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Sori disinfection in cultivation of *Saccharina latissima*

Evaluation of chemical treatments against diatom contamination

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ABSTRACT

Diatom contamination is a problem in the early cultivation stages of *Saccharina latissima*. Macro- and microalgae compete for the same abiotic resources, and diatoms may overgrow and eliminate seedlings of *S. latissima* if introduced to the macroalgae cultivation system. Germanium dioxide (GeO_2), which blocks the cell division in diatoms, has been used as a diatom controller in the growth medium in cultivation of macroalgae. However, the chemical is very expensive and it has been suggested that the chemical inhibit growth of *S. latissima* seedlings. In the present study, it was desirable to establish a sori disinfection method for large-scale cultivation systems that eliminate diatoms prior to *S. latissima* spore release. It was called for a purely chemical disinfection method as mechanical removal of contamination is labour-intensive and costly.

Five chemicals, including 130 different trials, were tested on diatoms in free suspension. Acetic acid, sodium hypochlorite and ethanol eliminated diatom growth independent of concentration, exposure time and exposure temperature. Formaldehyde and Lugol's solution revealed surviving diatoms in the weaker treatments. GeO_2 eliminated growth of *Chaetoceros muelleri* and *Skeletonema costatum*, but monocultures of *Phaeodactylum tricornutum* were found at both concentrations (0.1mL L^{-1} and 0.5mL L^{-1}) eleven days after inoculation. Acetic acid, Lugol's solution and sodium hypochlorite were tested as sori disinfectants, and GeO_2 was used as a negative control for diatom growth. Acetic acid treatments were lethal to both diatoms and *S. latissima* spores, and GeO_2 treatments appeared to have negative influence on fertility and growth of young seedlings of *S. latissima*.

Disinfection with 600ppm sodium hypochlorite or 2% Lugol's solution, with an exposure time of 2 minutes and exposure temperature of 10°C, followed by two rinsing baths of sterile seawater, gave total elimination of diatoms. No notable effects on spore release, sporophyte growth or early development of young seedling were seen. This implies that disinfection of sori can be done safely by use of these treatments, without concern about reduced quality or quantity of cultivated seaweeds. Sori disinfection was done on disks cut from ripe sori in a disinfecting bath, and no mechanical removal of sori contamination was performed. Sodium hypochlorite was suggested as a new sori disinfectant based on an apparently good safety margin between lethal doses to diatoms and a harmful dose for *S. latissima*. The chemical also appears to be a widespread disinfectant in aquaculture systems, and can easily be neutralized with thiosulphate.

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1 INTRODUCTION

1.1 Global aquaculture of macroalgae

Aquatic plants accounted for 24% of the global aquaculture production, and more than 40% of marine aquaculture (mariculture) in 2010 (FAO, 2010a). The production is highly dominated by marine macroalgae, often referred to as seaweeds, cultivated in East and Southeast Asia. (FAO, 2010b). In Japan and China, cultivation of seaweeds for food consumption has been undertaken for several hundred years. Algae were traditionally cultivated extensively in polyculture systems by rock cleaning just prior to the growth season of the seaweeds; in that way the spores was offered a substrate to settle on (Tseng and Borowitzka, 2003). The first production of *Saccharina japonica* on artificial substrate was performed in 1952, and it has been referred to as the birth of scientific aquaculture in China (Tseng, 1993). Since then, China has developed into the absolute, dominant producer of seaweeds and was responsible for as much as 62.8% of the global production in 2010 (FAO, 2010b). Commercial production has been introduced to Europe and North America, but the quantity and scale of production is at an early stage compared to Asian systems (Bartsch et al., 2008; Kraan, 2010).

Global aquaculture, with China in the lead, has grown rapidly since the 1950s, and an annual increase of 7.7% since 1970 makes aquaculture today the fastest growing food production sector in the world (FAO, 2010b). Monocultures of high value finfish and crustaceans, produced in intensive systems, has increased strongly over the last decades (FAO, 2010c). A potential problem of intensive farming is discharges from sea cages, this has been estimated to be 60-80% of the total nutrient added through feed (Islam, 2005). The aquaculture industries have received massive criticism from media and research due to environmental impact including eutrophication and unsustainable feed resources (Naylor et al., 2000; Drakeford and Pascoe, 2008; Diana, 2009). As a consequence of this debate, a new interest for environmental friendly aquaculture methods has been born (Chopin et al., 2001; Troell et al., 2003; Neori et al., 2004). Integrated multi-tropic aquaculture (IMTA) is suggested as an approach to solve the problem of eutrophication. The method involves recycling of nutrients into valuable biomass by cultivating species from different tropical levels in the same system (Chopin et al., 2001). Cultivation of inorganic extractive species and organic extractive species in short distance from fish farms is believed to reduce the nutrient release to the environment. In the North Atlantic region large-

scale IMTA systems of Atlantic salmon (external fed), seaweed e.g. *Saccharina latissima* (inorganic extractive) and mussels e.g. *Mytilus edulis* (organic extractive) have been suggested (Chopin et al., 2001; Sanderson et al., 2008; Troell et al., 2009; Reid et al., 2010; MacDonald et al., 2011). It is believed that by an ecological approach, the aquaculture production will get greater acceptance among stakeholders and media. Meanwhile, production of extractive organisms may increase the economical sustainability of fish farms (Neori et al., 2007; Neori, 2008).

1.2 Macroalgae biomass as raw material for biofuel production

Raw material for production of biofuel has been suggested as a market for large-scale cultivation of macroalgae, and *S. latissima* and *Alaria esculenta* appears to be very well suited for such production (Handå et al., 2009; Kraan, 2010; John et al., 2011; Forbord et al., 2012). Algae have been suggested as raw material for energy production as early as in the 1950's, and during the 1970's oil crisis, the concept attracted serious attention (Chen et al., 2009). In light of the CO_2 related climate changes and increasing fuel costs, the search for alternatives to fossil fuel has increased and the awareness of biofuel have grown the last decades (Nigam and Singh, 2011). Bioethanol produced from sugarcane and corns, together with biodiesel produced from rapeseeds, are often referred to as first generations biofuel and are the only commercially available biofuels today. Lignocellulos biomass, such as woods and agriculture waste which cannot be utilized as human food, has been suggested as a second generation biofuel. However, as the demand for biofuel increases, it is not sustainable to use either biomass (as in first generation biofuel) or water supplies and arable land (both first and second generation biofuel) in production of fuel (Kraan, 2010; Nigam and Singh, 2011). Seaweed is regarded an interesting source for biofuel production due to the fact that the plants grows two to three times faster than sugarcane (Handå et al., 2009), and approximately 60% of the dry weight of macroalgae is carbohydrates that can be fermented into bioethanol, yielding close to 0.3 litre of ethanol per kilo dry weight biomass. High content of carbohydrates and biomass yield, together with absent need for land areas, make marine macroalgae promising as biomass for biofuels production (Handå et al., 2009; Kraan, 2010; Nigam and Singh, 2011).

Natural populations of seaweed are not able to supply the biomass needed for large-scale production of biofuel, and large-scale cultivation systems needs to be developed. An area of

700km^2 for cultivation of *S. latissima* has been suggested necessary to cover 5% of the Norwegian annual consume of transport fuel (calculations based on fuel consume anno 2006; Handå et al., 2009). Large-scale production systems of *S. japonica* are well established in China (Tseng, 1993; Troell et al., 2009) and IMTA systems using *S. latisima* and *A. esculenta* are under development in Canada (Ridler et al., 2007; Troell et al., 2009). Yet, several challenges like cultivation technology and year around supply of seedlings need to be solved if large-scale production of seaweeds shall be a reality in Norway and Europe. In Asia, harvesting are mainly done by hand in cultivation systems, but that will not be economical sustainable in Norway due to higher labour costs (Handå et al., 2009). Offshore cultivation techniques and infrastructure must be developed as aquaculture activities tend to be moved further offshore (McHugh, 2003; Buck and Buchholz, 2004; Bartsch et al., 2008). Another bottle-neck in development of large-scale systems is production of juvenile sporophytes on ropes, via gametophytes cultures (Bartsch et al., 2008; Handå et al., 2009; Kraan, 2010). Recent research has shown that year around supply of sori can be obtained by manipulation of culture conditions in *S. latissima* hatcheries (Forbord et al., 2012), but diatom contamination may eliminate juvenile *S. latissima* seedlings in such hatcheries (Chapman, 1973; Markham and Hagmeier, 1982; Merrill and Gillingham, 1991 ; S. Forbord, SINTEF Fisheries and Aquaculture, pers. com.)

1.3 Biology and cultivation of *Saccharina latissima*

The life cycle of Laminariaceae, which *Saccharina latissima* belongs to, is an alteration between diploid sporophytes and haploid gametophytes. Macroscopic sporophytes produce spores by meiosis in special spore holding cells, called sporangia, on adult sporophytes (Kain, 1979). Dense area of sporangia, called sori, are produced naturally from October to December in Norwegian waters (Forbord et al., 2012). Sori production can also be artificially triggered by meristem removal (Buchholz and Lüning, 1999) and exposure to short day conditions (SD; 8 hours light day^{-1}) throughout the year (Lüning, 1988; Forbord et al., 2012). The blade meristem is believed to produce sporulation inhibiting factors and removal of the lower part of the lamina relives the tissue from the inhibiting effect (Pang and Lüning, 2004; Kai et al., 2006).

Sporangia are produced in the outermost layer of the lamina. Each sporangium produce 32 spores (Kain, 1979), that are soaked in a polysaccharide mucilage of alginic acid and fucoidan (Toth, 1976). Spore release can be induced artificially by osmotic shock and preferably a

temperature change. Dehydration overnight in a cold environment (< 10°C), followed by rehydration in cool seawater (~10°C) is a well-established method for artificial spore release (Merrill and Gillingham, 1991; Arbona and Molla, 2006; Edwards and Watson, 2011). It is suggested that hydration of the mucilaginous polysaccharides increase pressure on the sporangial cell walls. Paraphyses, thin hair-like filaments, are present in high numbers around the sporangia. The pressure from hydrated polysaccharides and the paraphyses may lead to burst of the sporangial tip so that spores are released to the environment (Toth, 1976).

In large-scale cultivation of Laminariaceae spores are seeded onto ropes (Merrill and Gillingham, 1991; Arbona and Molla, 2006). After settlement the spores germinate to microscopic, haploid gametophytes which extrude a germination tube and the first cell of the gametophyte ("primary cell") is formed at the end of the tube. Half of the gametophytes develop into sperm producing males and half is egg producing females. Male gametophytes soon divide, while the primary cell of female gametophytes swells to the size of an oogonium (Kain, 1979; Lüning, 1981). Blue light induce female gametophytes to become fertile and egg producing (Lüning and Dring, 1972), with strongest response at wavelengths close to 430nm and 450nm (Lüning and Dring, 1975). Fertilization can be inhibited by exposure to red light (Lüning and Dring, 1972) or high temperatures (Kain, 1979), and under such suboptimal conditions gametophytes of both sexes show vegetative growth. *S. latissima* gametophytes kept under optimal growth conditions produce eggs 8 to 10 days after spore settlement (Lüning and Dring, 1975), and spermatozoids can be observed shortly after (Lüning, 1981). The spermatozoids are released and attached directly to the female gametophyte in response to a pheromone called lamoxirene released by the female gamete. Only one sperm cell are needed to fertilize an egg, actually, polyspermy is believed to be lethal (Lobban and Harrison, 1994). After fertilization the zygote swells spherically, followed by elongation and eventually cell division. Young sporophytes perform cell division over the whole area, on equal rate, until the sporophyte is about 1mm. At some point beyond this size the base of the lamina becomes multi-layered and cell division localized to the meristematic region between stipe and lamina (Kain, 1979). The seeded ropes are kept in hatcheries until the sporophytes are approximately 4 weeks old or 1-2 mm in length (Merrill and Gillingham, 1991).

Harvest of the cultivation stock is recommended during late spring, after three to five months in the sea and before elevated water temperature and epiphytic growth degenerate the sporophyte (Merrill and Gillingham, 1991; Arbona and Molla, 2006; Broch and Slagstad, 2011; Edwards and Watson, 2011). *S. latissima* growth is reduced during summer when the sporophyte store

carbohydrates, and stays low through the autumn. From late winter until late spring, the sporophytes grow rapidly on stored carbohydrates (Sjøtun, 1993). However, the growth performance of seaweeds is dependent on several abiotic factors like light, temperature, available nutrients and seasonal interactions among these factors (Bartsch et al., 2008). Seasonal variations in *S. latissima* growth have been simulated by a high resolution model. The gross daily production of frond area of *S. latissima* was estimated to increase from <0.1 dm^2 day^{-1} in September, to 0.8 dm^2 day^{-1} in May. From May to September the area production per day decreased rapidly (Broch and Slagstad, 2011). A year-around cultivation experiment of *S. latissima* on the coast of central Norway (Bjugn) reported by Forbord et al. (2012) is in agreement with the model; fastest growth was obtained in the period February to June.

1.4 Problems with contaminating microalgae

Competition by other organisms for nutrients and substrate are the most common causes of failure in early stages of seaweed cultivation. Diatoms are thought to be the most threatening algae group for overgrowth and elimination of young seedlings (Merrill and Gillingham, 1991). Adult sporophytes are today collected from nature at the beginning of a cultivation cycle (Merrill and Gillingham, 1991; Arbona and Molla, 2006; Edwards and Watson, 2011) and are often hosting a rich epiphytic flora (Bartsch et al., 2008) that will be introduced to the production line if not treated. In addition to the sori, seawater is an important source for contamination (Merrill and Gillingham, 1991). Different methods have been suggested for treatment of contamination.

1.4.1 Chemical disinfection of sori

Lamina has an outer coating of mucilage. It is assumed that the mucilaginous covering will protect the spores from a disinfecting treatment so that the disinfection will work only on the contaminating organisms located on the surface of the lamina (Druehl and Hsiao, 1969). Disinfection is described as the process that destroys microorganisms or harmful diseases, and usually the disinfectant is a chemical agent (Block, 2001). Effectiveness of a chemical disinfectant is dependent on the contact time between the microorganism and the disinfectant during exposure, together with the initial concentration of the disinfectant and the microorganisms (Chick, 1908). Inactivation ability of a given concentration-time relationship may

vary with environmental factors such as pH, temperature, organic material in the solution and the accessibility to the target organism (Weavers and Wickramanayake, 2001). Increased temperature usually increase inactivation rates of microorganisms in agreement with the known Arrhenius equation, but temperature may also affect properties like solubility and stability of a chemical agent (Weavers and Wickramanayake, 2001; Chang, 2006). Chemicals used in sori disinfection should therefore have good effect in the optimal temperature range for seaweeds and for the pH of seawater.

A couple of exclusively chemical methods for sori disinfection have been suggested. Kientz et al. (2011) bathed fronds of several seaweed species twice in sterile artificial seawater, followed by a 30 seconds bath in a mixture of 40-50% ethanol (dependent on the water content of the algae) and 1% sodium hypochlorite. The treatment eliminated all growth of microorganisms on *Fucus serratus* and *Chondurus crispus*, but a small amount of microorganisms were left on the frond of *S. latissima*. Druehl and Hsiao (1969) established totally axenic cultures of *S. latissima*, *Alaria marginata* and *Nereocyctis luetkeana* by bathing sori in an antibiotic solution for three hours followed by a 20-30 minutes soak in 1% sodium hypochlorite. For both methods the aim was to produce axenic cultures. Axenic cultures should however not be necessary as long as microalgae are eliminated (Merrill and Gillingham, 1991, K. Lüning, pers.com., 2010). It has even been implied that totally axenic cultures of *S. latissima* may inhibit the transition from vegetative to reproductive phase (Druehl and Hsiao, 1969).

1.4.2 Chemical and mechanical disinfection of sori

Mechanical cleaning of sori with paper soaked in sterile or natural seawater is probably the simplest method for sori disinfection and is widely used (Shi et al., 2005; Shea and Chopin, 2007; Edwards and Watson, 2011). It has however, been claimed that a combination of mechanical and chemical removal of contamination is necessary to obtain unialgal cultures (Fernandes et al., 2011). Merrill and Gillingham (1991) suggested a combination of mechanical and chemical disinfection of *Nereocyctis luetkeana* sori by bathing of the sori in an iodine solution (0.5% Betadine) for 30 seconds and thereafter in filtered seawater for 5-10 minutes. After the baths, the sori were wiped dry. Hsiao and Druehl (1971) suggested to wipe sori with paper soaked in a 5% sodium hypochlorite solution, followed by five baths of sterile seawater. Fernandes et al. (2011) suggested a decontamination protocol for *Hypena musciformis* that included washing in detergent solution, brushing of the lamina, rinsing in distilled water, and use

of GeO_2 as a diatom controller in the growth medium after decontamination. Mechanical removal of contamination is labour-intensive and time-consuming, and in development of European large-scale production systems it is desirable to find a purely chemical method for treatment of diatom contamination.

1.4.3 Use of GeO_2

Filtered, sterile water are recommended for all phases of macroalgae cultivation to avoid contamination from the water source. Besides that, germanium dioxide (GeO_2) can be added to the growth medium to avoid diatom contamination of the macroalgae cultures (Merrill and Gillingham, 1991; Arbona and Molla, 2006; Edwards and Watson, 2011). Germanium dioxide (GeO_2) is reported to be a specific inhibitor of silicate utilization, and has been demonstrated to reduces diatom cell division, cell wall formation, DNA synthesis and silicate uptake (Lewin, 1966; Darley and Volcani, 1969; Azam et al., 1973). Merrill and Gillingham (1991) recommended a concentration of 1-2mL saturated GeO_2 solution L^{-1} growth medium in cultivation of *Nereocystis luetkeana*, without reporting any negative effects. On the other hand, Markham and Hagmeier (1982) and Shea and Chopin (2007) found lower concentrations of GeO_2 to have an inhibiting effect on sporophyte growth of *S. latissima*. In general, though, seaweeds are far less sensitive to GeO_2 than diatoms (Markham and Hagmeier, 1982). Another drawback is that GeO_2 is a very costly chemical. In 2012 GeO_2 cots between 1000 and 1200$ kg^{-1} and it would be desirable to find an alternative to GeO_2 in large-scale production (Metal-Pages-Ltd, 2012).

1.5 Experimental chemicals

a) Acetic acid

Acetic acid ($HC_2H_3O_2$) is a weak, organic acid and show an incomplete dissociation in aquatic solutions (Chang, 2006). It has been suggested that acetic acid, which is lipid soluble, penetrate the plasma membrane of cells and lower the internal pH of microorganisms with a lethal outcome (Greenacre et al., 2003). Weak, organic acids have long history in food preservation due to its microbial inhibiting effects (Theron and Lues, 2011). E.g. acetic acid diluted in water, is the main ingredient in vinegar (5% or 7% acetic acid), which is inexpensive and sold in common grocery shops in Norway.

b) Ethanol

Ethanol (CH_3CH_2OH) is a widely used nonspecific antimicrobial disinfectant that has been reported to cause protein coagulation, dehydration and cell lysis (Ali et al., 2001). Ethanol for industrial uses is relatively cheap (Solomons and Fryhle, 2008), and has been reported effective in sori disinfection in combination with sodium hypochlorite (Kientz et al., 2011).

c) Formaldehyde

Formaldehyde (HCHO) exists as gas, but is highly soluble in water (Solomons and Fryhle, 2008). The chemical causes protein denaturation with lethal outcome for microorganisms (Hawley and Eitzen, 2001), and aqueous solutions of the chemical are used in disinfection (Favero and Bond, 2001) and preservation (Douglas and Rogers, 1998; Marie et al., 2005). However, it is recommended to limit the use of formaldehyde as the chemical can lead to cancer and is potential explosive. Environmental release of formaldehyde is highly regulated (Hawley and Eitzen, 2001).

d) Lugol's solution

Lugol's solution is a high-iodine solution with typically 10% potassium iodide (KI) and 5% iodine (I_2) which is used in preservation of microorganisms (Guillard and Sieracki, 2005). Iodide (I^-) increases the solubility of elemental iodine (I_2) in water, and the high content of iodide in Lugol's solution makes the mixture completely soluble in water. The iodine-water system is complex, and iodide concentration and pH has influence on the iodine species represented in an aquatic solution (Gottardi, 2001). Iodine (I_2) and hypoiodous acid (HOI) are the forms with lethal effect on microorganisms, and in the pH of seawater I_2 will be the most important disinfectant. However, iodine can react with organic matter which is undesirable as such reactions consume available iodine. Equation 2 gives the dominant iodine species for Lugol's solution, (Gottardi, 1985):

$$I_2 + I^- \leftrightarrow I_3^- \qquad [1]$$

It has been suggested that Lugol's solution adds iodine to unsaturated fatty acids of the cell membrane and in that way damage the membrane so that intracellular material is lost to the surrounding water masses (Apostolov, 1980), or that iodine oxidize or iodinate amino acids so that DNA and protein synthesis of the cells is damaged (Gottardi, 2001). Betadine, an iodine

solution which release free iodine, has been reported to be an effective sori disinfectant (Merrill and Gillingham, 1991).

e) Sodium hypochlorite

Sodium hypochlorite (NaOCl) is a widespread disinfectant in medicine (Dychdala, 2001), water treatment (Clasen and Edmondson, 2006) and aquaculture (Hsiao and Druehl, 1971; Douillet, 1998; Khomvilai et al., 2006). In contact with water, NaOCl release free chlorine in the form of hypochlorous acid (HOCl). Hypochlorous acid together with hypochlorite ion (OCl^-) has a lethal effect on microorganisms, with hypochlorite ion (OCl^-) being the less effective disinfecting agent. The two exists in equilibrium (Equation 1), depending on pH.

$$HOCl \leftrightarrow H^+ + OCl^- \quad [2]$$

In seawater (pH ~ 8) more than 70% of the free chlorine is in the form of hypochlorite ion (OCl^-). However, it is unclear if the OCl^- is the decisive disinfectant at this pH, or if the equilibrium in Equation 1 constantly shifts to the left to replace hypochlorous acid (HOCl) which is believed to be a stronger disinfectant than OCl^-. Why HOCl is such an effective disinfectant has not been truly understood. It has been suggested that the compound release nascent oxygen, a short-lived oxygen radical, which destroy organisms by combining with compound in the protoplasm. Another theory is that HOCl reacts with ammonia in the water and form chloramines which act directly on the microorganism. Chlorine is an oxidative compound, and organic material together with inorganic reducing substances (e.g. Fe^+, $Mn2^+$, $NO2^-$) in a solution will consume available chlorine and reduce the efficiency of the chemical disinfectant (Dychdala, 2001).

1.6 Experimental organisms

1.6.1 Macroalgae: Saccharina latissima

Saccharina latissima (former *Laminaria saccharina*) (Lane et al., 2006) has a North Atlantic and North Pacific distribution. North Atlantic populations show high growth during winter and early spring, and reduced growth during summer (Lüning, 1979; Sjøtun, 1993), and photosynthesis is saturated at a photon flux above 150 µmol m^{-2} s^{-1} (Lüning, 1979). Water turbidity influences the maximum depth of seaweeds, and distribution down to 25 m has been reported for Spitsbergen populations of *S. latissima* (Bartsch et al., 2008). Optimal sporophytes growth has been reported

in in the range 10-15°C, but the gametophytes can survive temperatures up to 22°C without cell damage (Bolton and Lüning, 1982). The life cycle of *S. latissima* is presented in details in Section 1.3.

1.6.2 Diatoms; Chaetoceros muelleri *and* Skeletonema costatum, Phaeodactylum tricornutum

Most diatoms are unicellular, eukaryotic microorganisms, with a cell wall highly incorporated with silica (SiO_2). The cell wall consists of two large, sculptured units (valves) at each end of the cell which are bound together of thinner, linked structures (girdles). Together the wall components are called a diatom frustule. Diatoms are usually pigmented and photosynthetic, even though some species can live heterotrophic. A few species have also been described as apochlorotic (colourless), and these are solely heterotrophic (Round et al., 1990). Most diatoms are pelagic, but several genus are have be classified as benthic living on moist or submerged surfaces, e.g. seaweed (Jones, 2007).

Chaetoceros muelleri Lemmermann, *Skeletonema costatum* (Greville) Cleve and *Phaeodactylum tricornutum* Bohlin were chosen as experimental diatom species. All three species are present in Norwegian waters (Throndsen et al., 2003), and should be seen as representatives for Norwegian phytoplankton community. Limited literature seems to be available on species composition of epiphytic diatoms on *S. latissima*. Diatom species were therefore chosen to reflect the variable life forms of diatoms; benthic versus pelagic, centric versus pennate, chain forming versus unicellular. Reports of these species as epiphytes on *S. latissima* have not been found. However, Totti et al. (2009) found *Cocconeis scutellum,* a benthic diatom of the same superorder as *P. tricornutum*, to be the most abundant epiphytic diatom on *S. latissima* collected on Iceland.

P. tricornutum is an unicellular, pennate diatom that is found in ponds on the rocky shore (Throndsen et al., 2003), and is relatively rare in natural phytoplankton communities (Nelson et al., 1979; Round et al., 1990). The species occurs in three forms; oval, fusiform and triradiate, and it has been suggested that the oval form is benthic while the two others may be planktonic because they are more buoyant than the oval form (Round et al., 1990). *P. tricornutum* show high tolerance for variations in salinity (Abdullahi et al., 2006) and light intensity , and was chosen as an experimental organism because the species is very tolerant and regarded a weed in aquaculture systems (Nelson et al., 1979).

The genus *Skeletonema* have centric diatoms with a global distribution, and the diatoms of the genus are easy to keep in culture and are therefore often used as experimental organism (Round et al., 1990). Cells of *S. costatum* are linked together in chains by a circle of tubes attached to the outer edge of the valves (Throndsen et al., 2003). *S. costatum* was chosen because it is a dominant species in the Norwegian spring bloom (Sakshaug and Myklestad, 1973; Throndsen et al., 2003).

Chaetoceros ssp. are dominate species in the Norwegian spring bloom (Sakshaug and Myklestad, 1973), and *C. muelleri* is a centric, unicellular diatom of this important pelagic genus. The species is reported to occur in brackish waters close to shore (Throndsen et al., 2003), but is not considered to be among the common *Chaetoceros* species in Norwegian waters (J. Arff, SINTEF Fisheries and Aquaculture pers. com., 2012). *C. muelleri* was chosen as a representative for the genus *Chaetoceros* which is common in Norwegian waters (Sakshaug and Myklestad, 1973), due to its frequent use in aquaculture experiments (e.g. Liang et al., 2006a; Michels et al., 2010; Reitan, 2011) and relatively high tolerance for variations in abiotic factors (Fujii et al., 1995; Rousch et al., 2004).

1.7 Study aims and approach

The aim of this study was to establish a disinfecting method that relieves sori from diatom contamination, without damaging spores or affect early development of young sporophytes. Disinfection was undertaken in agreement with Druhel and Hsiao's (1969) assumption that the mucilaginous covering of lamina protected spores from the disinfectant. The intention was to suggest a disinfecting method suitable for large-scale production of *S. latissima*. Two adjustments were made to fit the industrial demands; a) mechanical removal of contamination was avoided and b) disinfection of punched, circular, sori pieces was tested in order to standardize the disinfection dose.

Three sub-objectives were formulated:

1. Evaluate the lethal effect on diatom after exposure to different disinfecting chemicals (ethanol, formaldehyde, sodium hypochlorite, acetic acid and Lugol's solution).
2. Evaluation of *S. latissima* spore survival and germination after exposure to effective diatom disinfectants found in 1.
3. Evaluation of early development of *S. latissima* seedlings after sori disinfection.

2 MATERIALS AND METHODS

Three experiments were performed from February to November 2011. First, a laboratory experiment was conducted on the lethal effect of diatoms after exposure to 130 different disinfection trials. Second, an experiment with sori disinfection was performed to evaluate the effect on *S. latissima* sori and spores of the disinfecting treatments that successfully eliminated diatoms in the former experiment. Third, an experiment was conducted to evaluate the early development of young *S. latissima* seedlings after sori disinfection on a two to three days interval over a period of three weeks.

2.1 Effect of chemical treatments on diatoms

2.1.1 Diatom species

Skeletonema costatum (Greville) Cleve (NIVA BAC 1), *Pheaodactylum tricornutum* Bohlin (CCAP 1052/1A) and *Chaetceros muelleri* Lemmermann (CCAP 1010/3) were chosen as test organisms for the chemical survey. The three species were taken from dense and healthy stock cultures that were checked for contamination by a microscope survey before the experiment was started. It has been suggested that the *S. costatum* strain, NIVA BAC 1 should be allocated it to *S. pseudocostatum* (ISO, 2008), the present study will however, refer to it as *S. costatum*.

2.1.2 Culture conditions

S. costatum, *P. tricornutum* and *C. muelleri* were cultivated semi-continuously in 1,5L soda bottles under constant light exposure (70-90µmol photons $m^{-2}s^{-1}$) and temperature (20°C). Cultures were bubbled with air added 0.2% CO_2, and pH of the cultures was measured during the first cultivation week to ensure that the rate of bubbling gave acceptable gas exchange. Two cultures were run for each diatom species, and the cultures were diluted on a two or three day's interval in order to keep algae in a steady growing phase. One replicate of each species were

restarted every tenth day to ensure the supply of healthy and steadily growing cultures at all times. Diatoms were inoculated by 20mL algae stock solution to 1L growth medium.

2.1.3 Growth medium

Sterile seawater (SSW) was used for cultivation of diatoms. Sand filtered seawater, taken from 90m depth in the Trondhjemsfjord, was autoclaved in bottles (NALGENE 5GAL, 10L) in a high pressure stem sterilizer (TOMY SX-700E) for 20min at 120°C. A modified version of the standard Conwy medium, with less manganous chloride than in the Walne (1979) standard version, was used in cultivation (Table 1). The modified version is the default medium used at NTNU Centre of Fisheries and Aquaculture and SINTEF SeaLab (D. Altin pers.com., 2012). Metasilicate (0.3%) was used as silica source for diatoms, and was obtained by dissolving 3g $Na_2SiO_3 \bullet 9H_2O$ in 100mL distilled freshwater. For cultivation, 1mL of Conwy medium and 1mL of metasilicate (0.3%) were diluted in 1L of SSW.

2.1.4 Sampling

Optical density (750nm), a measurement of diatom biomass, was measured daily in the cultures by use of a spectrophotometer (UviLine 9100), and the cell concentrations of the cultures were calculated according to Equation 3-5. Equations found by Jørgensen (1998) were used for cell concentrations of *S. costatum* and *C. muelleri*. For *S. costatum*, the number of cells (*y*) in millions mL^{-1} was calculated as (Jørgensen, 1998):

$$y = 10.7x - 0.5 \quad [3]$$

where *x* is the measured optical density at 750nm and *y* is number of cells in millions mL^{-1}. For *Chaetoceros muelleri*, number of cells (*y*) in millions mL^{-1} was calculated as (Jørgensen, 1998):

$$y = 8.15x - 0.66 \quad [4]$$

where *x* is the measured optical density at 750nm and *y* is number of cells in millions mL^{-1}.

For *P. tricornutum*, a relationship between cell density and optical density was established prior to the main experiment. Cell density in *P. tricornutum* cultures were estimated from manual counting of cells in a Brükner Chamber along with measurements of optical density (750nm). An

equation for the relationship between cell density and optical density was established by linear regression (Equation 5), giving the relationship:

$$y = 20.0x - 2.0 \quad [5]$$

where x is the measured optical density at 750nm and y is number of cells in millions mL^{-1}.

An algae stock solution (referred to as the mixture of diatoms) of the three diatom species was prepared for the chemical survey; 40.000 cells of each species were diluted in 90mL of SSW.

Table 1: *Modified Conwy growth medium used in cultivation of diatoms*

Chemical	Amount
$NaNO_3$ (Sodium Nitrate)	100.0g
Na-EDTA (EDTA disodium salt)	45.0g
H_3BO_3 (Boric Acid)	33.6g
$NaH_2PO_4 \cdot 2H_2O$ (Sodium Phosphate, monobasic)	20.0g
$FeCl_3 \cdot 6H_2O$ (Ferric Chloride, 6–hydrate)	1.3g
$MnCl_2 \cdot 4H_2O$ (Manganous Chloride, 4–hydrate) *	0.136g
Vitamin B_1 (Thiamin HCl)	0.1g
Vitamin B_{12} (Cyanocobalamin)	0.05g
Trace Metal Solution **	1mL
Distilled water	1000mL
*modified concentration.	
**Trace Metal Solution:	
$ZnCl_2$ (Zinc Chloride)	2.1g
$CoCl_2 \cdot 6H_2O$ (Cobalt Chloride, 6–hydrate)	2.1g
$(NH_4)_6Mo_7O_{24} \cdot 6H_2O$ (Ammonium Molybdate, 4–hydrate)	2.1g
$CuSO_4 \cdot 5H_2O$ (Copper Sulphate)	2.0g
Distilled water	100mL

2.1.5 Chemical survey

Five chemicals, in total 130 different trials, were tested in order to identify a disinfecting treatment that mitigates diatom growth. The trials were a combination of five chemicals at three concentrations (four for acetic acid), two reaction temperatures and four reaction times (Table 2), together with a positive (untreated diatoms) and two negative controls (GeO_2 enriched growth medium) for diatom growth. A saturated solution of GeO_2 was prepared by dissolving 0.894 g GeO_2 in 200mL distilled water. The saturated solution of GeO_2 was added to the Conwy growth medium of unexposed diatoms in two concentrations (0.1mL L^{-1} Conwy medium and 0.5mL L^{-1} Conwy medium) that were chosen based on previous research (Shea and Chopin, 2007). Lugol's solution was prepared from 45g potassium iodide (KI) and 30g iodine (I) that was diluted with distilled water until the total weight of the solution was 750g. The concentration of free iodine was approximately 9.5% in the undiluted Lugol's solution, and the sodium hypochlorite solution had approximately 12% free chlorine. The pH was measured in all chemical stock solutions.

Table 2: *Chemical treatments used in diatom exposure and control treatments*

Chemical	Concentration	Exposure time	Exposure temperature
Acetic acid	1%, 7%, 35%, 50%	2, 4, 10, 30min	10°C, 15°C
Ethanol	25%, 50%, 70%	2, 4, 10, 30min	10°C, 15°C
Formaldehyde	0.04%, 0.4%, 4%	2, 4, 10, 30min	10°C, 15°C
Lugol's solution	0.02%, 0.2%, 2%	2, 4, 10, 30min	10°C, 15°C
Sodium hypochlorite	6ppm, 60ppm, 600ppm	2, 4, 10, 30min	10°C, 15°C
GeO_2*	0.1mL L^{-1} , 0.5mL L^{-1}		
Control			

*Added to the Conwy growth medium of unexposed diatoms

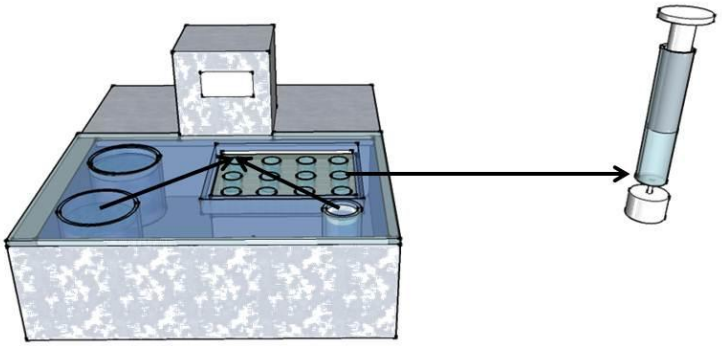
The different treatments will be named in the following order; first letter of chemical - concentration - exposure time - exposure temperature. For instance samples treated with 1% acetic acid and an exposure time of 2 minutes and exposure temperature of 10°C will be named A-1%-2min-10°C. Exposure of the diatoms to the different chemical trials (Table 2) was carried out in wells on a multi-well plate (see Section 2.1.6), and the initial concentration of diatoms after dilution of the mixture of diatoms with the chemical solution was 0.33 cells μL^{-1} with an equal distribution of the three diatom species. Six replicates were used for each trial.

2.1.6 Experimental setup

A Heto water bath with a circulator (HAAKE DC 30), covered by a Plexiglas plate, was used to establish stabile reaction temperature throughout the experiment (Figure 1). Two bottles (PYREX®, 80mL) for chemicals and one Erlend Meyer flask (90mL) containing the mixture of diatoms, were immersed into the water bath through holes in the Plexiglas plate. The right temperature in the solutions was established after approximately 30 minutes.

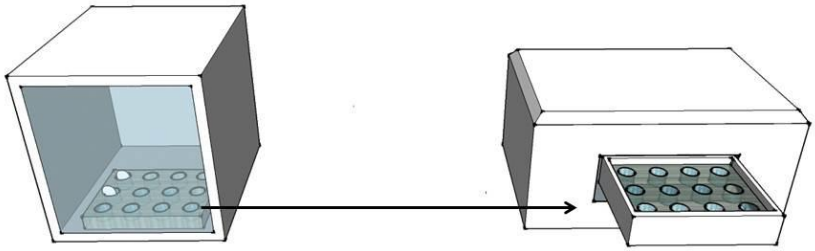
For each treatment 4,5mL of the chemical and 1,5mL diatom mixture were added to six, out of twelve, wells on a FALCON MULTIWELL^{TM} 12 Well plate; giving place for two treatments to be done simultaneously. Stock solutions of chemicals were prepared so that the desired concentrations (given in Table 2) were obtained in the well after dilution with the mixture of diatoms. The well-plate was placed on an aluminium block in the water bath and a hole was made in the Plexiglas plate above it. In that way the well-plate was made available for pipetting and the temperature was kept stabile. Acetic acid stock solutions were added to the well after the mixture of diatoms; to keep the reaction temperature as stable as possible. For the rest of the treatments the mixture of diatom was added to the chemical stock solutions. The temperature was measured (by a DuaLogR^{TM} THERMOCOUPLE THERMOMETER) in each well when the chemical stock solution was diluted with the mixture of diatoms, in that way it was registered if the exposure temperature was disturbed by the mixing. A FINPIPETTE® pipette (1-5mL) was used throughout the experiment.

A HUGER timer was used to keep the reaction time. After completed reaction time 4,5mL of the sample was transferred to a hand filter unit (Millipore swinnex®-25), and the solution was filtered through a MF-Millipore Membrane filter (pore size 0.45µm). The filters were rinsed with 10mL SSW (20mL SSW for Lugol's solution due to colouring of the filter) before they were placed in individual wells on a new well-plate. Each well contained 4,5mL Conwy and silica enriched seawater (1mL Conwy and 1mL Matasilice-stoc solution L^{-1} SSW), and all samples were incubated in a cabinet holding 20±1˚C and constant light (100µmol photons $m^{-2}s^{-1}$).



1. 4.5mL chemical solution and 1.5mL algae mixture were added to each well on a multiwell plate. Six replicates were accomplished for every treatment giving place to two treatments on every plate.

2. 4.5mL were filtered through a hand filter unit after ended exposure time. Filters were rinsed with 10mL SSW before transfer to a new multiwell plate



3. Multwell plates were incubated in a cabin holding constant light and temperature

4. Optical density (750nm) was measured two to four times

Figure 1: *Experimental set-up for chemical exposure of diatoms.* ***1.*** *Chemical solutions of two concentrations and the mixture of diatoms were immersed into the water bath to ensure stable temperature. The multiwell plate in which the chemical exposure was performed was located on an aluminium block to maintain the reaction temperature.* ***2.*** *A hand filter unit was used to filter end rinse diatoms after the chemical exposure.* ***3.*** *Growth of surviving diatoms was stimulated in an incubation cabinet.* ***4.*** *Optical density (750nm) was measured two to four times in a plate reader.*

2.1.7 Analysis of diatom growth after exposure

Optical density (750nm), of the samples on the well-plate, was measured on a plate reader (Epoch BioTek®) after minimum one day of incubation (Figure 1). The filter was removed from the well by use of tweezers prior to the first measurement. Measurements of optical density were repeated 1-3 times within a maximum of 14 days to detect any growth of surviving diatoms. Visual changes in the colour of the samples were registered, but only samples with reaction temperature 15°C and GeO_2 treated samples were checked in the light microscope when visual changes and changes in optical density were observed.

2.1.8 Evaluations of growth rates

Specific growth rate (SGR) of populations of diatoms, which resulted from both cell division and growth in size of existing cells, is defined by Equation 6 (Støttrup and McEvoy, 2002). In calculation of SGR, the optical density (OD) was used as a measurement of biomass.

$$SGR = [\ln(OD_t) - \ln(OD_{t0})] / (t - t_0) \quad [6]$$

where OD_t is the optical density at the end of the incubation (t) and OD_{t0} the first optical density measurement at day t_0. Equation 6 can be rewritten so that the equation gives a linear increase of lnOD with SGR expressed as the slope and $\ln OD_0$ as the intercept

$$\ln(OD) = SGR\ (t - t_0) + \ln(OD_0) \quad [7]$$

Equation 7 states a linear relationship between SGR of diatoms and measurements of the natural logarithm of optical density. The slope (SGR) can therefore be found by linear regression. Linear regression allows all measurements during the period of exponential growth to be included in the SGR estimate (Vadstein and Olsen, forthcoming). A generalized linear model (GLM, a non-parametric regression model) was used to find an estimate of mean SGR as described in Section 2.3.1.

2.2 Effect of chemical treatments on *S. latissima* sori and spores

Two experiments were performed from September to November 2011 on the tolerance of *S. latissima* to the disinfectants. The first experiment (referred to as Experiment 1) was a test of spore survival and success, and the second experiment (referred to as Experiment 2) was a survey on early development of *S. latissima* seedlings after the disinfection. Treatments used in the Experiment 2 were chosen based on the results in Experiment 1. A test of the method that compared the disinfection of sori disks contra disinfection of whole sori was performed in June, prior to Experiment 1 and 2.

2.2.1 Origin of sorus

S. latissima sporophytes, for all surveys, were collected by divers from the rocky, sublittoral zone at Vanvikan (63°52'N, 9°37'E) in the Trondheimsfjord. Sori production was induced by removal of meristem (15cm above the stipe) and light manipulation. The light regime was set to 8 hours light and 16 hours darkness (short day conditions), which together with meristem removal simulated late autumn conditions; when sori is produced naturally by the seaweed (see Section 1.3). Sporophytes were kept in heavy aerated tanks (160L), holding sand filtered water from 90m depth with a water exchange rate of 2L min^{-1}, until sori appeared. The light intensity was kept at 100µmol photons $m^{-2}s^{-1}$ at the surface of the tank, and the tank was located in a room with a constant temperature of 10°C.

2.2.2 Test of method; disinfection of sori disks

It was intended to use disks cut from ripe sori to standardized disinfection method. Natural sori are of variable size, and in order to produce true replicates it was necessary to standardize the area of disinfected sori (Disks; ø=12mm). For large-scale production, it will be desirable to avoid disinfection of surplus sori, both in light of the environment and the costs of disinfectants; hence the test on sori disks was also important for development of large-scale systems. The test was performed to investigate whether disks of sori exposed spores to the disinfectant (through edges of the sori) with a lethal outcome for the spores.

Disks (ø=12mm, n=6) were cut from ripe sori, and disinfected, seeded and incubated after the method presented in Section 2.2.3-2.2.4 and Figure 2. Whole sori were used as control, and these were disinfected, seeded and incubated in the same way as the disks. However, a bigger volume of the disinfectant was needed due to the larger surface area of a whole sorus. Sodium hypochlorite in two concentrations (6ppm or 60ppm), an exposure time of 10 minutes and an exposure temperature of 10°C was used in the test. Growth of juvenile sporophytes were registered as positive or negative after two weeks of incubation by use of an inverted microscope (Nikon eclipse TE2000-S). The disk method proved successful and was utilized in the experiments presented in Section 2.2.4 - 2.2.6.

2.2.3 Method for sori disinfection and spore release used in Experiment 1 and 2

Three plastic buckets (1000mL) were immersed into a Heto water bath for the disinfection of sori (Figure 2A). The water bath was covered by a Plexiglas plate which together with a circulator (HAAKE DC 30) ensured stable temperature (10°C) in the buckets. Disks (ø=12 mm) were cut from ripe sori, and immersed within a sieve into the first bucket holding 400mL of a given disinfectant (Table 4 or Table 6 for Experiment 1 and 2, respectively). The sieve was left undisturbed, as long as the sori were not clustered, throughout the exposure time of 2 or 10 minutes. Sori were rinsed twice by immersing the sieve into two plastic buckets (30 seconds in each bucket) holding 400mL sterile seawater (SSW). The sieve was shaken during rinsing so that all parts of the sori were thoroughly rinsed.

Disinfected sori were stored separately in empty wells on a well-plate (FALCON MULTIWELL^{TM} 12 Well plate). The plate was covered by a plastic bag and left overnight in a fridge holding 6±1˚C. Strong dehydration of sori was avoided by placing a wet paper (soaked in SSW) underneath the well-plate, inside the plastic bag.

Spore release was induced by rehydration of the sori; 5mL SSW (10^0C) was added to each well on the well-plate (Figure 2B). After 15 minutes of spore release, 1mL spore solution was transferred to a dish (Nunclon^{TM} Δ surface, squared (2x2mm) dish) holding 15mL precooled (10^0C) Conwy enriched SSW (1mL Conwy L^{-1} SSW). Some adjustments of the seeding procedure were done in Experiment 1 and these are described in Section 2.2.5.

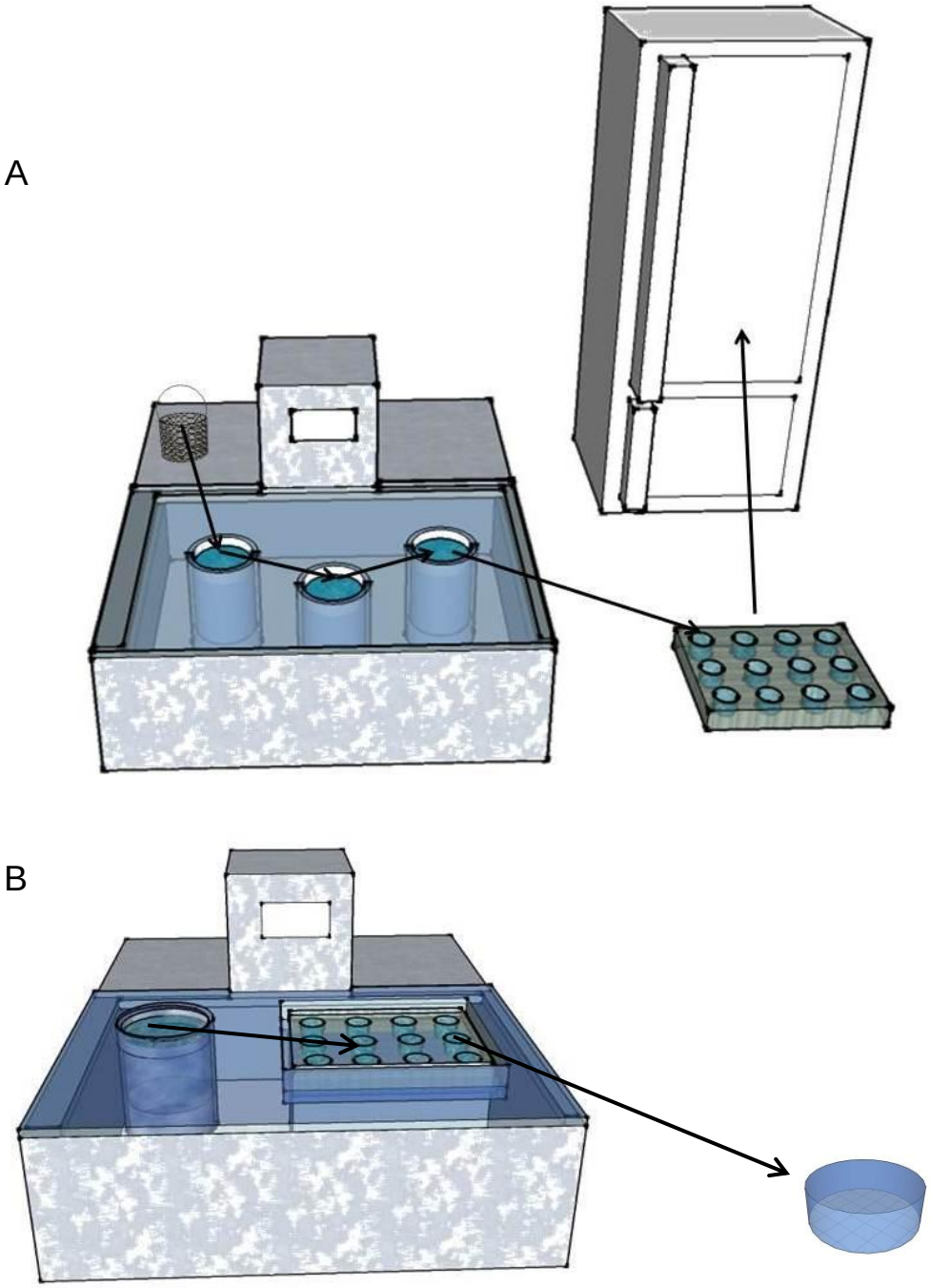


Figure 2: *Setup for sori disinfection and spore release.* ***A:*** *Sori were immersed into the disinfectant and the two rinsing baths in a perforated bucket, and placed individually in empty wells on a multiwell plate.* ***B:*** *after one night in the fridge spores were released by adding 5mL SSW to each well. 1mL of spore solution as transferred to a Nunclon dish holding 15mL of growth medium for incubation.*

2.2.4 Incubation of S. latissima *seedlings in Experiment 1 and 2*

The dishes (Nunclon™ Δ) with newly seeded spores were sealed with parafilm and left undisturbed in a room holding 10˚C. The light intensity over the dishes was 30µmol photons $m^{-2}s^{-1}$ and the light regime was set to 16:8 (day:night). The Conwy growth medium was prepared in the way presented in Section 2.1.3, but metasilicate was not added to the final growth medium. Incubation conditions were slightly different for the two experiments, and the differences are given in Table 3.

Table 3: *Incubation conditions of during Experiment 1 and Experiment 2*

Growth conditions	Experiment 1	Experiment 2
Incubation period	4 weeks	3 weeks
Addition of nutrients (Conwy medium)	Once (at seeding)	Twice (at seeding and after two weeks)
Distance of the dishes to the light source	Not given any attention	Equal

2.2.5 ***Experiment 1;*** *Effect of different disinfection treatments*

The aim of the experiment was to evaluate spore survival and growth of juvenile sporophytes after sori disinfection with the most promising diatom disinfectant from the experiment on diatom survival. Based on the results from the diatom experiment, 19 different trials were chosen for sori disinfection. The trials included acetic acid (1% or 7%), sodium hypochlorite (6ppm, 60ppm or 600ppm) and Lugol's solution (0.02%, 0.2% or 2%) with an exposure time of 2 or 10 minutes at an exposure temperature of 10°C (Table 4). Untreated sori were used as a positive control for sporophyte growth and diatom contamination. GeO_2 (0.1mL L^{-1} and 0.5mL L^{-1}), added to the growth medium after spore release from untreated sori, was used as a negative control for diatom contamination.

Six replicates were performed for each trial. Eight different sporophytes were used in the experiment, arranged to include sori from six sporophytes in each trial. Hence, every replicate (n=6) of a given treatment had sori from a different sporophyte.

Table 4: *Chemical treatments used in sori disinfection and control treatments*

Chemical	Concentration	Exposure time	Exposure temperature
Acetic acid	1%, 7%	2, 10 min	10°C
Lugol's solution	0.02%, 0.2%, 2%	2, 10min	10°C
Sodium hypochlorite	6ppm, 60ppm, 600ppm	2, 10min	10°C
GeO_2*	0.1mL L^{-1} , 0.5mL L^{-1}		
Control			

* in the Conwy medium of spores released from untreated sori

Three parameters were registered for Experiment 1:

1. Relative spore density, during spore release
2. Sporophyte density, four weeks after seeding of the spores
3. Presence of diatom contamination, four weeks after seeding of the spores

A sample of spore solution was observed under the microscope after 15 minutes of spore release, and 1mL of spore solution was transferred to a Nunclon dish if swimming spores were seen. If swimming spores was absent, the spore release was continued for another 15 minutes. Delayed spore release, as a consequence of the disinfection, was in that way registered. If non swimming spores were observed after 15 minutes of spore release, spores were seeded both after 15 and 30 minutes. Table 5 shows a relative scale established for recording the amount of swimming spores after 15 or 30 minutes of spore release.

Table 5: *Relative scale for spore density during spore release*

Number of swimming spores (counted at 40x magnification)	Degree of spore release
<5	Limited
5-10	Medium
10-20	Great
>20	Vast

After four weeks, the number sporophytes larger than 3 cells were counted in an inverted microscope (Nikon eclipse TE2000-S). Ten squares in the Nunclon dish were counted for each replicate of the different trials. Hence, 60 squares were counted for each trial. Presence of diatoms was recorded as positive or negative for each Nunclon dish.

2.2.6 ***Experiment 2;*** *Early development after sori disinfection*

The aim of the experiment was to examine if disinfection affected early development and growth of *S. latissima* seedlings. The highest concentration of Lugol's solution (2%) and sodium hypochlorite (600ppm) with an exposure time of 2 minutes and an exposure temperature of 10°C (L-2%-2min and S-600ppm-2min) were chosen for this survey (Table 6) based on previous results (Experiment 1). Untreated sori were used as a positive control for sporophyte growth and diatom contamination. GeO_2 (0.1mL L^{-1}) added to the growth medium after spore release from untreated sori, was used as a negative control for diatom contamination.

Table 6: *Chemical treatments used in sori disinfection and control treatments*

Chemical	Concentration	Exposure time	Exposure temperature
Lugol's solution	2%	2min	10°C
Sodium hypochlorite	600ppm	2min	10°C
GeO_2*	0.1mL L^{-1}		
Control			

* in the Conwy medium of spores released from untreated sori

All samples had sori disks (n=3, ø=12mm) cut from the same sorus (the same sporophyte). Any variations in spore release from different individuals were in this way eliminated. Disks were cut from the centre of the sorus as near each other as possible to ensure as identical conditions as possible for each replicate and treatment. Disinfection and spore release was performed after the method described in Section 2.2.3 - 2.2.4 and Figure 2.

The incubation period was three weeks, and pictures of the replicates were taken on a two or three day's interval. Four parameters were registered in Experiment 2:

1. Appearance of first egg
2. Number of female and male gametophytes
3. Sporophyte density, three weeks after seeding of the spores
4. Length of sporophytes, three weeks after seeding of the spores

From day 8, the samples were checked for egg production, as an indication of reproductive phase in the female gametophyte. Numbers of male and female gametophytes were counted for replicates on day 11. Ten squares per replicate were counted, and the Female: Male ratio was calculated. The numbers of sporophytes were counted after 21 days, and the length of 10 sporophytes per replicate (n=3, except GeO_2 n=1) of the trials was measured by use of the free software ImageJ. Statistic tests were not run on results from Experiment 2 due to low sample size (n=3).

2.3 Statistics

2.3.1 Tolerance of diatoms

Normality and log-normality was rejected for optical density by a Shapiro-Wilk test, at a significant level of $p \leq 0.05$. Hence a generalized linear model (GLM), a non-parametric regression method, was used to estimate SGR for the phase of exponential growth. OD data were ln-transformed and plotted against days of incubation in order to locate the period of exponential growth. The slopes of the curves expressed SGR, and values were estimated for each replicate of a treatment in the period of constant, exponential increase. Evaluation of OD data included in the SGR estimate for the control treatment is given as an example in Section 3.1.1. If the exponential growth phase were absent after the chemical exposure, a SGR estimate was made for the entire period of incubation.

Kruskal-Wallis significance test were run on groups consisting of control and the four treatments run at the same concentration and reaction temperature (Appendix I). A significance level of $p \leq 0.05$ was used. The Krusal-Wallis tests were followed up by a visual inspection of the graph and Mann-Whitney tests when the figures revealed SGR close to the SGR of the control treatment. Non-parametric tests were chosen due to non-normal distribution and low sample size (Siegel, 1957). Statistical analyses were performed and figures were created by use of IBM SPSS statistics 19.

2.3.2 Experiment 1: Survival and growth of S. latissima *seedlings after disinfection*

Normality was rejected for sporophyte density by a Shapiro-Wilk test. A significance level of $p \leq 0.05$ was used. Kruskal-Wallis significance test was run on groups consisting of control and treatments with the same disinfecting chemical. Krusal-Wallis tests were followed up by a visual inspection of the graph and Mann-Whitney tests when the figures revealed sporophyte density close to the density of the control treatment. The significance level used was $p \leq 0.05$ for both tests. Non-parametric tests were chosen because of non-normal distribution and low sample size (Siegel, 1957). Statistical analyses were performed and figures were created by use of IBM SPSS statistics 19.

3 RESULTS

3.1 Effect of chemical treatments on diatoms to the disinfectants

3.1.1 Growth of diatoms in the control treatment

Alteration in optical density (OD, 750nm), a measure of diatom growth, in the control treatments (n=6) during an incubation period of eight days is shown in Figure 3. Visual inspection of the OD data revealed stagnation in growth, most likely a lag-phase, during the first three days. The lag-phase was followed by constant, an apparent exponential growth phase from day 3 to 7, followed by declining growth rate from day 7 to 8. Replicate 3 showed a slightly different pattern with a lag-phase from day 2 to 3, exponential growth from day 3 to 4, a plateau phase from day 4 to 7, and declining growth from day 7 to 8.

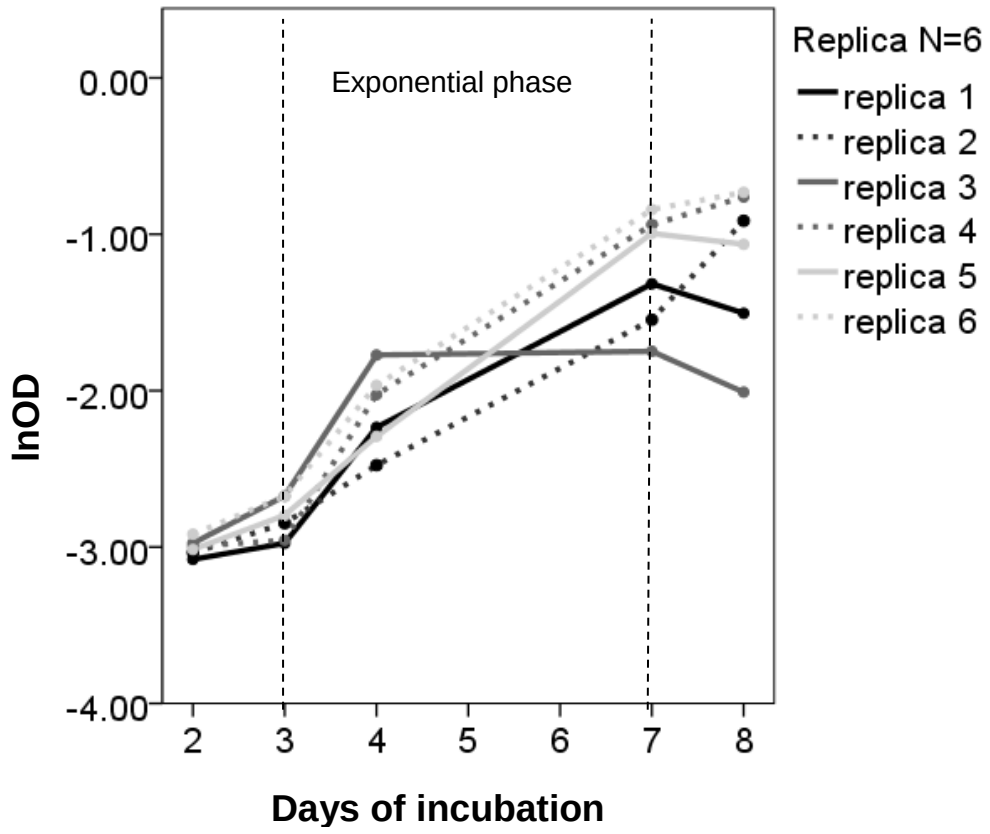


Figure 3: *ln-transformed data of optical density (750nm) of unexposed diatoms (the control, n=6) over eight days of growth.*

A Mann-Whitney test gave significantly higher mean OD on day 8 compared to day 2 ($p < 0.005$), and ln-transformation of the OD data revealed an overall exponential growth of diatoms from day 3 to 7. The exponential growth estimate (Mean SGR = 0.45±0.09day^{-1}, Figure 4) was hence calculated from day 3 to 7, with exception of replicate 3 (SGR calculated from day 3-4). SGR for all the 130 treatments was based on OD data evaluated in the same way as given above for the control treatment; mean SGR, standard error of mean SGR, p-values of significance tests, period of exponential growth, number of OD measurements and number of replicates for the 130 different treatments are given in Appendix I.

3.1.2 Growth of diatoms in treatments with germanium dioxide

SGR estimated for the two treatments with GeO_2 enriched growth medium (0.1mL L^{-1} and 0.5mL L^{-1}) together with untreated diatoms (control) are given in Figure 4. Both treatments with GeO_2 enriched growth medium (the negative control for diatom growth) showed SGR well below the SGR estimate for untreated diatoms. Diatom growth was considerable faster in the higher concentration of GeO_2 (0.5mL L^{-1}) than for the lower GeO_2 concentration (0.1mL L^{-1}). Monocultures of *P. tricornutum* were found in both treatments by a microscope survey after eleven days of incubation. This observation indicated that GeO_2 did not completely eliminate growth of *P. tricornutum*, but appears to eliminate growth of *C. muelleri* and *S. costatum*.

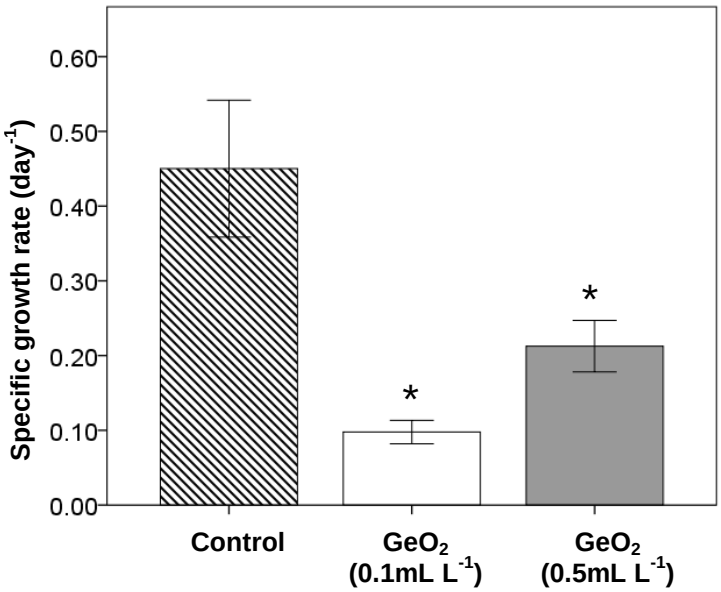


Figure 4: *SGR (day^{-1}) for untreated diatoms and when GeO_2 was added to the growth medium after spore release. All treatments were significantly different from each other. Values are mean of all replicates (n=6). Error bars indicate ±1SE. *= significantly different from the control.*

A Kruskal-Wallis test revealed significant differences among the three control treatments ($p< 0.005$), and when followed up by a pairwise Mann-Whitney test showed that both concentrations of GeO_2 showed significantly lower SGR than the untreated control ($p <0.005$). Samples treated with GeO_2 (0.5mL L^{-1}) showed significantly higher SGR than those treated with GeO_2 (0.1mL L^{-1}). The diatoms showed exponential growth from day 3 to 7 in the highest concentration of GeO_2 (0.5mL L^{-1}), and day 2 to 9 in the lower concentration (0.1mL L^{-1}). Hence, SGR were calculated based on measurements from these time periods (Appendix I).

3.1.3 Growth of diatoms in treatments with acetic acid

Figure 5 shows SGR for diatoms exposed to treatments with acetic acid. Panel A gives treatments with an exposure temperature of 10°C and Panel B treatments with an exposure temperature of 15°C. SGR of the control treatment is given in the same figure (striped bar). All treatments with acetic acid were lethal to diatoms independent of exposure time and exposure temperature. SGR were estimated based on measurements from the entire incubation period (varying from five to eleven days) as no variation in growth or colour was observed after exposure (Appendix I). White needle-like precipitations were seen in the pre-diluted bottles of acetic acid and seawater. However, the precipitation did not appear to influence the results.

3.1.4 Growth of diatoms in treatments with ethanol

SGR of diatoms exposed to ethanol treatments are given in Figure 6, together with SGR of the control treatment. Ethanol treatments were lethal to diatoms. At exposure temperature 10°C, the SGR ~ 0.1 was estimated for several treatments, but no visual change in colour was observed in the samples. However, a change towards pale white was seen when ethanol was diluted with seawater and precipitation of what was most likely salt crystals were observed in the microscope. There was presumably a reaction between seawater and ethanol, and the precipitation may have disturbed the growth measurements so that SGR was estimated from other particles than diatoms. A Kruskal-Walis test confirmed that SGR estimated for all ethanol treatments, independent of exposure temperature and time, were significantly lower than SGR of the control treatment. SGR were estimated from the entire incubation period (varying from six to eleven days) (Appendix I).

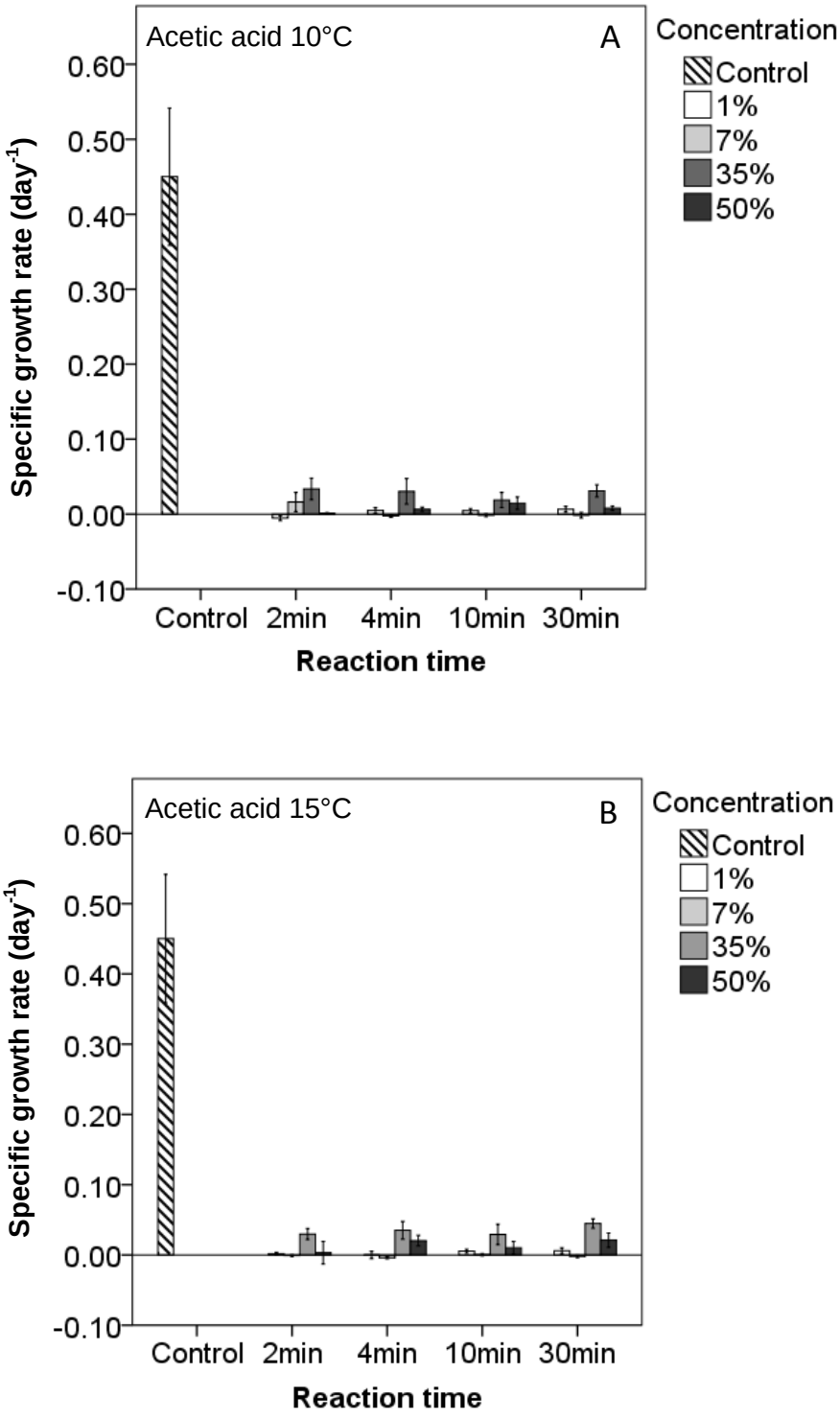


Figure 5: *SGR (day^{-1}) for untreated diatoms (control) and diatoms treated with different concentrations of acetic acid at reaction temperature 10°C (A) and 15°C (B). Values are mean of all replicates (number of replicates for the individual treatments are given in Appendix I). Error bars indicate ±1SE.*

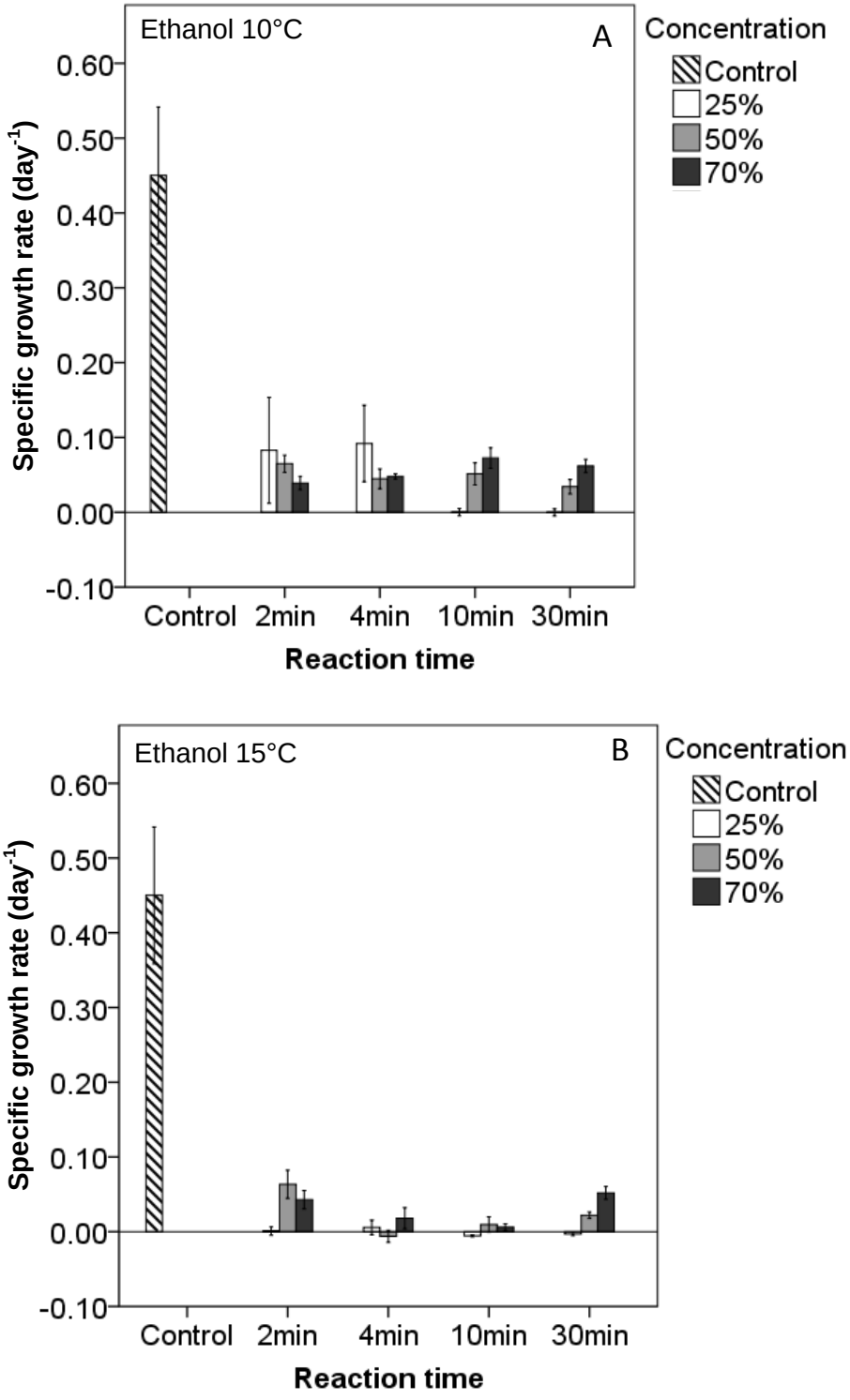


Figure 6: *SGR (day^{-1}) for untreated diatoms (control) and diatoms treated with different concentrations of ethanol at reaction temperature 10°C (A) and 15°C (B). Values are mean of all replicates (number of replicates for the individual treatments are given in Appendix I). Error bars indicate ±1SE.*

3.1.5 Growth of diatoms in treatments with formaldehyde

Figure 7 shows SGR of diatoms exposed to formaldehyde along with SGR of the control treatment. Growth of diatoms was found in all treatments with the lowest concentration of formaldehyde (0.04% formaldehyde); in all exposure times and both exposure temperatures. The medium concentration (0.4% formaldehyde) revealed growth in the two shortest exposure times (2 and 4min) at the highest exposure temperature (15°C). Visual change in colour of the samples and growth was registered after minimum one and maximum four days of incubation for the above mentioned treatments, and highest SGR was observed at an exposure temperature of 10°C. Diatoms were killed by all other treatments with formaldehyde.

Mann-Whitney tests were run on the treatments that revealed growth of diatoms. The treatment with 0.04% formaldehyde, an exposure time of 10min and an exposure temperature of 10°C (F-0.04%-10min-10°C) showed significantly lower SGR than the control. The three other treatments of this concentration and exposure temperature (F-0.04%-2min-10°C, F-0.04%-4min-10°C, F-0.04%-30min-10°C) showed a SGR similar to the control treatment. In treatments with an exposure temperature of 15°C, all treatments that showed growth (F-0.04% all exposure times, and F-0.4%-2min-15°C and F-0.4%-4min-15°C) showed significantly lower SGR than the control treatment with exception of treatment F-0.04%-4min-15°C. SGR were calculated for the period of exponential growth (varying from three to seven days) in samples with diatom growth, and for the entire incubation period for the treatments with lethal effect on diatoms (Appendix I).

P. tricornutum and one unknown, contaminating diatom species, most likely *Navicula* ssp., were found in all treatments with an exposure temperature of 15°C after 7 days of incubation. Unfortunately, samples with exposure temperature 10°C were not checked in the light microscope.

3.1.6 Growth of diatoms in treatments with Lugol's solution

Diatom growth after exposure to Lugol's solution is shown in Figure 8, together with growth of diatoms in the control treatment. Exposure to the lowest concentration of Lugol's solution (0.02% Lugol's solution), at the lowest exposure temperature (10°C) and all four reaction times, gave visual change in colour of the samples and diatom growth similar to the growth in the control treatment. Elevated concentration (0.2% or 2% Lugol's solution) or temperature (15°C) eliminated diatom growth in all other treatments.

Significance tests supported the result; all treatments, except for the treatments with the lowest concentration (0.02%) and an exposure temperature of 10°C (L-0.02%-2min-10°C, L-0.02%-4min-10°C, L-0.02%-10min-10°C and L-0.02%-30min-10°C), showed significantly lower SGR than the control treatment ($p < 0.01$). SGR were calculated for the period of exponential growth (varying from two to three days) in samples with diatom growth, and for the entire incubation period for the treatments with lethal effect on diatoms (Appendix I).

3.1.7 Growth of diatoms in treatments with sodium hypochlorite

Growth of diatoms exposed to sodium hypochlorite treatments, together with the control treatment, is given in Figure 9. A distinct difference between growth of diatoms exposed to sodium hypochlorite and unexposed diatoms was seen at all concentrations (6ppm, 60ppm or 600ppm sodium hypochlorite), at all exposure times (2, 4, 10 or 30min) and for both exposure temperatures (10°C and 15°C). Diatom growth was significantly affected ($p <0.005$) by exposure to sodium hypochlorite according to a Kruskal-Walis test. A visual inspection of the graph revealed a notable difference in SGR from the control treatment and pairwise Mann-Whitney tests were not considered necessary. SGR were estimated from the entire incubation period (varying from 5 to 8 days) as no variation in growth or visual changes in colour of the samples were observed after exposure (Appendix I).

3.1.8 pH of experimental chemicals

The pH in the different dilutions of the exposure chemicals is given in Figure 10; Panel A to E represent the different chemicals. All dilutions of acetic acid had a $pH < 2.8$. For the other chemicals, pH ranged from 7.7 to 9, with highest pH measured in the highest concentration of sodium hypochlorite (600ppm sodium hypochlorite) and lowest pH in the medium concentration of Lugol's solution (0.2% Lugol's solution).

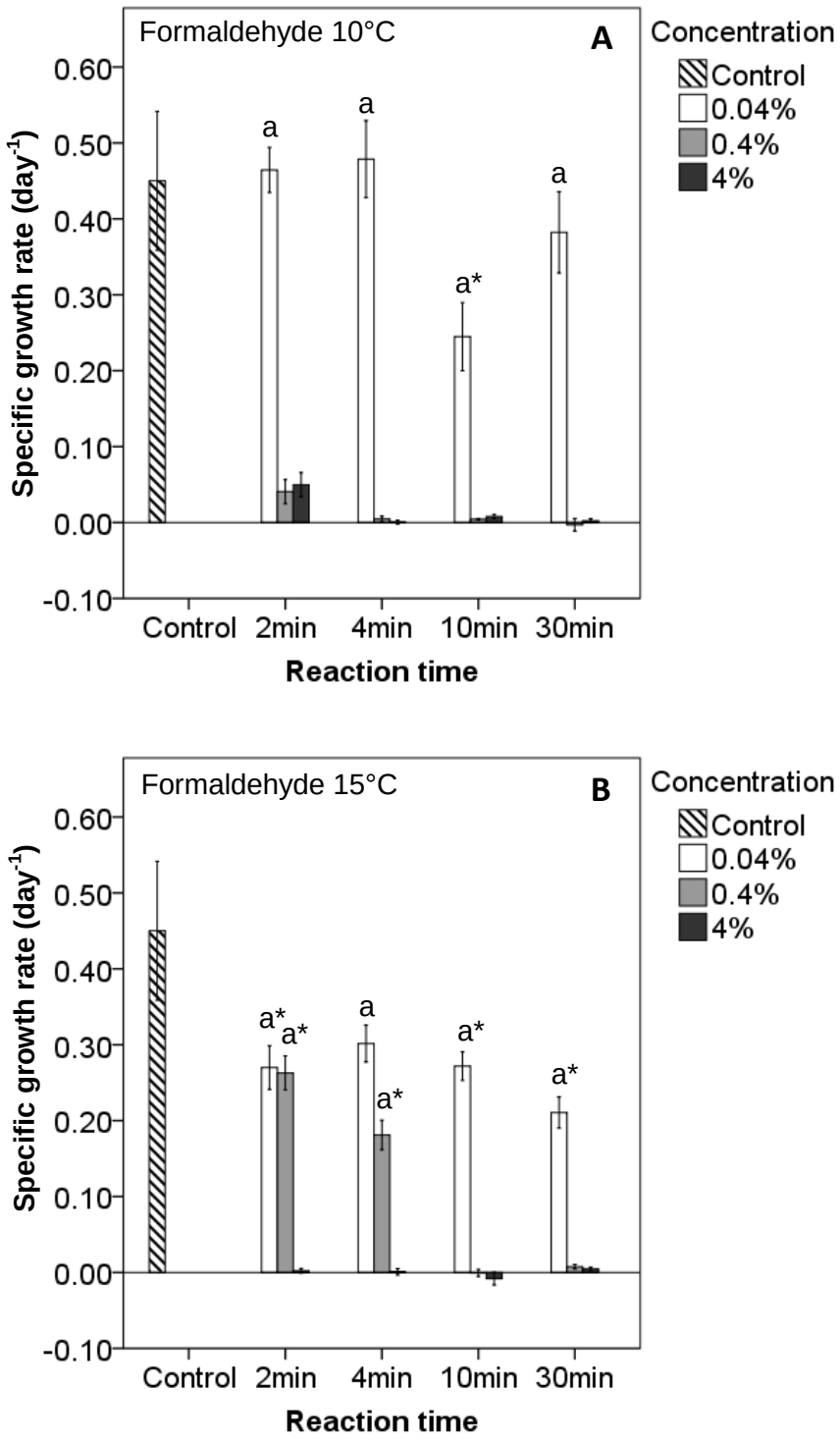


Figure 7: *SGR (day^{-1}) for untreated diatoms (control) and diatoms treated with different concentrations of formaldehyde at reaction temperature 10°C (A) and 15°C (B). Values are mean of all replicates (number of replicates for the individual treatments are given in Appendix I) Error bars indicate ±1SE. a = Mann-Whitney test, *= Mann-Whitney test gave significant difference from the control.*

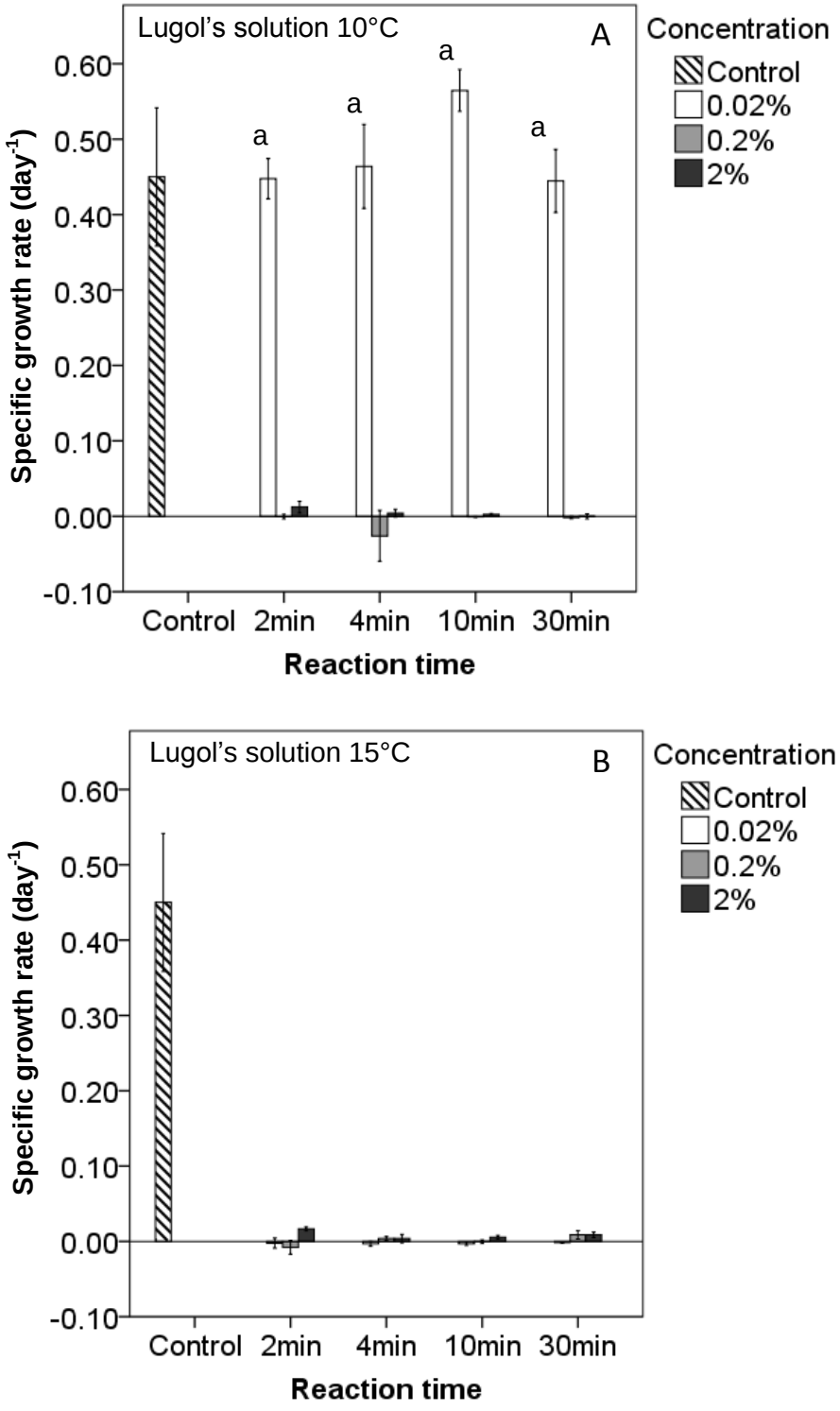


Figure 8: *SGR (day^{-1}) for untreated diatoms (control) and diatoms treated with different concentrations of Lugol's solution at reaction temperature 10°C (A) and 15°C (B). Values are mean of all replicates (number of replicates for the individual treatments are given in Appendix I) Error bars indicate ±1SE. a = Mann-Whitney test, *= Mann-Whitney test gave significant difference from the control.*

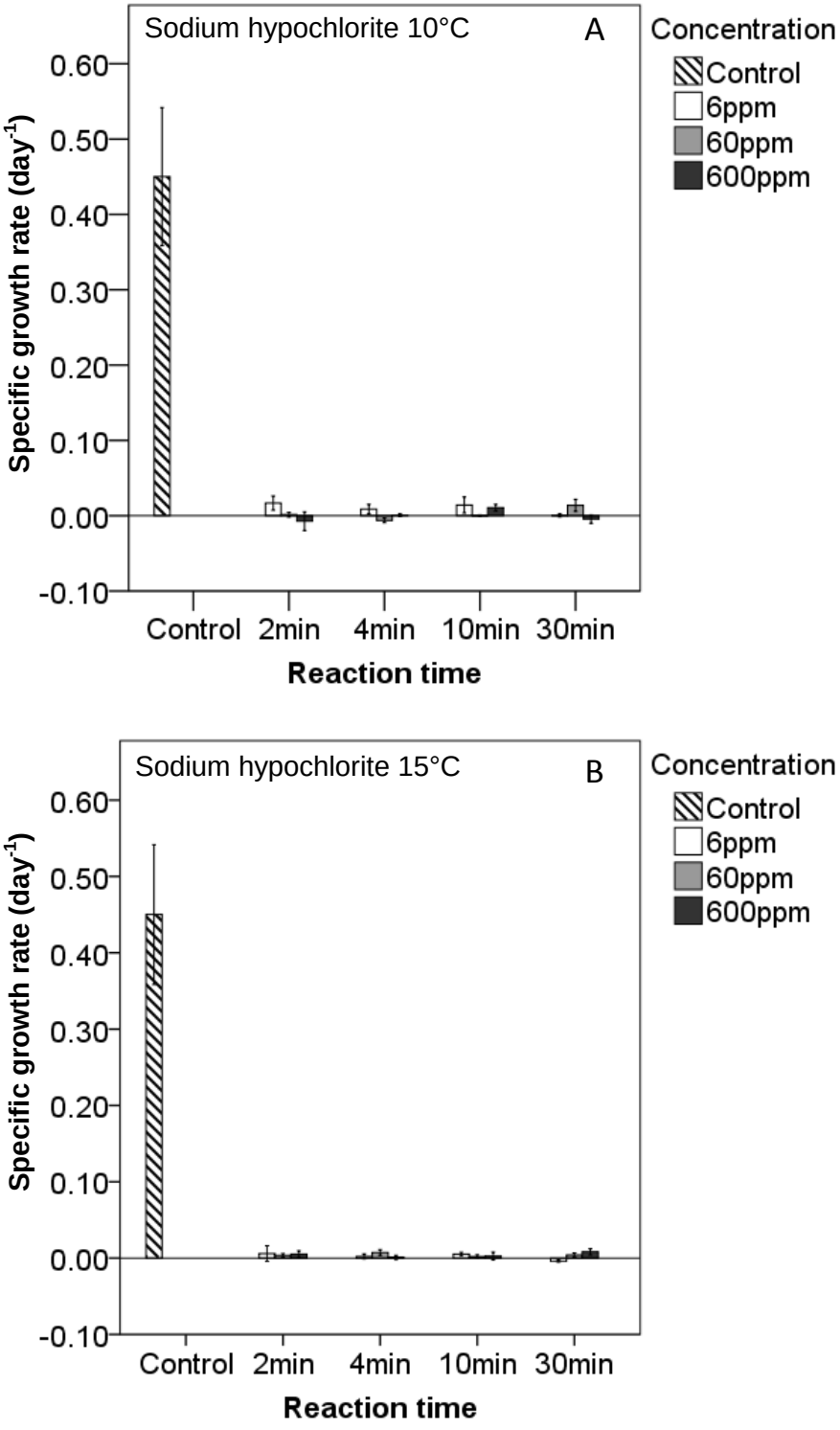


Figure 9: *SGR (day^{-1}) for untreated diatoms (control) and diatoms treated with different concentrations of Sodium hypochlorite at reaction temperature 10°C (A) and 15°C (B). Values are mean of all replicates (number of replicates for the individual treatments are given in Appendix I) Error bars indicate ±1SE.*

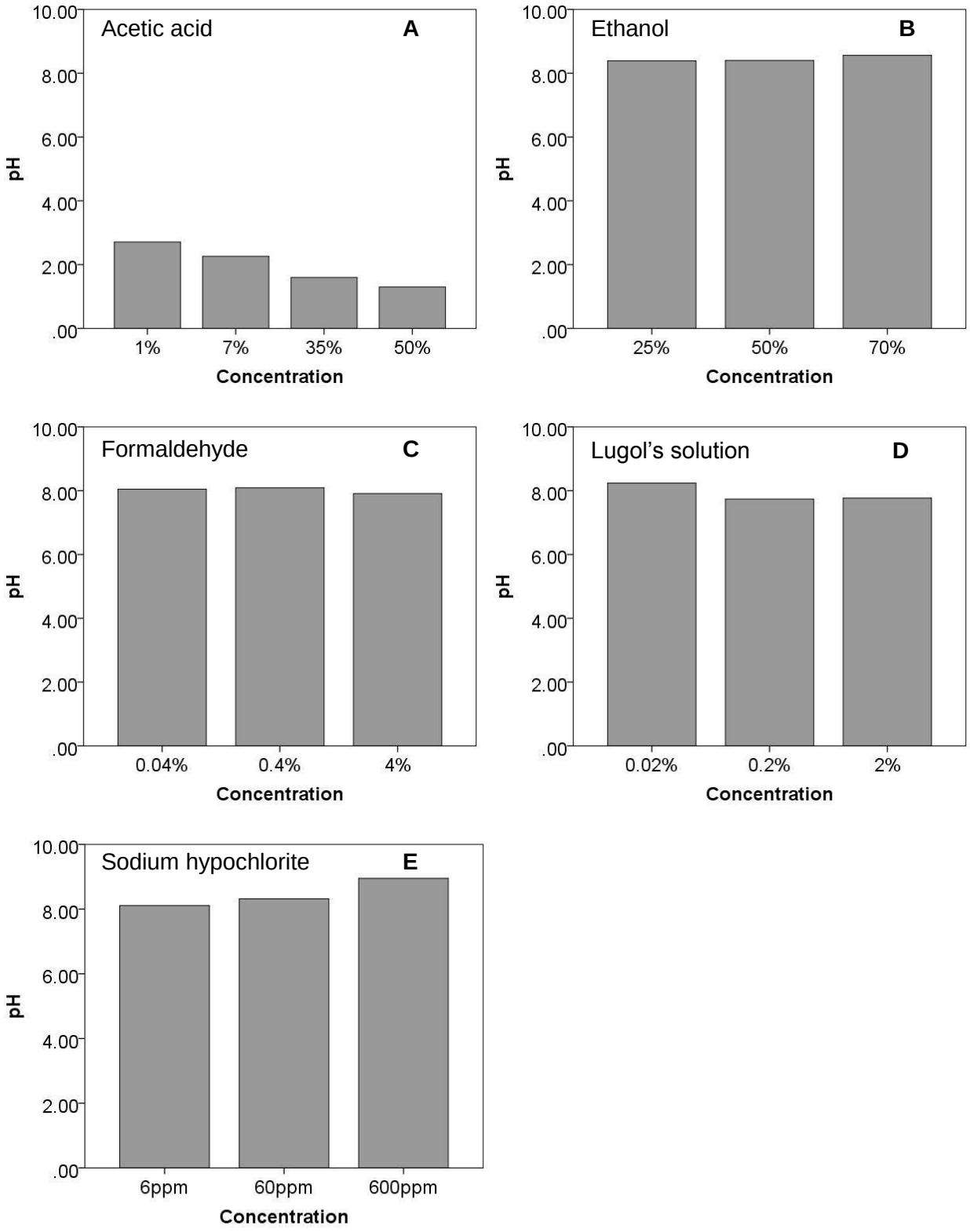


Figure 10: *pH of dilutions of Acetic acid (A), Ethanol (B), Formaldehyde (C), Lugol's solution (D) and Sodium hypochlorite (E) used in exposure of diatoms.*

3.2 Test of method for sori disinfection

A test on spore survival from sori disks (cut from mature sori) disinfected with sodium hypochlorite was performed prior to the sori disinfection experiments (see Material and Method Section 2.2.2).

3.2.1 Disinfection of sori disks; a test of method

Swimming spores were released from the disinfected disks of mature sori, and sporophyte growth was registered as positive in all replicates of the treatments. No noticeable differences in spore release and number of sporophytes (after two weeks of growth) were found between samples that were disinfected with the two concentrations of sodium hypochlorite (6ppm or 60ppm). However, the density of sporophytes (after two weeks of growth) appeared to be higher when spores were released from sori disks, than when spores were released from whole sori (control treatment). The observed difference was probably caused by a lower density of spores in the spore solution at inoculation of the control samples, because the spores were released in a larger volume of sterile seawater. The results indicated that spores inside disks of sori survived disinfection of sori disks. Sori disks may therefore be used in standardization of a disinfection method for large-scale production of *S. latissima*.

3.3 Experiment 1: *Survival and growth of* S. latissima *seedlings after disinfection*

The experiment on diatom survival after exposure to potential disinfectants (presented in Section 3.1) was followed by a survey on the tolerance of *S. latissima* spores. Sori were disinfected with the most promising diatom disinfectants; acetic acid, Lugol's solution and sodium hypochlorite (see Material and Methods Section 2.2.5). Spore density was registered during spore release, and sporophyte density and diatom contamination were registered after four weeks of growth.

3.3.1 Control treatments

The registrations of spore densities (during spore release), sporophyte densities (four weeks after spore release) and diatom contamination (four weeks after spore release) of the control treatments are shown in Figure 11. The line and the right y-axis in Panel B gives the relative spore density (mean of n=6), on a relative scale during spore release (see Materials and Methods, Section 2.2.5). The bars and the left y-axis in Panel B gives the mean number of sporophyte (number mm^{-2}, n=6) four weeks after seeding of the spores. Panel A, gives the percent of replicates (n=6) per treatment that were contaminated by diatoms four weeks after spore release. The different treatments are given on the x-axis.

Untreated sori (positive control for sporophyte growth and diatom contamination, referred to as the control) showed an average sporophyte density of 3±1.2 sporophytes mm^{-2}, and 50% of the replicates were highly contaminated by diatoms. Spores released from untreated disks and grown in GeO_2 enriched Conwy medium (negative control for diatom contamination in sporophyte cultures), showed a slightly lower sporophyte density than samples from the control treatment. Number of sporophytes decreased with increasing concentration of GeO_2. However, no significant differences in sporophyte density were found between treatments (Kruskal-Wallis test, $p>0.5$). Surprisingly, diatoms were only present in the highest concentration of GeO_2 (0.5mL GeO2 L^{-1}), yet in limited amounts (33%of the replicates) and at low cell density.

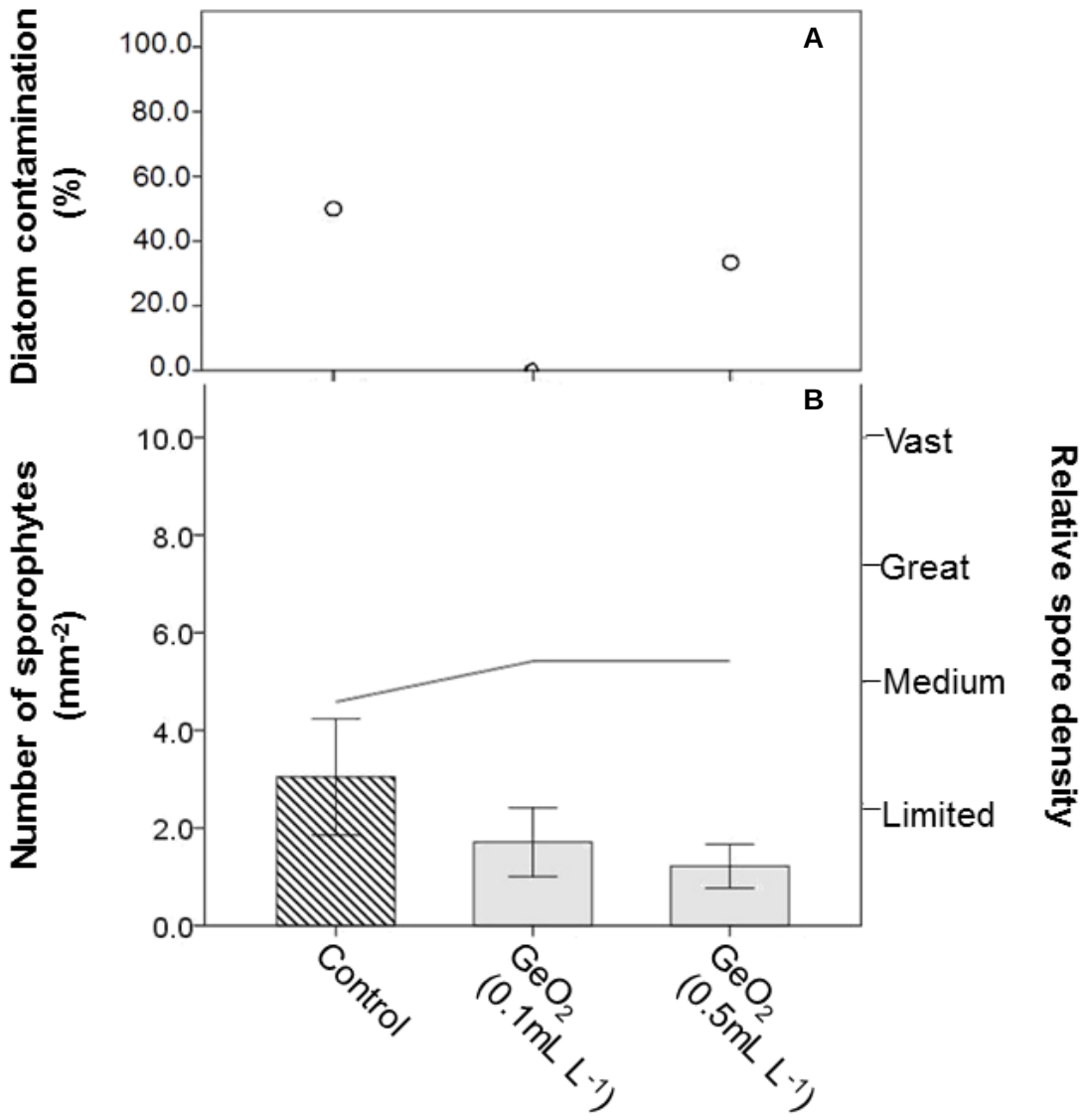


Figure 11: *Bars indicate number of sporophytes (sporophytes mm^{-2}) after four weeks of growth (mean values ± 1SE, n=6). Relative spore density is shown as a line and right y-axis (Vast, Great, Medium, Limited). Upper panel shows contamination of diatoms (% of replicates with diatom contamination after four weeks of growth).*

3.3.2 Treatments with acetic acid

Spore density (at spore release), sporophyte density (four weeks after spore release) and diatom contamination (four weeks after spore release) after disinfection with acetic acid are given in Figure 12. The different treatments are given on the x-axis, and are named after the system introduced in Section 2.1.5 (first letter of chemical - concentration - exposure time; exposure temperature was 10°C for all treatments and is therefore excluded from the lables). Spores were seeded both after 15 minutes and 30 minutes (indicated with * in the text on the x-axis) of spore release, in case the release was delayed by the disinfecting treatment (Section 2.2.5).

However, no swimming spores or sporophytes were seen in any of the samples (panel B), and it is clear from Figure 12 that acetic acid treatments were lethal to both diatoms and *S. latissima* spores. Diatom contamination (panel A) was only found in one replicate (16%) of the lowest concentration and reaction time (A-1%-2min). The results showed that acetic acid in the concentrations tested here, can be eliminated in the search of a sori disinfectant for mitigation of diatom contamination in *S. latissima* cultures.

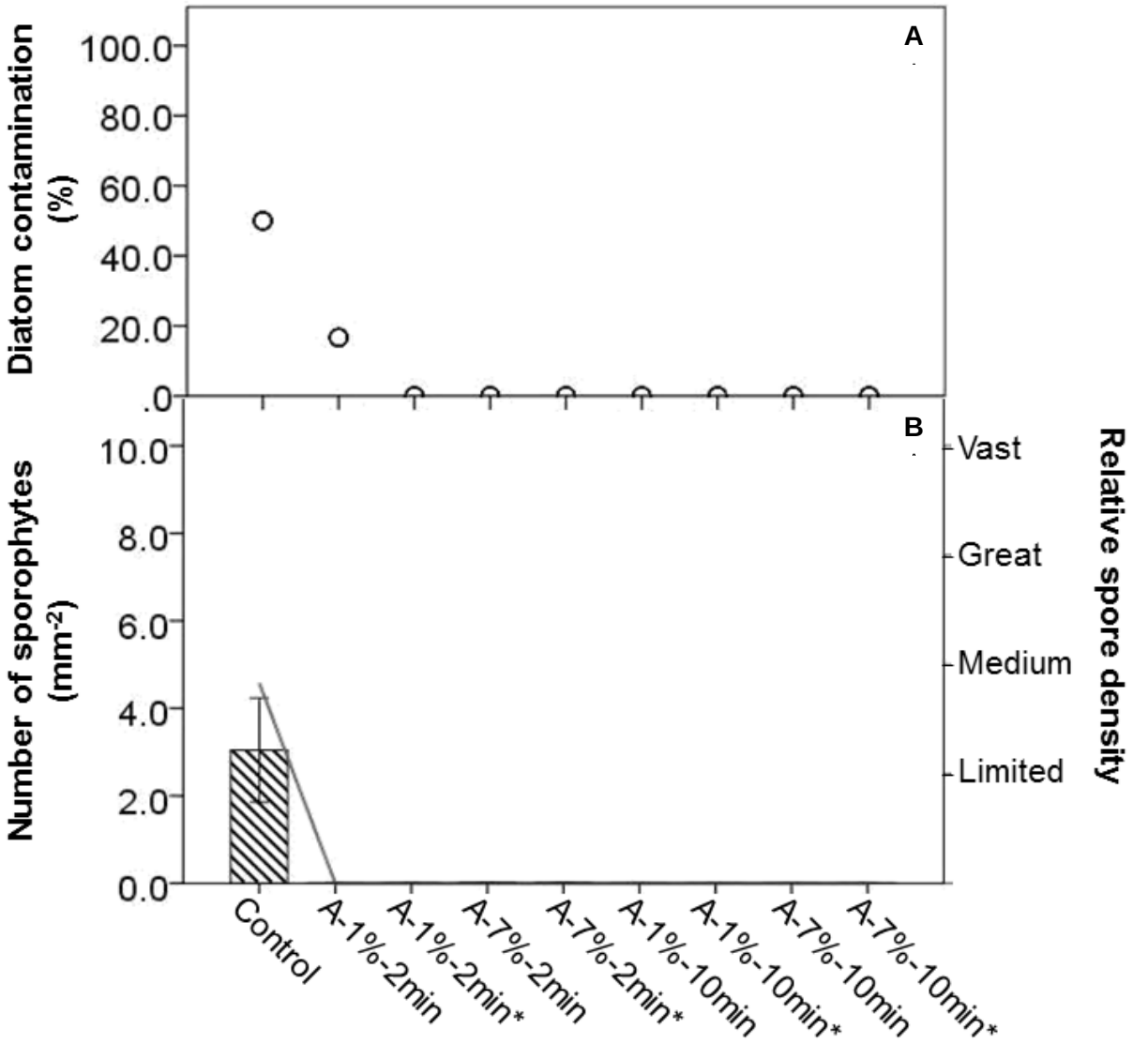


Figure 12: *Bars indicate number of sporophytes (sporophytes mm^{-2}) after four weeks of growth (mean values ± 1SE, n=6). Relative spore density is shown as a line and right y-axis (Vast, Great, Medium, Limited). Upper panel shows contamination of diatoms (% of replicates with diatom contamination after four weeks of growth). The x-axis gives the different treatments with acetic acid (first letter of the chemical - concentration - exposure time), *= spores were seeded after 30 minutes of spore release.*

3.3.3 Treatments with Lugol's solution

Spore density (at spore release), sporophyte density (four weeks after spore release) and diatom contamination (four weeks after spore release) from treatments disinfection with Lugol's solution are given in Figure 13. Sori disinfected with the highest concentrations and shortest exposure time of Lugol's solution (L-2%-2min) showed the highest mean number of sporophytes (n=6, bars and left y-axis in Panel B). Spores were seeded both after 15 and 30 minutes of spore release when disinfected with the highest concentration (both exposure times). Slightly higher sporophyte densities were registered in the samples that was seeded after 30 minutes of spore release (indicated with * in the text on the x-axis). However, no significant differences in sporophyte density from the control, or between concentrations of Lugol's solution, were revealed (Kruskal-Wallis test, $p>0.5$).

For both exposure times, the intermediate concentration (0.2% Lugol's solution) showed the highest percent of diatom contamination; 100% and 50% for exposure time 2 minutes and 10 minutes, respectively (Panel A). Whenever diatoms were present, the density of cells was high. Treatments with 2% Lugol's solution were free of diatom at both exposure times.

From Figure 13 it appears as the highest concentration of Lugol's solution gave the lowest spore densities (during spore release) at both exposure times. It should, however, be kept in mind that spore density was measured on a relative scale that is associated with high uncertainty. Based on these results L-2%-2min was chosen for the second experiment.

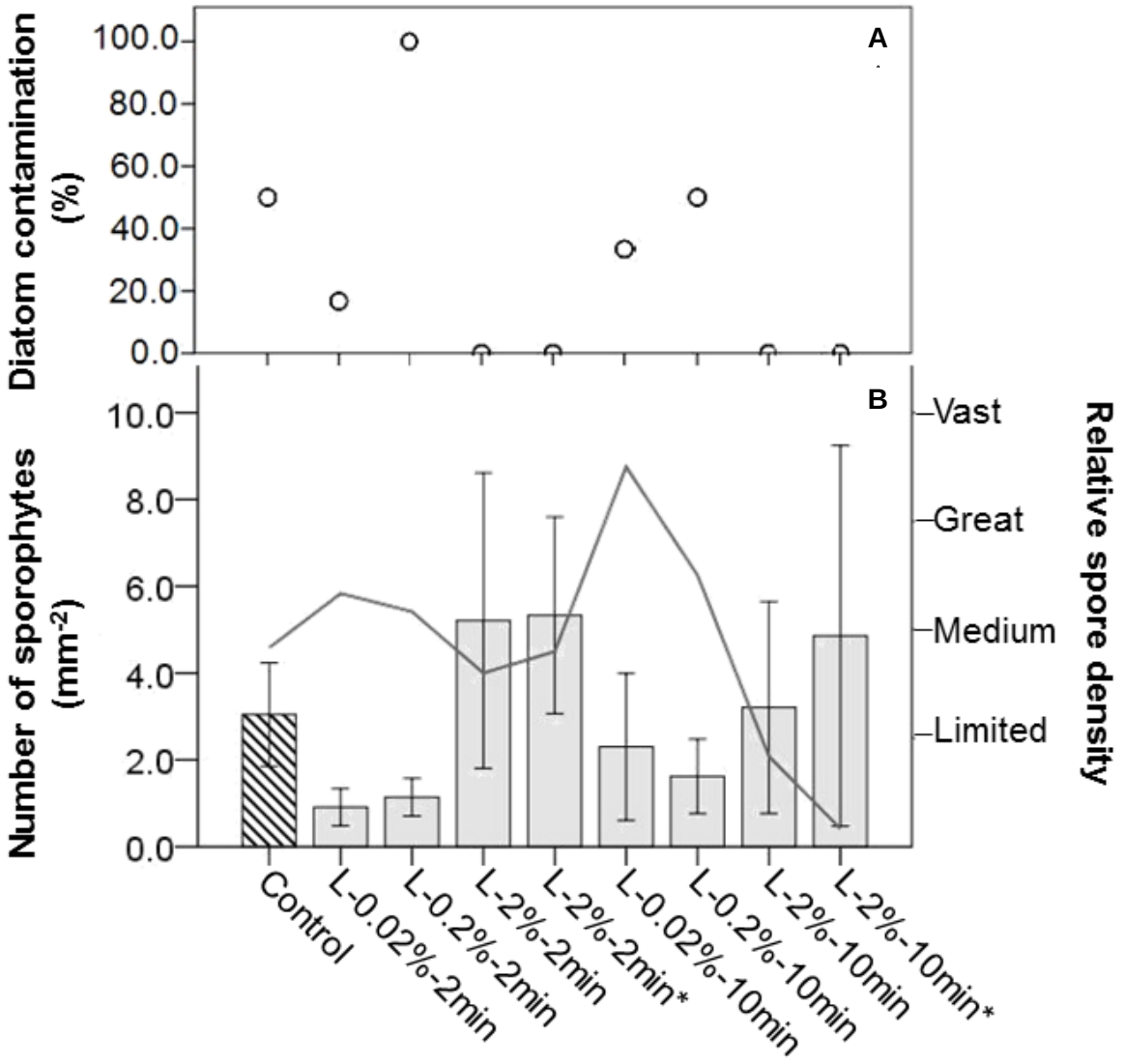


Figure 13: *Bars indicate number of sporophytes (sporophytes mm^{-2}) after four weeks of growth (mean values ± 1SE, n=6). Relative spore density is shown as a line and right y-axis (Vast, Great, Medium, Limited). Upper panel shows contamination of diatoms (% of replicates with diatom contamination after four weeks of growth). The x-axis gives the different treatments with Lugol's solution (first letter of the chemical - concentration - exposure time), *= spores were seeded after 30 minutes of spore release.*

3.3.4 Treatments with Sodium hypochlorite

Figure 14 shows the spore density (during spore release), sporophyte density (four weeks after spore release) and diatom contamination (four weeks after spore release) after exposure to sodium hypochlorite. The sporophyte density increased with increasing concentration of the sori disinfectant; sodium hypochlorite (Panel B). The highest sporophyte density was found after sori disinfected with the highest concentration (600ppm) and shortest reaction time (2 minutes). Significantly lower sporophyte density (Mann-Whitney test, $p<0.005$) was found in the samples from sori disinfected with the lowest concentration (6ppm sodium hypochlorite) and longest exposure time (10 minutes). Samples of this treatment (S-6ppm-10min) were incubated in the outer range of the light source and the low density of sporophytes may have been caused by light limitation.

The treatments with the highest concentration of sodium hypochlorite (600ppm) were free of diatom at both exposure times (Panel A). The medium concentration (60ppm) gave samples free of diatom only at the shortest exposure time (2 minutes). The density of contamination was high whenever diatoms were present.

Spore density (during spore release) was lowest when sori were disinfected with the highest concentration of sodium hypochlorite and highest after disinfection with the lowest concentration of sodium hypochlorite.

The results indicated that higher concentrations of sodium hypochlorite were lethal to diatoms, without having a significant effect on the sporophyte density. Based on the results, 600ppm sodium hypochlorite with exposure time 2 minutes was chosen for the second experiment.

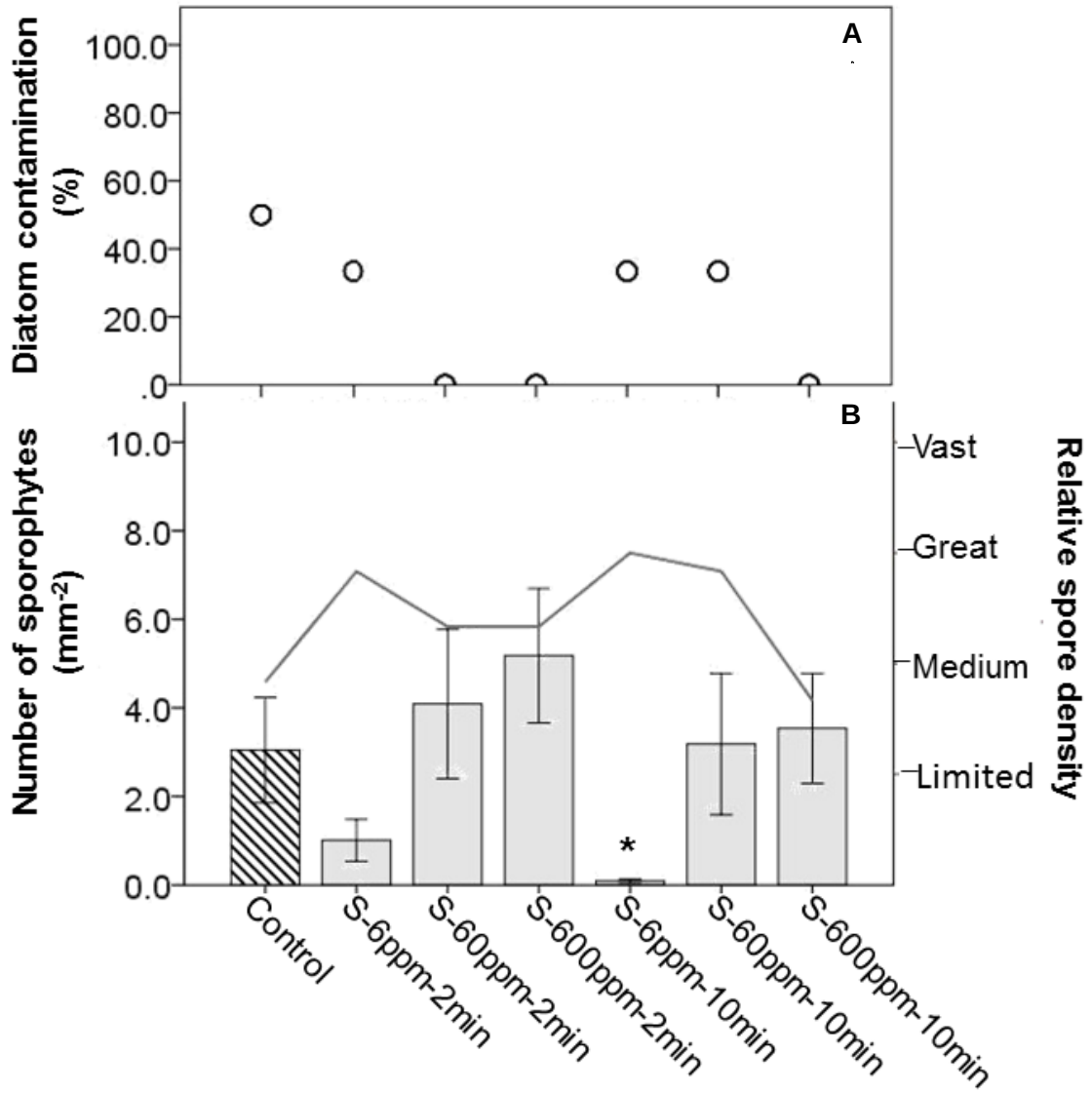


Figure 14: *Bars indicate number of sporophytes (sporophytes mm^{-2}) after four weeks of growth (mean values ± 1SE, n=6). Relative spore density is shown as a line and right y-axis (Vast, Great, Medium, Limited). Upper panel shows contamination of diatoms (% of replicates with diatom contamination after four weeks of growth). The x-axis gives the different treatments with sodium hypochlorite (first letter of the chemical - concentration - exposure time), *= significantly lower sporophyte density than the control treatment.*

3.3.5 Variations between replicates of the same treatment

Notable differences in sporophyte size and development were observed between replicates of the same treatment. Figure 15 A-B shows two replicates from the control treatment after four weeks of growth, and is an example of difference in growth observed among replicates of the same treatment. The size difference of the sporophytes was remarkable. On the sporophytes in Figure 15 A pale cells can be seen, which may imply low concentration of photosynthetic pigments and can be a sign on nutrient deficiency, which may explain the difference in growth.

Figure 15 C-D shows an example of differences in development stage among replicates of the same treatment (L-0.02%-2). Figure 15C shows a relatively big sporophyte, while Figure 15D shows gametophytes in early reproductive phase. The differences in development may have been caused by unequal light conditions.

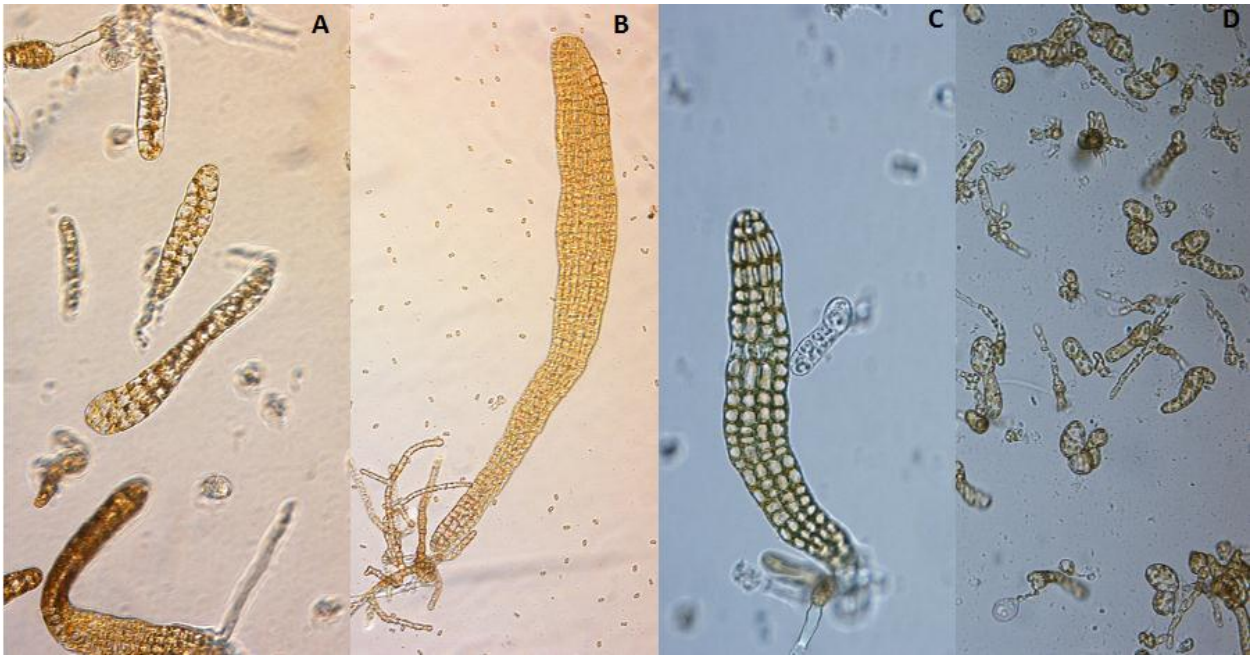


Figure 15: *Young seedlings of S. latissima after four weeks of growth.* ***A-B:*** *Replicates of untreated control, A: magnification 20x, B: magnification 10x.* ***C-D:*** *Replicates of L-0.02%-2, magnification 20x.*

Based on knowledge gained in this experiment some adjustments were done for the second experiment. Nutrients were resupplied after two weeks of growth, Nunclon^{TM} Δ dishes were placed in equal distance to the light source during incubation and all sori disks were taken from the same ripe sorus to eliminate natural variations in spore release among different sori.

3.4 Experiment 2: Effect on early development of S. latissima

A final experiment was carried out to describe the early development of *S. latissima* seedlings when sori were disinfected with the disinfectants described in Section 2.2.6.

3.4.1 Gametophyte development

Figures 16-17 show the development of seedlings during 21 days of growth after disinfection of sori. Primary cells were visible in all treatments after three days of incubation, and from day 6 it was possible to determine the gender for the main part of the gametophytes. Female:Male ratios were calculated on day 11 when the gender easily was determined for all gametophytes (Table 7). The Female:Male ratio was close to 1, yielding 50% of females for all treatments. This suggests that disinfection did not affected germination and determination of gender.

Gametophytes from sori disinfected with Lugol's solution or sodium hypochlorite used eleven days after spore release to produce eggs, while seedlings from untreated sori used eight days (Table 7). Only one replicate of sporophytes grown in GeO_2 (0.1mL L^{-1}) enriched growth medium entered reproductive phase during the 21 days of survey, and for this sample eggs were registered after 11 days. The delay in egg production suggested a negative effect of sori disinfection on gametogenesis.

Table 7: *Female: Male ratio at day 11 and time of egg release for the different treatments.*

Treatment	Nr. Replicates	Female: Male	Day of first visible egg
Control	3	1.04±0.56	8
GeO_2 (0.1mL L^{-1})	3	1.01±0.60	11*
L-2%-2min	3	0.98±0.66	11
S-600ppm-2min	3	0.91±0.45	11

The Female: Male ratio are means (n=3, ±SE) for all treatments. *n=1.

3.4.2 Sporophyte growth and survival

Only one replicate of the GeO_2 treatment was able to produce eggs and sporophytes, and the growth in that sample was slow compared to the control (Figures 16-17); sporophytes were not observed before day 21, and the density of sporophytes and their mean length was the lowest recorded (Table 8). However, diatoms were not observed in any of the replicates of GeO_2 treated samples.

At day 14, most seedlings in the control and from the two disinfecting treatments (L-2%-2min and S-600ppm-2min) entered the sporophyte generation. Among those treatments, samples disinfected with sodium hypochlorite showed the lowest density of sporophytes, but exhibited the highest mean length (Table 8). Sori disinfected with Lugol's solution showed a similar sporophyte mean length as the control, however, the mean sporophyte density was only 50% of the control density. Sporophytes in one of the control replicates were lumpy and irregular at day 21, and the sporophyte length measurements could not be accomplished. However, pale cells as observed in Experiment 1 were not seen in this experiment, presumably due to the addition of nutrients after two weeks.

Table 8: *Sporophyte density and length for the different treatments.*

Treatment	Nr. of replicates	Mean nr. of sporophytes mm^{-2}
Control (untreated)	3	14.0±6.5
GeO_2 (1mL L^{-1})	3	1.0±1.1
L-2%-2min	3	7.0±1.5
S-600ppm-2min	3	2.5±0.1
Treatment	**Nr. of replicates**	**Mean length of sporophytes (mm)**
Control (untreated)	2	0.2±0.0
GeO_2 (1mL L^{-1})	1	0.1
L-2%-2min	3	0.2±0.1
S-600ppm-2	3	0.4±0.1

Values are means (±1SE, except GeO_2 (0.1mL L^{-1}) length data).

From Figures 16-17 it was evident how the diatoms contamination was developing over time in the control, while diatoms are absent in the other treatments. The results showed that the two

disinfecting treatments were effective in killing contaminating diatoms, without notably influence on sporophyte density and length. Both S-600ppm-2min and L-2%-2min may be sufficient as sori disinfectants in *S. latissima* cultivation.

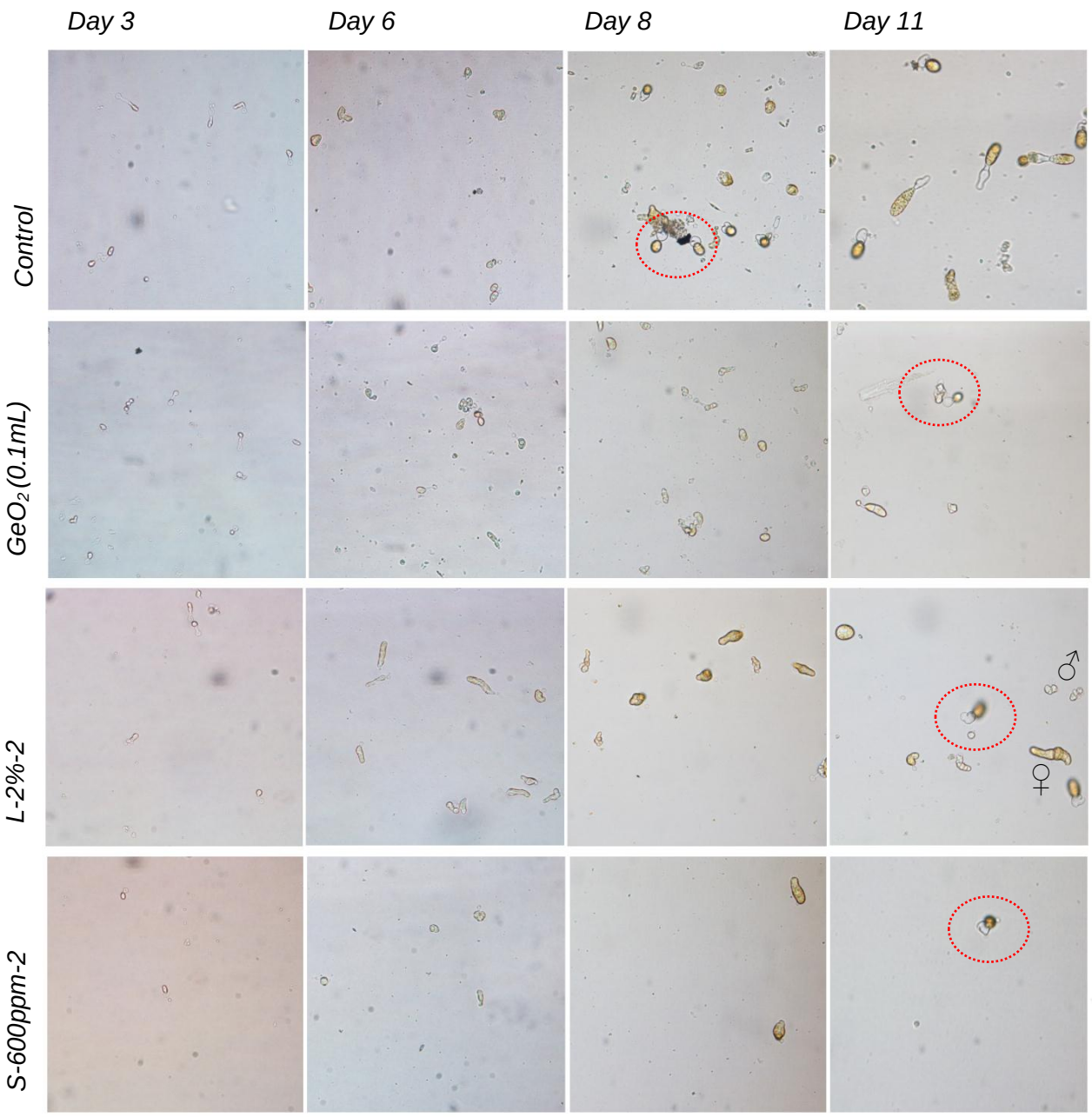


Figure 16: S. latissima *growth day 3-11. The red, dotted line encircles eggs. ♀=Female gametophyte. ♂=Male gametophyte. Magnification 20x.*

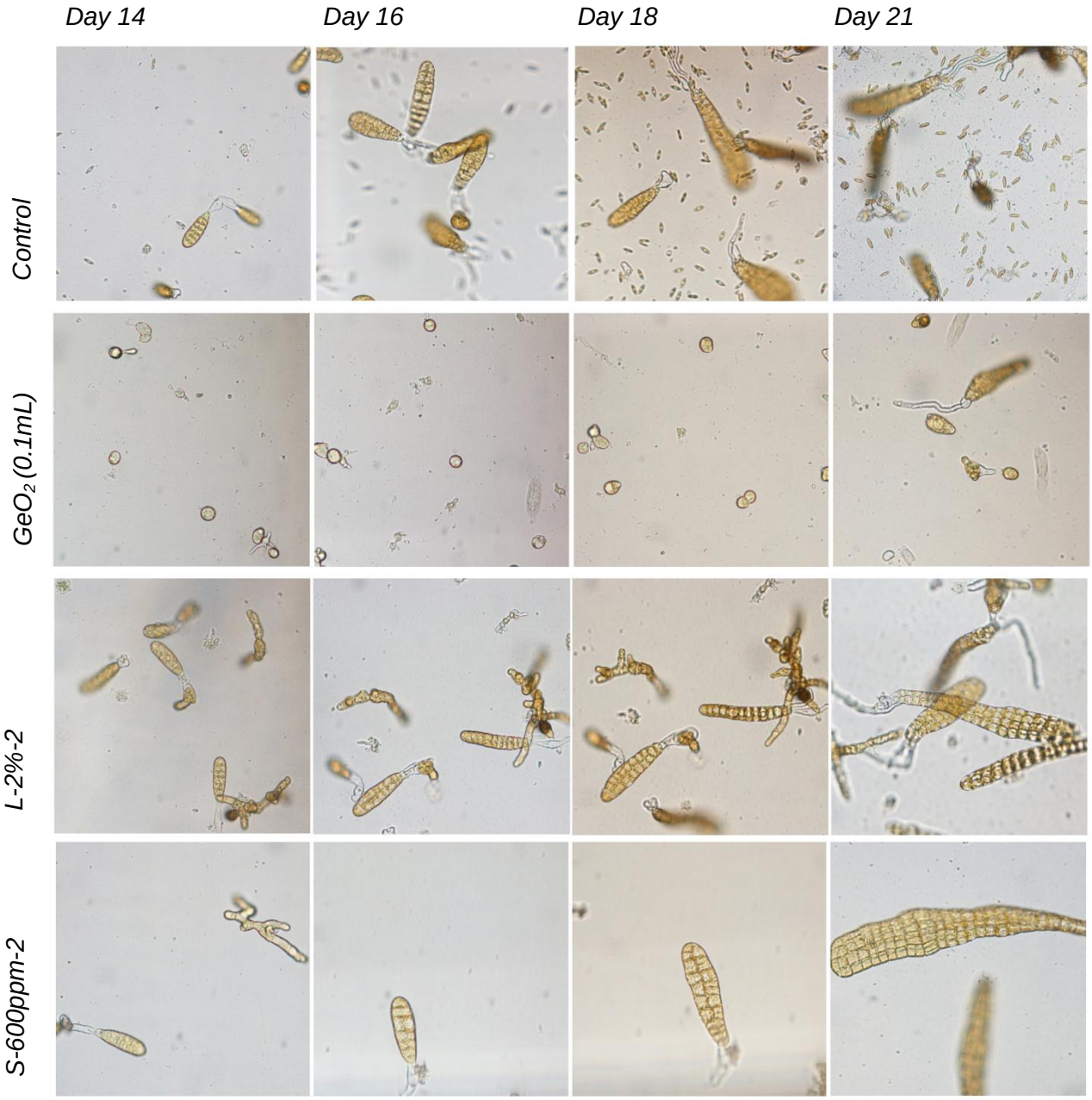


Figure 17: S. latissima *growth day 14-21. Magnification 20x.*

4 DISCUSSION

4.1 Successful mitigation of diatoms after sori disinfection

The present study has demonstrated that sodium hypochlorite and Lugol's solution can be promising chemicals for a disinfecting method that mitigate diatom contamination from *S. latissima* sori. Sodium hypochlorite (600ppm) and Lugol's solution (2%), with an exposure time of 2 minutes and an exposure temperature of 10°C, followed by two rinsing baths in sterile seawater for 30 seconds (10°C), gave total elimination of diatoms in cultures of *S. latissima*. No notable effects on spore release, sporophyte growth or early development of young seedling were registered after sori disinfection. This suggests that disinfection of *S. latissima* sori can be done safely, by use of these treatments, without concern about reduced quality or quantity of cultivated seaweeds. Sori disinfection with acetic acid was lethal to *S. latissima* spores at all concentrations and exposure times tested. The other treatments with Lugol's solution and sodium hypochlorite, with exception of S-60ppm-2min, revealed diatom contamination in some samples after 4 weeks of growth.

4.1.1 Control of diatom growth after disinfection

All the treatments with sodium hypochlorite were lethal to diatoms when the treatments were done to diatoms alone (Figure 9). However, when sori were disinfected with sodium hypochlorite, diatom contamination was found in samples with both exposure times of the lowest concentration (6ppm, 2 minutes or 10 minutes), and in the medium concentration (60ppm) with an exposure time of 10 minutes (Figure 14). Jeong et al. (2002) found a sodium hypochlorite concentration close to 3ppm to be lethal to 50% of the cells in a culture of *S. costatum* and *Thalassiosira rotula* after 10 minutes of exposure, while much higher concentrations (10,000ppm) have been suggested for sori disinfection (Druehl and Hsiao, 1969; Hsiao and Druehl, 1973; Kientz et al., 2011). The results found in those studies, seem to be in agreement with the present study; higher concentration of sodium hypochlorite seem to be necessary for elimination of diatoms from sori, compared to diatoms in free suspension. As mentioned in the introduction, organic material in a solution will consume available chlorine. Extracellular polysaccharides from microbial aggregates has been reported to consume and interact with

hypochlorite (Characklis and Dydek, 1976), and it is possible that the mucilage of polysaccharides consumed available chlorine. The sporophytes, from which the sori were taken, were kept in heavy contaminated water prior to the disinfection. Organic detritus may therefore, have been attached to the mucilage and consumed active chlorine during the disinfecting treatment. Additionally, the mucilage may have worked as a matrix that hindered the disinfectants in reaching the target organism. Accessibility to the target organism is important for how efficient the disinfectant work (Weavers and Wickramanayake, 2001). Armstrong et al. (2000) demonstrated that the heterotrophic diatom *Nitzschia alba* borrows itself into the mucilage of *Laminaria digitata* and may even penetrate the cell wall. By immersing into the mucilage, diatoms are less available for disinfection and by such behaviour the effect of the disinfectants on the diatom cells may have be reduced. However, literature on such performance by autotroph diatoms has not been found, yet it is likely that diatoms to a certain degree will be protected by the surrounding mucilage. Debeer et al. (1994), reported that only 20% or less of total chlorine in a liquid penetrates biofilms (a layer of mucilage that covers microorganisms), and suggested that to be the reason for a higher chlorine demand for inactivation of cells in a biofilms compared to planktonic cells.

Diatoms in free suspension that were exposed to treatments with Lugol's solution (Figure 8), were eliminated in all treatment except for treatments with the lowest concentration and exposure temperature (0.02% Lugol's solution, all exposure times, 10°C). However, diatoms were completely eliminated from sori only when 2% Lugol's solution used as disinfectant (Figure 13). A survey on cell shrinking as an effect of preservation with Lugol's solution, reported successful preservation of diatom cells with a concentration down to 0.5% (Menden-Deuer et al., 2001). Other has reported preservation by use of 1% Lugol's solution (Ehrenhauss and Huettel, 2004; Guillard and Sieracki, 2005), which is in the range of the lethal concentrations (0.2%-2%) for diatoms found in the present study. Betaiodine has been used as sori disinfectant in combination with mechanical removal at a concentration of 0.5% yielding <0.005% free iodine (Merrill and Gillingham, 1991). In the present study, diatom contamination was revealed in treatments disinfected with 0.2% Lugol's solution yielding close to 0.002% free iodine. Fries (1963) obtained axenic cultures of red algae by disinfection with 0.01% free iodine for 2 minutes (cited in Chapman, 1973), while Kientz et al. (2011) found 4.5% iodine to stain the tissue of *Palmaria palmata* and suggested that the iodine penetrated the mucilage with potential harm to the seaweed. It may therefore, be assumed that the critical level of iodine lies between 0.002% and 0.01% for diatoms, and presumably below 4.5% for seaweeds. As for sodium hypochlorite,

stronger concentrations of Lugol's solution were needed in the present study to control diatom contamination in sori disinfection compared to pure cultures of diatoms. The thick polysaccharide mucilage that covers ripe sori may have reduced the disinfecting capacity of the disinfectant. Iodine is known to interact with polysaccharides (Gaillard et al., 1969; Gaillard and Thompson, 1971), and Brown et al. (1995) mentioned a non-significant decrease in disinfecting activity when alginic acid was added to planktonic cultures of the bacteria *Pseudomonas aeruginosa*. Alginic acid, together with fucoidan, is the dominate polysaccharides of seaweed mucilage, and the results may strengthen the assumption of iodine being consumed by the seaweed mucilage. Additionally, Brown et al. (1995) found the diffusion of iodine into biofilms to be restricted, and suggested that to be the main reason for increased resistance of cells in a biofilm compared to cells in free suspension. It is therefore likely that mucilaginous protection of sori may have increased the demand for Lugol's solution.

The concentration of epiphytes is crucial for the disinfecting efficiency, and the required reaction time for successful disinfection will increase with the initial concentration of diatom cells, according to Chicks law about disinfection (Chick, 1908). The density of epiphytes on sori was not investigated in the present study. Hence, sori may have contained a higher cell density of epiphytes than the mixture of diatoms, which may help to explain the higher need for free chlorine and iodine in sori disinfection. Epiphytic growth increases with the age of the sporophyte (Christie et al., 1998) and the water temperature (Laycock, 1974). Stronger disinfecting treatments than those tested in this study, may therefore be needed if the epiphytic contamination is more dens. Increased concentration, exposure times and exposure temperature implies stronger disinfection according to the theory presented in the introduction. However, exposure time and temperature appeared to have little effect on diatom survival in the present study. Only diatoms exposed to Lugol's solution showed reduced survival when the exposure temperature was elevated to 15°C in the diatom experiment, and the overall impression was that concentration of the disinfectants was the decisive variable for diatom elimination.

The SGR of diatoms presented in this study was estimated from a mixture of three diatom species, making comparison to SGR achieved from other studies difficult. When cultivated individually, under constant light and temperature close to 20°C, a SGR of 2.3 day^{-1} has been reported for *P. tricornutum* (Ben-Amotz and Gilboa, 1980) and a SGR of 2.0 day^{-1} for *S. costatum* (Mortain-Bertrand et al., 1988). Another study, with cultivation temperature of 18°C

and 12h light day^{-1}, reported a SGR of 2.5 day^{-1} for *S. costatum* isolated from the Oslofjord (Paasche, 1980). For *C. muelleri*, Liang et al. (2006b) found a SGR of 0.8 day^{-1} under PAR light 16:08 (light: dark) and nitrate enriched f/2 medium. Størseth et al. (2005) on the other hand, reported SGR of *C. muelleri* to be 0.45 day^{-1} after one day of growth in f/2 medium at 18-20°C under continuously light of 140µmol photons m^{-2}s^{-1}. SGR in all these studies, except the study by Størseth et al. (2005), were high compared to SGR estimated for the control in the present study (0.45±0.09 day^{-1}). The growth conditions on the multi-well plate were likely suboptimal for the selected diatoms. Temperature was stable at 20±1°C and the light (100µmol photons m^{-2}s^{-1}) was constant, but cultures were left without mixing and nutrients renewal. As a consequence of absent mixing, limited gas exchange possibly occurred in the cultures which may have contributed to elevated pH, oxygen supersaturation and probably a nutrient and gas gradient evolved around the cells. The absent mixing can also have caused light limitation in parts of the well, giving less photosynthetic activity and growth.

4.1.2 Sporophyte density

Experiment 1 and 2 gave contradicting results with regard to the effect of sori disinfection on sporophyte density. In Experiment 1 all treatments that were free of diatom four weeks after seeding, showed a mean sporophyte density that was higher than in the control treatments (which had diatom contamination in 50% of the samples). The highest sporophyte densities were registered in the samples treated with the highest concentration of sodium hypochlorite (600ppm), at an exposure time of 2 minutes. The treatment gave 42% higher sporophyte density than in the control treatment. Disinfection with 2% Lugol's solution for 2 minutes and a spore release of 15 minutes gave a sporophyte density that was 32% higher than in the control treatment. However, no significant differences in sporophyte density were found between treatments in the first experiment (p-values in Appendix II), but it should be kept in mind that small sample sizes increase the chance of type II errors (Mittendorf et al., 1995). In this particular case this means an increased chance for concluding with non-significant differences in sporophyte density when there actually are significant differences.

The results from Experiment 1 may give the impression that high sporophytes densities were caused by improved culture conditions in absent of diatoms. Contamination has been asserted to be the most common cause of failure in gametophyte cultures, and diatoms to be the most threatening algae for overgrowth and elimination of young seedlings (Chapman, 1973; Markham

and Hagmeier, 1982; Merrill and Gillingham, 1991; Fernandes et al., 2011). Most algae are photosynthetic and will compete for the same abiotic factors like nitrogen and phosphate, light and substrate. Commercial production of *Phorpyra* ssp. (Nori) in Japanese coastal waters is on a regular basis subject to nitrogen deficiency caused by blooms of the diatom *Eucampia zodiacus*, and demonstrate a very expensive incident of how diatoms and seaweeds compete for the same nutrients (Nishikawa et al., 2007). Succession studies on colonization of hard substrate have shown that diatoms are a primary colonizer on substrate that is suitable for seaweeds (Niell, 1979; Campana et al., 2009), and microalgae tend to outgrow seaweed due to higher growth rates (Fernandes et al., 2011). All these studies support the assumption of improved culture conditions for macroalgae in the absent of diatoms.

Nevertheless, Experiment 2 showed a sporophyte density that was considerably higher in the control treatment than in the disinfected samples, even though Figures 16-17 show diatom contamination only in the control treatment. Sporophyte density in samples disinfected with 600ppm sodium hypochlorite for 2min was 52% lower in Experiment 2 than Experiment 1. The control treatment on the other hand, showed nearly five times higher density in the second experiment, while samples treated with Lugol's solution were 37% higher in the second experiment than in first experiment. It should be kept in mind that only three replicates were performed for experiment two, and it is problematic to conclude anything from the experiment due to low statistical power. Hence, the results should only be seen as indications on how the disinfectants may affect *S. latissima* seedlings.

Unfortunately, little literature has been found on sporophyte densities of young sporophytes of *S. latissima*. However, Chapman (1984) reported a maximal number of sporophytes to be 0.24 sporophytes mm^{-2} for *Laminaria digitata*, and 6.6 sporophytes mm^{-2} for *Saccharina longicruris* in a year-around experiment with spore traps on the floor of a kelp bed. The density found for *S. longicrucis* in that study, is comparable to densities found for samples given the disinfecting treatment S-600ppm-2min and L-2%-2min in Experiment 1 of this thesis. The highest sporophyte densities in the present study is in agreement with Contreras et al. (2007) which in a culture experiment found sporophyte density of *Lessonia nigrescens* to be close to 10 sporophytes mm^{-2} after approximately 5 weeks of growth.

It has been found that number of sporangia per mm^2 in *Laminaria hyperborea* sori decrease over the time period of ripe sori (Kain, 1975), which may indicate that sori will hold gradually less sporangia and release gradually less spores per area during the sporangial period. The mean sporophyte densities in Experiment 1 had wide standard errors, which indicated high variability

in sporophyte density among the different replicates of each treatment. It was not registered how long the different sori had been mature in the present study, and the variable spore release and sporophyte density may have been caused by unregistered differences in the age of sori. To avoid this effect, all sori disks were therefore taken from one sorus, as near each other as possible, in Experiment 2. The density of the spore release was not registered in Experiment 2, but mean sporophyte density showed high standard errors also in that experiment.

Spore density during spore release seem to vary among different sporophytes, but also internal in one sorus. Higher spore densities are usually obtained from the centre than from the edges of a sorus, yet two adjacent sori pieces may release very variable amount of spores (S. Forbord, SINTEF Fisheries and Aquaculture, pers. com., 2011). In a survey on *in situ* spore release in the macroalgae *Ecklonia maxima*, Joska and Bolton (1987) recorded high variability in spore release among sporophytes, but also with seasons. Both these observations strengthen the suggestion that natural variation in spore release may lead to high variability in sporophyte density. It is therefore likely that the contradicting results on sporophyte density in the two experiments were caused by natural variations in spore release from mature sori. The results of the present study implies that it will be difficult to standardize an area of sori necessary to seed e.g. one meter of rope, and it should be a priory to establish the relationship between sporophyte and spore densities in *S. latissima* as the relationship is important for a predictable large-scale production of the seaweed.

4.1.3 Development after sori disinfection

Sori disinfection with S-600ppm-2min and L-2%-2min did not seem to depress the development and length of the sporophytes, regardless of the differences seen in sporophyte density. Sporophytes from sori disinfected with sodium hypochlorite in Experiment 2 showed about the double length of the sporophytes in the control, despite a sporophyte density nearly 1/5 of the control treatment. Low-density conditions apparently, led to better culture conditions for the individual sporophytes when 600ppm sodium hypochlorite was used as disinfectant. The mean length of the control in the present study, was very similar to results obtained from untreated sori by Shea and Chopin (2007), and S-600ppm-2min treatments in the present study had comparable length with their longest sporophytes (GeO_2 treated; 0.5mL L^{-1}) giving an indication of normal growth after disinfection in the present study. Density-dependent survival of young sporophyte was not considered in the present survey; sporophyte density was registered only

one time (after four weeks). Studies have, however, showed that high population densities have negative impact on growth and survival of sporophytes (Schiel, 1985; Creed et al., 1998; McConnico and Foster, 2005), and small, slow growing individuals are more likely to be ripped loose by wave motions because of smaller haptera (Dean et al., 1989). Figures 12-14 implies inverse relationship between sporophyte density and spore release, and it would be interesting to look further into that as the result obtained in Experiment 1 was based on a relative scale and therefore associated with high uncertainty. It appears that longer sporophytes can be obtained by lower sporophyte density, which seems to be desirable for a large-scale macroalgae production due to stronger haptera and reduced loss of biomass loss at sea, and production methods favouring low densities may therefore be advantageous.

Seedlings from disinfected sori appeared to develop normally (Figures 16-17). Eggs were found in the control treatment after 8 days of growth, which is suggest to be the earliest possible appearance of eggs under optimal conditions (Lüning and Dring, 1975). For sodium hypochlorite and Lugol's solution disinfected samples, eggs were not seen before day 11. However, as samples were checked every third day eggs could have been present already at day 9 which is in the time interval of 8-10 days defined as normal egg release under optimal conditions (Lüning and Dring, 1975; Lüning, 1981). The Female:Male ratio is a measure of normal development, and for all treatments the ratio was close to 1 which indicates that the treatments did not influence the distribution of *S. latissima* gender. Hsiao and Druhel (1971) found Female:Male ratios close to 1 for a variety of culture conditions, and it may be assumed that the gender ratio is a relatively robust parameter. It can be argued that the S-600ppm-2min and L-2%-2min disinfection of sori did not influence the early stages of development of *S. latissima* seedlings. However, the low sample size in the experiment (n=3) should be kept in mind.

4.1.4 The recommended disinfectant in the new method

Sodium hypochlorite emerges as a better disinfecting agent in the mitigation of diatom contamination in large-scale *S. latissima* cultivation There are indications on much higher tolerance for sodium hypochlorite by *S. latissima* than tested in this survey (10,000ppm for 10-30 minutes, Druehl and Hsiao, 1969; Kientz et al., 2011), and a good safety margin between lethal doses for diatoms and a harmful dose for *S. latissima* will be desirable if the contamination on sori is more dens than tested in the present study. The upper limit for sodium hypochlorite exposure should be established so that severe amount of epiphytes can be treated without

concern about the quality of the seedlings. Additionally, the chemical appears to be a more widespread disinfectant in aquaculture systems (Druehl and Hsiao, 1969; Merrill and Gillingham, 1991; Douillet, 1998; Støttrup and McEvoy, 2002; Hirvela-Koski, 2004; Kientz et al., 2011) than Lugol's solution do. Also, it has a lower toxicity and easier waste handling than Lugol's solution. Actually, sodium hypochlorite can easily be neutralize by addition of thiosulphate and thereafter be discharged directly to nature (Merrill and Gillingham, 1991; Støttrup and McEvoy, 2002). The direct interaction between diatom cells and the disinfectant was not investigated in the present study, and it is therefore difficult to draw any conclusions regarding the disinfecting mechanics in this survey. It may be important to understand how the disinfectants inactivate diatom cells to avoid any potential negative influence on *S. latissima*, and that should probably be looked into in further research.

4.2 GeO_2 as a diatom controller in *S. latissima* cultivation

The present study demonstrated that the germanium dioxide (GeO_2) is efficient in control of the diatoms *C. muelleri* and *S. costatum*, but *P. tricornutum* show a higher tolerance for the chemical. However, the results suggest that GeO_2 may have an inhibiting effect on the growth and development of *S. latissima* seedlings.

Monocultures of the diatom *P. tricornutum* were registered at both concentrations of GeO_2 (0.1mL or 0.5mL per L Conwy medium), the negative control for diatom growth, when the mixture of diatoms was grown in GeO_2 enriched Conwy medium. The results are in agreement with Lewin (1966) who found *P. triconutum* to be less sensitive to GeO_2 inhibition in a survey of several diatom species. *P. triconutum* is a gently silicified diatom with less than 1% silica in the cells (Lewin and Guillard, 1963), and the low silica requirement probably explains why *P. tricornutum* show higher tolerance for GeO_2 than *S. costantum* and *C. muelleri* in the present study. The SGR of diatoms in free suspension in both concentrations of GeO_2 were significantly lower ($p < 0.005$) than in the control, but surprisingly, the treatment with GeO_2 (0.1mL L^{-1}) showed significantly lower ($p < 0.05$) SGR than the treatment with GeO_2 (0.5mL L^{-1}). When GeO_2 was used to control diatom growth in cultures of *S. latissima*, diatoms were found in 33% of the replicates of the higher concentration (GeO_2 0.5mL L^{-1}), while the lower concentration (GeO_2 0.1mL L^{-1}) was free of diatom in both cultivation experiments. Other studies have suggested an increased inhibition of diatom growth with increasing GeO_2 concentration in the growth medium

(Lewin, 1966; Azam et al., 1973). The contradicting results in this study compared to Lewin (1966) and Azam et al. (1973), may be explained by difference in initial cells concentration or improved culture conditions for *P. tricornutum* in the highest concentration. Diatoms cells are known to form aggregates (Round et al., 1990; Hansen et al., 1995), and *S. costatum* produce particularly sticky cells during exponential growth (Kiørboe and Hansen, 1993). Even though the diatom stock cultures and the mixture of the three diatom species were properly mixed prior to the experiments; an uneven distribution of cells may have caused higher initial concentration of cells in the treatment with highest concentration of GeO_2. Another possible explanation is that the higher concentration gave a stronger and more rapid inhibition of *S. costatum* and *C. muelleri* growth, and in that way released nitrogen and phosphorus sources to be used by *P.tricornutum*. The species is very tolerant and has been reported to tolerate a wide salinity (Abdullahi et al., 2006) and light intensity range, which makes it a robust contaminator that is hard to eliminate if introduced to the aquaculture systems (Nelson et al., 1979). However, *P. tricornutum* is relatively rare in natural, marine phytoplankton communities (Nelson et al., 1979; Round et al., 1990), and it is unlikely that the diatom is a common epiphytic species on *S. latissima* sori.

The sporophyte density in the treatments with GeO_2 appeared to decrease with an increasing concentration of GeO_2, however, no significant differences between concentrations or the control treatment was found ($p>0.1$), but as mentioned before, low samples sizes ($n=6$) increases the chance for type II errors. Sporophytes grown in GeO_2 enriched growth medium developed slowly (Figure 16-17) and the sporophyte density was relatively low in both experiments (<2 sporophytes mm^{-2}). The second experiment suggested reduced fertility and sporophyte length for the seedlings grown in GeO_2 enriched growth medium. In Experiment 2, only one out of three replicates had seedlings that entered reproductive phase during the three weeks of growth. And the mean length of sporophytes in that replicate was 50% of the mean length in the control treatment. Five out of six replicates produced sporophytes in the first experiment, hence the seedling must have entered reproductive phase, but the day by day development or length of sporophytes in the samples were not registered. Therefore, the reproductive phase could have been delayed compared to the control also in the first experiment. Markham and Hagmeier (1982) demonstrated a moderate reduction in growth rate of young *S. latissima* seedlings when exposed to 0.5mL GeO_2 per liter growth medium, which is in agreement with the present study, and a strong reduction in growth rate was seen in treatments with to 2mL GeO_2 per liter growth medium in that study. Shea and Chopin (2007) on the other hand, found 0.1mL or 0.5mL GeO_2

per liter growth medium to give significantly longer sporophytes than in the control treatment, and suggested normal development and timing of the life cycle of *S. latissima* grown in GeO_2 enriched medium. Merrill and Gillingham (1991) recommended a much higher concentration of GeO_2 (1-2mL L^{-1}) in cultivation of *Nereocystis luetkeana*, yet no deformities or retained growth were discussed in that report. The timing of GeO_2 addition in the different studies may explain the contradicting results. In this study GeO_2 was added at time zero while Merrill and Gillingham (1991) waited 24 hours, and Shea and Chopin (2007) postponed the addition until day 8 which is after the time of normal egg release. If GeO_2 have an inhibiting effect on the transition from vegetative to reproductive phase the problem may be avoided by postponing the addition of GeO_2 until after fertile females have been observed. However, the results in this thesis imply that use of GeO_2 should be unnecessary if the culture medium is sterile and sori are disinfected after the method introduced here.

4.3 Evaluation of the other disinfecting treatments

Ethanol, acetic acid and formaldehyde were tested as potential disinfectants on diatoms in free suspension. Ethanol and acetic acid eliminated growth of diatoms at all concentrations (25%, 50% or 70% ethanol, 1%, 7%, 35% or 50% acetic acid) all exposure times (2, 4, 10 or 30min) and both exposure temperatures (10°C or 15°C). Diatoms that was exposed to formaldehyde revealed growth after exposure to the lowest concentration of the chemical (0.04%), at both exposure temperatures and at all exposure times, and to the two lower exposure times (2 or 4 minutes) of the medium concentration (0.4%) at an exposure temperature of 15°C. However, all samples with an exposure temperature of 15°C revealed contamination of another diatom together with growth of *P. tricronutum*.

Acetic acid was the only treatment of the three that was tested as a sori disinfectant, but appeared to be lethal to the spores. The general pH tolerance of marine microalgae is between 6 and 9 (Støttrup and McEvoy, 2002), while the optimum for daitoms are reported to be in the range 8.0 to 8.5 (Taraldsvik and Myklestad, 2000; Søgaard et al., 2011). It has been suggested that phytoplankton are unable to maintain the intracellular pH when extracellular pH is extreme (Coleman and Colman, 1981; Nimer et al., 1994), hence the acetic acid treatments with pH<2.8 probably led to denaturation of DNA and proteins (Puppels et al., 1994; Nelson and Cox, 2008). Still, it would be interesting to explore in more detail the tolerance limits for diatoms and *S.*

latissima to acetic acid, as the low toxicity and costs of the chemical makes it attractive in large-scale production of *S. latissima*.

Ethanol has been suggested as an effective sori disinfectant in combination with sodium hypochlorite (Kientz et al., 2011). In the present study, ethanol seemed to react with seawater and was therefore not tested as sori disinfectant. Further research may be done on ethanol as a potential disinfecting agent. However, disinfecting properties of ethanol seem to be dependent on the water content of the algae as high water content tend to dilute the ethanol on the surface of the algae (Kientz et al., 2011), and it may therefore be difficult to standardize a disinfecting dose. Combinations of ethanol and sodium hypochlorite have been suggested as powerful disinfectants (Kientz et al., 2011), and it may be interesting to compare combinations of sodium hypochlorite and ethanol to the disinfection with sodium hypochlorite demonstrated in this study.

The contamination of the samples exposed to formaldehyde made it difficult to suggest a lethal dose of the chemical, and formaldehyde was not chosen for further investigation. A concentration of 5% has been suggested for fixation of microalgae (Guillard, 1973) and preservation of sori (Chapman, 1984), and the results in this study indicate that 4% is lethal to diatoms. Presumable there may be a limited safety margin between lethal dose to diatoms and harmful dose to *S. latissima*. Glutaraldehyde has been used successfully in disinfection of fish eggs (Salvesen and Vadstein, 1995), and is a widely used fixative in microalgae studies (Menden-Deuer et al., 2001). However, aldehydes are toxic (Hawley and Eitzen, 2001; Scott and Gorman, 2001), and if it is possible to use other chemicals as sori disinfectants that would be desirable in a large-scale production, due to environmental and health issues.

4.4 Concluding remarks

The concentration of acetic acid, ethanol, formaldehyde, Lugol's solution and sodium hypochlorite seemed to be the decisive variable for elimination of diatom in the present study. Since the study was conducted on concentration intervals at a power of ten, further testing with concentrations of higher resolution may in time reveal a more optimal concentration for sori disinfection. Epiphytic growth varies with the season and the need for a stronger disinfection may appear to be necessary. The upper tolerance of *S. latissima* to sodium hypochlorite should therefore be established.

It would also be interesting to investigate low concentration (<1%) of acetic acid as a potential alternative sori disinfectant to sodium hypochlorite. Combinations of ethanol and sodium hypochlorite may also be looked into. Potential inhibiting effects on *S. latissima* seedlings after use of germanium dioxide should be investigated in more detail due to contradicting results. The potential need of a diatom controller in *S. latissima* cultures after sori disinfection with the method presented in this study together with harmless GeO_2 concentration for *S. latissima* seedlings should be further investigated.

The method used for disinfection in the present study, one disinfecting bath followed by two rinsing baths of sterile seawater (SSW) holding 10°C, can be used for small scale disinfection of *S. latissima* sori. If large-scale cultivation systems of *S. latissima* are developed it may be necessary to develop a larger disinfection system. A production line with one container holding sodium hypochlorite followed by two containers holding sterile seawater (all precooled to 10°C) may be suitable for production on an industrial scale. Sori should be placed in a perforated bucket and immersed into the baths. Optimal seeding density and methods to estimate spore release from mature sori should also be investigated.

5 CONCLUSION

The present study demonstrated that sori disinfection in 600ppm of sodium hypochlorite for 2 minutes at an exposure temperature of 10°C, followed by two baths of sterile seawater (10°C) for 30 seconds, can be a successful disinfection method to mitigate diatom contamination from *S. latissima* sori. Disinfection of disks cut from ripe sori proved to be successful. The method in the present study implies that diatoms contamination can be removed by a purely chemical disinfection, and mechanical removal can be avoided. Sodium hypochlorite is suggested as sori disinfectant due to an apparently large safety margin between lethal dose for diatoms and harmful dose for *S. latissima*, together with easy neutralization by use of thiosulphate and a widespread use in aquaculture systems.

Diatoms in free suspension were significantly affected ($p < 0.05$) by all treatments except the lower concentrations of Lugol's solution and formaldehyde. Ethanol and formaldehyde were not tested as sori disinfectants, but acetic acid proved to be lethal to spores even at the lowest concentration (1%) tested. The concentration of the chemical agent seemed to be the decisive variable for diatom elimination while exposure time and exposure temperature appeared to have limited effect on the diatoms.

Sori disinfection with both 600ppm sodium hypochlorite and 2% Lugol's solution gave total elimination of diatoms. Swimming spores were released from the disinfected sori, and the sporophyte densities were comparable to the control treatment after four weeks of incubation. A delay was seen in egg release in samples from disinfected sori, but no influence was seen on the gender ratio 11 days after seeding, or sporophyte length measured three weeks after seeding. The results in the present study indicated that a stronger disinfecting treatment was needed to disinfect *S. latissima* sori than to control microalgae growth in pure cultures of diatoms.

Germanium dioxide eliminated growth of *C. muelleri* and *S. costatum*. However, *P. tricornutum* seemed to survive GeO_2 treatments. Sporophytes grown in a GeO_2 enriched growth medium showed retarded growth, and there were indications on delayed transition from vegetative to reproductive phase. S-600ppm-2 appears to be an effective disinfectant in the mitigation of diatom contamination from sori, and the need for GeO_2 to control diatom contamination should be limited after use of the disinfecting method.

6 REFERENCES

Abdullahi A.S., Underwood G.J.C., Gretz M.R. (2006) Extracellular matrix assembly in diatoms (Bacillariophyceae). V. Environmental effects on polysaccharide synthesis in the model diatom, *Phaeodactylum tricornutum*. *Journal of phycology,* **42**, 363-378.

Ali Y., Dolan M.J., Fendler E.J., Larson E.L. (2001) Alchohols. In: *Disinfection, Sterilization, and Preservation* (ed Block S.S.). Lippincott Williams &Wilkins, Philadelphia, USA, pp. 229-253.

Apostolov K. (1980) The effects of iodine on the biological activities of myxoviruses. *Journal of Hygiene,* **84**, 381-388.

Arbona J.F., Molla M. (2006) Cultivation of brown seaweed, *Alaria esculenta* In: *Aquaculture Explained*. BIM, Dublin, Irland, pp. 1-47.

Armstrong E., Rogerson A., Leftley J.W. (2000) Utilisation of seaweed carbon by three surface-associated heterotrophic protists, *Stereomyxa ramosa*, *Nitzschia alba* and *Labyrinthula* sp. *Aquatic Microbial Ecology,* **21**, 49-57.

Azam F., Hemmingsen B.B., Volcani B.E. (1973) Germanium incorporation into the silica of diatom cell walls. *Archives of Microbiology,* **92**, 11-20.

Bartsch I., Wiencke C., Bischof K., Buchholz C.M., Buck B.H., Eggert A., Feuerpfeil P., Hanelt D., Jacobsen S., Karez R., Karsten U., Molis M., Roleda M.Y., Schubert H., Schumann R., Valentin K., Weinberger F., Wiese J. (2008) The genus Laminaria sensu lato: recent insights and developments. *European Journal of Phycology,* **43**, 1-86.

Ben-Amotz A., Gilboa A. (1980) Cryopreservation of marine unicellular algae. I. A survey of algae with regard to size, culture age, photosynthetic activity and chlorophyll-to-cell ratio. *Marine Ecology Progress Series,* **2**, 157-161.

Block S.S. (2001) Definitions of terms. In: *Disinfection, Sterilization, and Preservation* (ed by Block S.S.). Lippincott Williams & Wilkins, Philadelphia, USA, pp. 19-29.

Bolton J.J., Lüning K. (1982) Optimal growth and maximal survival temperatures of Atlantic Laminaria species (Phaeophyta) in culture. *Marine Biology,* **66**, 89-94.

Broch O.J., Slagstad D. (2011) Modelling seasonal growth and composition of the kelp *Saccharina latissima*. *Journal of Applied Phycology*, 1-18.

Brown M.L., Aldrich H.C., Gauthier J.J. (1995) Relationship between glycocalyx and povidone-iodne resistance in *Pseudomonas aeruginosa* (ATTC-527853) biofilms. *Applied and Environmental Microbiology,* **61**, 187-193.

Buchholz C., Lüning K. (1999) Isolated, distal blade discs of the brown alga Laminaria digitata form sorus, but not discs, near to the meristematic transition zone. *Journal of Applied Phycology,* **11**, 579-584.

Buck B.H., Buchholz C.M. (2004) The offshore-ring: A new system design for the open ocean aquaculture of macroalgae. *Journal of Applied Phycology,* **16**, 355-368.

Campana G.L., Zacher K., Fricke A., Molis M., Wulff A., Quartino M.L., Wiencke C. (2009) Drivers of colonization and succession in polar benthic macro- and microalgal communities. *Botanica Marina,* **52**, 655-667.

Chang R. (2006) *General Chemistry: The essential concepts,* McGraw-Hill, New York, USA.

Chapman A. (1973) Methods for macroscopic algae. In: *Handbook of phycological methods, culture methods and growth measurments* (ed by Stein J.R.). Syndics of the Cambridge University press, New York, USA, pp. 87-105.

Chapman A. (1984) Reproduction, recruitment and mortality in two species of Laminaria in southwest Nova Scotia. *Journal of Experimental Marine Biology and Ecology,* **78**, 99-109.

Characklis W.G., Dydek S.T. (1976) The influence of carbon-nitrogen ratio on the chlorination of microbial aggregates. *Water research,* **10**, 515-522.

Chen P., Min M., Chen Y., Wang L., Li Y., Chen Q., Wang C., Wan Y., Wang X., Cheng Y., Deng S., Hennessy K., Lin X., Liu Y., Wang Y., Martinez B., Ruan R. (2009) Review of the biological and engernering aspect of algae to fuel approach. *International Journal of Agricultural and Biological Engineering,* **2**, 1-30.

Chick H. (1908) An investigation of the laws of disinfection. *Journal of Hygiene,* **8**, 92-158.

Chopin T., Buschmann A.H., Halling C., Troell M., Kautsky N., Neori A., Kraemer G.P., Zertuche-Gonzalez J.A., Yarish C., Neefus C. (2001) Integrating seaweeds into marine aquaculture systems: A key toward sustainability. *Journal of phycology,* **37**, 975-986.

Christie H., Fredriksen S., Rinde E. (1998) Regrowth of kelp and colonization of epiphyte and fauna community after kelp trawling at the coast of Norway. *Hydrobiologia,* **375-76**, 49-58.

Clasen T., Edmondson P. (2006) Sodium dichloroisocyanurate (NaDCC) tablets as an alternative to sodium hypochlorite for the routine treatment of drinking water at the household level. *International Journal of Hygiene and Environmental Health,* **209**, 173-181.

Coleman J.R., Colman B. (1981) Inorganic carbon accumulation and photosyntesis in a blue-green algae as a function og external pH. *Plant Physiology,* **67**, 917-921.

Contreras L., Medina M.H., Andrade S., Oppliger V., Correa J.A. (2007) Effects of copper on early developmental stages of *Lessonia nigrescens* Bory (Phaeophyceae). *Environmental Pollution,* **145**, 75-83.

Creed J.C., Kain J.M., Norton T.A. (1998) An experimental evaluation of density and plant size in two large brown seaweeds. *Journal of phycology,* **34**, 39-52.

Darley W.M., Volcani B.E. (1969) Role of silicon in diatom metabolism: A silicon requirement for deoxyribonucleic acid synthesis in the diatom *Cylindrotheca fusiformis* Reimann and Lewin. *Experimental Cell Research,* **58**, 334-342.

Dean T.A., Thies K., Lagos S.L. (1989) Survival of juvenile giant kelp: the effects of demographic factors, competitors, and grazers. *Ecology,* **70**, 483-495.

Debeer D., Srinivasan R., Stewart P.S. (1994) Direct measurement of chlorine penetration into biofilms during disinfection. *Applied and Environmental Microbiology,* **60**, 4339-4344.

Diana J.S. (2009) Aquaculture production and biodiversity conservation. *Bioscience,* **59**, 27-38.

Douglas M.P., Rogers S.O. (1998) DNA damage caused by common cytological fixatives. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* **401**, 77-88.

Douillet P. (1998) Disinfection of rotifer cysts leading to bacteria-free populations. *Journal of Experimental Marine Biology and Ecology,* **224**, 183-192.

Drakeford B., Pascoe S. (2008) Substitutability of fishmeal and fish oil in diets for salmon and trout: A meta-analysis. *Aquaculture Economics and Management,* **12**, 155-175.

Druehl L.D., Hsiao S.I.C. (1969) Axenic culture of Laminariales in defined media. *Phycologia,* **8**, 47-49.

Dychdala G.R. (2001) Chlorine and chlorine compounds. In: *Disinfection, Sterilization, and Preservation* (ed by Block S.S.). Lippincott Williams & Wilkins, Philadelphia, USA, pp. 135-158.

Edwards M., Watson L. (2011) Cultivating *Laminaria digitata*. In: *Aquaculture explianed*. BIM Irish Sea Fisheries Board, Irland, pp. 1-71.

Ehrenhauss S., Huettel M. (2004) Advective transport and decomposition of chain-forming planktonic diatoms in permeable sediments. *Journal of Sea Research,* **52**, 179-197.

FAO (2010a) FIGIS - online query panel - Global Production 1950-2010. FAO, http://www.fao.org/fishery/statistics/global-aquaculture-production/en, accessed 4th of April 2012.

FAO (2010b) The State of World Fisheries and Aquaculture. FAO Fisheries and Aquaculture Department Rome, Italy.

FAO (2010c) World aquaculture 2010. FAO Fisheries and Aqaculture Department, Rome, Italy.

Favero M.S., Bond W.W. (2001) Chemical disinfection of medical and surgical materials. In: *Disinfection, Sterilization, and Preservation* (ed Block S.S.). Lippincott Williams & Wilkins, Philadelphia, USA, pp. 881-917.

Fernandes D.R.P., Yokoya N.S., Yoneshigue-Valentin Y. (2011) Protocol for seaweed decontamination to isolate unialgal cultures. *Revista Brasileira De Farmacognosia-Brazilian Journal of Pharmacognosy,* **21**, 313-316.

Forbord S., Skjermo J., Arff J., Handå A., Reitan K.I., Bjerregaard R., Lüning K. (2012) Development of *Saccharina latissima* (Phaeophyceae) kelp hatcheries with year-round production of zoospores and juvenile sporophytes on culture ropes for kelp aquaculture. *Journal of Applied Phycology,* **19**, 1-7.

Fujii S., Nishimoto N., Notoya A., Hellebust J.A. (1995) Growth and osmoregulation in *Chaetoceros muelleri* in relation to salinity. *Plant and Cell Physiology,* **36**, 759-764.

Gaillard B.D., Thompson N.S. (1971) Interaction of polysaccarides with iodine. 2. Behavior of xylans in different salt solutions. *Carbohydrate Research,* **18**, 137-146.

Gaillard B.D., Thompson N.S., Morak A.J. (1969) Interaction of polysaccharides with iodine. 1. Inverstigation of general nature of reaction. *Carbohydrate Research,* **11**, 509-519.

Gottardi W. (1985) The influence of the chemical behaviour of iodine on the germicidal action of disinfectant solutions containing iodine. *Journal of Hospital Infection,* **6, Supplement 1**, 1-11.

Gottardi W. (2001) Iodine and iodine compounds. In: *Disinfection, Sterilization, and Preservation* (ed by Block S.S.). Lippincott Williams & Wilkins, Philadephia, USA, pp. 159-184.

Greenacre E.J., Brocklehurst T.F., Waspe C.R., Wilson D.R., Wilson P.D.G. (2003) *Salmonella enterica* Serovar Typhimurium and *Listeria monocytogenes* acid tolerance response induced by organic acids at 20^{o}C: Optimization and modeling. *Applied and Environmental Microbiology,* **69**, 3945-3951.

Guillard R.R. (1973) Division rate. In: *Handbook of phycological methods, culture methods and growth measurments* (ed by Stein J.R.). Syndics of the Cambridge University Press, New York, USA, pp. 289-313.

Guillard R.R.L., Sieracki M.S. (2005) Counting cells in cultures with the ligth microscope. In: *Algal culturing techniques* (ed Andersen R.A.). Elsevier academic press, Oxford, UK, pp. 239-252.

Handå A., Forbord S., Broch O.J., Richardsen R., Skjermo J., Reitan K.I. (2009) Dyrking og anvendelse av tare, med spesielt fokus på bioenergi i nordområdene. In: *SINTEF report*. SINTEF on assignment for Fiskeri- og kystdepartementet, Trondheim, Norway, pp. 1-32.

Hansen J.L.S., Timm U., Kiørboe T. (1995) Adaptive significanca of phytoplankton stickness with emphasis on the diatom *Skeletonema costatum*. *Marine Biology,* **123**, 667-676.

Hawley R.J., Eitzen E.M.J. (2001) Protection against biological warfare agents. In: *Disinfection, Sterilization, and Preservation* (ed by Block S.S.). Lippincott Williams & Wilkins, Philadelphia, USA, pp. 1161-1167.

Hirvela-Koski V. (2004) Renibacterium salmoninarum: effect of hypochlorite treatment, and survival in water. *Diseases of Aquatic Organisms,* **59**, 27-33.

Hsiao S.I.C., Druehl L.D. (1971) Environmental control of gametogenesis in *Laminaria saccharina*. I. The effects of light on culture media. *Canadian Journal of Botany,* **49**, 1503-1508.

Hsiao S.I.C., Druehl L.D. (1973) Environmental-control of gametogenesis in *Laminaria saccharina*. 4. In-situ development of gametophytes and young sporophytes. *Journal of phycology,* **9**, 160-164.

Islam M.S. (2005) Nitrogen and phosphorus budget in coastal and marine cage aquaculture and impacts of effluent loading on ecosystem: review and analysis towards model development. *Marine Pollution Bulletin,* **50**, 48-61.

ISO (2008) Water quality - Scientific and technical aspects of batch algae growth inhibitation tests. ISO (International Organization for Standarization), Switzerland, pp. 1-28.

Jeong H.J., Kim H.R., Il Kim K., Kim K.Y., Park K.H., Kim S.T., Yook Y.D., Song J.Y., Kim J.S., Seong K.A., Yih W.H., Pae S.J., Lee C.H., Do Huh M., Lee S.H. (2002) NaOCl produced by electrolysis of natural seawater as a potential method to control marine red-tide dinoflagellates. *Phycologia,* **41**, 643-656.

John R.P., Anisha G.S., Nampoothiri K.M., Pandey A. (2011) Micro and macroalgal biomass: A renewable source for bioethanol. *Bioresource Technology,* **102**, 186-193.

Jones V. (2007) Diatom introduction. In: *Encyclopedia of Quaternary Science* (ed by Editor-in-Chief: Scott A.E.). Elsevier, Oxford, UK, pp. 476-484.

Joska M., Bolton J. (1987) In situ measurement of zoospore release and seasonality of reproduction in *Ecklonia maxima* (Alariaceae, Laminariales). *British Phycological Journal,* **22**, 209-214.

Jørgensen H.Y. (1998) Optimalisert algeproduksjon som fôr til kamskjellyngel, Hovedoppgave i Akvakultur. In: *Biologisk Institutt*. Norwegian University of Science and Technology, NTNU, Trondheim, Norway.

Kai T., Nimura K., Yasui H., Mizuta H. (2006) Regulation of sorus formation by auxin in laminariales sporophyte. *Journal of Applied Phycology,* **18**, 95-101.

Kain J.M. (1975) The biology of *Laminaria Hyperborea* VII. Reproduction of the sporophyte. *Journal of the Marine Biological Association of the United Kingdom,* **55**, 567-582.

Kain J.M. (1979) A view of the genus Laminaria. *Oceanography and Marine Biology, Annual Review,* **17**, 101-161.

Khomvilai C., Kashiwagi M., Sangrungruang C., Yoshioka M. (2006) Preventive efficacy of sodium hypochlorite against water mold infection on eggs of chum salmon *Oncorhynchus keta*. *Fisheries Science,* **72**, 28-32.

Kientz B., Thabard M., Cragg Simon M., Pope J., Hellio C. (2011) A new method for removing microflora from macroalgal surfaces: an important step for natural product discovery. In: *Botanica Marina,* pp. 457-469.

Kiørboe T., Hansen J.L.S. (1993) Phytoplankton aggregate formation - observations of patterns and mechanisms of cell sticking and the significance of exopolymeric material. *Journal of Plankton Research,* **15**, 993-1018.

Kraan S. (2010) Mass-cultivation of carbohydrate rich macroalgae, a possible solution for sustainable biofuel production. *Mitigation and Adaptation Strategies for Global Change*, 1-20.

Lane C.E., Mayes C., Druehl L.D., Saunders G.W. (2006) A multi-gene molecular investigation of the kelp (Laminariales, Phaeophyceae) supports substantial taxonomic re-organization. *Journal of phycology,* **42**, 493-512.

Laycock R.A. (1974) Detrital food-chain based on seaweeds. 1. Bacteria assosiated with the surface of Laminaria fronds. *Marine Biology,* **25**, 223-231.

Lewin J.C. (1966) Silicon metabolism in diatoms. V. Germanium dioxide, a specific inhibitor of diatom growth. *Phycologia,* **6**, 1-12.

Lewin J.C., Guillard R.R. (1963) Diatoms. *Annual Review of Microbiology,* **17**, 373-414.

Liang Y., Beardall J., Heraud P. (2006a) Changes in growth, chlorophyll fluorescence and fatty acid composition with culture age in batch cultures of *Phaeodactylum tricornutum* and *Chaetoceros muelleri* (Bacillariophyceae). *Botanica Marina,* **49**, 165-173.

Liang Y., Beardall J., Heraud P. (2006b) Effects of nitrogen source and UV radiation on the growth, chlorophyll fluorescence and fatty acid composition of *Phaeodactylum tricornutum* and *Chaetoceros muelleri* (Bacillarlophyceae). *Journal of Photochemistry and Photobiology B-Biology,* **82**, 161-172.

Lobban C.S., Harrison P.J. (1994) *Seaweed ecology and physiology,* Cambridge University Press, New York, USA.

Lüning K. (1979) Growth strategies of 3 Laminaria species (Phaeophyceae) inhabiting different depth zones in the sublittoral region of Helgoland (North-Sea). *Marine Ecology Progress Series,* **1**, 195-207.

Lüning K. (1981) Egg release in gametophytes of *Laminaria saccharina* - induction by darkness and inhibition by blue light and UV. *British Phycological Journal,* **16**, 379-393.

Lüning K. (1988) Photoperiodic control of sorus formation in the brown alga *Laminaria saccharina*. *Marine Ecology-Progress Series,* **45**, 137-144.

Lüning K., Dring M.J. (1972) Reproduction induced by blue light in female gametophytes of *Laminaria saccharina*. *Planta,* **104**, 252-256.

Lüning K., Dring M.J. (1975) Reproduction, growth and photosynthesis of gametophytes of *Laminaria saccharina* grown in blue and red-light. *Marine Biology,* **29**, 195-200.

MacDonald B.A., Robinson S.M.C., Barrington K.A. (2011) Feeding activity of mussels (*Mytilus edulis*) held in the field at an integrated multi-trophic aquaculture (IMTA) site (*Salmo salar*) and exposed to fish food in the laboratory. *Aquaculture,* **314**, 244-251.

Marie D., Simon N., Vaulot D. (2005) Phytoplankton cell counting by flow cytometry. In: *Algae culturing techniques* (ed by Andersen R.A.). Elsevier academic press, Oxford, UK, pp. 253-269.

Markham J.W., Hagmeier E. (1982) Observations in the effects of germanium dioxide on the growth of marcro-algae and diatoms. *Phycologia,* **21**, 125-130.

McConnico L.A., Foster M.S. (2005) Population biology of the intertidal kelp, *Alaria marginata* Postels and Ruprecht: A non-fugitive annual. *Journal of Experimental Marine Biology and Ecology,* **324**, 61-75.

McHugh D.J. (2003) A guide to the seaweed industry In: *FAO Fisheries technical Paper NO.441*. FAO, Rome, Italy

Menden-Deuer S., Lessard E.J., Satterberg J. (2001) Effect of preservation on dinoflagellate and diatom cell volume and consequences for carbon biomass predictions. *Marine Ecology-Progress Series,* **222**, 41-50.

Merrill J.E., Gillingham D.M. (1991) Bull kelp cultivation handbook. (ed National Coastal Resources R.a.D.I.). National Coastal Resources, Research and Development Institute, Portland, USA pp. 1-70.

Metal-Pages-Ltd (2012) Metal-prices germanium dioxide. http://www.metal-pages.com/metalprices/germaniumdioxide/, accessed 5th of May 2012.

Michels M.H.A., van der Goot A.J., Norsker N.H., Wijffels R.H. (2010) Effects of shear stress on the microalgae *Chaetoceros muelleri*. *Bioprocess and Biosystems Engineering,* **33**, 921-927.

Mittendorf R., Arun V., Sapugay A.M.V. (1995) The problem of the type II statistical error. *Obstetrics and Gynecology,* **86**, 857-859.

Mortain-Bertrand A., Descolas-Gros C., Jupin H. (1988) Growth, photosynthesis and carbon metabolism in the temperate marine diatom *Skeletonema costatum* adapted to low temperature and low photon-flux density. *Marine Biology,* **100**, 135-141.

Naylor R.L., Goldburg R.J., Primavera J.H., Kautsky N., Beveridge M.C.M., Clay J., Folke C., Lubchenco J., Mooney H., Troell M. (2000) Effect of aquaculture on world fish supplies. *Nature,* **405**, 1017-1024.

Nelson D.L., Cox M.M. (2008) *Principles of biochemistry,* W.H. Freeman and Company, New York, USA.

Nelson D.M., D'Elia C.F., Guillard R.R.L. (1979) Growth and competition of the marine diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* . II. Light limitation. *Marine Biology,* **50**, 313-318.

Neori A. (2008) Essential role of seaweed cultivation in integrated multi-trophic aquaculture farms for global expansion of mariculture: An analysis. *Journal of Applied Phycology,* **20**, 567-570.

Neori A., Chopin T., Troell M., Buschmann A.H., Kraemer G.P., Halling C., Shpigel M., Yarish C. (2004) Integrated aquaculture: Rationale, evolution and state of the art emphasizing seaweed biofiltration in modem mariculture. *Aquaculture,* **231**, 361-391.

Neori A., Troell M., Chopin T., Yarish C., Critchley A., Buschmann A.H. (2007) The need for a balanced ecosystem approach to blue revolution aquaculture. *Environment,* **49**, 37-43.

Niell F.X. (1979) Structure and succession in rocky algal communities of a temperate intertidal system. *Journal of Experimental Marine Biology and Ecology,* **36**, 185-200.

Nigam P.S., Singh A. (2011) Production of liquid biofuels from renewable resources. *Progress in Energy and Combustion Science,* **37**, 52-68.

Nimer N.A., Brownlee C., Merrett M.J. (1994) Carbon-dioxide availability, intracellular pH and growth rate of the coccolithophore *Emiliania huxleyi*. *Marine Ecology-Progress Series,* **109**, 257-262.

Nishikawa T., Hori Y., Tanida K., Imai I. (2007) Population dynamics of the harmful diatom *Eucampia zodiacus* Ehrenberg causing bleachings of Porphyra thalli in aquaculture in Harima-Nada, the Seto Inland Sea, Japan. *Harmful Algae,* **6**, 763-773.

Paasche E. (1980) Silicon content of five marine plankton diatom species measured with a rapid filter method. *Limnology and Oceanography*, 474-480.

Pang S.J., Lüning K. (2004) Breaking seasonal limitation: year-round sporogenesis in the brown alga *Laminaria saccharina* by blocking the transport of putative sporulation inhibitors. *Aquaculture,* **240**, 531-541.

Puppels G.J., Otto C., Greve J., Robertnicoud M., Arndtjovin D.J., Jovin T.M. (1994) Raman microspectroscopic study of low-pH-induced changes in DNA-structure of the polytene chromosomes. *Biochemistry,* **33**, 3386-3395.

Reid G.K., Liutkus M., Bennett A., Robinson S.M.C., MacDonald B., Page F. (2010) Absorption efficiency of blue mussels (*Mytilus edulis* and *M. trossulus*) feeding on Atlantic salmon (*Salmo salar*) feed and fecal particulates: Implications for integrated multi-trophic aquaculture. *Aquaculture,* **299**, 165-169.

Reitan K.I. (2011) Digestion of lipids and carbohydrates from microalgae (*Chaetoceros muelleri* Lemmermann and *Isochrysis aff. galbana* clone T-ISO) in juvenile scallops (*Pecten maximus* L.). *Aquaculture Research,* **42**, 1530-1538.

Ridler N., Wowchuk M., Robinson B., Barrington K., Chopin T., Robinson S., Page F., Reid G., Szemerda M., Sewuster J., Boyne-Travis S. (2007) Integrated multi-trophic aquaculture (IMTA): A potential strategic choice for farmers. *Aquaculture Economics and Management,* **11**, 99-110.

Round F.E., Crawford R.M., Mann D.G. (1990) *The diatoms, biology and morphology of the genera,* Press syndicate of the University of Cambridge, Cambridge, UK.

Rousch J.M., Bingham S.E., Sommerfeld M.R. (2004) Protein expression during heat stress in thermo-intolerant and thermo-tolerant diatoms. *Journal of Experimental Marine Biology and Ecology,* **306**, 231-243.

Sakshaug E., Myklestad S. (1973) Studies on the phytoplankton ecology of the Trondheimsfjord. III. Dynamics of phytoplankton blooms in relation to environmental factors, bioassay experiments and parameters for the physiological state of the populations. *Journal of Experimental Marine Biology and Ecology,* **11**, 157-188.

Salvesen I., Vadstein O. (1995) Surface disinfection of eggs from marine fish: evaluation of four chemicals. *Aquaculture International,* **3**, 155-171.

Sanderson J.C., Cromey C.J., Dring M.J., Kelly M.S. (2008) Distribution of nutrients for seaweed cultivation around salmon cages at farm sites in north–west Scotland. *Aquaculture,* **278**, 60-68.

Schiel D.R. (1985) Growth, survival and reproduction of two species of marine algae at different densities in natural stands. *Journal of Ecology,* **73**, 199-217.

Scott E.M., Gorman S.P. (2001) Glutaraldehyde. In: *Disinfection, Sterilization, and Preservation* (ed by Block S.S.). Lippincott Williams & Wilkins, Philadelphia, USA, pp. 361-383.

Shea R., Chopin T. (2007) Effects of germanium dioxide, an inhibitor of diatom growth, on the microscopic laboratory cultivation stage of the kelp, *Laminaria saccharina*. *Journal of Applied Phycology,* **19**, 27-32.

Shi C., Kataoka H., Duan D. (2005) Effects of blue light on gametophyte development of *Laminaria japonica* (Laminariales, Phaeophyta). *Chinese Journal of Oceanology and Limnology,* **23**, 323-329.

Siegel S. (1957) Nonparametric statistics. *American Statistician,* **11**, 13-19.

Sjøtun K. (1993) Seasonal lamina growth in 2 age-groups of *Laminaria saccharina* (L) Lamour in Western Norway. *Botanica Marina,* **36**, 433-441.

Solomons T.W.G., Fryhle C.B. (2008) *Organic chemistry* John Wiley & Sons, Inc., USA.

Størseth T.R., Hansen K., Reitan K.I., Skjermo J. (2005) Structural characterization of β-d-(1→3)-glucans from different growth phases of the marine diatoms *Chaetoceros mülleri* and *Thalassiosira weissflogii*. *Carbohydrate Research,* **340**, 1159-1164.

Støttrup J.G., McEvoy L.A. (2002) *Live feeds in marine aquaculture,* Blackwell Science Ltd, Oxford, UK.

Søgaard D.H., Hansen P.J., Rysgaard S., Glud R.N. (2011) Growth limitation of three Arctic sea ice algal species: effects of salinity, pH, and inorganic carbon availability. *Polar Biology,* **34**, 1157-1165.

Taraldsvik M., Myklestad S. (2000) The effect of pH on growth rate, biochemical composition and extracellular carbohydrate production of the marine diatom *Skeletonema costatum*. *European Journal of Phycology,* **35**, 189-194.

Theron M.M., Lues J.F.R. (2011) *Organic acids and food preservation,* Taylor & Francis, Boca Raton, USA.

Throndsen J., Hasle G.R., Tangen K. (2003) *Norsk kystplankton flora,* Almater forlag AS, Oslo, Norway.

Toth R. (1976) A mechanism of propagule release from unilocular reproductive structures in brown algae. *Protoplasma,* **89**, 263-278.

Totti C., Poulin M., Romagnoli T., Perrone C., Pennesi C., De Stefano M. (2009) Epiphytic diatom communities on intertidal seaweeds from Iceland. *Polar Biology,* **32**, 1681-1691.

Troell M., Halling C., Neori A., Chopin T., Buschmann A.H., Kautsky N., Yarish C. (2003) Integrated mariculture: Asking the right questions. *Aquaculture,* **226**, 69-90.

Troell M., Joyce A., Chopin T., Neori A., Buschmann A.H., Fang J.-G. (2009) Ecological engineering in aquaculture - Potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems. *Aquaculture,* **297**, 1-9.

Tseng C.K. (1993) Notes on mariculture in China. *Aquaculture,* **111**, 21-30.

Tseng C.K., Borowitzka M. (2003) Algae Culture. In: *Aquaculture, Farming Aquatic Animals and Plants* (ed. by Lucas J.S., Southgate P.C.). Blackwell Publishing, UK, pp. 253-266.

Vadstein O., Olsen Y. (forthcoming) Some thoughts on calculation of growth rates. Norwegian University of Science and Technology, NTNU, Trondheim,Norway.

Walne P.R. (1979) *Culture of bivalve molluscs: 50 years' experience at Conwy,* Fishing News Books Ltd., Farnham, UK.

Weavers L.K., Wickramanayake G.B. (2001) Kinetics of the inactivation of microorganisms. In: *Disinfection, Sterilization, and Preservation* (ed by Block S.S.). Lippincott Williams & Wilkins, Philadelphia, USA, pp. 65-78.

APPENDIX I

Table 9: *Raw data for specific growth rate estimation for all treatments with exposure temperature 10°C.*

Treatment (10°C)	Period of exp. growth	# measurements	# replicates	Mean	SE (mean)	K-W test	M-W test
Control	3-7/3-4	3/2	6	0.450	0.092		
G-0.01%	2-9	4	6	0.098	0.016	p<0.005	p<0.005
G-0.05%	3-7	3	6	0.213	0.034		p<0.005
A-1%-2	3-10	3	5	-0.005	0.003	p<0.005	
A-1%-4	3-10	3	6	0.005	0.004		
A-1%-10	3-10	3	6	0.005	0.003		
A-1%-30	3-10	3	6	0.007	0.004		
A-7%-2	1-6	3	5	0.016	0.013	p<0.005	
A-7%-4	2-7	3	6	-0.002	0.002		
A-7%-10	2-7	3	6	-0.002	0.002		
A-7%-30	2-7	3	6	-0.002	0.004		
A-35%-2	1-7	3	5	0.034	0.014	p<0.010	
A-35%-4	2-8	3	5	0.030	0.017		
A-35%-10	2-8	3	5	0.019	0.010		
A-35%-30	2-8	3	6	0.031	0.008		
A-50%-2	1-7	3	6	0.001	0.001	p<0.005	
A-50%-4	2-8	3	6	0.007	0.003		
A-50%-10	2-8	3	6	0.015	0.008		
A-50%-30	2-8	3	6	0.008	0.003		
E-25%-2	3-9	3	5	0.083	0.071	p<0.010	
E-25%-4	3-9	3	6	0.092	0.051		
E-25%-10	3-9	3	6	0.000	0.005		
E-25%-30	3-9	3	6	0.000	0.005		

Treatment (10°C)	Period of exp. growth	# measurements	# replicates	Mean	SE (mean)	K-W test	M-W test
E-50%-2	1-9	4	6	0.065	0.011	p<0.005	
E-50%-4	1-9	4	6	0.045	0.013		
E-50%-10	1-9	4	6	0.051	0.015		
E-50%-30	1-9	4	5	0.034	0.010		
E-70%-2	1-9	4	5	0.039	0.009	p<0.005	
E-70%-4	1-9	4	6	0.048	0.004		
E-70%-10	1-9	4	6	0.073	0.014		
E-70%-30	1-9	4	6	0.062	0.009		
F-0.04%-2	4-7	3	6	0.464	0.030	p<0.050	p>0.100
F-0.04%-4	4-7	3	5	0.479	0.051		p>0.100
F-0.04%-10	1-6	3	6	0.245	0.045		p<0.050
F-0.04%-30	4-7	3	5	0.382	0.054		p>0.500
F-0.4%-2	3-6	2	6	0.041	0.016	p<0.005	
F-0.4%-4	4-8	3	5	0.005	0.004		
F-0.4%-10	4-8	3	6	0.004	0.001		
F-0.4%-30	4-8	3	5	-0.003	0.008		
F-4%-2	3-6	2	5	0.050	0.016	p<0.005	
F-4%-4	4-8	3	6	0.001	0.002		
F-4%-10	4-8	3	6	0.008	0.003		
F-4%-30	4-8	3	6	0.002	0.003		
L-0.02%-2	4-7	3	6	0.448	0.027	p>0.100	p>0.100
L-0.02%-4	4-6	2	5	0.464	0.056		p>0.500
L-0.02%-10	4-6	2	5	0.565	0.028		p>0.050
L-0.02%-30	4-6	2	6	0.445	0.042		p>0.100

Treatment (10°C)	Period of exp. growth	# measurements	# replicates	Mean	SE (mean)	K-W test	M-W test
L-0.2%-2	2-8	3	6	-0.001	0.003	p<0.010	
L-0.2%-4	2-8	3	5	-0.026	0.034		
L-0.2%-10	2-8	3	5	-0.001	0.001		
L-0.2%-30	2-8	3	5	-0.002	0.002		
L-2%-2	2-8	3	6	0.012	0.008	p<0.010	
L-2%-4	2-8	3	6	0.004	0.005		
L-2%-10	2-8	3	6	0.003	0.001		
L-2%-30	2-8	3	6	0.000	0.003		
S-6-2	1-6	3	5	0.017	0.009	p<0.005	
S-6-4	2-7	3	6	0.009	0.007		
S-6-10	2-7	3	6	0.014	0.011		
S-6-30	2-7	3	6	0.001	0.002		
S-60-2	1-9	4	5	0.001	0.003	p<0.005	
S-60-4	1-9	4	6	-0.006	0.003		
S-60-10	1-9	4	6	0.000	0.001		
S-60-30	1-9	4	6	0.014	0.008		
S-600-2	1-9	4	6	-0.003	0.006	p<0.005	
S-600-4	1-9	4	6	0.000	0.002		
S-600-10	1-9	4	6	0.011	0.004		
S-600-30	1-9	4	6	-0.005	0.006		

Table 10: *Raw data for specific growth rate estimation for all treatments with exposure temperature 15°C.*

Treatment (15°C)	Period of exp. growth	# measurements	# replicates	Mean	SE (mean)	K-W test	M-W test
Control	3-7/3-4	3/2	6	0.450	0.092		
G-0.01%	2-9	4	6	0.098	0.016	p<0.005	p<0.005
G-0.05%	3-7	3	6	0.213	0.034		p<0.005
A-1%-2	2-9	3	5	0.002	0.002	p<0.010	
A-1%-4	2-9	3	6	0.000	0.005		
A-1%-10	2-9	3	6	0.005	0.003		
A-1%-30	2-9	3	6	0.006	0.004		
A-7%-2	2-13	3	6	-0.001	0.001	p<0.010	
A-7%-4	2-13	3	6	-0.004	0.002		
A-7%-10	3-14	3	6	0.000	0.002		
A-7%-30	3-14	3	5	-0.002	0.002		
A-35%-2	1-8	2	5	0.030	0.008	p<0.010	
A-35%-4	1-8	2	6	0.035	0.013		
A-35%-10	1-8	2	5	0.029	0.015		
A-35%-30	1-8	2	6	0.045	0.007		
A-50%-2	1-8	2	6	0.003	0.016	p<0.010	
A-50%-4	1-8	2	6	0.020	0.008		
A-50%-10	1-8	2	6	0.010	0.009		
A-50%-30	1-8	2	6	0.021	0.010		
E-25%-2	4-11	2	6	0.006	0.010	p<0.010	
E-25%-4	4-11	2	6	0.006	0.010		
E-25%-10	4-11	2	6	-0.006	0.001		
E-25%-30	4-11	2	6	-0.003	0.002		

Treatment (15°C)	Period of exp. growth	# measurements	# replicates	Mean	SE (mean)	K-W test	M-W test
E-50%-2	1-12	3	6	0.064	0.019	p<0.005	
E-50%-4	2-13	3	5	-0.006	0.008		
E-50%-10	2-13	3	5	0.010	0.010		
E-50%-30	1-12	3	6	0.022	0.004		
E-70%-2	1-12	3	5	0.043	0.012	p<0.001	
E-70%-4	2-13	3	6	0.018	0.014		
E-70%-10	2-13	3	6	0.006	0.005		
E-70%-30	1-12	3	6	0.052	0.009		
F-0.04%-2	2-9	2	4	0.270	0.029	p<0.010	p<0.050
F-0.04%-4	2-9	2	6	0.302	0.024		p>0.050
F-0.04%-10	2-9	2	6	0.272	0.019		p<0.010
F-0.04%-30	2-9	2	6	0.211	0.021		p<0.005
F-0.4%-2	3-10	2	6	0.263	0.022	p<0.001	p<0.010
F-0.4%-4	3-10	2	6	0.181	0.019		p<0.050
F-0.4%-10	3-10	2	6	-0.001	0.005		
F-0.4%-30	3-10	2	6	0.008	0.003		
F-4%-2	3-10	2	5	0.002	0.003	p<0.005	
F-4%-4	3-10	2	6	0.001	0.004		
F-4%-10	3-10	2	6	-0.008	0.008		
F-4%-30	3-10	2	6	0.005	0.002		
L-0.02%-2	2-13	3	6	-0.002	0.007	p<0.005	
L-0.02%-4	2-13	3	6	-0.003	0.003		
L-0.02%-10	3-14	3	6	-0.003	0.002		
L-0.02%-30	3-14	3	6	-0.002	0.001		

Treatment (15°C)	Period of exp. growth	# measurements	# replicates	Mean	SE (mean)	K-W test	M-W test
L-0.2%-2	1-7	3	5	-0.008	0.009	p<0.005	
L-0.2%-4	1-7	3	6	0.004	0.002		
L-0.2%-10	2-8	3	6	0.000	0.002		
L-0.2%-30	2-8	3	5	0.009	0.006		
L-2%-2	1-7	3	6	0.017	0.003	p<0.005	
L-2%-4	1-7	3	6	0.004	0.005		
L-2%-10	2-8	3	5	0.005	0.002		
L-2%-30	2-8	3	6	0.009	0.003		
S-6-2	4-11	2	5	0.006	0.010	p<0.005	
S-6-4	4-11	2	5	0.002	0.003		
S-6-10	4-11	2	6	0.005	0.003		
S-6-30	4-11	2	6	-0.004	0.001		
S-60-2	1-9	3	5	0.004	0.002	p<0.010	
S-60-4	1-9	3	6	0.007	0.004		
S-60-10	1-9	3	5	0.002	0.002		
S-60-30	1-9	3	6	0.004	0.003		
S-600-2	1-9	3	6	0.005	0.005	p<0.005	
S-600-4	1-9	3	5	0.001	0.003		
S-600-10	1-9	3	6	0.003	0.005		
S-600-30	1-9	3	6	0.008	0.004		

APPENDIX II

Table 11: *Sporophyte density and diatom contamination in Experiment 2.*

Treatment	Mean number of Sporophytes (mm^2)	SD (mean)	SE (mean)	Mann-Whitney test ($p \leq 0.05$)	Diatoms present
Control	3.0	2.9	1.2		50.0
G-0.01%	1.7	1.7	0.7	p>0.5	0.0
G-0.05%	1.2	1.1	0.5	p<0.5	33.3
A-1%-2	0.0	0.0	0.0	p<0.005	16.7
A-1%-2, SR 30min	0.0	0.0	0.0	p<0.005	0.0
A-7%-2	0.0	0.0	0.0	p<0.005	0.0
A-7%-2, SR 30min	0.0	0.0	0.0	p<0.005	0.0
A-1%-10	0.0	0.0	0.0	p<0.005	0.0
A-1%-10, SR 30min	0.0	0.0	0.0	p<0.005	0.0
A-7%-10	0.0	0.0	0.0	p<0.005	0.0
A-7%-10, SR 30min	0.0	0.0	0.0	p<0.005	0.0
L-0.02%-2	0.9	1.0	0.4	p<0.5	16.7
L-0.2%-2	1.1	1.1	0.4	p<0.5	100.0
L-2%-2	4.4	7.1	2.9	p>0.5	0.0
L-2%-2, SR 30min	4.4	5.0	2.1	p>0.5	0.0
L-0.02%-10	2.3	4.2	1.7	p<0.5	33.3
L-0.2%-10	1.6	2.1	0.9	p<0.5	50.0
L-2%-10	3.2	6.0	2.4	p<0.5	0.0
L-2%-10, SR 30min	4.9	10.7	4.4	p<0.5	0.0
S-6ppm-2	1.0	1.2	0.5	p<0.5	33.3
S-60ppm-2	4.1	4.1	1.7	p>0.5	0.0
S-600ppm-2	5.2	3.7	1.5	p<0.5	0.0
S-6ppm-10	0.1	0.1	0.0	p<0.005	33.3
S-60ppm-10	3.2	3.9	1.6	p>0.5	33.3
S-600ppm-10	3.5	3.0	1.2	p>0.5	0.0

n=6 for all treatments, SR 30 min = spores were seeded after 30 minutes of spore release