extract (H / L)
- 0.50 ml *A. glauca* spore suspension.

The controls had 0.25 ml of Milli Q water added instead of algal extracts. For each algae screened, one lipophilic (**L**) and one hydrophilic (**H**) extract were tested in two different concentrations, 1 and 5% of total culture volume (250 µl extract added equals 5%, 50 µl equals the 1% concentrations with 200 µl of additional Milli-Q water. Extracts of cyanophytes were concentrated to 200 µl, therefore adding only 125 and 25µl adjusted to total 250 µl using Milli-Q water gave the same extract to assay ratio as for greenalgae). All extracts were measured at time 0 and after 6 hours (terminate end of experiment), the control cultures also in periods in between to ensure that swelling proceeded as normal.

## 4 RESULTS

In this section, general growth and morphological / physiological observations of the experimental organisms will first be presented, followed by a description of the work leading to the final construction of the bioassay employed. Then all results regarding spore investigations will be presented (assessment of spore handling, spore behaviour and effects of the different additives), before ending with a presentation of the screening results.

### 4.1 *Microalgal growth*

#### 4.1.1 Greenalgae

Greenalgae were mostly fast growing, with some few exceptions. The biomass produced and harvested is listed in table 4.1 together with days of growth. Slow growing species were allowed to stand for an extended time before they were harvested.

As can be read from table 4.1, all cultures produced sufficient biomass for screening (a minimum of 50.0 mg were needed for preparation of extracts), except strain G 18, which barely produced enough for screening. On average, 62 % of the biomass harvested was preserved as freeze dried biomass (effective harvest) when compared to that obtained directly as dry weight from the fresh culture. Growth efficiency of the different Chlorophytes can be interpreted from table 4.1, when growth rates are compared with culture densities at harvest. It should be noted that inocula varied slightly (10-20 ml of different concentrations), and might have affected growth rates. Only G 61 and G 63 are regarded as slow growing species (28 days respectively). Tendency to migrate out of the culture medium was observed (especially G 61), and was considered as a sign of non-vigorous growth. Fast growing species were G 8, G 45, G 46 (all harvested after 11 days) and G 48 (8 days). Densities obtained by these algae are higher than what usually are observed when growing algae. The growth of G 45 and G 46 was especially smooth without any clumping, growth on the glass-walls or above the medium, nor at the bottom. Remaining species displayed a medium to low growth rate. G 13 tended to be slightly filamentous. One strain (ISO X, designated by the author) was

isolated from contaminated cyanophycean cultures. This strain was cultured in the same manner as all the cyanobacterial strains (i.e. closed system, page 34).

**TABLE 4.1**  
**Greenalgae strains.**

<b>BIOMASS PRODUCED BY GREENALGAE</b>					
<b>Strain</b>	<b>Days of growth</b>	<b>Biomass mg/ml (dry weight)</b>	<b>Biomass mg/ml (freeze-dried)</b>	<b>Effective harvest</b>	<b>Total biomass obtained (mg)</b>
<b>G 8</b>	11	3.891	1.417	36.4 %	198.4
<b>G 9</b>	11	2.150	0.827	38.5 %	124.0
<b>G 13</b>	8	0.850	0.590	68.9 %	87.7
<b>G 17</b>	10	0.652	0.450	69.0 %	58.5
<b>G 18</b>	10	0.432	0.298	69.0 %	35.7
<b>G 20</b>	8	1.520	1.042	68.6 %	156.3
<b>G 23</b>	8	1.720	0.640	37.1 %	95.8
<b>G 44</b>	10	0.689	0.605	87.8 %	72.6
<b>G 45</b>	11	4.160	2.548	61.3 %	331.3
<b>G 46</b>	11	4.347	2.614	60.1 %	339.8
<b>G 48</b>	8	3.190	1.624	50.9 %	243.6
<b>G 52</b>	13	0.950	0.442	46.5 %	88.3
<b>G 61</b>	28	1.550	1.210	78.1 %	204.9
<b>G 63</b>	28	1.710	1.390	81.3 %	208.3
<b>G73</b>	10	0.829	0.637	76.8 %	76.4
<b>ISO X</b>	23	1.287	1.207	93.8 %	330.8

Table 4.1 presents biomass productions of all the greenalgae used.

### 4.1.2 Cyanobacteria

All the different strains grown were inherently slow growing. They also had to be grown at low light intensities. Their stock cultures were also exceptionally slow growing, and recultivation could therefore be done at 3-4 month intervals or even later. To increase growth rates without stressing the algae, they were slowly adapted to higher light and temperature intensities (as described in section 3.2.4). This optimised growth by slowly leading the algae into adaptation to the new growth environment and entering the exponential growth phase without stressing them but still allowing photoadaptation for further growth. Table 4.2 list time of cultivation and biomass obtained from the different strains at harvest. Growth was generally better in room temperature and in this illumination regime than in the refrigerator for all stock cultures of the five strains. Throughout the year of cultivating these cyanobacteria, growth patterns, both morphologically and physiologically changed. 209

displayed a much better growth than 207 in the last months, opposite to the initiation of cultivation where 207 was the better growing one. None of the cultures produced gas-vacuoles, making the harvesting simple.

**TABLE 4.2**  
**Cyanobacteria strains.**

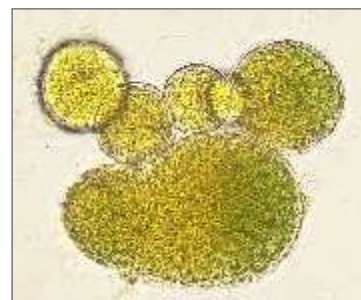
**BIOMASS PRODUCED BY CYANOBACTERIA**

Strain	Day of growth	Biomass mg/ml (dry weight)	Biomass mg/ml (freeze-dried)	Effective harvest	Total biomass obtained (mg)
<b>192</b>	28	1.470	1.211	82.4 %	387.3
	25	2.220	1.816	81.8 %	468.6
	37	1.900	1.760	92.6 %	351.0
<b>199</b>	36	1.660	1.498	90.2 %	367.0
*	40	—	1.089	—	217.0
<b>181</b>	53	1.030	0.789	77.5 %	247.5
	31	1.080	0.788	73.0 %	191.4
*	18	1.350	-----	-----	-----
*	40	—	0.532	—	106.0
<b>207</b>	33	—	1.080	—	324.1
	47	—	0.560	—	137.0
<b>209</b>	33	—	1.383	—	408.0
	47	—	0.980	—	238.0

NOTE: The first presented biomass of each species represents the biomass applied for all subsequent work. The following results represent biomass production and harvesting that were preliminary to the final production for further extractions, and is only included in the table for comparison of results. \* indicate that an open cultivation system was used (page 34).

The first attempt in cultivating these strains lead to the contamination of several of the tubes. Due to these problems, it was decided to grow them in the closed system. Also high bacterial growth in the cultures was a major problem. All problems were related to the low growth rates, and it was only after more than 6 month's of cultivation it was possible to harvest biomass for screening. In earlier experiments Hansen and Knutsen experienced very slow initial growth by cultures obtained from NIVA. Using closed tubes and conditioning of the strains solved growth problems. Following is a general description of the five cyanophytes screened in this work:

**Nostoc sp.192:** Fast growing when first adapted to growth conditions. Light filters necessary for the first step of cultivation, but could be removed after a short time of growth. When new cultures were started from exponential growing cultures, less exo-polysaccharide were produced than initially. Filaments tended to produce small clusters, but these were easily shaken into smaller fragments, and were not regarded to be large enough to make dry-weight measurements an improper means of expressing biomass. When cultures became dense, the strain migrated out of the medium and created a layer a few centimetres above the surface that had to be shaken into the medium again daily. Therefore, cultures were harvested at this point, as it indicated that no more growth could be obtained, and cultures would produce high amounts of exo-polysaccharides.



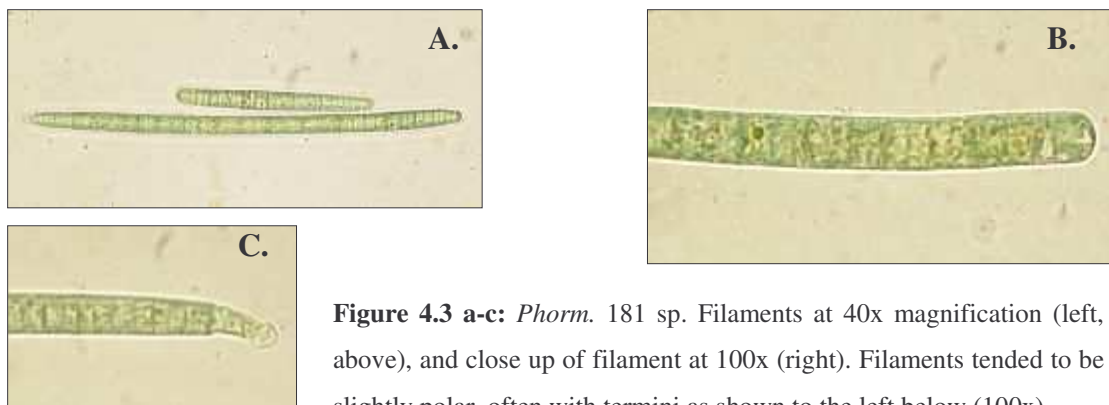
**Figure 4.1:** *Nostoc* 192 sp. (40 x magnification).

**Merismopedia sp.199:** Slow growing. When cultures reached some densities, they were growing in large “leaves”, two dimensionally aggregates of cells that could be as large as 2-3 cm<sup>2</sup>. These aggregates also varied in coloration, suggesting a uniform growth within, but the explanation for differences between filaments are tentative. The last culture (the one used for preparation of extracts) did however not display such growth patterns. Here cell aggregates were much smaller (typically only 4-16 cells), and thus more evenly distributed. No differences in pigmentation was observed, all were fresh green.



**Figure 4.2:** *Meris. Angularis* 199 sp. (100 x magnification).

**Phormidium sp. 181:** Fast growing when first adapted to growth conditions. Filters absolutely necessary for the first step of cultivation, and the adaptation period lasted for a long duration of time when inoculums were prepared from stocks. Cultures consisted of filaments (3-15 μm long) that did not cluster, and dry-weight measurements were obtained without any problems. The strain gained a fresh green coloration in its exponential growth phase, but was pale and more or less without pigmentation if not adapted to growth conditions under low light intensities.



**Figure 4.3 a-c:** *Phorm.* 181 sp. Filaments at 40x magnification (left, above), and close up of filament at 100x (right). Filaments tended to be slightly polar, often with termini as shown to the left below (100x).

**Phormidium sp. 207:**

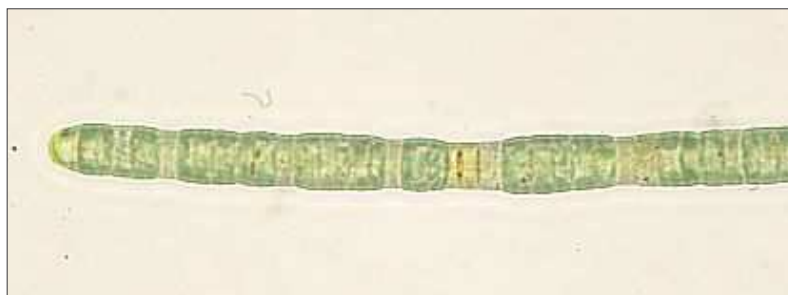
Slow growing. Filters were absolutely necessary for the first step of cultivation. Growth characterized by filaments of dark green pigmentation growing mostly on the walls of the growth tube, covering this as a sheet. This created mats of alga, and in the last stage of growing, some crude clusters were also to be found to a larger extend growing free in the medium. The last cultures did however grow much faster, probably better adapted.



**Figure 4.4 a-b:** *Phorm.* 207 sp. at 40 x (right) and 100 x (left) magnification.

**Phormidium sp. 209:**

Slow growing. Filters necessary for the first step of cultivation. Growth characterized by filaments of some dark green pigmentation growing mostly on the walls of the growth tube. Growth and morphology much similar to *Phormidium* sp. 207 but differed some in pigmentation. This created mats of alga, and in the end of exponential phase, many clusters of algae were found in the culture.



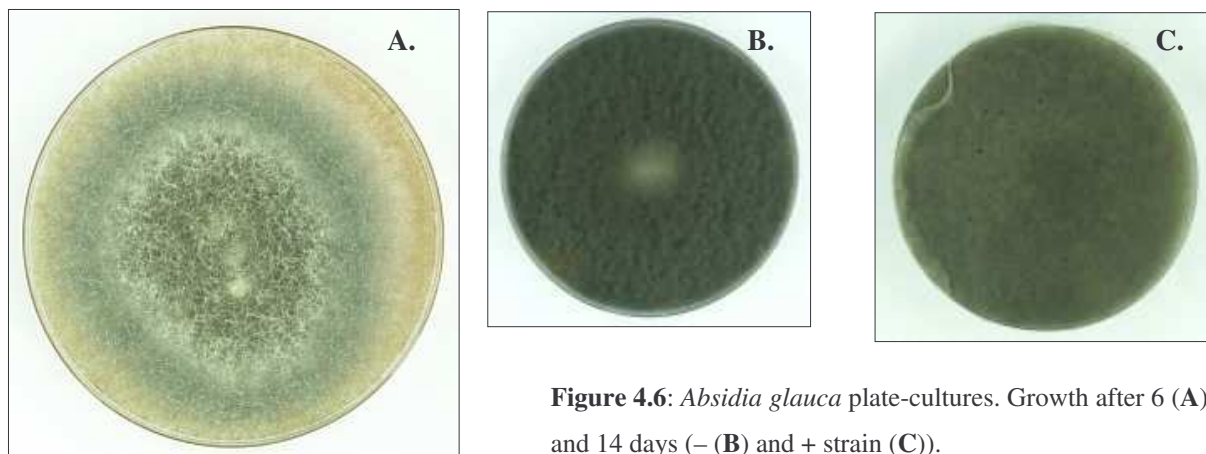
**Figure 4.5:** *Phorm.* 209 sp. at 100x magnification.



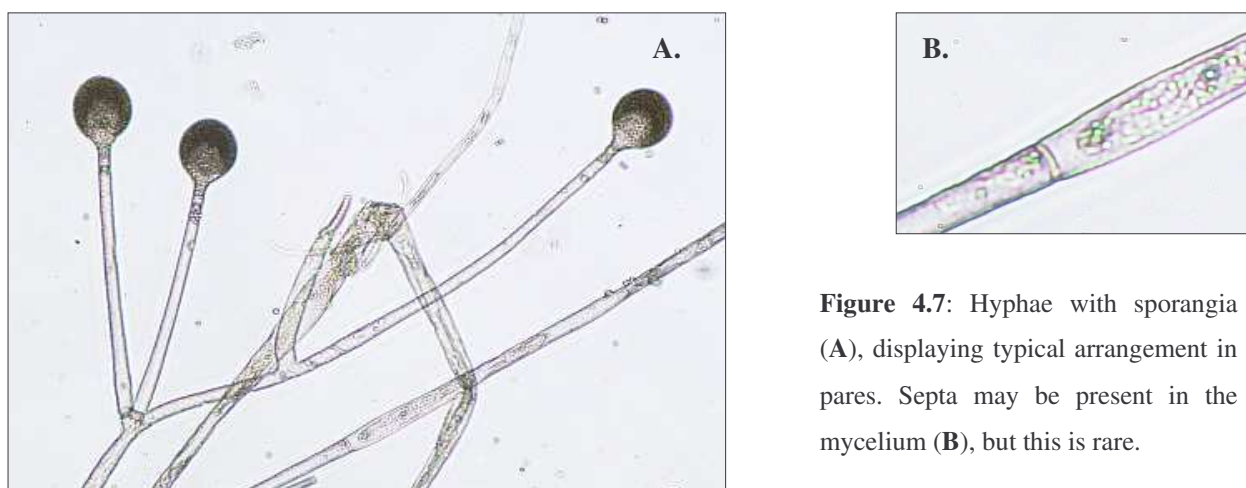
## 4.2 Fungal growth

Growth proceeded as expected for the genus (see section 2.2). First white hyphae emerged from the site of inoculation, and stayed at the edge of the growing colony. Older hyphae would in a matter of few days become more lightly green, until the first formation of sporangiophores, when the colour turned much darker green (figure 4.6). Stolons rose high above the colony, touching the underside of the lid of the Petri dish. Hyphae were almost without septa and contained many nuclei. A slightly different growth pattern was observed for the two different mating types. The mating type designated + had a visibly stronger growth, filling the entire dish. This was not the case for the – type, which showed a slower growth, almost not reaching the lid. Despite this, the – type produced a higher amount of spores, and thus became the obvious choice for further experiments. This decision was also based on the experience that these spores were evidently easier to separate from each other, and had a more even spore size distribution (see below). It was decided that cultures would be incubated for 14 days based on general growth observations (i.e. mainly based on the sporulation process). This served to standardize the method, as all spores used were produced and harvested under the same conditions. A seasonal-dependent growth pattern was observed for both strains, most expressed in the – mating type. This was displayed as a much denser and higher mycelial growth during spring and early summer months. No change in incubation conditions occurred, and the phenomenon could thus not be explained by known external factors. The plates were closed from all external light and temperature sources. However, seasonal growth variation did not change the properties of the spores, which were harvested in the same quantities as before, and seemingly possessed the same qualities also though this was not further studied. It is worth noticing as a factor that could possibly affect results. Based on, and due to differences in properties outlined here and below, the – mating type was selected for all further investigations and tests. After considering the two strains, selection was based especially on spore production, behaviour and handling. Mating types were checked by cross breeding, and proved to be of the same species. This was achieved by inoculating on the same agar dish but at different sides, allowing them to grow towards each other. The formation of a zygosporangium confirmed that they were actually two mating types of the same species.

**ABSIDIA GLAUCA MYCELIUM, SPORANGIA AND SPORANGIOSPORES:**



**Figure 4.6:** *Absidia glauca* plate-cultures. Growth after 6 (A) and 14 days (– (B) and + strain (C)).



**Figure 4.7:** Hyphae with sporangia (A), displaying typical arrangement in pares. Septa may be present in the mycelium (B), but this is rare.



**Figure 4.8** (left) **and 4.9** (right): Germinated sporangiospores, approximately 6-7 hours after initiation of the swelling phase. Several dark spots (organelles and nuclei) (4.8 and 4.9) are present inside the spores and the mycelium, and spore walls are clearly visible (4.9).

**Figure 4.10:** Fully matured sporangium, yet to release its sporangiospores.

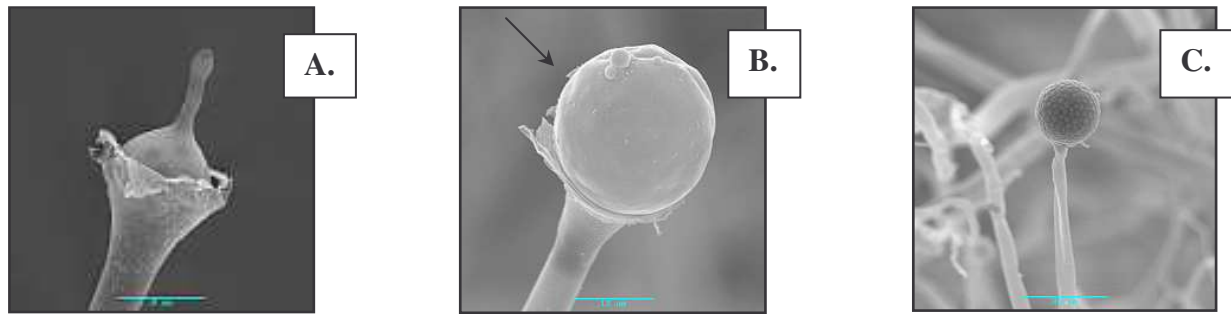


### 4.3 Electron microscopic preparations and observations

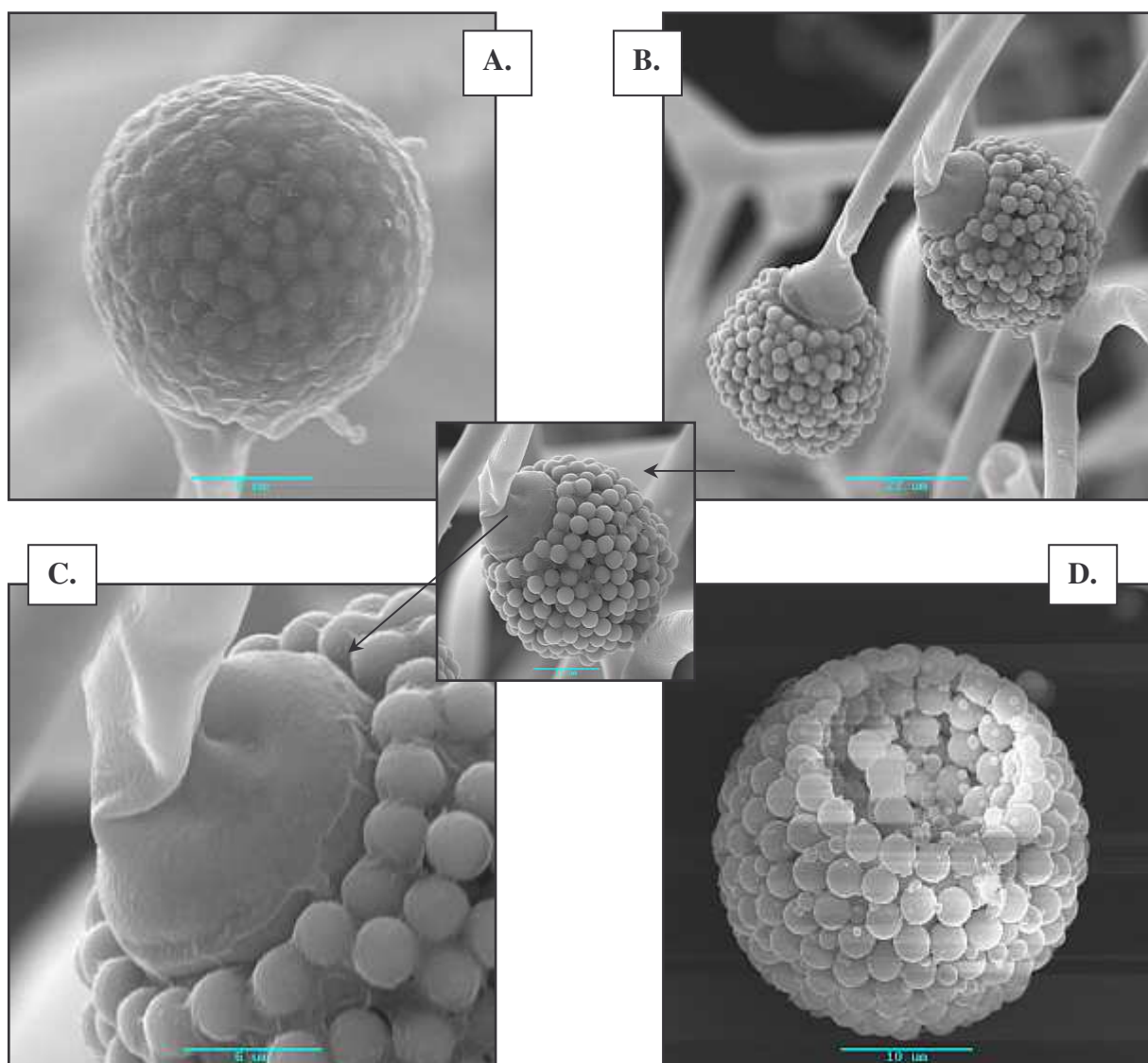
All samples prepared were studied in the SEM. Pictures were successfully taken of all species except *Phorm.* 207 and *Nostoc* 192, and images of spores, sporangia and mycelium were successfully obtained in addition to details of the cyanobacterial strains.

It proved to be the *A. glauca* strain that was not prepared according to the standard method (i.e. grown on filters) that was best qualified and used for further preparations for SEM. These parallels proved to be the better choice due to the natural occurrence of a strong mat of leathery consistence between the agar medium and the growing mycelium. The presence of this mat under the culture, made the mycelium coherent and could therefore easily be removed from the surface of the agar and be further divided into suitable fractions. This had the advantage of producing uniform growth samples, instead of filters with more mycelium and stolons than sporulated sporangia, as was the case for the agar dish where the *A. glauca* strain had been inoculated at the middle of each filter. Growth here, however, even though there was a “with mark” at the sight of inoculation in the middle of each filter, was firm and contained enough sporulated mycelium at the edges for preparations. This produced five parallels of good quality, and the gradient dish therefore became redundant.

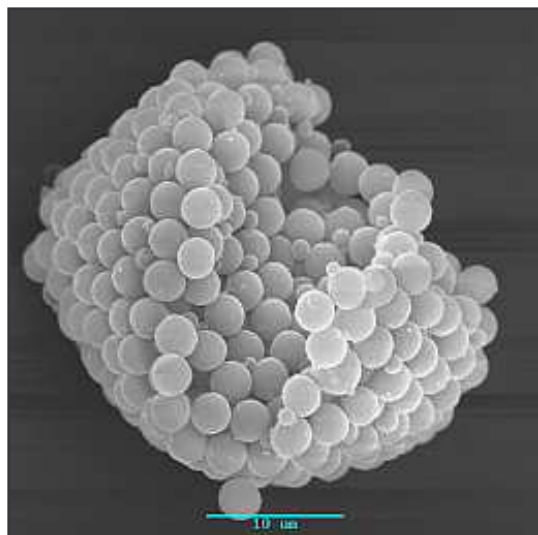
Some problems emerged during preparations of samples during the step of critical point drying and also during the coating procedure. The Sputter coater failed repeatedly. This led to the uncalculated consuming of time, and consequently samples had to be stored for a longer duration in 100% ethanol before they were dried and coated (approximately 24 hours past after drying before coating could be accomplished). This should however not lead to any severe damage or altering of the samples. When critical point drying was performed on *A. glauca* and the two cyanobacteria *Phormidium* sp. 207 and 209, a leakage in the pressure chamber appeared, and the CO<sub>2</sub> gas escaped. It was therefore not possible to raise the pressure in the chamber after the ethanol had been exchanged with liquid CO<sub>2</sub>, and the process failed to dry the material in a proper way. This might have caused damage to the material due to tensions created in the liquid / gas interface, consequently leading to the collapse of internal cavities. However, no severe damage was observed for any of the three specimens. As a consequence of lack of time no images of spores in different phases of the swelling process were performed as scheduled. Images of un-swelled spores from the mycelium preservatives were therefore regarded as satisfactory.



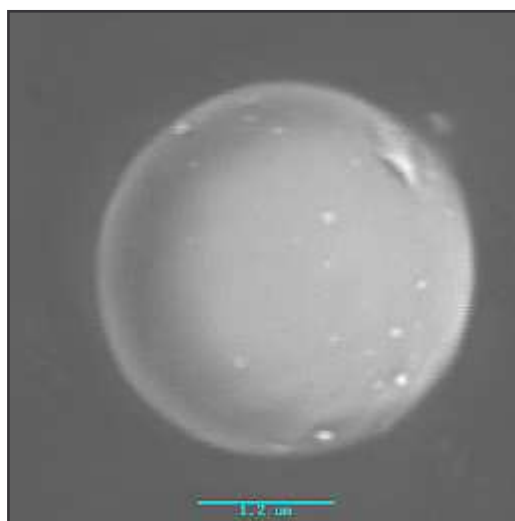
**Figure 4.11 A-C:** Hyphae where a sporangium has been located (A/B). Left is the sterile columella. A spheroid mass covers the columella and mature into sporangiospores (C). Sizes of objects are 8, 12 and 30  $\mu\text{m}$ , respectively (blue line).



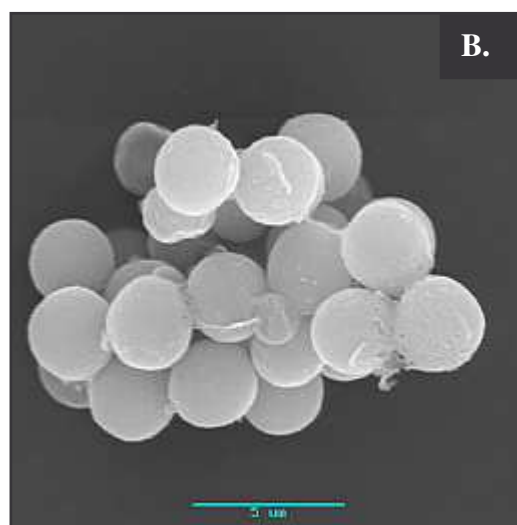
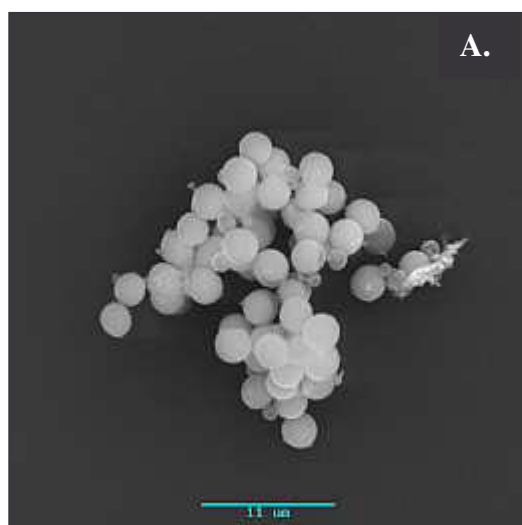
**Figure 4.12. A-D:** Sporangium of *A. glauca*. Spores are kept within the sporangium covered by a sheet (A). They occur most often in pares (B) that each bears several hundred sporangiospores situated on the sterile columella (C), which penetrate inside the spheroid sporangium (D and figure 4.1.B). Objects are 8, 22, 6 and 10  $\mu\text{m}$ , respectively (blue line).



**Figure 4.13:** Cluster of newly released sporangiospores. Under normal conditions they would have been scattered, not attached. The presence of some smaller spores is visible. Size of assemblage is 10  $\mu\text{m}$  (blue line).



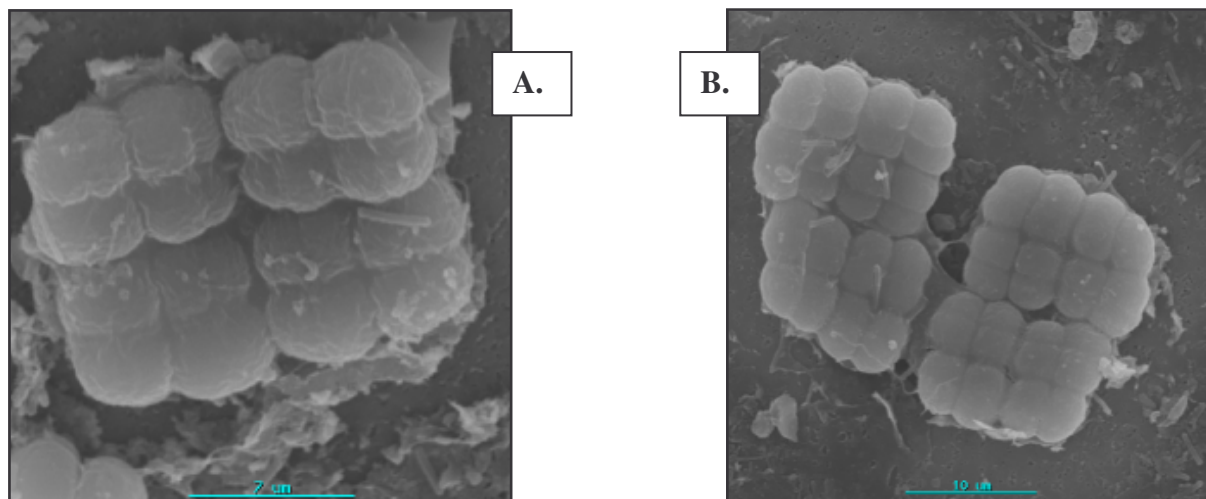
**Figure 4.14:** Sporangiospore. The surface is smooth; no other morphological features are visible. Protective mucus is present, and is most likely causing spores to cling in pairs or clusters. Size is 1.2  $\mu\text{m}$  (blue line).



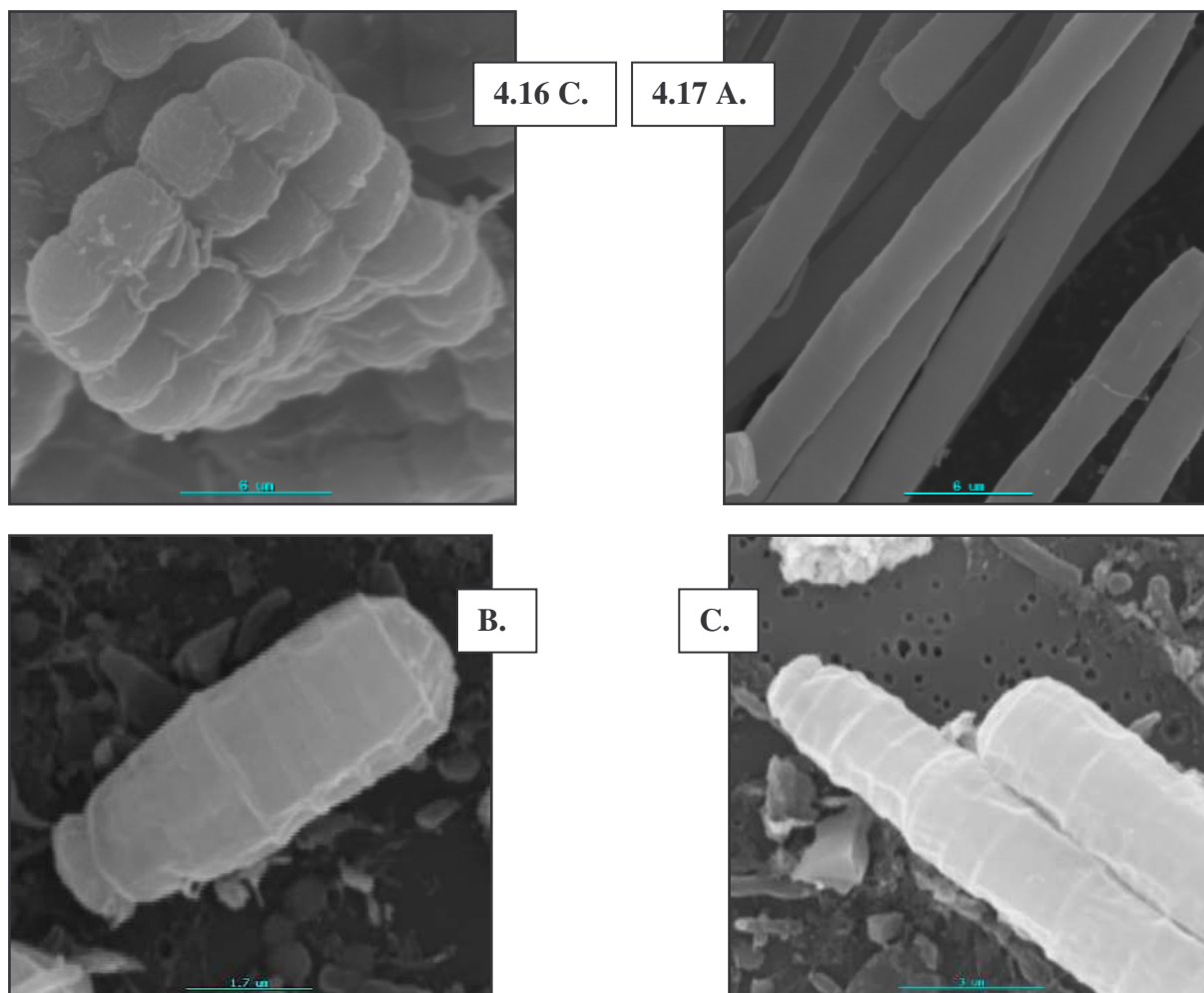
**Figure 4.15 A and B:** Two pictures showing sporangiospores, 11 and 5  $\mu\text{m}$  (blue line).

**Comment to SEM images:** Samples may suffer from damages or morphological changes, caused by the forces that the electron beam exposes them for in the SEM. This can lead to the shrinkage of surfaces, like cell membranes and sheets. The risk is higher the longer the material is exposed under the electron beam. This was avoided as far as possible, but must be considered when studying details of the material. Comments under each picture generally describe the true nature of the sample.

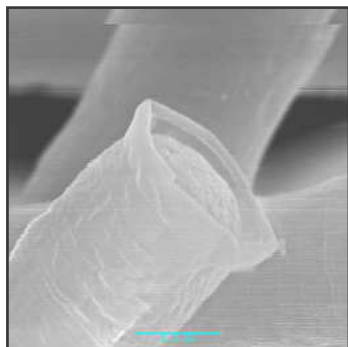




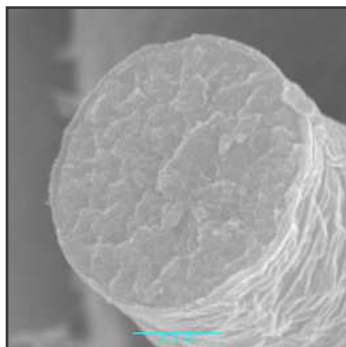
**Figure 4.16 A-C:** *Merismopedia Angularis* 199. Cell aggregates grouped in 4x4 due to growth in two planes. Cells are within sheets, clearly visible in picture C. Viewed from the side cells prove to be rounded (C). Size bars are 7, 10 and 6  $\mu\text{m}$ , respectively (blue line).



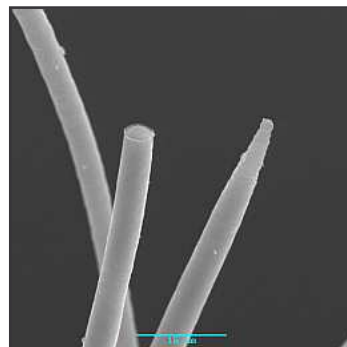
**Figure 4.17 A-C:** *Phormidium* sp. 181. Filaments consisting of disk-shaped cells covered within a sheet, fragmented filaments displaying typical polar orientation (B). Ends of filaments are either a growth region (C) or flat broken region after fragmentation. Size bars are 6, 1.7 and 3  $\mu\text{m}$ , respectively (blue line).



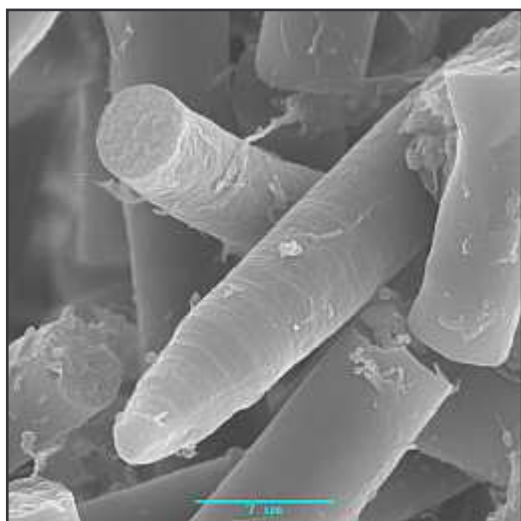
**Figure 4.18:** *Phorm.* sp. 209. Fragmented filament, where sheet is visible.



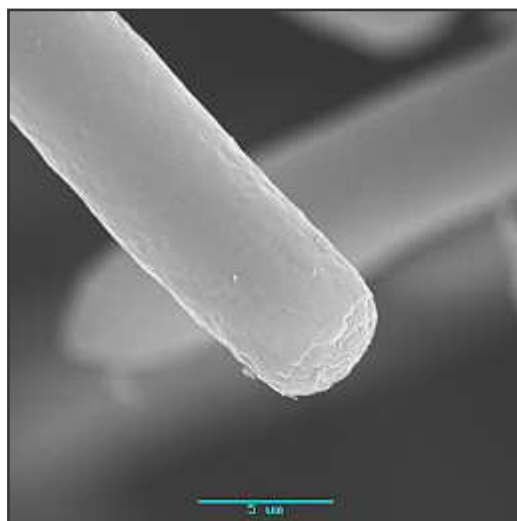
**Figure 4.19:** *Phorm.* sp. 209. Cross section of a filament.



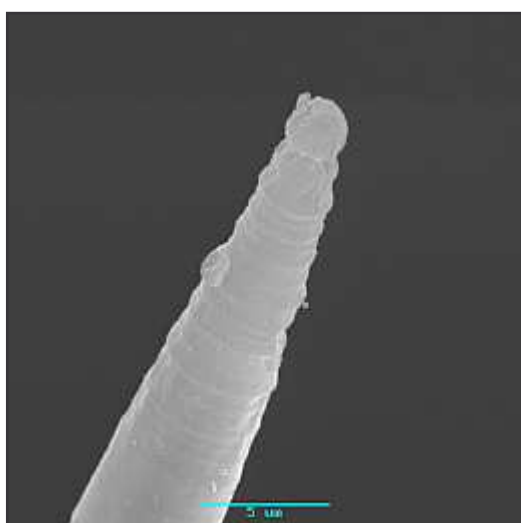
**Figure 4.20:** *Phorm.* sp. 209 filaments.



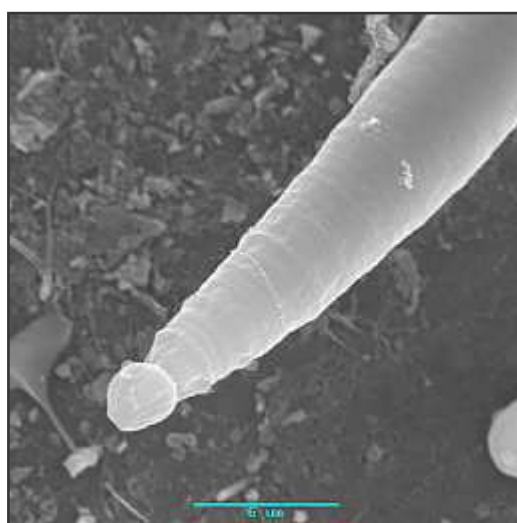
**Figure 4.21:** *Phormidium* sp. 209.



**Figure 4.22:** *Phormidium* sp. 209.



**Figure 4.23:** *Phorm.* sp. 209 filament with typical polar end.



**Figure 4.24:** *Phormidium* sp. 209. Characteristic end of an unbroken filament.



#### 4.4 Experimental set-up of the Bioassay

Growth condition needed to be optimal for incubation of the spores. All growth conditions for the production, harvest and preparation of spore suspension were already standardized (see section 3). The spore bioassay had to be done at the optimal temperature and with an optimal growth medium, and in suitable containers. Reproducible sampling of spores for measurements required homogenous spore suspensions. Without the necessary temperature gradient system to establish the optimum temperature, that found by Frøyen (1975) for the same strain was used, i.e. 27.0°C. The use of air bubbling to keep the spores in suspension was first tried (figure 4.25), but was abandoned after many trials due to problems like spores accumulating at the culture surface due to increasing buoyancy during the late stages of swelling, as well as accumulation of spores on the tube walls above the culture (mostly due to Tween 80). Most of the spore germination tests described in this Thesis was run in the air bubbling assay, so it was well tested before the decision to abandon it was taken. The assay system was therefore changed from glass tubes with aeration, to shaking of Erlenmeyer flasks in a temperature controlled shaken water bath. In the final assay a small culture volume, i.e. 5.0 ml, was used with 50 ml Erlenmeyer flasks, with a bottom diameter of 45 mm. Small volumes were also considered necessary to allow the small amounts of extracts to be screened efficiency, and to reduce space and consumption of media and other ingredients. None of the many tests done to reach the final procedure are described here.



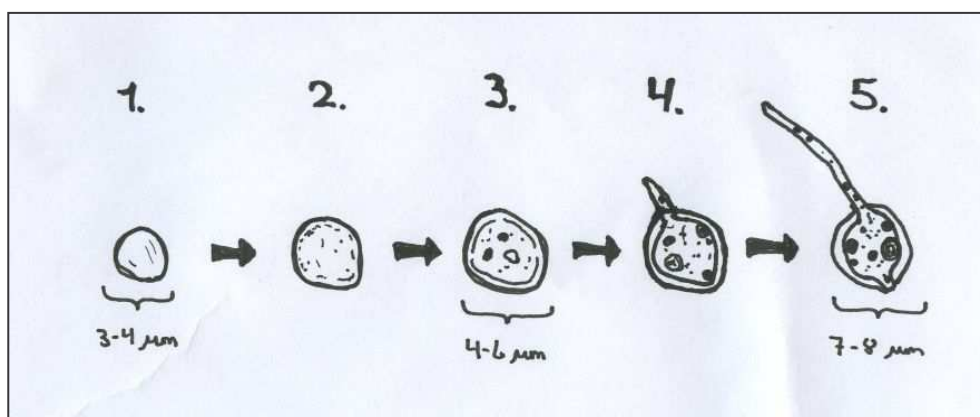
**Figure 4.25:** Preliminary construction of the Bioassay implemented the use of air to provide mixing.

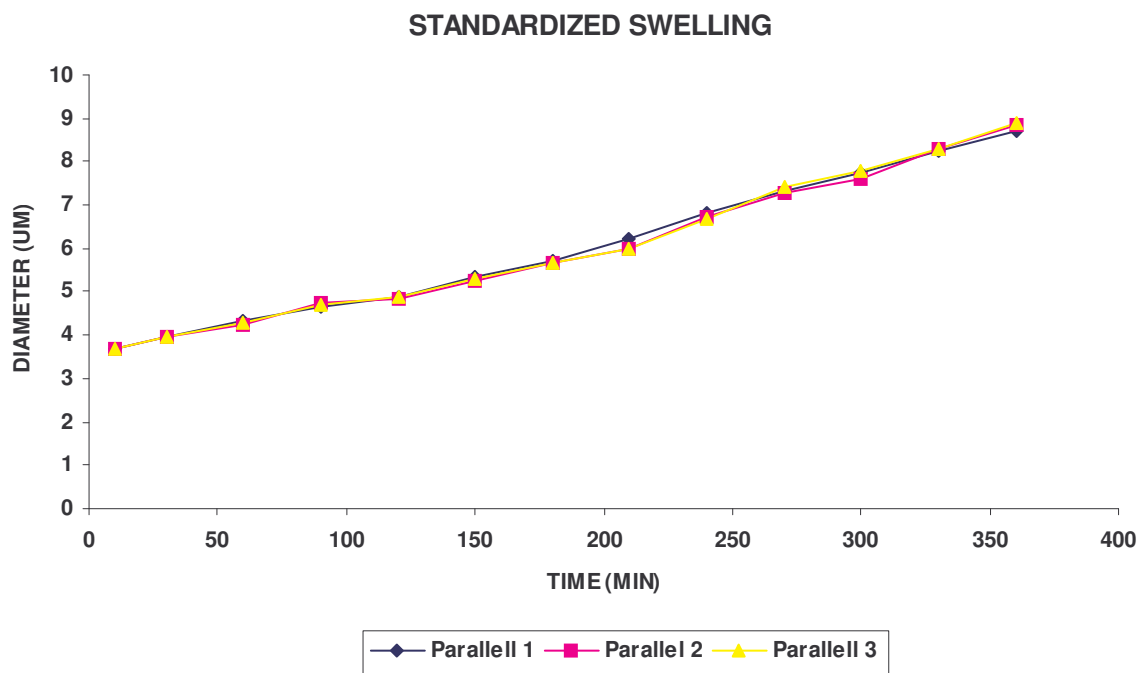
## 4.5 Spore swelling and germination

### 4.5.1 Swelling and germination

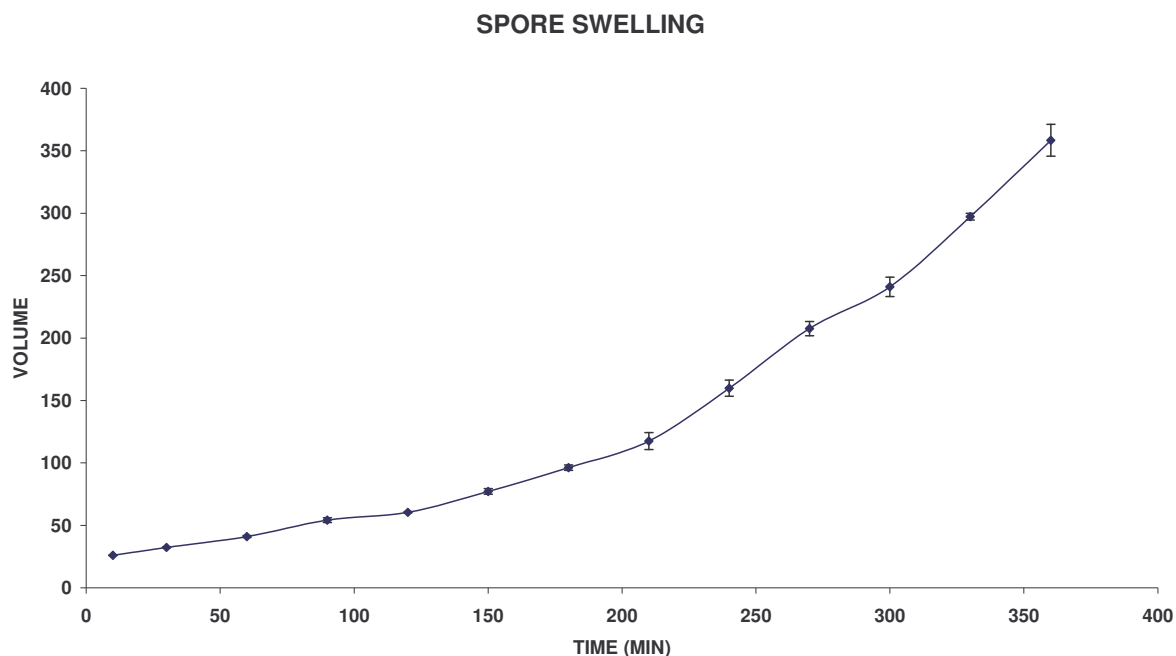
Swelling of *Absidia glauca* spores began immediately when the spores were introduced to the growth medium. No lag period was observed. No swelling was seen when spores were introduced to distilled water, even after 14 days of storage (figure 4.32). It was not observed any difference in volumes between spores investigated at the moment of harvest (test referred to as “absolutely zero”) and for those stored for 7 days. This was valid for both strains (-/+). Swelling was observed as a linear increase of diameter with time, or a logarithmic increase of volume with time. After approximately 2-3 hours, the first signs of the formation of the second spore wall were visible in the light microscope, and then dark spots (1-4 nuclei) would be visible inside the spores (figure 4.26). Prior to germination, these would move towards the new inner wall and settle there, before they became the starting point of the formation of germination tubes. These germination tubes would form from the inner wall, and then break through the outer wall after 4-5 hours. One to three (or in some cases even more) germination tubes would normally germinate in each spore. Germination was initiated after approximately 5-6 hours after introduction to the growth medium, whereas swelling would impede (figure 4.29). Growth proceeded then by rapid extension of mycelium. Results of the swelling progression can be viewed in figure 4.27 and 4.28. Spores increased their volume by approximately  $330 \mu\text{m}^3$  in a matter of 5-6 hours (figure 4.30). Swelling measured in the Coulter showed a smooth size distribution till spores entered the germination phase, whereas sizes would be greatly influenced by the presence of germination tubes (figure 4.31).

**Figure 4.26:** The different stages of spore swelling and germination; (1) a spore before (2) and after swelling has started. (3) First visible formation of a second inner wall and organelles, (4) the spore then germinates and (5) the germ tube grows as a hyphae.



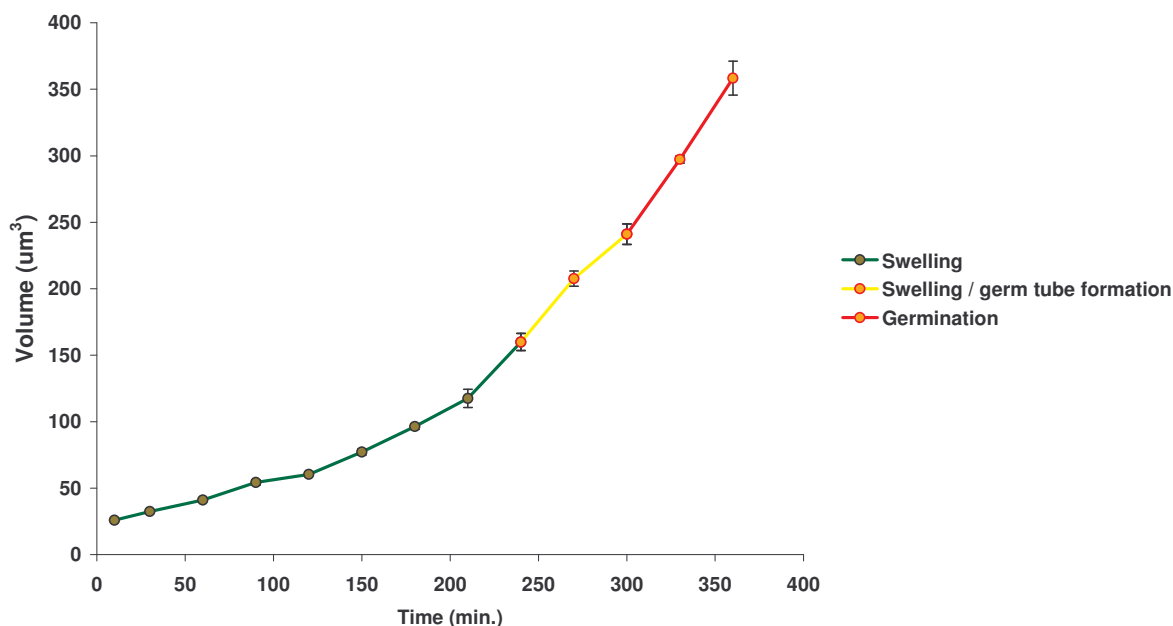


**Figure 4.27:** Swelling of spores. Three parallel cultures were measured every 30 minutes over a period of 6 hours. Increase of the diameter of spores is a linear function with time, or a logarithmic increase of volume with time (see below, figure 4.28).



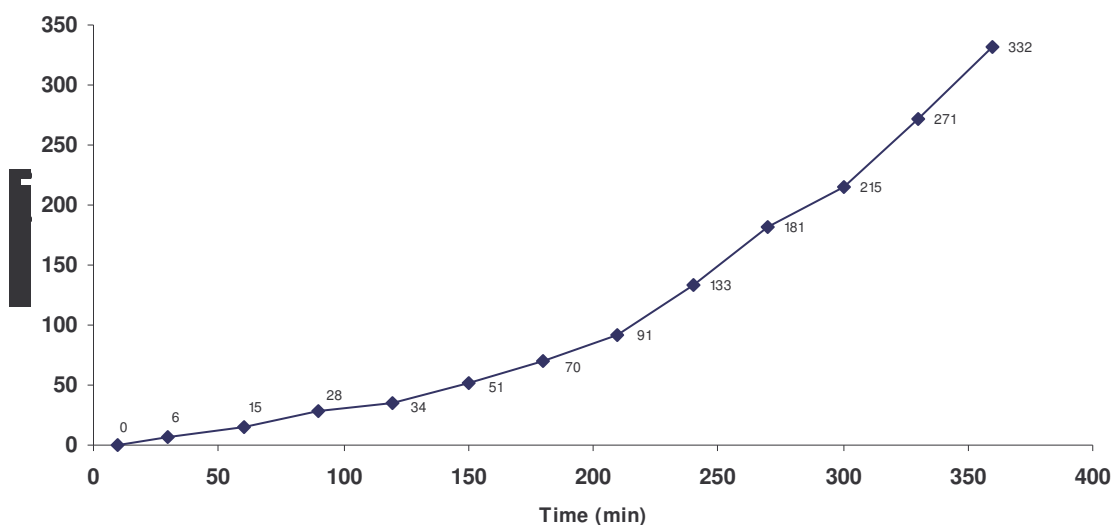
**Figure 4.28:** Swelling of spores expressed as increase of volume with time. The trend line is average of three parallels (from figure 4.27). Increase of volume is a logarithmic function.

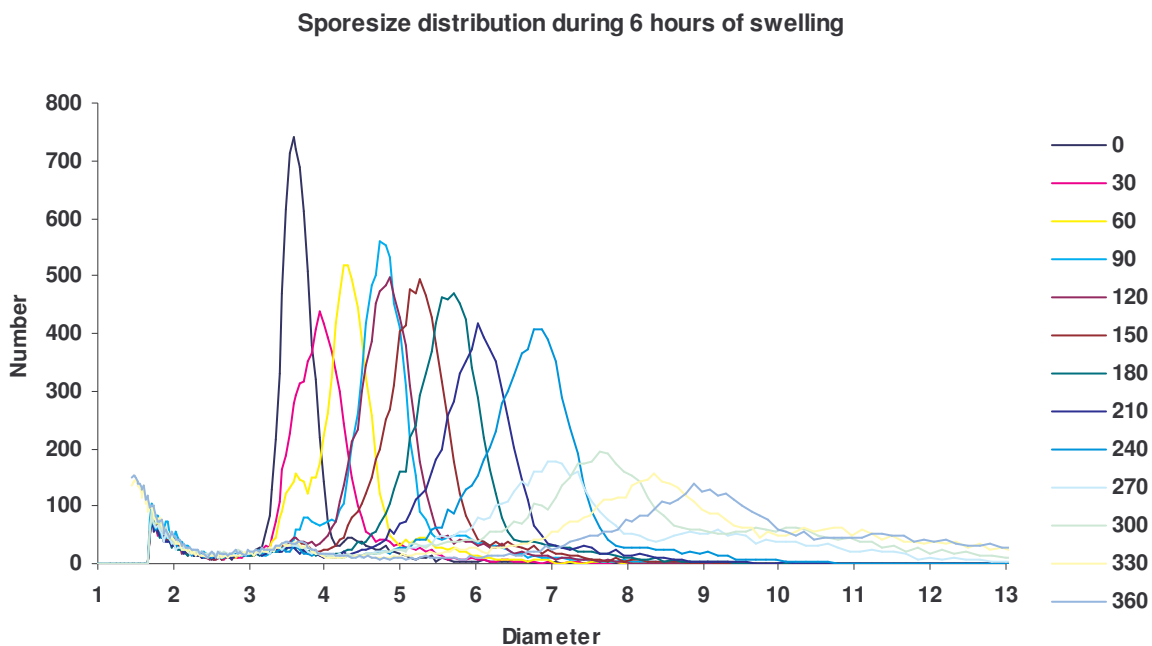
## STAGES OF SPORESWELLING



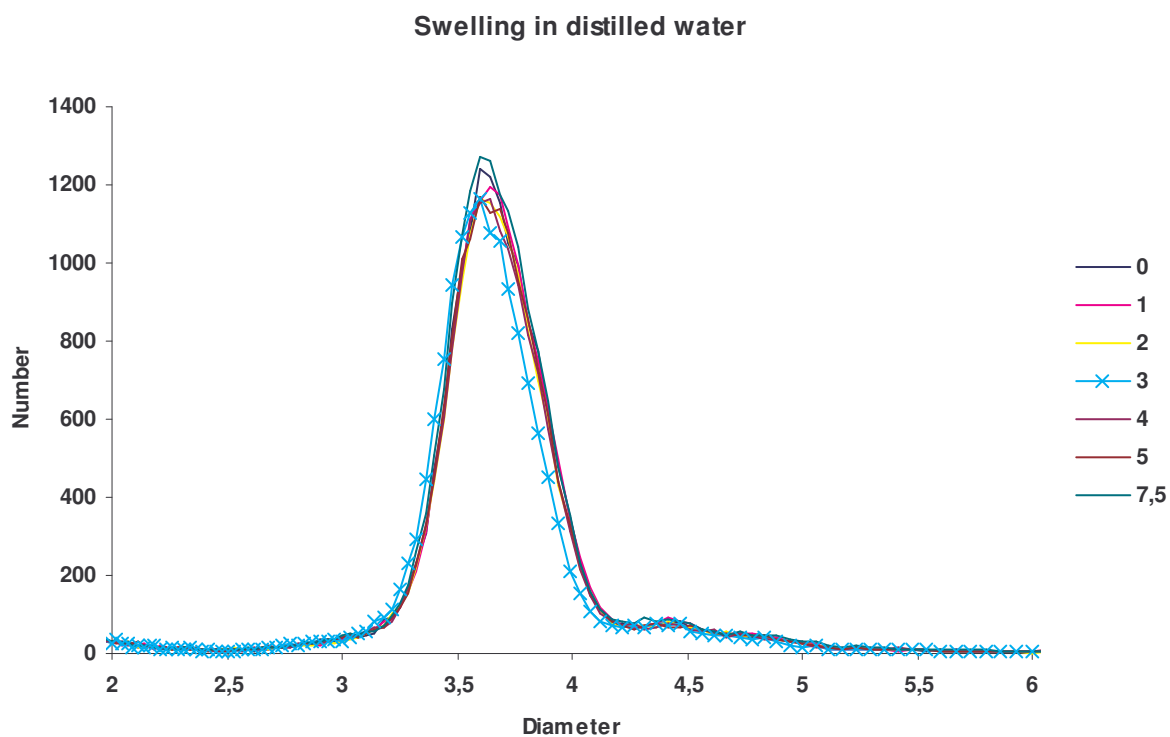
**Figure 4.29 and 4.30:** Different stages of sporangiospores (**4.29, above**). First, only swelling occurs (green), then the first spores reach the point of germination after approximately 240 min. (indicated by yellow). The red line are volumes measured after most spores have formed germ tubes or germinated. After 6 hours, practically all viable spores have germinated, and swelling no longer occurs. Even though the last hour (time 300-360 min) of these two figures is greatly influenced by the germination, the displayed dots represent more or less the average true volume of spores at the given time, as it was to some degree possible to distinguish between the earliest germinated spores (i.e. those with germ tubes) from the not fully germinated ones. Increase of volumes is shown in **4.30** (below).

## Total increase of volume





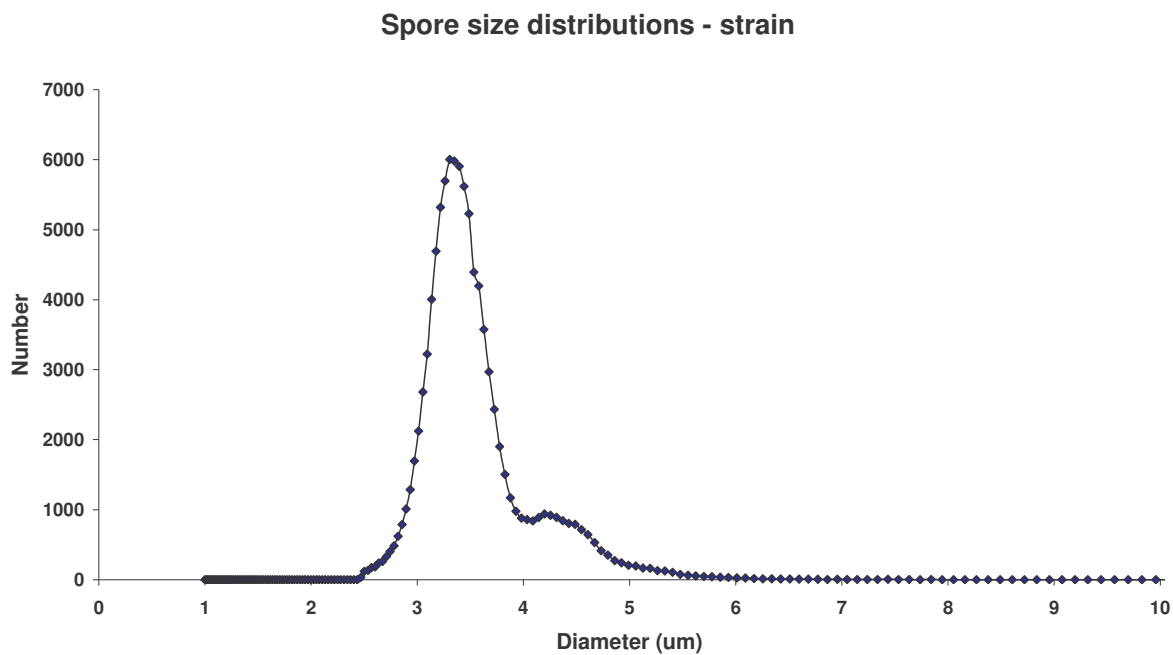
**Figure 4.31:** Spore size distribution measured every 30 min. for 6 hours. After 4-5 hours, germination occurs and leaves the distribution patterns less smooth due to the formation of germination tubes. Spores did not have a diameter more than 8-9  $\mu\text{m}$ , and sizes over this represent spores with germination tubes.



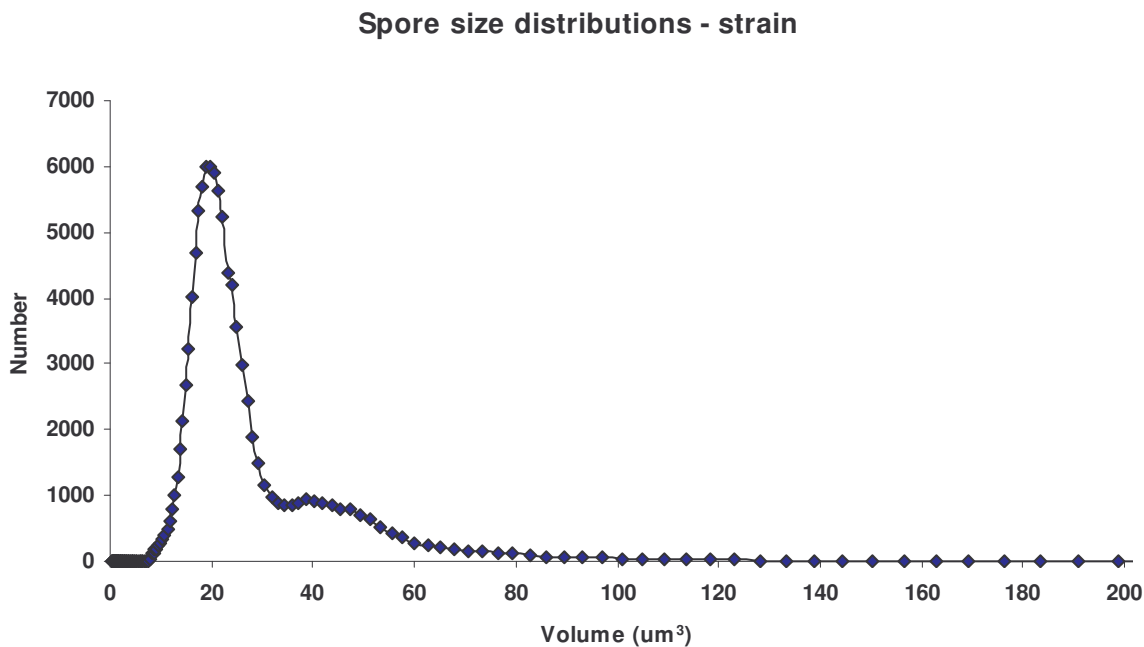
**Figure 4.32:** Swelling of spores under standard incubation conditions, suspended in distilled water without media. No swelling occurred, and no sign of passive swelling was either seen during 7.5 hours.

#### 4.5.2 Morphology and size distribution of spores

Size distribution of un-swelled spores of – and + strains can be viewed in figures 4.33 – 4.36 (diameter / volume). Spores had an average diameter of 3.7  $\mu\text{m}$  (- strain), with the highest number of spores (the top/mode of the distribution curve) at 3.5  $\mu\text{m}$  diameter. Some very few spores had a diameter of 4-5  $\mu\text{m}$  (about the double volume of the other spores), and comprised a second size distribution for the + strain (mode 3.0 and 3.8  $\mu\text{m}$ , average 3.5  $\mu\text{m}$ ). This second size cluster would increase in number if not thoroughly homogenized, as the Coulter Counter would measure two spores that cling together as one particle with the volume of two spores. It was found that even after thoroughly treatment, 10.91 % of the spores would still cling in pairs, and about 4.68 % in groups of four or more (table 4.3). This means that 15.59 % of the spores (- strain) were present not as single, but paired or grouped particles. This is a considerable fraction of the spores, an effect possibly enhanced by the haemocytometer, so the true numbers may be lower. Treated spore suspensions that showed only single spores by inspection in the microscopic, still gave the two size peaks by Coulter analysis. Some spores would also be found as immature small sized spores, in the range of 1.5– 2.5  $\mu\text{m}$  in diameter. These were not included in any results. They would also be below the measured size window in the Coulter Counter, and would thus not interfere with the spore measurements. If included, they would comprise 7.04 % of the total number of spores. They did not swell during experiments, and were assumed to be residues from the sporulation process and not to be viable. All spores proved to be spherical, both observed in light microscope and in the electron microscope. The surface seemed to be covered by a small layer of mucous, which is probably the explanation for why spores so easily did cling to each other. A fraction of the spores had a rounded outgrowth on the surface, which first was believed to be the formation of a germination tube when observed in the light microscope (figure 4.37). This abnormality always occurred as one per affected spore, and 4.23 % of the spores were found to bear this outgrowth when estimated in the haemocytometer. Another explanation examined was that it was a small spore that did cling to the averaged sized spores. This phenomenon did not resemble the properties of a germination tube, as the spores had not swelled, nor metabolised a second inner spore wall. No continuity between the cytoplasm of the spore and this outgrowth could be observed. When observed in the electron microscope, it resembled budding. The same distribution patterns were always observed for both strains through the preparation of several spore suspensions during this work.

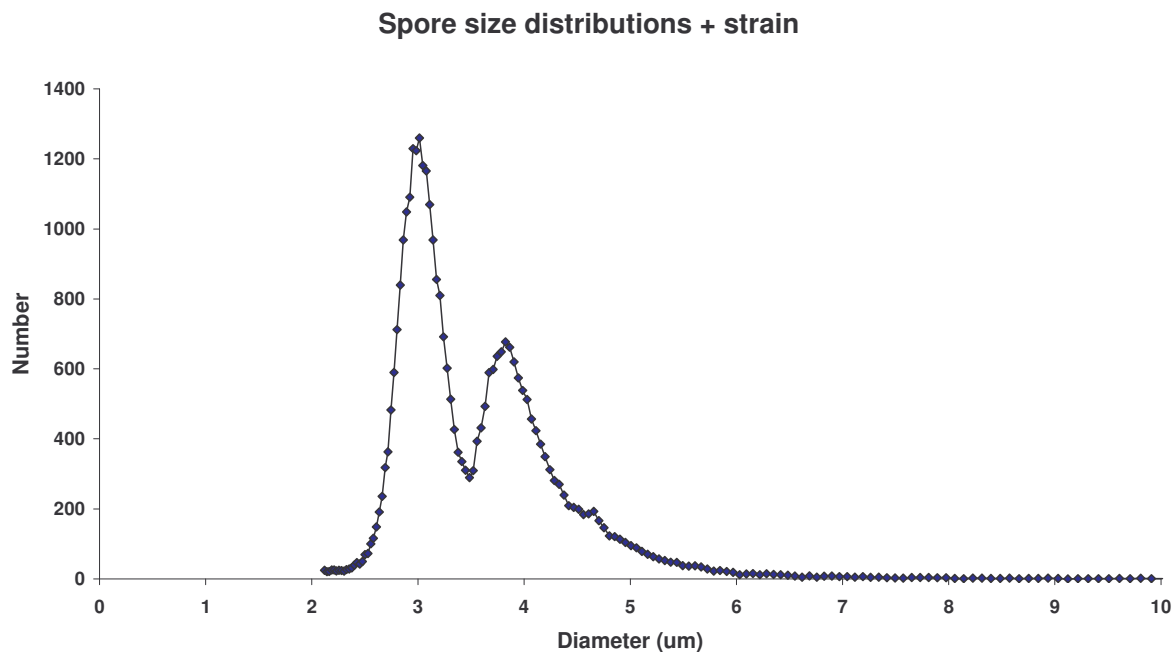


**Figure 4.33:** Spore size distribution of *Absidia glauca* - spores. A total of  $4.04 \times 10^8$  spores were counted and sized in this diagram. The second size distribution is mainly caused by clustering of spores.

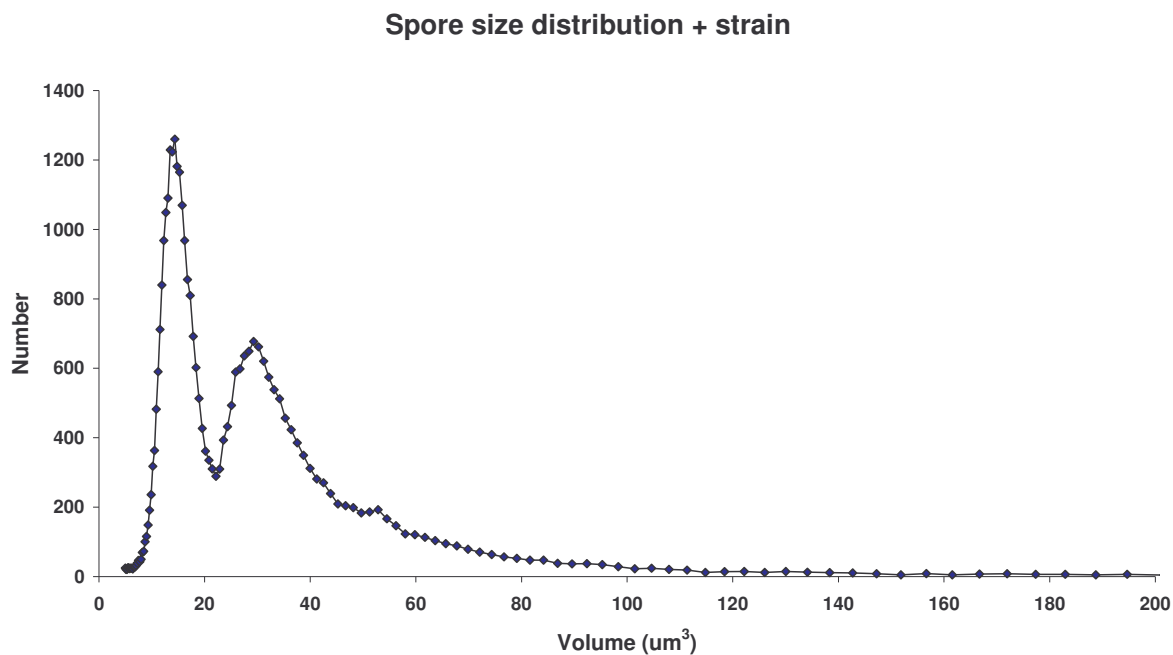


**Figure 4.34:** Spore volume distribution of *Absidia glauca* – spores from the same suspension as those in figure 4.33.





**Figure 4.35:** Spore size distribution of *Absidia glauca* + spores expressed as diameter. Two modes of size distributions clearly stand out as separate, verified by light microscope observations. Approximately  $1.0 \cdot 10^7$  particles were measured. Two separate distributions can be seen, partially overlapping.

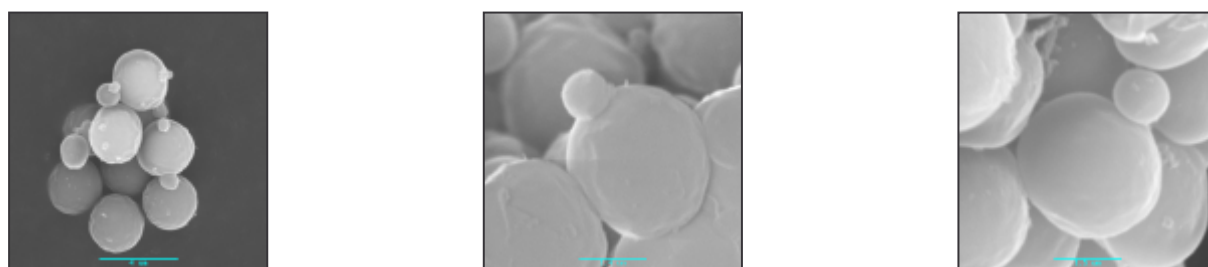


**Figure 4.36:** Spore volume distribution of *Absidia glauca* + spores from the same suspension as those in figure 4.35.

**Table 4.3**  
**Properties of *Absidia glauca* spores**

Properties:	Small sized	Larger sized	Budding	Paired	Clusters	Total clinging
<b>Strain –</b>	100 %	0.00 %	4.23 %	10.91 %	4.68 %	15.59 %
<b>Strain +</b>	70.48 %	29.52 %	0.00 %	21.95 %	15.46 %	37.41 %

Distribution patterns clearly differ between the two strains of *Absidia glauca*. Most profound is the absence of budding in the + strain, and the occurrence of two size distributions in the same strain. The tendency of spores to cling was attempted estimated. Clusters are defined as three or more spores.

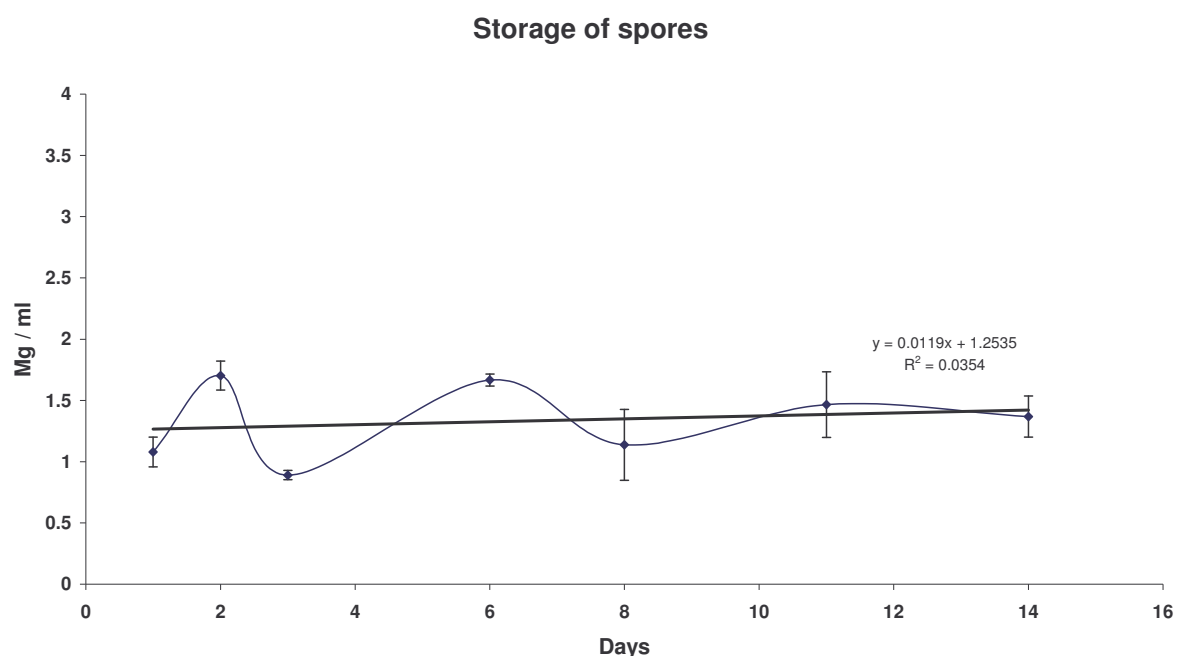


**Figure 4.37:** SEM images of the observed phenomenon believed to be budding. This was only observed on the sporangiospores of the – strain. Spores would develop these when they were attached to the sporangium. The blue size bars are 4.0, 1.4 and 1.5  $\mu\text{m}$ , respectively.

### 4.5.3 Storage of spores

Storage of the spores was considered absolutely necessary for an efficient use of the screening method. Growing and harvesting spores is time consuming, so it was therefore desirable to keep harvested spores at store for later use, and also to make the experimental aspects more flexible. The experiment was performed to imitate the handling of a suspension used over a period of several days, including the stress of handling. To approach this problem, first elements that could initiate the spore swelling had to be eliminated. This was done already in the step of harvesting the spores; through washing the spores thoroughly, all remains of the medium of which the fungus was grown on are removed. Concentrating the spores in distilled water and keeping them cold at all stages was the next step before storage in refrigerator at 4.0°C. The experiment would further establish the viability of the stored spores. Observations indicated that a small fraction of the spores germinate after some days (approximately after 6 days storage). Counts in the haemocytometer demonstrated that this

was valid for less than 0.5-1.0 % of the spores, a fraction considered to be too small to constitute an error, and could be ignored. The greater portion of spores stayed dormant throughout the experimental period. The Coulter Counter was used to determine any changes in the size distribution of the spores. Any changes in the distribution would appear as spores that had begun swelling, indicating that they would germinate after some time in dormancy regardless of the storage environment. No change in the size distribution was found for the spores during the 14 days experimental period, proving that spores would not swell nor germinate exposed to water alone. It also proved that spores did not exhibit a passive swelling phase that is triggered by water alone. All spores swelled immediately after exposure to MEX, and no difference in swelling or germination patterns were found. Dry weight measurements after an incubation period of 24 hours were used to investigate the viability of the spores, if affected but not visible in swelling properties (i.e. could increase sensibility in screens, false positive). A decrease in dry weight would be interpreted as a declined viability due to poorer growth. This can be assumed, as fungal mycelial growth is a linear process when the medium is not a limiting factor. Results can be viewed in table 4.4 and figure 4.38. Despite uneven results, it is clear that there is no trend in either direction (that be poor or better / enhanced growth).



**Figure 4.38:** Biomass measurements after 24 hours of growth over a period of 14 days. Dry weight represents the growth of the culture, and reflects the viability of the spores (i.e. success of swelling and germination to initiate growth of mycelium). Black line is the trend line.

Table 4.4

**Growth measures of stored spores incubated for 24 hours.**

Day	Spores	Day 1	Day 2	Day 3	Day 6	Day 8	Day 11	Day 14
<b>Mg/ml</b>	1.73	1.08	1.70	0.89	1.67	1.14	1.47	1.37

Dry weight expressed as increase of biomass after 24 hours (weight of harvested spores subtracted from total culture weight). Spores are weight of un-swelled spores, all other values increase of biomass after incubation for 24 hours.

As can be interpreted from the graph, spores can be stored to some extent without any decrease of viability. Nevertheless a limit was set to maximum 7 days of storage (preferable safe limit 5 days), to be within a safe margin and due to uncertainties whether they would be increasingly sensitive to inhibition of extracts or fungicides during longer periods of storage. The test performed had also a secondary objective, which was to investigate the use of dry weight as a measurement for biomass during spore swelling. This was found not to be an alternative, due to the amount of work, the use of a toxic component (paraformaldehyde, proved to be necessary for the handling of excess numbers of parallels) affecting spore qualities (table 4.6), and based on the results that turned out to be less stable (figure 4.38). Even though biomass was easily collected and measured, results showed too high degree of variation to be a valid measure for a bioassay and to estimate inhibition effects.

#### 4.5.4 The effect of spore concentration on the germination

It was necessary to investigate if spore concentrations had any effects on the germination process. The following experiment was done to investigate this. Growth measured as accumulated biomass after a 24 hours incubation period, turned out to be decreasing to all increasing spore concentrations applied (table 4.5 and figure 4.39). Figure 4.40 show the same results displayed with a logarithmic x-axis, and the increased weight estimated per spore. This decrease of growth is most probably a consequence of the exhaustion of nutrients in the MEX medium, rather than an effect caused by spores themselves. Results could be explained with a slower swelling and germination, leading to a lower biomass after 24 hours. But since no difference in number or time of germinating spores was observed in the Coulter, results must be interpreted as a reduced growth after spores has germinated, most likely when some

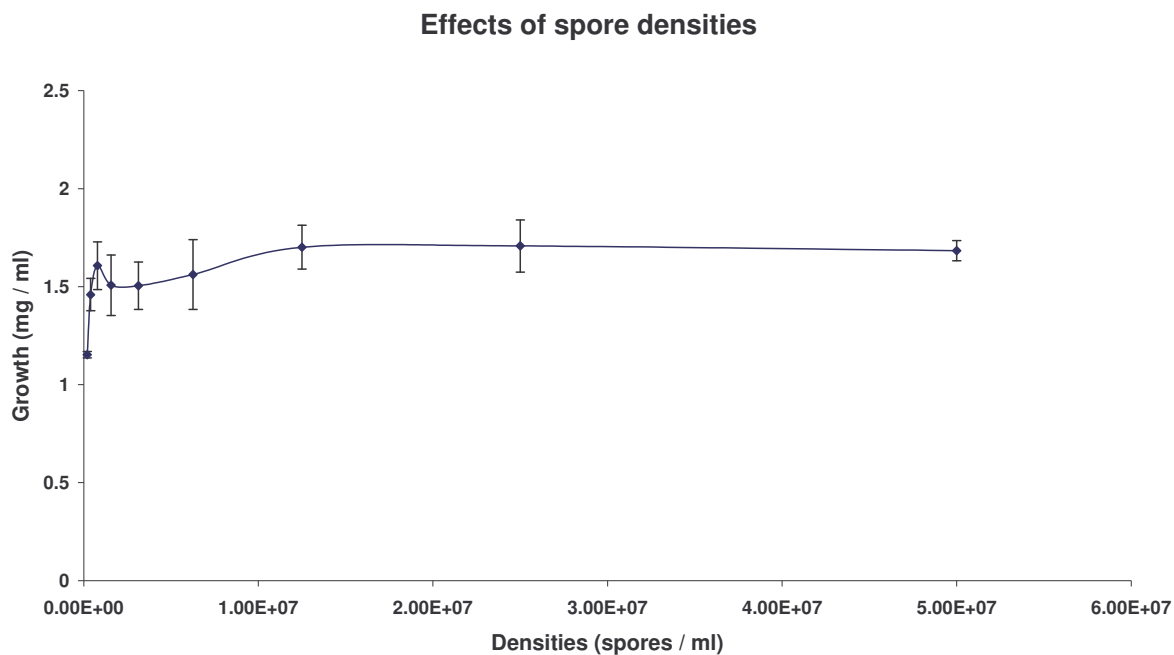
growth already has taken place in the cultures. Quorum sensing will also be expected to be a phenomenon occurring in the swelling / germinating phases of spores, and could therefore be more easily investigated by other means (e.g. Coulter Counter). These results do not understate nor disprove densities effects earlier reported by Frøyen on this strain (earlier germination with increasing concentrations, see page 94). But it should be noted that concentrations at this densities rarely if ever is to be found in natural conditions, and all quorum sensing responses must be expected to be seen on much lower spore densities. No effects on the swelling patterns and volumes were observed in the Coulter. No further investigations were performed on the subject in this work, as it was not in the aim of the Thesis. As long as the medium would not be limiting under swelling and germination, i.e. 6 hours after addition of spores to the growth medium, and spores did not inhibit each other at these high concentrations, it was concluded to be defendable to use high spore concentrations. This experiment is the foundation for the spore densities used in all subsequent assay work being set to  $1.0 \cdot 10^7$  spores / ml. The test performed here also had a secondary objective, which was to investigate the use of dry weight as a measurement for biomass during spore swelling. The results here do not necessary represent a good estimate of spore germination, even though this can be interpreted, as also mycelial growth is constant when environments allow this (i.e. not limiting). All further use of dry weight to assess growth patterns of spores was abandoned based on the results and considerations of laboratorial work in this spore density experiment and the experiment investigating storage of spores. This led also to the discarding of a scheduled experiment regarding biomass accumulation during swelling.

**Table 4.5**

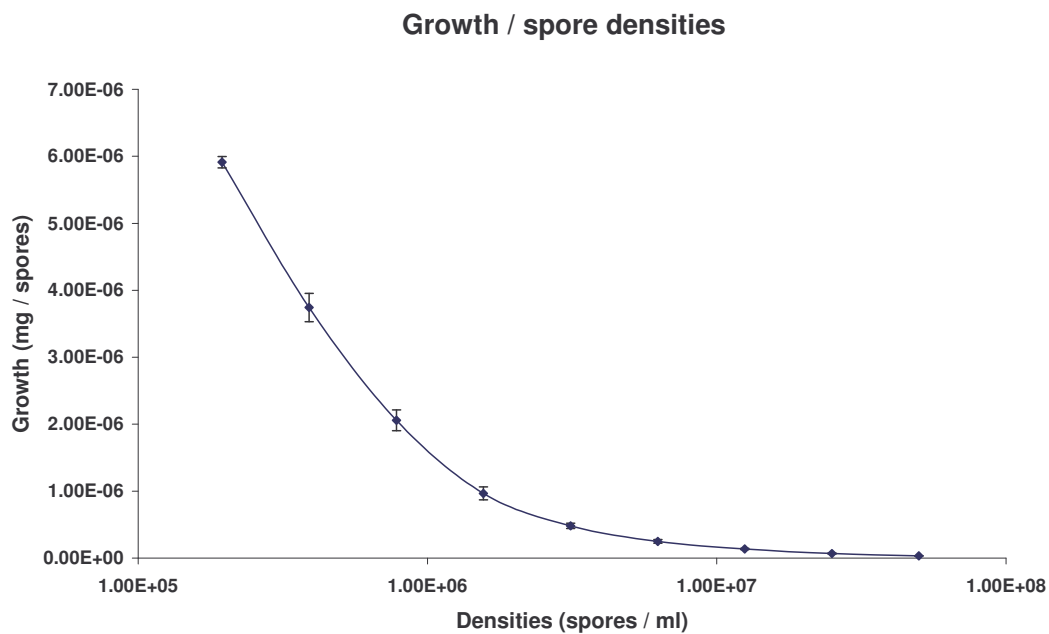
**Growth measures of spores incubated at different concentrations.**

<b>D</b>	$5.0 \cdot 10^7$	$2.5 \cdot 10^7$	$1.25 \cdot 10^7$	$6.25 \cdot 10^6$	$3.125 \cdot 10^6$	$1.56 \cdot 10^6$	$7.81 \cdot 10^5$	$3.9 \cdot 10^5$	$1.95 \cdot 10^5$
<b>W</b>	1.68	1.71	1.705	1.56	1.51	1.51	1.61	1.46	1.15
<b>k</b>	$3.36 \cdot 10^{-8}$	$6.84 \cdot 10^{-8}$	$1.43 \cdot 10^{-7}$	$2.5 \cdot 10^{-7}$	$4.82 \cdot 10^{-7}$	$9.68 \cdot 10^{-7}$	$2.06 \cdot 10^{-6}$	$3.74 \cdot 10^{-6}$	$5.9 \cdot 10^{-6}$

Spores (i.e. un-swelled) were measured to weigh 1.50 mg/ml at a concentration of  $5.0 \cdot 10^7$  spores/ml (equals a weight of  $3.0 \cdot 10^{-5}$   $\mu$ g / spore), and weight were subtracted from results at each respective concentration. All weight is expressed as total weight of cultures per ml after 24 hours of growth. D = Spore densities; W =  $\Delta$  weight (mg/ml); k = (mg/ml) / (spores/ml) = mg / spores.



**Figure 4.39:** Growth expressed as mg of accumulated biomass per ml culture after 24 hours with increasing spore densities in the medium. Biomass increases until 1.75 mg / ml, from where no further increase in biomass occurs.

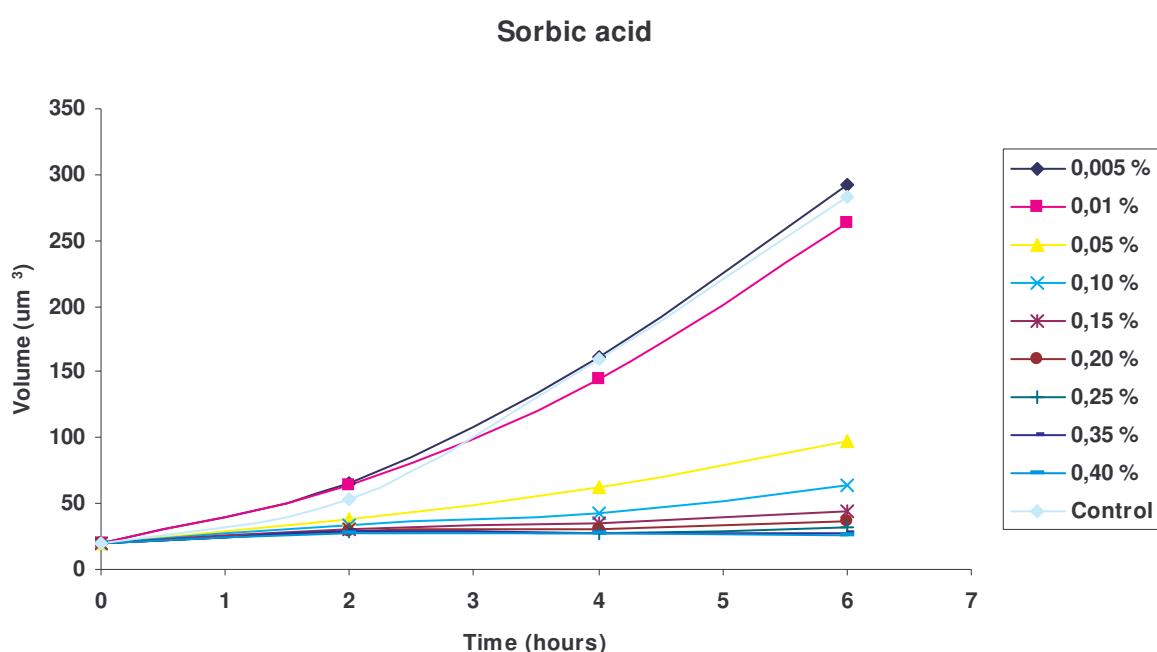


**Figure 4.40:** Growth expressed as mg increase of biomass of each spore after 24 hours of incubation. Biomass declines rapidly with increasing densities after 24 hours.

## 4.6 Inhibition by known fungicides

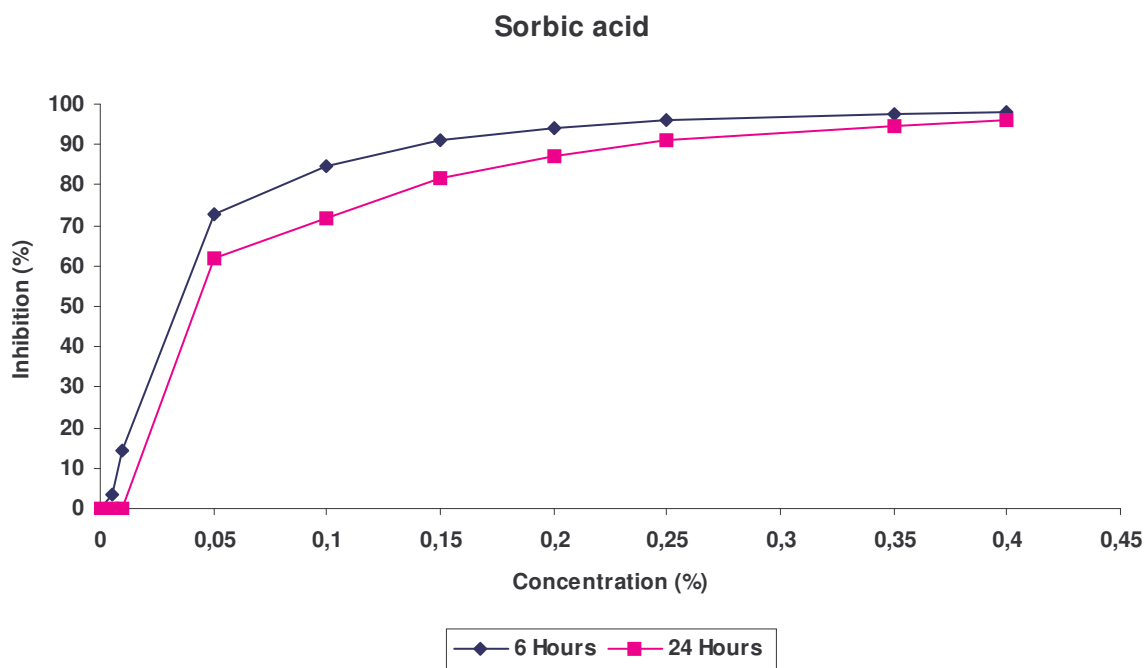
### 4.6.1 Sorbic acid

The assay was done according to the description in section 3.9 and 3.11, page 45 and 48. 3 standardization assays were run, until the right concentrations were found covering the range from no inhibition to full inhibition. Results from the third assay covered this range (9 sorbic acid concentrations from 0.005 % - 0.4 %, and one control) and is presented in the following. Minute amounts of sorbic acid present in the bioassay inhibited the spores. Inhibition after 2, 4, 6 and 24 hours was assessed after exposure to sorbic acid. Inhibition occurred as a slower swelling rate, until a certain concentration, where inhibition became more pronounced (as also observed in Benzoate, ethanol and DMSO assays, see below). Inhibition was measured as a decreased swelling rate until no swelling occurred, rather than a decrease in number of swelling and germinating spores as anticipated. Concentrations of 0.05 % and more were strongly inhibiting all spores. When spores were exposed to higher concentrations, some swelling still occurred, but never reached germination, even after 24 hours. Figure 4.41 illustrates spore volumes every second hour until 6 hours where viable spores were expected to germinate. This is swelling as preceded in the assay, where concentrations above 0.01 % all strongly affect swelling and inhibit germination. Figure 4.42 show inhibition percentages after

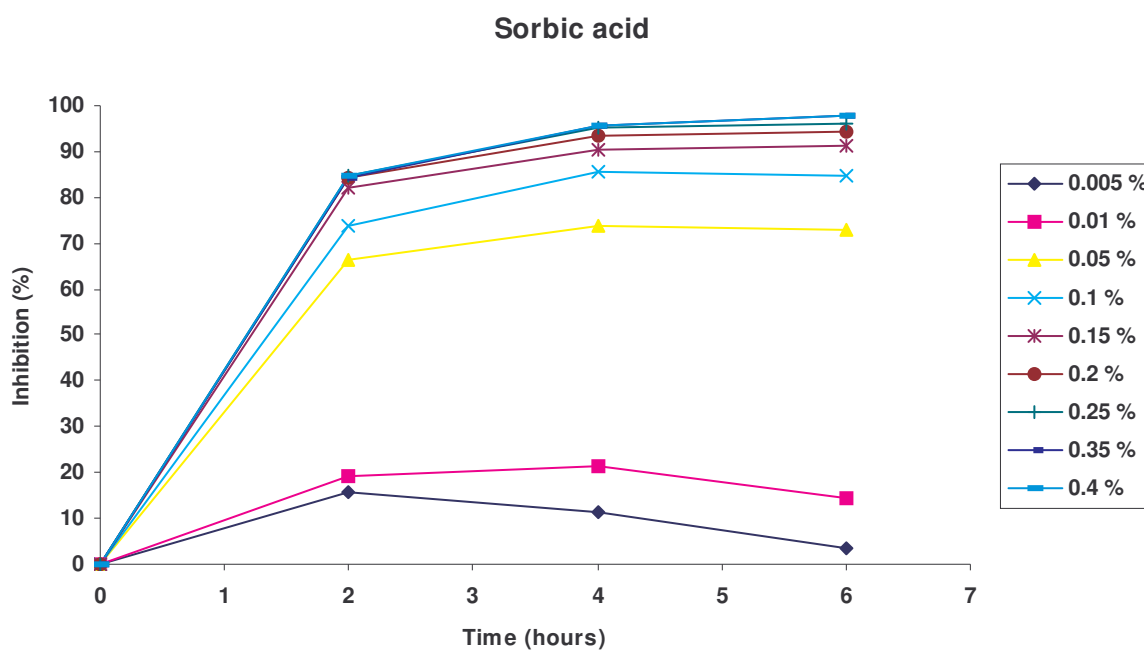


**Figure 4.41:** Effect of different concentrations of sorbic acid on volumes measured after 2, 4 and 6 hours.





**Figure 4.42:** Percent inhibition of spore swelling by sorbic acid after 6 and 24 hours.

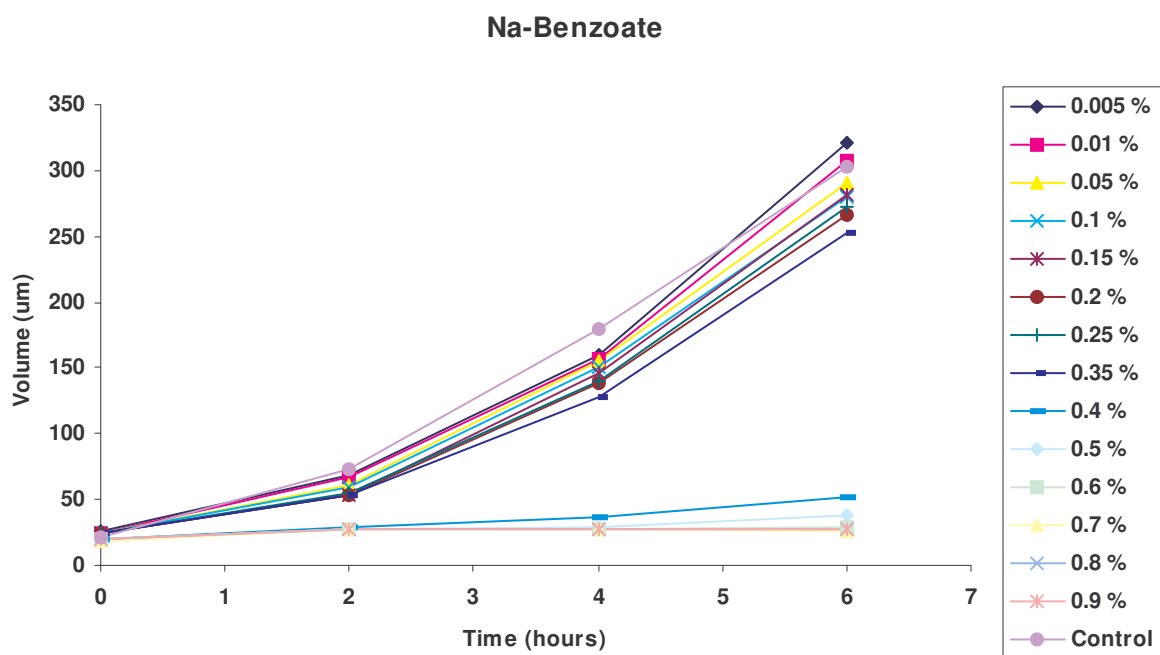


**Figure 4.43:** Percent inhibition after 2, 4, and 6 hours. Note the difference in inhibition patterns at time 2, 4 and 6 hours, especially for sorbic acid concentrations below 0.05 %.

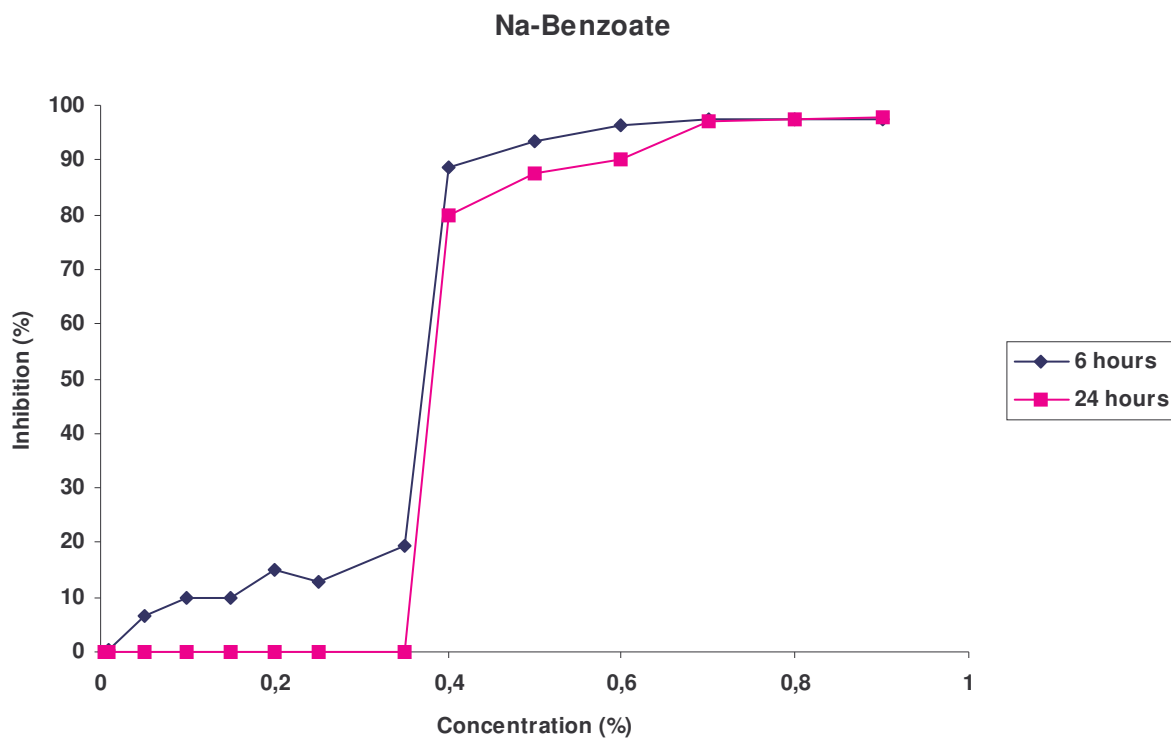
6 and 24 hours. Inhibition proves to be stable and permanent after 24 hours. Next figure (4.43) show that inhibition patterns were time-dependent, increasing with duration of swelling. This was especially clear for the less inhibited spores, i.e. at concentrations below 0.05 %. Spores are more inhibited early in their swelling phase than after 4 and 6 hours, probably caused by metabolic adaptation to the fungicide compound. Inhibition is relatively stable and can possibly be measured compared to the swelling state of controls at an earlier stage than after germination. However, those treated with low concentrations of inhibitor are best expressed at a later stage, which will probably be more like those situations met in screening algal extracts (i.e. low concentrations of inhibitors in extracts). This is a very interesting results, suggesting that time of sampling must be at the time when most viable spores would have been allowed to germinate, to obtain the best results reflecting the true inhibition. Minimum inhibitory concentrations (MIC) are listed in table 4.7.

#### **4.6.2 Na-Benzoate**

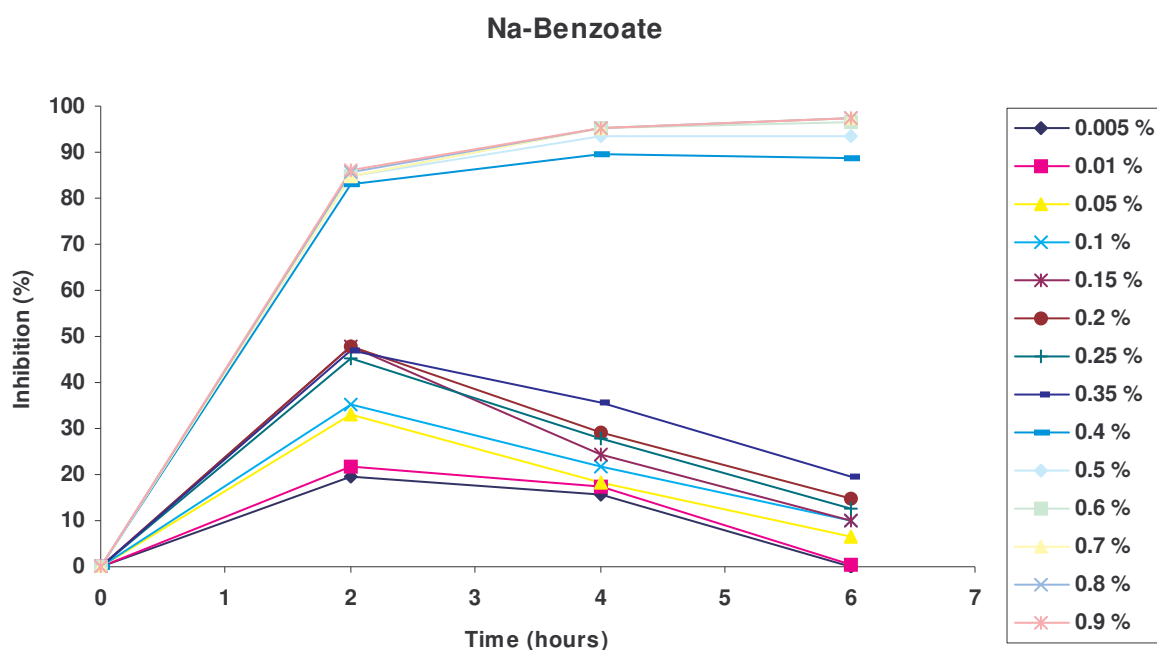
The assay was done according to the description in section 3.9 and 3.11, page 45 and 48. 2 standardization assays were run, until the right concentrations were found covering the range from no inhibition to full inhibition. Results from the second assay covered this range (14 Benzoate concentrations from 0.005 % - 0.9 %, and one control) and is presented in the following. Benzoic acid was a weaker inhibitor than Sorbic acid. Figure 4.44 show swelling in volumes as preceded. The same sudden inhibition caused by small changes in fungicide concentrations was also seen for this agent. Concentration above 0.4 % gave total inhibition, even after 24 hours, whereas several lower values gave some inhibition measured after 6 hours, but not after 24 hours (figure 4.45). This suggests an effect on spores from which they do recover from, an increase in resistance to the fungicide when not fully inhibited from the onset of the assay. Much lower MICs were found for this agent than for sorbic acid (table 4.7). Another striking feature also seen using sorbic acid, but most profoundly expressed by benzoic acid, is the difference in inhibition measured within different time intervals (figure 4.46). It is quite obvious that spores at first when exposed to lower concentrations of the agent reacts by a slower swelling rate within the first hours, before they rapidly recover and resume swelling until they reach the point of germination. This clearly shows that inhibition measured before spores reach the germination, might give inaccurate estimates, as already mentioned above. Overall, the same trends were found in inhibition of swelling of the two agents tested.



**Figure 4.44:** Effect of different concentrations of Na-Benzoate measured after 2, 4 and 6 hours.



**Figure 4.45:** Inhibition of swelling by Na-Benzoate expressed as percentage after spores has been allowed to swell for six hours. For the lower concentrations (below 0.4 %) a major change in inhibition were observed after 24 hours, from where all spores recover and germinate. For all concentrations above, inhibition is still present.



**Figure 4.46:** Percentage inhibition of swelling after 2, 4, and 6 hours. Note the clear difference in inhibition patterns at time 2, 4 and 6 hours, especially for Benzoic-acid concentrations from 0.35 % and lower. After 2 hours, most spores express some degree of inhibition. After 6 hours of swelling, this effect is much smaller.

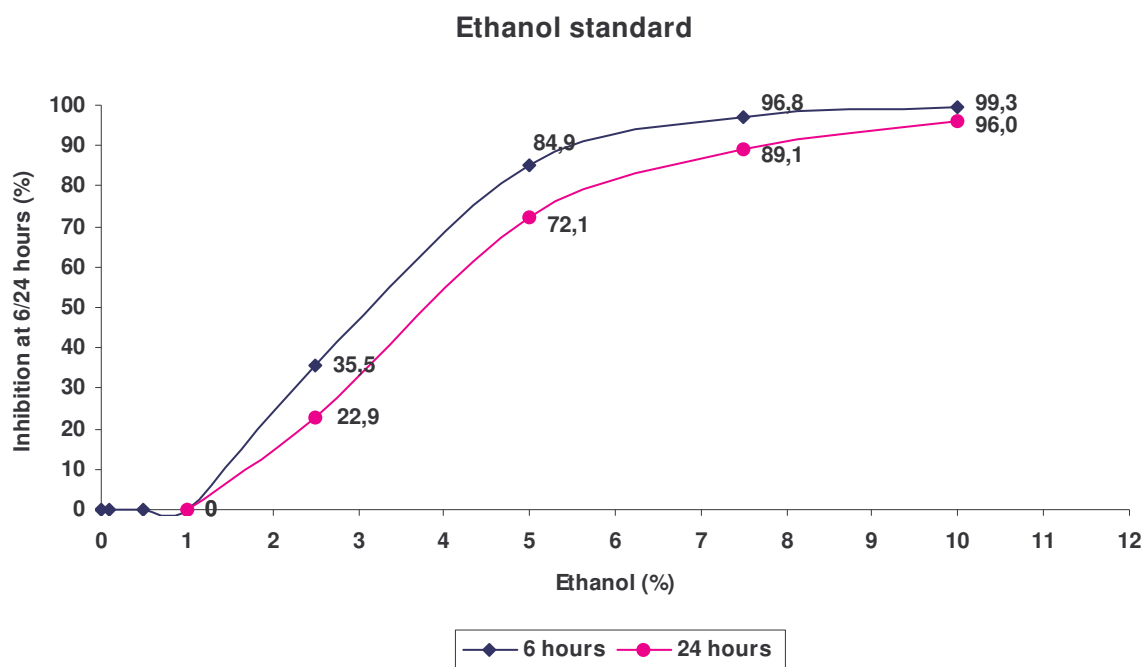
## 4.7 Control assays

Since ethanol and DMSO would be present in the algal extracts to be tested, their possible effects on the spores had to be determined. Acetone and methanol were also briefly investigated as candidates besides these two. Results of all four are summarized in the last part of this section.

### 4.7.1 Ethanol standard

The assay was done according to the description in section 3.9 and 3.12, page 45 and 49. 7 different concentrations (0.1 % – 10.0 %) were tested together with a control, covering the range from no inhibition to full inhibition. Spores were not inhibited by concentrations below 1.0 % ethanol. Above this concentration, inhibition increased rapidly with increasing

concentrations as can be seen from figure 4.47. A 98.0 % reduction of the swelling process was observed after 6 hours at a concentration of 10.0 % ethanol in total culture volume. Thus, 1.0 % seemed to be the threshold limit for inhibition. Only slight reduction of the inhibition was observed after 24 hours, which can be assumed to mean that the inhibition observed after 6 hours, where most spores not encountering any inhibiting factors would have initiated the germination phase, reflects the true overall inhibition by ethanol. This assay show that the two extract concentrations used when screening greenalgae (1.0 % and 5.0 %), would inhibit the spores. 5.0 % ethanol inhibited 84.9 % of the swelling, and therefore the inhibition detected in the screens must be assumed to originate from ethanol (see section 4.8). Estimated MIC values can be read in table 4.7.

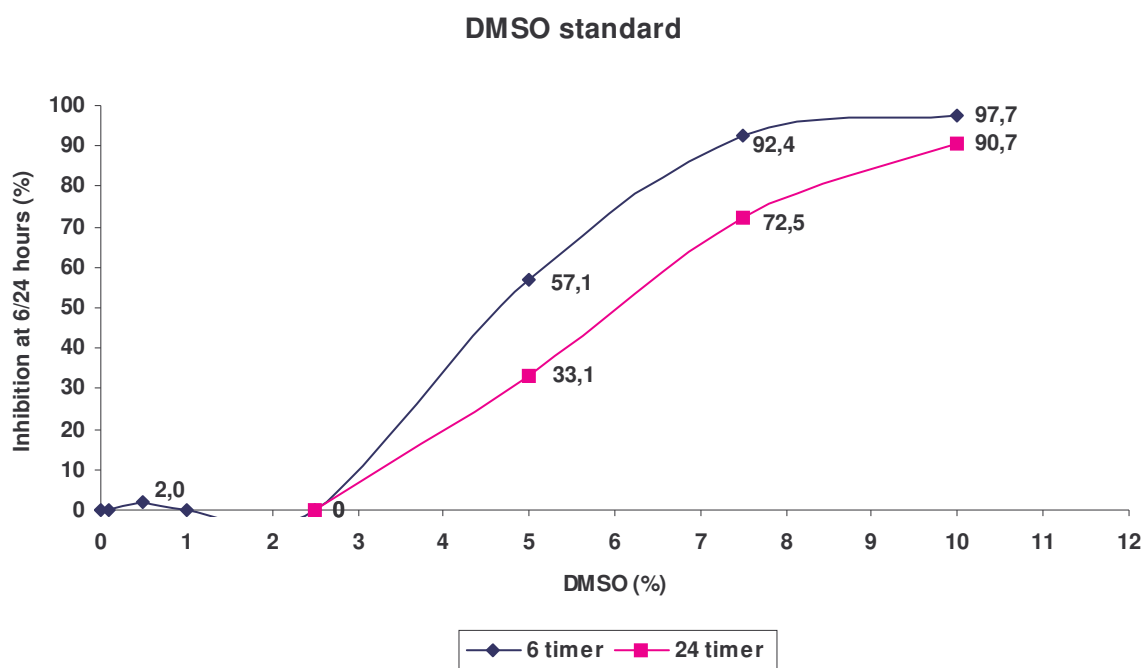


**Figure 4.47:** Standard ethanol inhibition curve exhibiting percentage of ethanol in total culture volume and the percentage of inhibition of the spore swelling after 6 and 24 hours.

#### 4.7.2 DMSO standard

The assay was done according to the description in section 3.9 and 3.12, page 45 and 49. 7 different concentrations (0.1 % – 10.0 %) were tested together with a control, covering the range from no inhibition to full inhibition. Inhibition of the swelling spores was observed in concentrations of 2.5 % DMSO and above (figure 4.48). Inhibition then increased rapidly

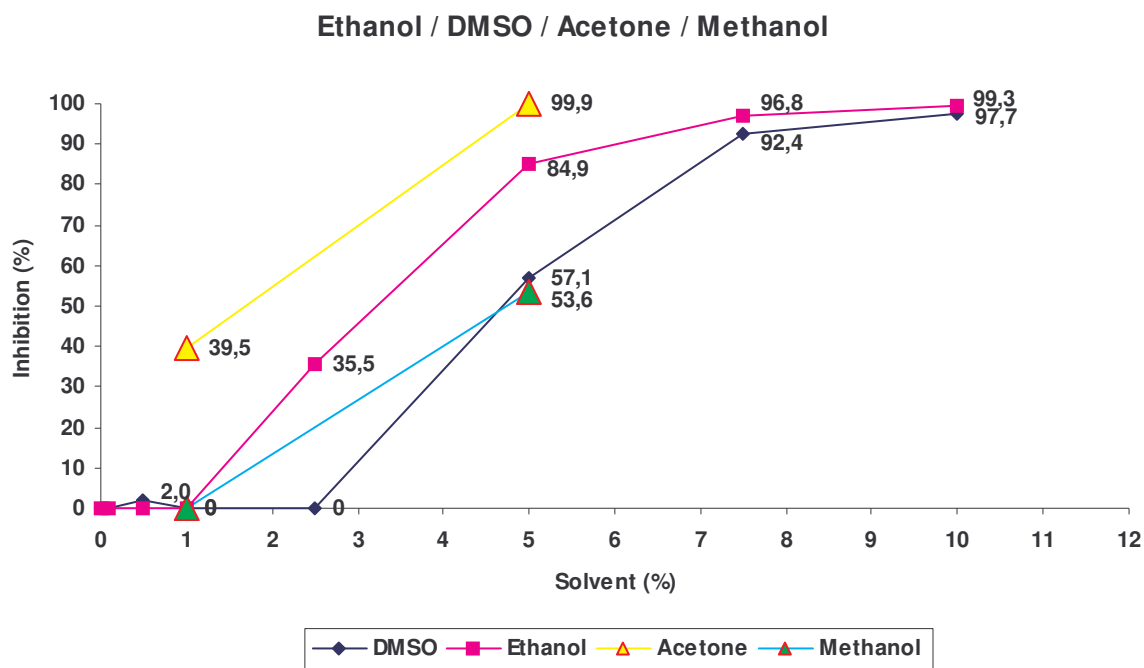
with increasing concentrations as can be seen from figure 4.48. At 10.0 % DMSO, 97.7 % of the swelling was inhibited. As also shown by the ethanol standardization, no significant reduction of the inhibition was observed after 24 hours, although some more pronounced change did occur. This might suggest that the viability of the spores is not affected as severe as for other solvents. Estimated MIC values at different endpoints can be read in table 4.7, where it is clear that DMSO has the closest value of inhibition of both swelling and germination ( $\Delta G$  %), where 10 % inhibition equals 40.5 % of the amount of DMSO required for 90 % inhibition.



**Figure 4.48:** Standard DMSO inhibition curve exhibit percentage of DMSO in total culture volume and the percentage of inhibition of the spore swelling after 6 and 24 hours.

The rapidity of inhibition at a certain concentration, in this case from 2.5 %, also suggests for DMSO as for ethanol, that there is a threshold limit from which below spores are tolerant. Then they rapidly get inhibited by increasing fungicide concentrations. Inhibition also seems to be constant when first a fungicide compound has affected the spores. Based on these results, it was decided to use a smaller volume of extracts in the assays, comprising 2.5 and 1 % of total culture volume (5.0 ml), but with the double strength of the ethanol extract used for Chlorophytes, as the same amount of extract (from 50 mg) were used and dissolved. Figure 4.49 compares inhibition by both ethanol and DMSO. Acetone and methanol are also

included, as they were briefly investigated as candidates beside DMSO to replace ethanol in only two concentrations (1.0 % and 5.0 %). As can be seen, acetone inhibited spores slightly more than ethanol, and was therefore not an option. Methanol inhibited within the same range as DMSO, and therefore clearly stands out as a candidate besides DMSO.



**Figure 4.49:** Comparison of inhibition by ethanol, DMSO, acetone and methanol. Acetone (tested in two concentrations, 1.0 and 5.0 %) is most inhibiting, whereas methanol (also tested in only two concentrations, 1.0 and 5.0 %) inhibits almost within the same range as DMSO.

#### 4.8 Inhibition of crude extracts from microalgae

Of the first 15 greenalgae screened for antifungal activity, three strains gave positive results or “hits” from the H and L extracts when inhibition of ethanol was subtracted (figure 4.50). All the remaining extracts showed no inhibition in the concentrations used here. It is adjacent to conclude that if some inhibition had been present, but hidden behind what was subtracted from the ethanol effect, it would be possible to either see some sign in the other concentration and in the H extracts or expect a higher column in the 5 % concentration. No inhibition was found in the H extracts. Two of the strains were difficult to measure due to the



creation of particles in the lipophilic extracts. Ethanol inhibited 84.9 % of the swelling at a 5.0 % concentration. As it was suspected that formaldehyde might influence screening results, as all three positive hits had been preserved due to experimental circumstances, this agent was tested against swelling spores (table 4.6). It was then clear as formaldehyde reduced the size of spores that what had been seen as inhibition could be explained by this effect alone. Therefore no antifungal bioactivity was detected in the Chlorophyte screens. However, as can be viewed in figure 4.50, another effect was pronounced. Several hydrophilic extract enhanced germination (not swelling) as germ tubes would grow exceptional in their present (negative values). This effect was so obvious that it clearly proves that H extract, and to some extend also L extracts possess factors that enhance germination even when grown in a rich media. This is also confirmed by the increased effect from 1 % to 5 % concentrations. G9, G13, G17, G44 and G 73 showed the best enhancement of growth.

**Table 4.6**

**Formaldehyde as a preservative – percent shrinking in volumes of spores**

Time of swelling:	2 Hours	4 Hours	6 Hours	Average
30 min. after addition	0.00 %	18.77 %	11.84 %	10.20 %
3 days after addition	48.42 %	46.39 %	50.70 %	48.50 %

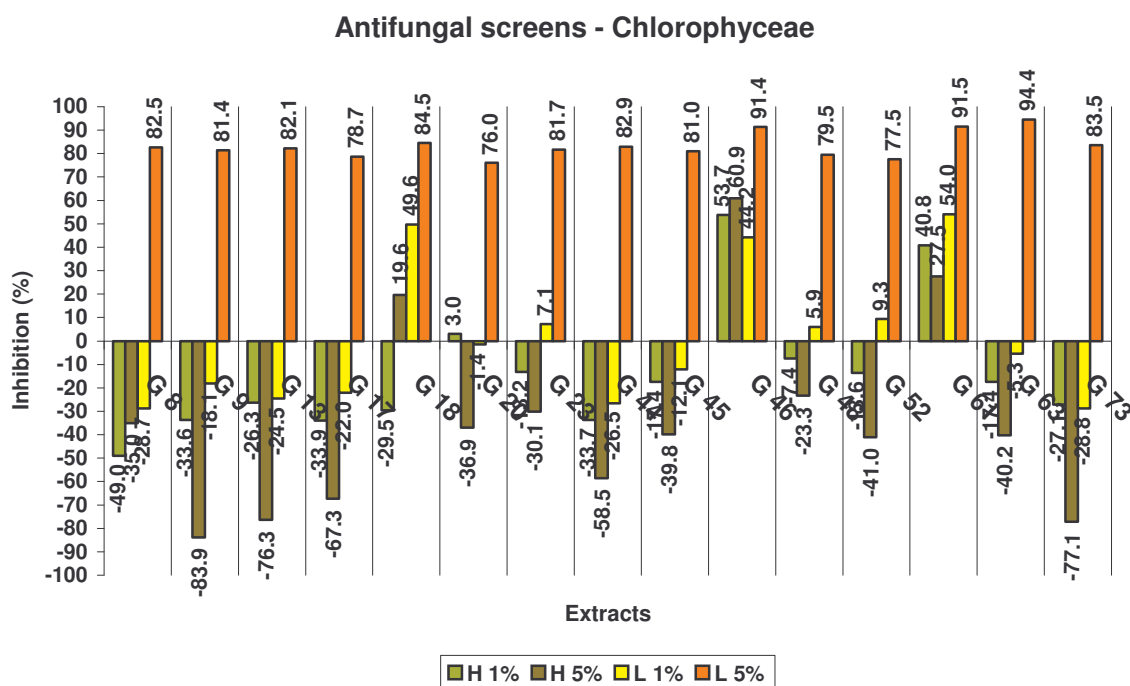
To assess the effect of formaldehyde on the volumes of spores, the scenario from the screening was replicated. To investigate if the stage of swelling did matter, the preservative was added at time 2, 4, and 6 hours (since any inhibition from extracts could not be replicated). All algal extracts was however preserved at time 6 hours. Sizes were measured right before formaldehyde was added, then again after 30 minutes to asses the swiftness of change, then again after 3 days, as was the case of the algal extracts.

Inhibition cannot be estimated accurately when it is weak (i.e. close to germination), something also shown by standardization to known fungicides. This is caused by the uncertainties regarding spore volumes when they germinate, compared to controls. It was therefore decided to use 90 % inhibition as an endpoint criteria for germination (where all germination is inhibited), and 10 % as endpoint for swelling (i.e. prevents all spores from germinating). Therefore, inhibition that is less than 10 % are not regarded as acceptable as true inhibition and excluded. Table 4.7 summarize estimated endpoint MICs for the two fungicides applied, and for the extraction solvents used. Values which gave 50 % inhibition are also included. These are the values available to comparison of results, and for the evaluation in differences of each substance in their ability to prevent germination (G) and swelling (S).

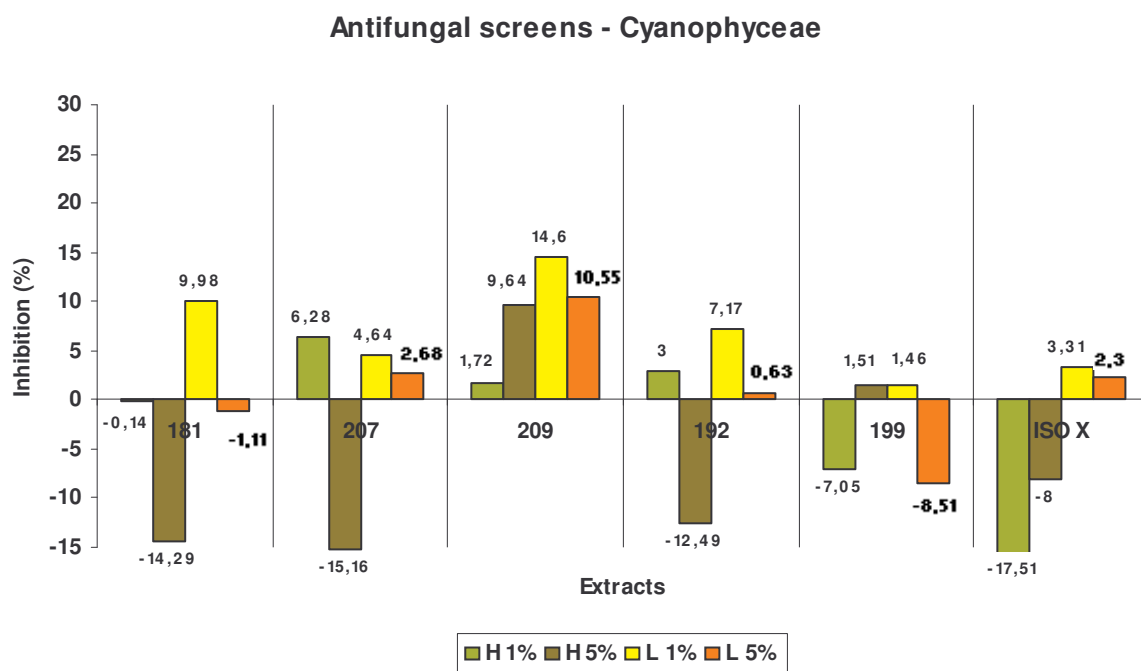
Results from the cyanobacterial screens can be viewed in figure 4.51. These were performed after screens of Chlorophytes were conducted in a modified bioassay, where DMSO was used as the solvating agent for the lipophilic extracts. These extracts were concentrated to be of the same concentration in the bioassay compared to amount of biomass originally extracted, but in half the volume of solvent to avoid all effects from DMSO. In addition, hydrophilic extracts were added at the double concentration of those used in green algal hydrophilic extracts. This would only make the detect ability of the screen better. No inhibition was found for three of the extracts. Of the two remaining, *Phormidium sp.* 209 clearly possessed inhibition properties (though expressed weak) both by analyses and by observation of the whole culture. Deviations were observed in the germinating phase of the second extract as well, but the effect where to low (less than 10 %) to be included as true inhibition. However, it is worth noticing that this strain (*Phormidium sp.* 181) has been reported to possess antifungal activities against *Aspergillus niger* among others (unpublished work, UiB). Effects from *P. sp.* 209 were present as a small change in germ tube formation patterns, observed as slightly slower growth rates at and right after the first occurrence of germ tubes compared to spores that were not inhibited. This could be observed both in the Coulter Counter and visually in the cultures (a smaller amount of “grains” were formed at germination compared to other cultures). *P. sp.* 209 seems also to be more or less inhibited in all extracts, as this is the only strain not enhancing growth by any means.

**Table 4.7**  
**COMPARED MICs OF DIFFERENT SUBSTANCES INVESTIGATED**

<b>MIC (minimum inhibitory concentrations) A: <math>\mu\text{g} / \text{ml}</math> and B: <math>\text{mmol L}^{-1}</math></b>					
<b>Endpoint MIC:</b>		<b><math>\geq 90 \%</math> (S)</b>	<b>50 %</b>	<b><math>\geq 10 \%</math> (G)</b>	<b><math>\Delta G \%</math> (G/S*100)</b>
<b>Sorbic acid</b>	<b>A</b>	1.4	0.3	0.08	5.71 %
	<b>B</b>	9.32	1.99	0.53	— —
<b>Benzoic acid</b>	<b>A</b>	4.5	3.5	1.0	22.22 %
	<b>B</b>	34.0	26.5	7.57	— —
<b>Ethanol</b>	<b>A</b>	63.0	32.0	12.0	19.05 %
	<b>B</b>	1367.7	694.7	260.5	— —
<b>DMSO</b>	<b>A</b>	74.0	46.0	30.0	40.54 %
	<b>B</b>	947.1	588.8	383.9	— —
<b><i>Phormidium sp.</i> 209</b>	1-5 % extract (equals extraction of 6.25 and 30.25 mg algal biomass, respectively) gave an estimated inhibition of $\geq 10 \%$				



**Figure 4.50:** Inhibition of crude extracts from Chlorophyceae strains. Negative values indicates enhancement of growth (i.e. after germination) of spores. 5 % lipophilic extracts are all inhibited by ethanol (84.92 %, value from the standardized assay).



**Figure 4.51:** Inhibition of crude extracts from Cyanophyceae strains. Negative values indicates enhancement of growth (i.e. after germination) of spores. Especially interesting is *Phormidium* sp. 209, which stands out as inhibited in all extracts.

## 5 DISCUSSION

This section first discusses results from growth and harvesting of all organisms applied. Then attention onto swelling and germination patterns of the fungal model organism will be given. The next main section will discuss results regarding screens (i.e. standardization, constructing and results), a final evaluation of the bioassay as a method for screening, and suggestions for further research.

### 5.1 *Discussion of Results*

#### 5.1.1 Cultivation and extraction of algal biomass for screening

Preceding the screening for bioactivity in algae is their growth, harvesting and extraction of biomass, and preparation of crude extracts for the bioassay. Factors that have to be considered when selecting species for this purpose have previously been presented in the introduction. Algae, and especially cyanobacteria, have proven to be a valuable source of bioactive compounds, including antimicrobial activities such as fungicides. All species investigated here are isolates from temperate or polar locations, and consequently cold climatic conditions. After selection of species, preparations for screens in a bioassay then follow. These steps are discussed below, with focus on treatment of species for best results (i.e. accurate and precise procedures for detecting true inhibition) in bioassay screens:

**Cultivation of species:** All algal cultures were cultivated on a general first crude prospecting basis. This means that no growth studies of any of the cultured species were performed, as the main objective was to cultivate enough biomass for the rapid screen. Production of algal biomass conducted on such a general basis, allows no effort in altering growth conditions before any bioactivity has been detected, these were attempted to be closest to optimal. Therefore, knowledge of the species and their physiology is crucial, so that the first screen will represent the properties of the species screened. This includes the information available of reported bioactivity or other active metabolites. It is also important to optimize growth conditions, so that biomass can be obtained in larger quantities. General observations on morphology to achieve knowledge of the species, and dry weight as means to establish

biomass densities were considered necessary. DW was chosen to estimate growth potential and quantities obtainable. None of the cultures were grown axenic. This is difficult to achieve without more time consuming maintenance of the species and demands a specialized growth system or bioreactor. Since they produce exo-polysaccharides, cyanobacteria are especially hard to grow axenic. Bacteria feed on the nutrition these provide and many actually live as epiphytes on species producing polysaccharide sheets. The problem with bacteria in the cultivation of algal biomass is first and foremost due to the possibility that it can be an error regarding bioactivity in an assay. This is already a known phenomenon when it comes to cyanobacteria living inside sponges (section 1.2.2). They can also make growth conditions in general affected and not favourable for algae through the release of different metabolites produced and released either during growth or by lysis. To keep the number of bacteria at a low level under biomass production, the strategy was to keep the algae in the exponential growth phase. This would limit the number of dead algal cells and other waste products produced under unfavourable conditions. Second, most of the bacterial population could be removed through centrifugation (see below). After this treatment, the algae would greatly outnumber the resisting bacterial biomass, and the extract could be regarded as suitable for further screening. If an inhibition of interest was to be found in one strain of algae, axenic cultures could be cultivated. This would also provide additional information whether bacterial populations influence or trigger the release of bioactive substances from algae. Then, further tests to investigate the effect of growth conditions on the produced amount and quality of inhibitors together with general growth tests to optimise growth rates, efficiency of biomass production and biocide production could be accomplished. Cultures grown at the institute are generally kept in semi closed growth tubes (figure 3.2.a), where the outgoing air would minimize the entering of contaminants from the outside. Some cyanophyte cultures were obviously not protected from the environment and this led to the cross contamination between cultures or contamination from the air by unknown species. This became a severe problem, and made it hard to obtain the desired amount of biomass in some cases. Slow growing species are more vulnerable due to longer growth times, and the probability of contaminants to take control over the cultures by rapid growth when introduced greatly increases. Therefore, a new system was applied, consisting of entirely closed tubes (figure 3.2.b) making it practically impossible for any contaminants to enter. When handling of cultures involved opening and exposure to air, 70 % ethanol was used at the rim of the cork to sterilize it, and the edge of the tube was burned for further sterilization. This solved the problem, as contamination never occurred in any of these closed tubes. Another advantage

with this system is that tubes can be placed closer to each other, taking more advantages of space and light without the risk of cross-contamination. The cyanobacteria used in this work were all fairly slow growing, and therefore fall into the category of species that cannot be used for large-scale cultivation for substances. It has however been shown here that growth rates can be altered by adapting algae to favourable conditions over a period of time. A problem that also is true for some species is that they are slow growing due to suboptimal culturing, and may therefore be in a dormant physiologic state. Adaptation, however, may also lead to the successive alteration of the strain. What this means for the properties and assemblage of the strain is worth mentioning because laboratorial conditions might change the species when grown for a longer time.

**Harvesting algal biomass:** Centrifugation was chosen as means for harvesting biomass. This is a common way of algal biomass recovery, and has been concluded to be the preferred technique of choice (Grima *et al.*, 2003). When harvesting, keeping algal biomass intact and at the same time washing the broth is wanted when concentrating cells into a pellet. Centrifugation always bears the risk of breaking cells and consequently loss of important cell content to the surrounding water. Time and speed are parameters that can be altered to harvest biomass intact. Another purpose for the centrifugation process was to wash cells so no remnants of the medium were left. All of the greenalgae were marine species, therefore washing and re-suspending the pellet with distilled water removed most of the salts remaining in the biomass. An important factor in the centrifugation procedure is to diminish the number of bacterial cells present. Using the lowest speed in which the algal broth can be centrifuged and still produce a firm pellet accomplished this, leaving the bacteria in the supernatant. Re-suspending and washing the pellets would further remove bacteria. This was not in all cases achieved completely through centrifugation alone. They were then removed manually with a Pasteur pipette (section 3.2.6). As can be viewed in table 4.1 and 4.2, a reduction of biomass from estimated DW and freeze-dried occurred. Loss of biomass before freeze-drying can largely be explained by the washing and centrifugation process. Debris and bacteria in the culture can add to the total DW results. DW does not distinguish between dead and living cells. This demonstrates that dry-weight might not always be an accurate measurement for biomass and densities in the culture volume. But all loss cannot be explained in this way only, and is likely to be a consequence of differences between the species when it comes to physiology. In worst case cells might be too fragile to withstand the centrifugation, and they might break open releasing cytoplasmic content to the supernatant. This would obviously also influence the screening itself, but all potential precautions were performed to avoid this.

Another possible explanation is that a fraction of the biomass is lost to the surroundings during depressurisation when freeze-drying.

**Preparation of crude extracts:** When biomass is obtained, it has to be decided what quantities are acquired for extraction. It should be considered that extracts have to be in proportion to the bioassay it is to be used in (i.e. not a too small extract volume in a large culture volume). Secondly, it also has to be considered that the bioactivity searched for has to be effective in small amounts in the bioassay to be of interest. It is not possible to estimate in advance of what magnitude the activity will be expressed in the bioassay, or how much of any potential substances the algae will contain if it produces any. Preliminary steps together with comparisons with known fungicides and their effect in the bioassay were necessary in the developing phase of the screens. Operating within these terms, it is likely that the screen can detect bioactivity, and results can further be thoroughly investigated in a later phase. After all, this first screening is only a crude screen for any sign of bioactivity. Another factor that has to be evaluated, is the solvent used to obtain extracts. It is important that the extracting solvent itself has no or only little effect on the assay organism, i.e. *A. glauca* in the present case. Screening of hydrophilic extracts were performed using Milli-Q water as extracting solvent, and all inhibition would thus arise from any inhibitors originating from the algae screened. Selecting the lipophilic solvent proved to be more difficult. When the greenalgae were screened, it was assumed that ethanol was a suitable solvent for use. It was also assumed that any inhibition originating from ethanol could be subtracted from the results after a standardization of its effects on spores had been performed. This is also valid to some extent, the concern being that ethanol then could give a “false” detected inhibition caused by a general weakening of the viability of spores, or by hiding effects of extracts. This concern was appropriate since ethanol inhibited swelling of the spores. Alternatively, acetone or methanol could be used, and they were assessed in two concentrations (figure 4.49). Acetone inhibited spores more than ethanol did, and was therefore not an option. Methanol inhibited within the same range as DMSO, and is clearly an alternative solvent. But it was decided to use DMSO as it proved to inhibit spores to a lower degree than did ethanol. DMSO has the unique capability to penetrate living tissues without causing significant damage, most probably related to its polar nature, its capacity to accept hydrogen bonds and its relative small and compact structure (Szmant, 2001-2003). It has been reported to possess a wide range of pharmacological actions in laboratory studies, including membrane transport of non-ionized molecules of low molecular weight (Jacob & Herschler, 1986). Also, spores seemed to recover slightly better at low inhibiting DMSO concentrations over the next passing 24



hours, indicating a less severe metabolic effect (compare figure 4.47 and 4.48). Based on this, DMSO was applied as solvent for all cyanobacterial lipophilic extracts. DMSO extracts also tended to be less aggregated when introduced to the assay medium. The occurrence of large amount of small particles and clustering of spores was a challenging problem encountered with several ethanol extracts of greenalgae. Whether this could be explained as simply being a consequence merely originating from differences in the properties of the algal species is not known, but must also be considered (not only different species, but also diverging highly in taxonomic classification). DMSO has been used in previously antifungal research (Piccardi *et al.*, 2000; Voda *et al.*, 2004), and also methanol is frequently applied (Demule *et al.*, 1991; Kellam *et al.*, 1988; Mahakhant, 1998; Zulpa *et al.*, 2003). After both hydrophilic and lipophilic extraction, the pellet was dried and re-suspended. The lipophilic extracts would normally have been dried *in vacuum*, but the volume was too small to fit into the equipment available. Therefore another approach was used, as described in section 3.3.3. Nitrogen was carefully blown on the surface of the liquid in the Nunc tube, removing dichloromethane and isopropanol (both evaporate easy at room temperature) used for extraction, and replacing them with ethanol or DMSO. No residues should therefore be left of dichloromethane or isopropanol. Nitrogen was used instead of air to avoid oxidation of the material.

It is reasonable to conclude that all was done to diminish the probability of effects originating from another source than the specimen investigated, and also to prevent deterioration of biomass after growth, harvest and extraction. All antifungal activity would thus be assumed to originate from the algae.

### 5.1.2 Spore harvesting and storage

It is necessary to have a way to effectively harvest spores intact and viable. It is also necessary to keep the spores and preserve them intact until they are used in the bioassay. Therefore, a proper method was developed for this purpose, and it was investigated whether storage of spores was possible, if time of storage would affect viability or affect properties (e.g. passive swelling) related to the bioassay.

**Spore harvesting:** An initial search in the literature did not provide any consistent methods for harvesting. Different approaches were therefore tried until a satisfactory method was found (section 3.1). These involved different techniques to remove mycelium and debris and separating the spores, like filtration through cotton and the use of sintered glass for

filtration. Filtrating paper (Gelman Instrument Company, filter paper type W-41, 47 mm diameter) was tested using different Büchner-funnels. Spores passed through easily, but the suspension was not filtrated. A major problem besides the failure of removing debris (e.g. mycelium residues) was foaming (enhanced by Tween 80). This also became a problem when different polycarbonate (Uni-pore<sup>tm</sup>, 8.0 µm pore size, 47 mm diameter) and cellulose filters (Sartorius, pore size 8.0 µm, 47 mm diameter) were tested. In addition, spores failed to pass through these. Sintered glass filters produced the same problems, both with and without filters. Spores did cling to each other and foam was created. All of these methods also increased efforts in preparing the spore suspension, not being as successful as the final method used, refined and described in section 3.1.4. Tween-80 was employed as a surface-active agent (0.1% v/v) to remove surface tensions in the suspension and to reduce clustering of spores. Support for this approach was searched for and found in the literature, and provided some more information on possible advantages or disadvantages of this agent, as both Tween 20 and 80 are widely recognized as appropriate surface active agents. The National Committee for Clinical Laboratory Standards (NCCLS) has recommended the use of concentrations between 0.5 – 1.0 % in 0.85 % saline for inoculum preparations, but no standardized amounts of these agents have been employed in most of the reports published until now. This might however be of great importance, as it has been found that significantly higher MICs were associated with higher Tween concentrations (Gomez-Lopez *et al.*, 2005). It was found that the effect of Tween apparently is antifungal dependent, and it was postulated that this could be related to the solubility of the antifungal compound in the medium used. It has been suggested that some surfactants interfere with antimicrobial agents within surfactant micelles, preventing the microorganism-agent interaction. Coulter Counter has been used to detect the inactivation of preservatives by a non-ionic surface-active agent (Polysorbate (Tween) 80). It was found that Tween alone (2 %) did not prevent swelling of spores, nor did it inhibit the effect of phenylmercuric nitrate or chlorocresol, but interfered with the action of chlorocresol hydroxybenzoate esters, Nipastat and cetrimide (Parker *et al.*, 1966). This emphasizes the need to investigate antagonistic effects in two phase systems, suspensions and bioassays using such agents. A description of a harvesting procedure similar to the one developed here was eventually found to be used by a team of French microbiologists (Dantigny *et al.*, 2002; Sautour *et al.*, 2003), where Tween-80 was briefly investigated and was found to have no effect on spores prepared for inoculums on agar dishes of a *Penicillium sp.* specimen. Considering the species used (including the Zygomycete *Mucor sp.*) and the similarities of the method, it is likely to conclude that the method of harvesting spores

developed here is suitable for the purpose. A similar method was also found to be used for preparing spores from *Rhizopus oligosporus* (Mucorales) grown on malt extract agar (Thanh & Nout, 2002). Other methods found in the literature used much simpler methods for spore harvesting that could not be considered used or developed for the purpose in this Thesis, like gently scraping with a pipette tip (Khunyoshyeng *et al.*, 2002). In addition to harvesting the spores, it is important that they do not initiate the swelling phase. Keeping them cold at all times assess this. Temperature is a factor that affects spore germination and might initiate spore swelling, as seen in the work of this Thesis. As described in the Methods in section 3.1, suspensions were also washed several times to remove all of the medium, debris and bacteria using centrifugation. Spores proved to be relative dense, and did easily sediment under low speed, allowing centrifugation to proceed without harming or damaging the spores, and at the same time effectively wash them. However, properties of spores must be expected to vary greatly between fungal species, and refined approaches have to be found for hydrophobic or less dense spores if used.

**Spore storage:** Storage for a shorter period, i.e. 7 days was found to be possible. Storage for a longer time resulted in some fraction of the spores germinating, even when suspended in cold distilled water. During several weeks of storage under these conditions the spore fraction that germinated increased considerably. This made spores unfeasible for the assay. Storing spores in distilled water with Tween 80, had more germinating spores than storage without this compound. No further tests were performed to assess this, but nevertheless raise questions whether Tween 80 might act as a nutrient source that causes this response. No indication that Tween-80 affected the viability of the spores in the concentrations used here was observed. Additional dilution was done as the spores were prepared for the bioassay, and therefore the remaining Tween-80 was present only in very small concentrations. It is therefore assumed that Tween 80 imposes no effect in the screens (see above). It was, however, finally decided to store the spores in distilled water only, and Tween 80 only used in the harvest (leaving only minute residues). No changes were found in the volume or swelling properties of spores after storage. When prepared for research, stock solutions were at all times kept on ice, to avoid temperature changes. Storing spores in high concentrations was not only done to keep stock solutions easily available for further dilutions, but also served the purpose of minimizing nutrients per spore or other factors left in the fluid that might initiate an early spore swelling. Storage suspensions with more than 1-5 % germinated spores were considered to be useless for testing purposes. Removing the germinated ones by filtration was not an option, because of uncertainties about the quality of the remaining spores. Storage is

absolutely necessary, due to all the time consuming work (e.g. cultivation and harvesting). Storage is highly species dependent and has to be evaluated for each species to be used in a bioassay, but the rule is that asexual spores are relatively short-lived and survive best at low temperatures near 0°C (Cochrane, 1958). The concentration of spores, if they would prove to have an effect on the germination as reported (Frøyen, 1975), was considered not to be an issue in the absence of growth medium, but could actually be of importance if self inhibition occurred (see section 5.1.3).

### 5.1.3 Spore concentration and inocula

Spore densities had to be kept at a certain level; If the concentration were too low relative high levels of noise would affect measures in the Coulter Counter. If the concentration were kept high, an accurate size distribution was obtained when measured, but on the other hand the medium might become a limiting factor during the germination. The ratio of algal extract to the number of spores would then also decrease. The spore concentrations eventually chosen in this assay based on results and experience are relative high, in the order of  $10^1$ - $10^2$  spores / ml higher than other comparable assays found in the literature (generally ranging from  $5 \cdot 10^5$ - $5 \cdot 10^6$  spores or CFU / ml). It has been reported that inoculum sizes ( $10^4$  CFU/ml versus  $10^5$  CFU/ml) did not have significant effects on antifungal susceptibility testing results (Gomez-Lopez *et al.*, 2005), but at the same time it is also stressed as an critical variable. Another scenario that also would affect the screening if present is *Quorum sensing*. This is the ability of bacteria of the same species to communicate and coordinate their behaviour via signalling molecules. Growth patterns resembling this have been reported for spores of *A. glauca* in earlier research on the same strain (Frøyen, 1975). Spores of *A. glauca*, and also *A. niger* germinated earlier with increasing spore concentrations. Spores of many fungi germinate poorly or not at all in a dense suspension or when crowded upon a surface, caused by sporostatic factors that inhibit spore germination (i.e. self-inhibitors) (Carlile, 1994b). These are produced at the time of sporulation. The crowding effect alone is not sufficient evidence for the presence of self-inhibitors, although it suggests that inhibitory materials from the spores might be present. In many fungi self-inhibition has been traced to inhibitory substances given off by the spores themselves, but in others competition for key substances such as oxygen or nutrients may be a factor (Cochrane, 1958; Macko, 1976). Therefore, a gradient of different spore concentrations was examined to reveal more information on spore behaviour,

and to investigate if the medium itself could become limiting under the concentrations used here. But no such effect was encountered, besides what could be explained as nutrient limitation some time after germination. Spores germinated at the same rate and number regardless of the concentration in the assay, no lag or premature swelling was observed. However, such growth behaviours caused by the concentration in the assay deserves more attention than given in this Thesis.

#### 5.1.4 Spore morphology and size distribution

Two separate distributions of spore sizes were observed. It became clear when volumes of these two were compared that the second distribution largely consisted of paired spores that were measured as a single particle (approximately twice the volume). These distribution patterns were diminished by thoroughly homogenizing suspensions. Still, some abnormal spores were found when observed in the microscope, which had twice the volume of the majority of spores. As described in the Results, this was most striking in the + strain, which clearly possessed two size distributions. This complicates the inhibition measurements performed with the Coulter Counter. Striking was also the spores having globular structures, what is at present believed to be buds. This phenomenon was only observed in the – strain. Some smaller spores were present with a diameter of approximately 1.5  $\mu\text{m}$ , that was not observed to be swelling. These were first believed to be un-maturated residues from the sporangia. But as many of the normal sized spores were observed to possess an “outgrowth”, these small spores might also originate from budding. This is however still unclear. Microcycle conidiation is an unusual growth form observed in some species (e.g. *A. niger*), where conidiophores are produced directly from the spore without prior mycelial formation (McRobbie *et al.*, 1972). This is associated by poor growth condition, suggesting that differences in this phenomenon related to age, incubation condition and media of *A. glauca* needs to be investigated to gain more insight in this matter. In earlier work where spores are sized by the ESZ method, it has been found that spores typically among several species are distributed like the - strain (i.e. symmetrical about the peak size except for an extension of the upper size limit), especially distinct for different *Penicillium* spores (ranging from about 2 – 40  $\mu\text{m}^3$ ). *Aspergillus sp.* and *Mucor plumbeus* showing the same trends, but with more convex curves at sizes below the peak (about 10 – 300  $\mu\text{m}^3$ ). It is clear that different species has its own volume distribution with characteristic values for the minimum and maximum sizes, and

the peak size. These differences is hardly detected in the light microscope, but enlarged when volumes are measured in the Coulter, thus might serve as a valuable tool in identification and classification and in other aspects of mycology (Barnes & Parker, 1968). When investigated with a light microscope, it was difficult to study anything but crude structures and developments due to the small size and pigmentation of the spores. In addition, the striking features of some spores (i.e. budding) observed were not easily explained through the light microscope alone. Spores were therefore investigated using SEM to study details more thoroughly. The value of SEM in the study of structures and development in fungi, and for the classification of species has been emphasized (Hawker, 1971). The SEM confirmed the smoothness of the surface of sporangiospores. It was also established that the outgrowth of some of the spores did not assemble that of a germination tube, but looked very much alike the budding of a second spore, as already mentioned.

### 5.1.5 Spore swelling and germination

Spore swelling and germination kinetics are often referred to as simply germination in the literature. This reflects the little attention onto the process of spore development preceding formation of hyphae (i.e. germination tube). Behavior of spores are therefore often neglected when it comes to fungal growth, which becomes interesting to people first when filamentous growth occurs. Spores undergo a period of both passive and active swelling, and metabolic changes and responses to the external environment before they finally germinate. Hyphal growth is measured as hyphal extension rate, which is usually reported as radial growth rate of colonies ( $\text{mm d}^{-1}$ ). Growth occurs at the tip of the hyphae, and is linear with time. Changes in spores can be measured as increase of volume in a matter of hours ( $\text{vol. h}^{-1}$ ) prior to germination, and it is assumed that when germ tubes emerge growth assembles that of the mycelium, but often occurs at an exponential rate after the onset of germination (Cochrane, 1958). This might be used in advantage of this assay for fungal susceptibility testing, if both swelling and growth of germ tubes are measured. In other words, susceptibility of three different stages (swelling, germination and early hyphal growth) of fungal growth can be investigated within 6 hours and in the same assay. Neither passive swelling nor lag phase was observed. This is supported by evidence from the stored spores, which exhibited the same spore volume and size distribution when measured in the Coulter Counter, even after 14 days in the refrigerator. Also by comparison of spores measured at the instance of harvest, which



did not differ from those prepared or stored. No change in volume was found when spores were incubated in distilled water. This proves that temperature and water is not enough to initiate swelling. Spores initiated swelling immediately when introduced to the growth medium, and germination occurred within 4-5 hours. This is a most rapid response to external environments. It reflects that the state of dormancy is left when nutrients are available, triggering a metabolic response. Swelling lasted for approximately 4 hours, before the first germination tubes emerged. Swelling would still occur, ending at the time of germination which then was replaced by mycelial growth. Swelling was proven to be a logarithmic increase of volume to time (linear increase of diameter, see below). Even though volumes no longer increased after germination, germination tube elongation might also be used as an indicator of growth (or inhibition of growth) as already mentioned above. It was however not possible to estimate percentage of germination using the Coulter, and it was assessed if DW measurements of total culture volumes might be used instead (subtraction of spores left in the media after germination at a given time was also assessed, but to many problems emerged; i.e. noise, excess particles from extracts and spores, problems with removing germinated spores). Counts in the haemocytometer were also difficult and was regarded to have too many errors. Plate dilution series were also regarded as unfitted for this purpose. For the screens, other approaches need to be used (see below). The main problem complicating this was the clumping of germinated spores. Much lower densities have to be applied to estimate the percentage of spores, which exclude the Coulter as an appropriate tool (except to discriminate between swelled and un-swelled ones). However, as spore germination tended to be close to 100 % and inhibition was expressed as a lower swelling rate rather than in the percentage of swelling / germinating spores, this approach was not needed. All inhibition was therefore measured as average volume at a given time compared to the average volumes of untreated spores. This is concluded to be satisfactory, and to describe the actual event. To avoid osmotic errors in volume measurements with the Coulter system, the measuring electrolyte was always kept isosmotic with the growth medium. Spore germination usually has a narrower pH range than growth, presumably because there is less time for metabolic products to modify an unfavourable medium. Spores of most fungi germinate best at pH 4.5 – 6.5, which limits at about pH 3 and pH 8 (Cochrane, 1958).



### 5.1.6 Standardization with known fungicides

It was shown that inhibition of swelling and germinating spores could be measured. Inhibition was manifested as lower overall swelling rates, until swelling came to a halt, rather than a decrease of percentage swelling or germinating spores. When inhibition was weak, spores would swell and reach the point of germination. Germination patterns then resembled that of swelling (e.g. slower growth rates) as growth rates were also affected if germination occurred. Inhibition occurred as a sigmoid curve with increasing fungicide concentrations. Inhibition was also found to be relative stable measured after 24 hours compared to 6 hours (both for known inhibitors and for the extraction solvents tested). It also seems to be necessary to assess inhibition when most untreated spores would have been allowed to germinate. As can be interpreted from figure 4.43 and 4.46, inhibition measured earlier tends to differ from values measured after 6 hours, being higher for low concentrations and lower for the higher ones. This was especially apparent for Benzoic acid, and can be explained by metabolic adaptations when growth environments possess harmful compounds. It is likely to assume that these trends will be more obvious when inhibition is characterized by reversible changes within the spores, whereas irreversible changes will be a more instant inhibition (e.g. sorbic acid), and can possibly be used in the preliminary interpretation of inhibition patterns of an antifungal screen. Degree of resistance is determined by the lowest concentration of inhibitor required to prevent growth, the minimum inhibitory concentration (MIC). Determination of susceptibility in an assay composed of swelling spores can be determined in two ways, by no swelling or no germ tube formation (both preventing growth). MICs of swelling can be difficult to estimate accurately, as long as it is unclear what mechanisms lay behind initiation of swelling, or whether spores afterwards first swell mainly by a passive mode (i.e. uptake of water, no metabolic growth). MICs will also be higher than those required for germination. MICs ( $\geq 90$ ) of approximately 1.4  $\mu\text{g} / \text{ml}$  for sorbic acid and 4.5  $\mu\text{g} / \text{ml}$  for benzoic acid were estimated to prevent swelling, and to inhibit germination MICs ( $\geq 10$ ) of 0.08  $\mu\text{g} / \text{ml}$  and 1.0  $\mu\text{g} / \text{ml}$  respectively (values listed in Table 4.7, also as  $\text{mmol L}^{-1}$ ) would be required. This enlightens the great differences of concentrations needed to inhibit swelling compared to germination. The latter scenario is most likely when it comes to investigating extracts in a screen. Any inhibitor would most probably be present in minute concentrations. Different species of fungi differs in their sensitivity to sorbic acid and benzoic acid. Many species show a co-resistance to weak-acid preservatives (e.g. acetic acid, benzoic

acid, sorbic acid, all common food preservatives), and has contributed to the widespread assumption that all weak acids have similar mechanisms of action (Steels *et al.*, 2002). The classical 'weak acid preservative theory' proposes that protons are released in the cytoplasm causing the cytoplasm to become acidic, thus inhibiting the cell / spore. This theory has been confirmed. However, the role of sorbic acid and benzoic acid as a cause of cytoplasmic acidification has been questioned (Stratford & Anslow, 1998). Alcohols, notably ethanol, are known to disrupt membrane structure, altering membrane fluidity and dissipating proton gradients (Cartwright, 1986). The similar degree of inhibition shown by sorbic acid and sorbic alcohol, together with reports of high ethanol (18 %) tolerance in preservative-resistant yeast (Thomas, 1985), suggest common mechanisms of action (Stratford & Anslow, 1998). *A. glauca* showed in the present research a high sensitivity to both ethanol and sorbic acid. It is speculated that inhibition by benzoic acid may represent a hybrid action, releasing protons into the cytoplasm but also inhibiting by another mechanism (Burlini, 1993). This means that summarized, inhibition of these two fungicides may be explained as weak-acid preservatives, as membrane-active compounds (i.e. sorbic acid) or as specific inhibitors of metabolism (Ferrand *et al.*, 1998). A question that emerges when discussing inhibition measured on swelling spores is whether there are differences in fungicide susceptibility between swelling and germinating fungal spores and growth by mycelial elongation. These are clearly different physiological states of the fungus, and thus must be expected to have different metabolic patterns. This would in turn lead to differences in inhibition by the same preservatives. This is assessable if standardized inhibition on swelling and germination are compared to radial growth of mycelium. This would give a foundation to compare inhibition, and make clear if there are high differences in sensitivity or resistance. This was not performed in this Thesis, but is highly recommended for the method. Inhibition was estimated and determined by differences in volumes of untreated spores compared to those treated. Volume increases exponential, and was assumed to reflect growth (i.e. metabolic activity). It is worth noting that inhibition could also be estimated as a function of the diameter of the swelling spores (an approach found in older literature). But this would in turn lead to different inhibition values compared to those estimated from volumes obtained from the same sample, as diameter is a linear function with time. Due to the plausible difference of metabolism in swelling spores compared to mycelial growth, it is questionable which of diameter and volume that will give the best measure of inhibition. It was assumed in this Thesis that volume would reflect this best. This, however, stresses the need to standardize this approach to estimate inhibition on swelling spores. Chemical preservatives and their inhibition of the development of the spores

of a spoilage mould (*P. spinulosum*) were found to differ in their effects. While propylhydroxybenzoate and Phenonip reduced the rate of swelling of germinating spores at its onset (suggested MICs (100 % inhibition) of 0.025 % and 0.125 % (w/v) respectively), benzoic acid increased the time before swelling was started (McCafferty, 1970; Parker, 1971), thus extending the lag period before which the onset of metabolic swelling was detected with the Coulter. Linear growth was prevented by a concentration of 0.1 %. The antifungal agent methyl *p*-hydroxybenzoate also prolonged the lag period before onset of germination (McRobbie *et al.*, 1972). It is seen that the effect of preservatives is to depress the rate of metabolic swelling according to their efficacy and ambient concentration. These results supports that suppression of spore swelling provides early indication of preservative potential of a given compound and the type of swelling curve produced indicates the mechanism of fungistasis. This shows that the method might also in addition to measure antifungal susceptibility, indicate its mechanism of action. A trend which was found is that the earlier the onset of detectable swelling the faster is the rate of that swelling and the greater is the resistance of the spore to the agents (note; *A. glauca* exhibited a rapid swelling response). The use of swelling and germinating spores investigated by a Coulter Counter to screen preservatives for pharmaceutical and cosmetic preparation showed that effects were measured as slower swelling rates. Several species were investigated, and they all exhibited linear swelling rates (diameter) with time, differing in lag time only (Parker, 1971). It was also found that time of addition of antifungal agents influenced upon their inhibition on fungal spores. In every case a lag time was imposed as soon as the agent was added, and the maximal lag resulted when their addition was 1 h after the onset of germination (McRobbie & Parker, 1975). It was suggested to explain the increased sensitivity of the germinating spore with time of addition that spores germinated in the presence of inhibitors from the onset of the process developed resistance, or that as germination proceeded increasing permeability of the spore wall renders it more susceptible to inhibitors. This is a critical parameter in the standardization of such a methodology. The fungicides used in this Thesis are used as food preservatives. These must be expected to have higher MICs (due to its application) than does drugs used in the medicine. Therefore, when screening for highly bioactive compounds, this spore assay should also be tested against relevant medical compounds in use today; Amphotericin B, itraconazole, voriconazole, terbinafine, flucanazole, miconazole or ketoconazole to name a few in common use. Fungicides are usually grouped by similarities in chemical structure and mode of action, and can be divided in site-specific fungicides (disrupt single metabolic processes or structural sites, including cell division, sterol synthesis, or

nucleic acid synthesis) and multi-site fungicides (interfere with many metabolic processes and are usually protective in activity. These typically inhibit spore germination and must be applied before infection occurs). Fungicide groups have different levels of resistance risk, and risk assessment is critical for newly developed fungicides. Maintaining an array of effective fungicides is thus critical in resistance management. The risk of resistance to multi-site fungicides is low or absent, whereas site-specific fungicides have a high risk of single- or multiple gene resistance (Damicone, 1996).

### 5.1.7 Screening of extracts

No high degree of inhibition was detected from any of the extracts used, except one that clearly possessed inhibiting properties. There might not be any more reason for this than that there simply was no antifungal activity present in these extracts screened. There might also be the explanation that fungicides were present, but would not show any detectable inhibition in this assay. This stresses the need to compare this method with results of others, to assess screen detection sensitivity. Standardization with known fungicides has shown that it is quite possible to detect any antifungal activity if present in high enough concentration. However, some of the extracts may contain active compounds, but their concentration in the extract was too low to be detected. The assay should perhaps have been compared to an algal species with known antifungal effects (a strain of *Chlamydomonas reinhardtii* has been shown to effectively inhibit radial growth of this fungal species on agar assays by work not included in this Thesis). Antifungal results from the strain of *Phormidium sp.* 209 is believed to be valid, and thus is a candidate for further investigations, but the effect is too low to give any indications about the inhibition mechanism. It might be a novel compound present in small quantities. But it can also be explained by simple alterations in the bioassay caused by other factors introduced in the medium, like changes in pH or availability of nutrients caused by the substances from the extracts. It is also very interesting what was observed in several H-extracts, where germination rates clearly improved compared to controls. Due to the difference compared to those spores not germinating in the presence of extracts, this effect can not be explained by simply being extracts that are not harmful in any way, but must be explained by factors that improve growth conditions. Plant growth regulators (PGRs) are common in algae (see introduction), and such substances might explain this phenomenon, as it is believed that the media should be satisfactory for germination to occur. The strain of

*phormidium* tested here and that was found to possess antifungal properties, might suggest that cyanophytes from earth samples (the habitual origin of this species) might be the choice in search and discovery tactics, as they might already possess such properties competing with fungal species. PGRs might be more broadly found among species, dependent on what kind of substances fungal species like *A. glauca* (saprophyte) are stimulated by. It may be general residues from the broken cells, or it is also quite possibly specific factors indicating the present of a nutrition source not found in a medium like the one employed here. Several antifungal activities have been detected from both microalgae and especially cyanobacteria (Begum *et al.*, 1999; Daranas *et al.*, 2001; Demule *et al.*, 1991; Edvardsen *et al.*, 2004; Fish & Codd, 1994; Golakoti *et al.*, 2000; Houdai *et al.*, 2004; Kajiyama *et al.*, 1998; Kellam *et al.*, 1988; Kulik, 1995; Mahakhant, 1998; Nagai *et al.*, 1993; Nagai *et al.*, 1995; Piccardi *et al.*, 2000; Sato & Sasaki, 2005; Skulberg, 2000; Soltani *et al.*, 2005; Zulpa *et al.*, 2003) (section 1.2.2).

## **5.2 Evaluation of the bioassay**

Evaluation of a method must be based on its success on being sensible, i.e. detect antifungal activities, rapid, efficient (amount of work spent preparing, performing and producing results) and reliable (i.e. stability, reproducibility of results). These four items will first be evaluated. Conclusions will be based on experience throughout this work as well as on results obtained. Important in the final evaluation is comparisons to other methods and the prospect of optimizing and refining the methodology from its present status as a prototype. There are two major factors that have to be considered in the overall evaluation of this method. First the use of spores as means of assessing fungicide screening contrary to filamentous fungal growth measurements. Second, the composition of the bioassay as a candidate for the rapid screening of extracts has to be evaluated. The use of Coulter Counter as a method to investigate spores and detect changes in swelling and germination has been proposed in earlier studies, first reported by Parker and Barnes (1966), and then later as a candidate for investigations on fungal and bacterial spore developments, and for the screening of preservatives and detection of antifungal activities. Several of these have been mentioned in the proceeding discussion (Barnes & Parker, 1966; Barnes & Parker, 1967; Barnes & Parker, 1968; McCafferty, 1970; McRobbie *et al.*, 1972; McRobbie & Parker, 1975; Parker,

1971), and have given background information contemplating the development of this method. None of these, however, have been found describing the bioassay proposed in this Thesis.

### **5.2.1 Fungal spore swelling and germination as a method**

It has been shown through the work in this Thesis that swelling of fungal spores can be effectively measured using a Coulter Counter. Further, it has been shown that antifungal effects when introduced as known fungicides can be detected and estimated on swelling spores. A disadvantage might be the sensitivity of the bioassay. Small inhibitory effects were hard to detect or distinguish when most spores were then in the germinating phase where some would have developed germination tubes or even germinated. It might be hard if not impossible to detect and make accurate estimates of inhibition below 5-10 % due to the problem of separating germinated spores from those not germinated. In an ideal screen, all spores would have germinated at the same time. This is not the case, and it was therefore decided to allow most spores to germinate in the control. It might be lowered to right in advance of germination, using endpoint criteria between 75 – 90 % to solve this problem. It has to be emphasized in this regard that earlier measurements may be premature for detection of true inhibition (page 98). When spores germinate, volumes are no longer reliable measures, but could still serve as an indicator for growth. Hyphal growth is not measurable in the Coulter Counter, but trends might be detected and even standardized. Therefore, the method seems to be limited to the swelling phase. A major problem after germination that highly affects the performance of the Coulter is the clustering of spores, as mycelium weaves into one another. This knitting makes grains of mycelium that are hard to dissolve. It was found that spores after germination had different growth rates exposed to different extracts. It was not found that spores germinated earlier even when they displayed a higher growth rate after germination (i.e. hydrophilic extracts). This shows that also factors enhancing growth (most visible after germination) can be detected. There is no accurate way of determining the percentage of germinated spores using this method due to clustering. It seems that other approaches using haemocytometer, colony counts on agar dishes or automatic imaging remains the best and possibly the only alternative. This is however not considered to be of great importance to the method. Due to problems determining the percentage of germinated spores, DW was tried as an alternative, but it was concluded not to be satisfactory (see



Results). Percentage of germinated spores also has to be a function of time, as more spores will have germinated after 6-7 hours than after 5 hours. This will lead to the occurrence of more elongated mycelium in the culture volume, and thus make the use of Coulter or counting chamber more difficult. Inhibition might be expressed as both inhibition of swelling and germination. Preventing germination will prevent fungal growth and will also be inhibited by less fungicide concentrations than what are needed to prevent all swelling. The question that ultimately has to be asked is when to define the start of swelling, if passive swelling will be more pronounced in some spores / species. It is proposed in this Thesis that start of germination could be the best endpoint criteria. Densities of spores might influence the percentage of germinated spores. This was investigated, and was found not to be a major source of concern. The Coulter Counter has proven to be a definite usable tool to study and describe the swelling and germination of fungal spores. This method has to be considered to be rapid allowing many extracts to be screened within a short time frame. A challenge to the method is the tendency of spores to cling in groups of two or more. Another problem is their increase of buoyancy, from being denser than water to become buoyant when germinating. They do not act as merely suspended particles, but change their properties according to development phase. This must be expected to vary among different species. These properties change according to physiology (e.g. swelling phase) and to external factors such as extracts (especially the lipophilic). These problems are diminished using homogenizing techniques during incubation and when sampling, which is also necessary to keep ingredients circulated. This is also important during preparations of spore suspensions (inoculums). Spherically properties (e.g. elongated or flattened contrary to absolute spheres) of spores of different species of fungus might contribute to special approaches to volume estimates, but would not affect relative antifungal susceptibility estimates. Spore dynamics are investigated in a fluid environment, and bioassays using spores consequently have to be based in a fluid medium, where extracts and other ingredients are added. This is advantageous in terms of handling, but offers a challenge in means of estimating effects. This approach makes it easy to control incubation conditions for spores, offers the ability to be more accurate in determining bioactivity at the germination level. It also can be more rapid than most other methods; results are present after 6 hours of incubation (species dependent). On the other hand, it has to be improved if it is to compare to automatic turbidity measures in terms of efficiency. Particles in the suspension or in the bioassay are a major problem when measured in the Coulter Counter. Especially large concentrations of particles were occurring in some of the lipophilic extracts. These were mostly confined to the lower region of the volume scale, but when they



did accumulate, they not only interfered with measurements as single particles, but they did also to a great extent contribute to the creation of clusters of spores. The MEX-medium consisted of a crude undefined part, namely the malt-extract. This part will always have some debris that could act as particles in the solution and so lead to an error or "noise" in the results. Solving this by filtering the medium would therefore also remove a part of the nutrients; this was not considered to be a problem as spores were only grown in a short time period, and did not exhaust the media within this time scale (see results). After filtering, further reduction of noise from both electrolyte and medium can be measured and subtracted by the software. Filtration was absolutely necessary to diminish the background noise. Importance of running controls simultaneously cannot be stressed enough. They will reveal any deviations during experiments, and is essential in the judgement of results. Bioassay parameters such as volumes, spore concentrations and parallels are easily adapted and optimised. Culture volumes used were rather small, but is comparable with other methods using fluid medium. It was desirable to keep culture volumes small to avoid extensive use of large quantities of spore suspensions and extracts. This is necessary in the larger screens. But some volume was consumed for the measurements in the Coulter Counter, especially when general research was done to access the germination and swelling dynamics, when measures had to be taken within small time lapses. The inoculum concentration was set to a relative high level to accommodate handling in the Coulter, but investigations showed that this should not affect the overall performance of the screen (section 5.1.3). Concentrations of  $1.0 \cdot 10^6$  spores / ml are however within the range and recommendable. This will also give access to unlimited supplies of spores from only one or two harvested Petri dishes (each producing more than  $2.0 \cdot 10^8$  spores / ml). Parallel cultures were not run simultaneously when screening. One culture was decided to be viewed as an entity, and three parallels were instead measured in the Coulter. This was not believed to constitute a potential source of error. A criterion for the method was that it had to be rapid. Therefore parallels were not considered as an option. However, if this is considered of great importance, the use of a fixative will allow this. Finally, the use of spores contrary to measure fungal susceptibility in means of suppressed hyphal growth has to be considered. It can be speculated whether physiology (e.g. diffusion of fungicides through spore walls vs. hyphae) and metabolism is too different to give inhibition measures that are comparable. This can be solved by comparison of same concentrations in respective appropriate bioassays comparing spores and hyphal growth. This approach used here is simple and reliable, based on investigations of effects on the range of many individual spores, contrary to turbidity methods that are based on the overall growth. Volumes of spores

did not change, nor did swelling properties. It is also possible that the use of this method will accommodate the further studies in the accurate physiological effect imposed by an extract, as the way spores are inhibited may well give leading information. As already mentioned, different fungistatic action may inhibit the spores at different stages or disrupt the swelling and germination by other means (e.g. excessively swelling during germination resulting in lysis, a deficient linked to a defect in cell wall biosynthesis (dEnfert, 1997). It has also been reported that temperature and media greatly influence on the swelling rates (McRobbie *et al.*, 1972), something that further stresses the importance of standardisation of assay parameters. Age of spores has also been reported to influence, as older spores tend to swell slower. Osmotic effects on mould spores were also detected in different saline solutions, which did not interfere with swelling rates, but altered the time at which swelling was first detected by the Coulter Counter (Barnes & Parker, 1967). This may enhance the need for a standardisation of media and the electrolyte used. The lag in detect ability with different saline electrolytes compared to optical measures in the media showed that it was caused by an immediate increase in the size of spores due to uptake of water. This experiment was conducted in what can be regarded as a transfer of spores from one media to another exhibiting lower osmotic pressure. This is highly species dependent, an effect increased by a longer swelling phase and a higher passive swelling portion of the overall swelling. Lag periods observed in some species may therefore be caused by the spore becoming isotonic with the media before swelling is detectable in the Coulter.

## 5.2.2 Comparison to other antifungal screening methods

As mentioned in the introduction, the increasing understanding of cell biology and new biotechnological techniques has replaced the non-specific assays of the past with approaches to drug discovery using mechanism-based screens directed at specific biochemical targets (Hertzberg, 1993; Riedlinger *et al.*, 2004; Shu, 1998). These falls into two categories; receptor- or enzyme based screens using a particular target of interest in a purified system; or cell based assays using engineered cells and microorganisms (Hertzberg, 1993). Cell function assays (*in vivo*) are to prefer over *in vitro* assays. It allows the screening of many crude extracts, for further evaluation of positives, rather than use purified molecules in an assay that might not possess the same properties *in vivo*. The factors that has to be considered selecting and developing a screen for revealing antifungal activity from crude algal extracts has been

presented in the introduction. The method developed and tested in this Thesis is shaped within these terms. The earliest classical method used to detect antifungals is the whole-cell bioassay technique. Minimum inhibitory concentrations (MIC) and minimum fungicidal concentration are determined and the best compound is selected. This classical method is still in use today, because it targets the growth of whole organism. However, this method cannot identify the target specificity or the mechanism of action of the compounds. With the greater knowledge of fungal metabolism, efforts are being made to inhibit specific enzymes in the metabolic pathway of the fungus (Gupte *et al.*, 2002). Targets can be summarized in specific inhibition of the cell wall biosynthesis (particularly the glucan synthesis, but also chitin), cell wall associated “adhesions” (mannoproteins in the cell wall periphery), inhibition of synthesis of specific lipids of plasma membrane, secreted aspartyl proteinase and translation elongation factor. The fungal cell membrane and cell wall are structures that are essential for the fungus to survive. They differ from those in the mammalian host and consequently present attractive targets for new antifungals. One reason for the slow progress compared to antibacterials is that, like mammalian cells, fungi are eukaryotes; and therefore agents that inhibit protein, RNA or DNA biosynthesis in fungi have greater potential for toxicity to the host as well. Amphotericin B (polyene macrolides), discovered in the 1950s and marketed in 1957 is still a “gold standard” for the treatment of the most severe invasive fungal infections. Bioassays involving fungus whether using recombinant strains or not to assess a target, are generally constructed using vegetative growing cultures exposed to extracts. Different assays available and in use for antifungal screening of crude extracts can be roughly divided into plate dish diffusion assays, turbidity assays, and methods using dry weight as a biomass measure of growth;

**Paper-disk plate method:** The double layer technique composed of 9 cm Petri dishes containing agar-based medium. Suspensions of spores or mycelium (i.e. cells, as c.f.u.) are inoculated on the top layer of the agar. Then microalgal extracts (e.g. 1 mg) are applied on paper discs (6 mm in diameter) for antibiotic assay, and incubated for some time to allow growth (e.g. 5 days). A commercial antifungal compound is used as a reference standard of bioactive compound, and the clear zone size produced by the crude extract is converted to the concentration of this anti-fungicide with a standard curve (e.g. expressed as mg equivalent to known fungicide  $\text{g}^{-1}$  algal dry weight) (Houdai *et al.*, 2004; Mahakhant, 1998; Soltani *et al.*, 2005). This method is by far the most common used in antifungal assays (Moore *et al.*, 1996).

**Plate method:** Petri dishes (9 cm) are prepared with agar medium mixed with extracts (e.g. ratio 1:1 or 1:5). These are inoculated at its center with an agar plug (5 mm diameter) cut

from actively growing plates of fungus and incubated for some time (e.g. 3-14 days). Growth is estimated by measuring the colonial mean radii compared to controls (Begum *et al.*, 1999; Demule *et al.*, 1991).

**Plate well method:** Cell or spore suspensions are mixed with agar medium before introduced to Petri dishes (9 cm). Wells are then stamped (e.g. 5 mm in diameter) in the agar when cooled, the algal extracts inserted in these and the plates incubated for some days. Filtrates or extracts that contain antifungal compounds will produce distinct, clear circular zones of growth inhibition around the wells, and the widths of these are then measured (Kellam *et al.*, 1988).

**Turbidity methods:** Based on optical densities of liquid cultures. Growth is measured efficiently using 96 microtiter plates in a microplate reader. Absorbance (630 nm) is normalized to dry weight, and is a rapid method (Langvad, 1999).

**Dry weight measures:** A suspension of either mycelium or spores are incubated in a fluid medium and separated by filtration after some time (less time consuming than the plate method). Growth is then evaluated by dry weight of the mycelium (Zulpa *et al.*, 2003).

**Others:** There are several other approaches, like the use of tubes or flasks for incubation for special purposes like construction of “aromatic chambers” to investigate volatiles in a saturated atmosphere (Jain & Agrawal, 2002), or released by cyanophytes directly by placing Petri dishes inoculated with fungus (plate method) on top of Petri dishes inoculated by the cyanophyte (Begum *et al.*, 1999).

Essential in all such assays are the use of controls (blanks and extraction solvents) and standardization to known fungicides, and also the use of different concentrations of extracts. Comparisons of antifungal susceptibility are not always, however, compared to known fungicides. These may be based on controls alone. Since these methods involve growth, inoculums of spores (when used) or mycelium (concentrations given as CFU/ml) do not need to be high, and are most often in the range of  $1.0 \times 10^4$  –  $1.0 \times 10^6$  unites per ml. Most often suspensions prepared of broken mycelium (i.e. cell suspension) are employed. Incubation time is an important element. Spore swelling assays are superior to all plate dish assays when it comes to the experimental time required, also when it comes to amount of medium and extracts needed for each screen. Therefore, the method presented in this Thesis has an advantage in that regard. This bioassay is based on the inhibition of swelling and germination of fungal spores rather than on the inhibition of fungal growth (mycelium). Another method that has been employed is the testing of extracts added to spores in different concentrations using 96 well microtiter plates and a microplate reader, which has been developed for

filamentous growth measurements (Langvad, 1999). A major problem has been errors due to uneven growth (i.e. growth at the edges of the wells) and subsequent incorrect turbidity measures (based on experimental experience at the Institute, UiB). Erlenmeyer flasks instead of wells offer the opportunity of using higher volumes, but have the disadvantage of being more space demanding. Such problems have already been discussed as a potential problem when measuring spores in the Coulter Counter. The method is however a prototype with the potential for further improvements. For determining the percentage of viability of spore preparations, the plate count technique has been a useful tool. It is based on the assumption that when a dilute suspension is spread on a suitable medium, each individual spore will grow and produce an isolated colony. Basic problems with this method are underestimations of percentage viabilities (more than one spore per colony or not visible colonies), lack of reproducibility (uneven distributions of spores), and long incubation times (2-4 days). This approach was used to assess viability and percentage of spores compared to measures in the Coulter Counter, but was found not to be an alternative for the method. The same conclusion was made for the use of dry weighs, thought it can be used in preliminary research. Thus, this assay is left with Coulter samples as the only effective means of assessing bioactivity.

Automatic image analysis is a method that has been developed for assessing the viability and the germination characteristics of fungal spores in submerged cultures. The method described (Paul *et al.*, 1993) offers a number of advantages over photomicroscopy or colony counting being rapid, accurate and consistent, can discriminate between non-germinated and just germinated spores, and in particular can be used on spores germinating in the actual submerged fermentation medium. Antifungal susceptibility testing has been limited by a lack of reproducibility and uncertain clinical relevance. In drug development the focus may be on selection of the most potent of a series of compounds for further development. In clinical epidemiological research the issue may be the tendency of resistance in initially susceptible isolates or species and to establish the local pattern of resistance. The area of antifungal susceptibility testing is now rapidly evolving with simultaneous (rather than sequential) development of standardized reference methods (broth macrodilution), of more readily applicable methods (broth microdilution) and of commercial systems. Antifungal susceptibility testing presents a few specific problems, which have been addressed in the standardization process (determination of endpoint for antifungals, complicated by the phenomenon of trailing, related to the fungistatic action of these agents which may result in a lag period of some hours before complete inhibition of growth is established). The proposed standard macrodilution broth method for yeast (M27-A2 reference method) and the M38-A

reference method for filamentous fungi (NCCLS, 2002) has formed the basis for development of a variety of antifungal susceptibility test methods. These include a standardized microdilution broth method and the Alamar, Sensititre, and Etest commercial systems. The NCCLS proposed standard for yeast susceptibility testing has also acted as a starting point for development of the standard method for susceptibility testing of the filamentous fungi. Elements in standardization are methodological questions, including assay media, standardized inoculums and stocks, harvesting spores, incubation temperature, pH and duration, and determination of endpoint criteria for different antifungal compounds. The most direct approach to development of a microdilution broth method is the miniaturization of the broth macrodilution method. Both methods estimate MICs after a period of 24 – 48 hours of incubation, or even longer. A relatively recent development in the field of antibacterial susceptibility testing is the stable gradient technology marketed as the Etest. The Etest is a plastic strip with the relevant antimicrobial on one side and a scale on the other side. When applied to an agar plate, a continuous gradient of antimicrobial is established in the surrounding agar. This technology is being applied also to antifungal susceptibility testing (Cormican & Pfaller, 1996) (Mallie 2005), and has proved to be in agreement with MICs found by comparing with the standard broth microdilution method (Serrano *et al.*, 2003). For the purposes of standardization, the inoculum concentration is a critical variable. The effect of increasing inoculum size causing an increase in the MIC of an antimicrobial compound is defined as the inoculum effect (related to the different mechanism of action of antifungal drugs, caused by e.g. enzymatic target and other processes). Inoculums are commonly prepared by spectrophotometric methods using OD to determine inoculums (CFU/ml) and have been widely studied in yeasts (Espinelingrof, 1991). The length of incubation has also been reported to affect MICs, something that was investigated in the present method in this Thesis. Extended incubation times may allow resistant organisms to overgrow the initially susceptible subpopulation, leading to higher MICs. On the other hand, during prolonged incubation a degradation of antifungal compounds also may occur. This offers an advantage to the proposed assay in this Thesis, due to the short time frames involved. Effect of inoculum size of medically important filamentous fungi on MICs of antifungal compounds was found to depend on the organism and the compound tested. The choice of assay media and incubation temperature and time as well as different approaches in scoring are important factors in determination of MICs. Little is known, however, about the effect of inoculum size on MICs for pathogenic filamentous fungi (Gehrt *et al.*, 1995). The method presented here, falls in between the two standard methods proposed by NCCLS and widely recognized as the



foundation of standardization of antifungal susceptibility testing, but is likely to be easily adapted within these terms. Several efforts have contributed to the standardization of a reference method for antifungal susceptibility testing of the filamentous fungi. Less stringent MIC endpoints criteria ( $\geq 75\%$  inhibition relative to growth control) were reported resulting in improved interlaboratory agreement. The less stringent (75%) inhibition criterion was necessary to allow for the trailing frequently seen with the azole antifungal agents (Espinelgroff *et al.*, 1995). Using defined endpoint criteria is also important in the standardization of this present method, besides those already mentioned (i.e. inoculum, time, temperature, media, stocks, harvesting and pH).

### 5.2.3 Improvements of the fungal spore assay

Refining the present method may involve different approaches. Optimising by lowering the time of inhibition endpoint criteria (by comparing standardized swelling trends with standardized fungicides to find optimal time of sampling). Use of fixation of spores prior to measuring, and perform these measurements alternately to the Coulter by image analysing or flow Cytometry. To improve the method by easing work efforts and assess sampling and screens, formaldehyde was tested to preserve cultures. This fixative was found to strongly influence spore volumes, thus not being a good preserving agent. Using a fixative that would both stop all growth and at the same time preserve spores fully intact (i.e. volumetric) would greatly increase the efficiency of the method. Both by increasing the amount of extracts run simultaneously and by making screens less dependent on strict time bound measures for accurate and reproducible results. An alternative to formaldehyde might be Lactophenol cotton blue. This fixative, commonly used to stain fungi, was not tested here, but was reported to not affect spore properties (Paul *et al.*, 1993). It is composed of lactophenol, which serves as the mounting fluid, and the dye, cotton blue. Organisms suspended are rapidly killed by the presence of the phenol, which acts as a cytoplasmic poison, precipitating cellular proteins and inactivating essential enzyme systems. At high concentrations of phenol, cells are not lysed due to the inactivation of lytic cellular enzymes. Cotton blue is an acid dye, which stains chitin and cellulose. Staining of fungi by cotton blue is due to the presence of chitin in their cell walls. Staining would however mean nothing for measures in the Coulter, but could assess other approaches to measure inhibition of spores (i.e. automatic image analyses). Testing against Amphotericin B is also recommended, to compare results from screens with



medical important drugs. Several different fungal species also need to be tested within the terms of the present bioassay in this Thesis, to further standardize the method and its application as a fungicide method for a variety of fungal species. Testing of the relationship between inhibitions on spores versus inhibition on disk diffusion assays is important to assess the differences in methods and physiology contrary to the different stages of lifecycle. When it comes to storage, investigations concerning the possibility to freeze spores are interesting, and would ease laboratory efforts preparing screens, a method not found described in the literature yet. Viability tests on the inhibited spores are also of great concern, to assess whether spores are dormant or dead in the presence of inhibitors. This could easily be tested by removing spores from the medium with inhibitors, wash them and transfer them to favourable conditions without inhibitory substances. Assays with the same fungicide concentration, but different inoculum sizes to investigate possible differences in MICs might also be useful for the standardization of the method.

### **5.3 Conclusion**

In this Thesis it has been shown that spores can be easily harvested and stored. This will to some extent depend on the species applied, and tests is necessary to investigate patterns. It has also been shown that a bioassay can be composed that will reveal any antifungal effects. Trough the standardization to known inhibitors, and the swelling and germinating patterns of the spores, screening can rapidly be performed on a larger basis, producing results the same day as tests are initiated without extensive work or handling. Harvesting and preparation of spore suspensions are easily done within the frame of one day, and as much as 10 algae (20 extracts) can be measured a day by the work of one person. Further optimising the work by automatic measurements can increase screens and decrease work. It has also been shown that applying Coulter Counter in fungal spore behaviour investigations can be of great value, not only to access amount of spores produced, but also to give accurate insights in the process of swelling and germination, as well as spore size distribution patterns. Results here serve as an example of the diversity of spores in behaviour and morphology. Swelling can be used as a first indicator of inhibition, and further research (e.g. disk diffusion assays) can be performed at a later stage to investigate fungicidal effects if necessary. The use of two different concentrations (or more) of each extract will ensure that effects are investigated at different

resolutions or scales, and will at the same time serve as a first indicator on the nature of positive hits if encountered. Running controls parallel with crude extracts will ensure that no unforeseen events have any effects on the screens. Coulter screening applications should also be transferable to screening of yeasts cells as well as on spores of higher fungi, due to their single cell nature. This applies to bacterial and actinomycetes as well. It is concluded after this examination that the bioassay is a worthy candidate for screening after antifungal effects from crude algal extracts. The degree and mode of inhibition can be used as an early indication on the kind of fungicidal action, a clear advantage over existing method. After having screened 21 algae (84 extracts) and tested spores to two known inhibitors and four extraction solvents, this method has been investigated thoroughly and is considered well tested within the terms of this Thesis. All advantages and disadvantages revealed have been reported and discussed. Factors regarding spore handling, behavior, algal biomass production and extracting, and the screening process have been documented. Most scenarios that could occur have been thought of and taken into account. Given the many extracts screened, it is likely that most of the possibilities this method offers has been investigated. This has been a preliminary (prototype) bioassay to prove its functionality or its failure as such, and is open for redesign, some of which has been proposed. To discover new and maintaining an array of effective fungicides is critical in resistance management. This new method can contribute to the challenges of developing new drugs, and at the same time bears the prospect of being rapid and allowing the fungistatic action of fungicides to be assessed early in the developing phase.

#### ***5.4 Suggestion for further research***

Besides the proposed improvement of the method as a bioassay (section 5.2.3), there are several other elements that deserves more attention. This involves investigation concerning bioactivities in algae. Screens based on the cultivation medium after harvest of algal biomass might reveal higher doses of substances with antifungal activities that might be present at higher concentrations in the medium due to releases from living or dead cells (lysis). These are expected to be hydrophilic, and might be lost during preparation of only biomass for screening. Screens based on algal strains cultivated in the presence of extracts from fungus (i.e. mainly the fungal species that are target for investigations) are also a modification that

can trigger a response from the algae, and contribute to the optimizing of active substances as algae might produce such substances in higher amounts when encountering fungal growth in their presence. In addition the findings in this Thesis regarding spore size distributions and the phenomenon described as probably a case of budding deserves more attention. Also the effect of spore densities are an area of great interest, whether spores inhibit or trigger each other to swell and germinate, and the relation to high or low concentrations. Further, their increase in buoyancy is an interesting topic (not essential to this method, when using homogenizing techniques), together with the increase in biomass (and general metabolism during swelling). This could also enlighten passive vs. active swelling, and the species dependent use of stored nutrition during germination.

## ***5.5 Epilogue***

The original aims of this Thesis did somehow change during my work. This was mostly due to the much greater challenge of developing the method than what was initially expected. There was not initially found much support in the literature, therefore most of the material and methodology had to be developed progressively. Cultivating the algal species also became a true challenge, consuming much time and efforts. In addition, several problems were encountered during general experimental work, which I will not give any more attention here. After all, the methodology was confined within wanted terms, and even though many things did not work out, they also presented a great challenge and the opportunity to observe new and interesting problems. Throughout this work, I have been introduced to a field of bioscience that I consider myself not to have been familiar to, but which have been mostly interesting and have enhanced my perspective of the wide field of bioprospecting. I have also learned so much more than what concern this Thesis. First of all, I have put a lot of effort in teaching myself how to use different software. Also, I have now perfected the art of “surfing the net”. Using Internet has become more and more important as a tool for information and communication, priceless in the field of science because it allows for the first time in history the rapid sharing of ideas and inventions. To summarize, this Thesis has been a true overall challenge, but when now finished also very much interesting and over all teaching. I am pleased to present the final work, which aims I find satisfactory met and solved.

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## APPENDIX

### 1. *Malt Extract Medium - MEX*

This is the medium used for all fungal growth in this work. The medium is a non-defined medium based on malt extract. Petri dishes were prepared as according to table A.1. All fluid cultures were grown on the same medium, but without agar added. Fluid medium was used for the investigation and screening of fungal germination, and prepared as according to table A.2. Since this medium was intended for the use in the Coulter Counter test system, it had to be filtered to remove any debris or fragments of the malt extract large enough to interfere with the measurements.

#### MALT EXTRACT MEDIUM (MEX):

**TABLE A.1**  
Solid MEX (Agar plates)

<b>Ingredients</b>	<b>g/L</b>	<b>Medium state</b>	<b>Area of use</b>
Agar malt-extract	50	Solid	Agar dishes
Agar	5	Solid	Agar dishes

All ingredients were mixed together in distilled water, and then warmed below boiling point until they were fully dissolved. Then the medium was autoclaved (125°C for 20 min.) before dispersed (still warm) into sterile Petri dishes and left for cooling for 24 hours. The dishes contained then about 0.5 cm of finished medium, and were kept upside down in darkness at room temperature until needed

**TABLE A.2**  
Fluid MEX

<b>Ingredients</b>	<b>g/L</b>	<b>Medium state</b>	<b>Area of use</b>
Peptone (Oxoid)	5	Fluid	Spore suspension
Malt-extract	30	Fluid	Spore suspension

All ingredients were mixed together in distilled water. Then after blending it was filtered through a layer consisting of a glass micro fibre filter (GF/C, diameter 47 mm) at the top, and a membrane filter (polycarbonate, pore size 0.2 µm, diameter 47 mm) at the bottom, before it was autoclaved (125°C for 20 min.). The finished medium was then stored in a refrigerator at 4°C until needed.

## 2. *Standard Chlamydomonas Medium*

This is the standard medium (Kuhl and Lorenzen, 1964) that was used to grow all greenalgae strains. The medium was based on freshwater (autoclaved distilled water), with 24 g/L NaCl added for the marine species grown here.

The medium was prepared from 5 different autoclaved stock solutions stored at 4°C as listed in table A.3.

**TABLE A.3**  
Medium for unicellular greenalgae (after Kuhl and Lorenzen, 1964).

Stock solutions	Component	g/L	Add to distilled water ml/L	Molarity in Medium
<b>A</b>	KNO <sub>3</sub>	101.1	10	1.0•10 <sup>-2</sup>
	CaCl <sub>2</sub> •2H <sub>2</sub> O	1.47		1.0•10 <sup>-4</sup>
<b>B</b>	MgSO <sub>4</sub> •7H <sub>2</sub> O	24.66	10	1.0•10 <sup>-3</sup>
<b>C</b>	NaH <sub>2</sub> PO <sub>4</sub> •H <sub>2</sub> O	62.1	10	4.5•10 <sup>-3</sup>
	Na <sub>2</sub> HPO <sub>4</sub> •2H <sub>2</sub> O	8.9		0.5•10 <sup>-3</sup>
<b>D</b>	H <sub>3</sub> BO <sub>4</sub>	0.061	1	1.0•10 <sup>-6</sup>
	MnSO <sub>4</sub> •H <sub>2</sub> O	0.169		1.0•10 <sup>-6</sup>
	ZnSO <sub>4</sub> •7H <sub>2</sub> O	0.287		1.0•10 <sup>-6</sup>
	CuSO <sub>4</sub> •5H <sub>2</sub> O	0.0025		1.0•10 <sup>-8</sup>
	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> •4H <sub>2</sub> O	0.0124		1.0•10 <sup>-8</sup>
<b>E</b>	FeSO <sub>4</sub> •7H <sub>2</sub> O	6.95	1	2.5•10 <sup>-5</sup>
	EDTA (Na <sub>2</sub> – salt)	9.30		

## 3. *Standard culture medium – Z8*

This is the standard medium (Staub 1961; modified Kotai 1972, NIVA 1976) that was used to cultivate all cyanobacteria strains. The medium is freshwater based (autoclaved distilled water), and was prepared from 4 different autoclaved stock solutions stored at 4°C in refrigerator. Vanadium (V<sub>2</sub>O<sub>5</sub>) was not in store at the Institute, and therefore not used in the medium.

All the ingredients of the Z8 medium are listed in table A.4, the ingredients of the trace element solution in table A.5.

**TABLE A.4**  
Standard culture medium Z8 after Staub 1961; modified Kotai 1972, NIVA 1976.

	Stock solutions	g/L	Add to distilled water ml/L
1.	NaNO <sub>3</sub>	46.7	10
	Ca(NO <sub>3</sub> ) <sub>2</sub> •4H <sub>2</sub> O	5.9	
	MgSO <sub>4</sub> •7H <sub>2</sub> O	2.5	
2.	K <sub>2</sub> HPO <sub>4</sub> (K <sub>2</sub> HPO <sub>4</sub> •3H <sub>2</sub> O)	3.1	10
	Na <sub>2</sub> CO <sub>3</sub>	2.1	
3.	FeEDTA *		10
4.	Trace element solution **		1

\* FeEDTA

10.0 ml from a FeCl<sub>3</sub> solution (2.80 g FeCl<sub>3</sub>•6H<sub>2</sub>O dissolved in 100 ml 0.1 N HCl) and 9.5 ml from an EDTA solution (3.90 g EDTA-Na<sub>2</sub> dissolved in 100 ml 0.1 N NaOH) are mixed and filled up to 1000 ml distilled water.

\*\* Trace element solution

All 13 different stock solutions were mixed together and kept in one trace element stock solution to ease the use.

**TABLE A.5**  
Trace element solution in the Z8 medium.

	Stock solutions	g/L	Add to distilled water ml/L
1.	Na <sub>2</sub> WO <sub>4</sub> •2H <sub>2</sub> O	0.330	10
2.	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> •4H <sub>2</sub> O	0.880	10
3.	KBr	1.20	10
4.	KJ	0.830	10
5.	ZnSO <sub>4</sub> •7H <sub>2</sub> O	2.87	10
6.	Cd(NO <sub>3</sub> ) <sub>2</sub> •4H <sub>2</sub> O	1.55	10
7.	Co(NO <sub>3</sub> ) <sub>2</sub> •6H <sub>2</sub> O	1.46	10
8.	CuSO <sub>4</sub> •5H <sub>2</sub> O	1.25	10
9.	NiSO <sub>4</sub> (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> •6H <sub>2</sub> O	1.98	10
10.	Cr(NO <sub>3</sub> ) <sub>3</sub> •9H <sub>2</sub> O	0.410	10
11.	V <sub>2</sub> O <sub>5</sub>	0.089	10
12.	Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> K <sub>2</sub> SO <sub>4</sub> •2H <sub>2</sub> O	4.74	10
13.	H <sub>3</sub> BO <sub>3</sub>	3.100	100
	MnSO <sub>4</sub> •4H <sub>2</sub> O	2.230	