Toxicity, autecology and DNA content in the marine flagellate *Pseudochattonella* (Dictyochophyceae, Heterokonta)

Birger Skjelbred

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Department of Biology
Faculty of Mathematics and Natural Sciences
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PREFACE

The presented work was funded by the University of Oslo. The experimental work has been conducted at the Department of Biology, University of Oslo, Norwegian Institute for Water Research (NIVA) and Norwegian School of Veterinary Science (NVH).

A number of people have accompanied and supported me during my PhD period.

First of all I would like to thank my wife, Anne, for her patience and my children Åse Marit, Veslemøy and Jonathan for just being wonderful small persons with great individualities.

I would like to thank my main supervisor, Tom Andersen for his knowledge, scientific contribution, guidance and interesting discussions during my PhD period. I would also like to thank my co-supervisor, Bente Edvardsen, for her knowledge scientific contribution and guidelines. Thanks also to my second co-supervisor, Dag Klaveness, especially for introducing me to the microscopic world of protists.

I wish to thank Sissel Irene Brubak, Carl Morten Laane, Per-Johan Færøvig, Tor Einar Horsberg and Knut Erik Tollefsen for helping me in many tasks related to my work at this thesis, and Oddbjørn Pettersen for the use of the facilities at Solbergstrand.

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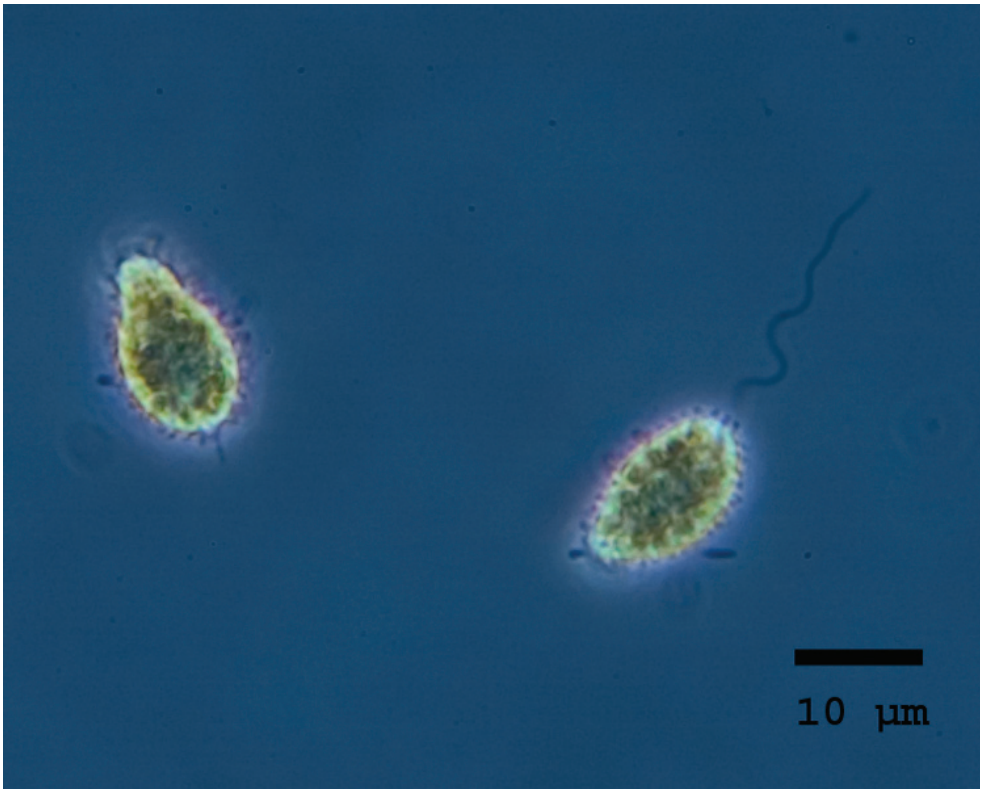

Living cells of *Pseudochattonella farcimen*.

SUMMARY

Phytoplankton is main producers of marine pelagic food webs, acting as link between the energy source, the sun, and consumers. Phytoplankton is an evolutionary diverse group which is, however, traditionally assembled due to their ability to fix carbon through photosynthesis.

Some phytoplankton taxa occur in a form of algal blooms that may cause problems for their environment. This phenomenon is often associated with the name harmful algal bloom (HAB) and it has important economical and health related implications. The ichthyotoxic genus *Pseudochattonella,* investigated in this thesis, is such taxon that can produce HAB. Primary goal of this work was to investigate mechanisms of the toxicity of *Pseudochattonella* and deliver a proper assay for detecting harmful effects in ichthyotoxic flagellates.

There are increasing interests for utilizing algae in topics like toxicity assays, biodiesel production, as food components and for medical usage. A new-developed assay for estimating optimal environmental conditions for growth, loss rates and compensation irradiance was carried out in microplates. Furthermore, the assay was used for comparisons of seven strains from the two known species of *Pseudochattonella*.

DNA content of organisms is strongly correlated with cell volume, nuclear volume, growth rates and cell cycle length. However, closely related taxa might have large differences in genome size. Two species of *Pseudochattonella* and several strains of these species were compared in respect of their genome size.

PAPERS INCLUDED

Paper I:

Skjelbred, B., Horsberg, T.E., Tollefsen, K.E., Andersen, T., Edvardsen, B. (2011) Toxicity of the ichthyotoxic marine flagellate *Pseudochattonella* (Dictyochophyceae, Heterokonta) assessed by six bioassays.
Harmful Algae 10, 144-154.

Paper II:

Skjelbred, B., Edvardsen, B., Andersen, T. A high-throughput method for measuring growth and loss rates in microalgal cultures.
Journal of Applied Phycology, in press.

Paper III:

Skjelbred, B., Edvardsen, B., Andersen, T. Environmental optima for seven strains of *Pseudochattonella* (Dictyochophyceae, Heterokonta).
Submittet to Journal of Phycology.

Paper IV:

Skjelbred, B., Edvardsen, B., Andersen, T. Variation in genome size among fifteen strains of marine bloom-forming *Pseudochattonella* (Dictyochophyceae, Heterokonta).
Manuscript

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1. INTRODUCTION

1.1 Background

Algae belong to evolutionary diverse groups (Cavalier-Smith 2004) which are traditionally identified as eukaryotes with a potential to fix carbon via photosynthesis (Keeling 2004). Phytoplankton consists of both algae and prokaryotic photosynthetic organisms (e.g. cyanobacteria). Although phytoplankton biomass constitutes only 0.2 % of the total plant carbon amount, it fixes approximately 40 % of the global total carbon (Falkowski et al. 1998). Integrating similar models of the growth of marine and terrestrial primary producers yielded a roughly equally contributions to fixed carbon from land and oceans (Field et al. 1998). Among the 4000 described marine phytoplankton species in the World (Sournia et al. 1991), about 300 species regularly form blooms with adverse effects and at least 80 species have the capacity to produce toxins (Hallegraeff 2003). Most of these harmful species of eukaryotic microalgae are flagellates (Wright and Cembella 1998), although some species of the diatom genus *Pseudo-nitzschia* produce the neurotoxic amino acid, domoic acid (Hasle 2002). Harmful effects occur when these algae produce noxious or toxic secondary metabolites that exhibit potent biological activities against other biota, including humans upon consumption of the seafood contaminated by toxic metabolites. Some important toxins affecting humans are okadaic acids causing diarrhetic shellfish poisoning (DSP), saxitoxins causing paralytic shellfish poisoning (PSP), brevetoxins causing neurotoxic shellfish poisoning (NSP), domoic acid causing amnesic shellfish poisoning (ASP) and ciguatoxins causing ciguatera fish poisoning (CFP). Algal species can be harmful to fish by producing neurotoxic, hepatotoxic, haemolytic substances (Bruslé 1995) or produce reactive oxygen species in fish gills (Tanaka et al. 1994, Marshall et al. 2005). However, non-toxic algae can also negatively affect fish by mechanically injuring or irritating gill membranes and causing suffocation by excessive mucus production (Hallegraeff 2003) or cause hypoxia mediated by decomposition of senescent phytoplankton blooms and consequently causing suffocation (Bruslé 1995). Higher susceptibility for diseases has been reported when fish were exposed to high concentrations of algae (Pearl 1988). The interactions between toxic phytoplankton and grazing zooplankton are more complex (Turner and Tester 1997).

The first registered bloom of *Pseudochattonella* in European waters occurred in April-May 1998 by killing 350 tons of farmed fish, whereas the Skagerrak blooms in 2001 killed 1100 tons of farmed fish (Backe-Hansen et al. 2001, Edvardsen et al. 2007). *Pseudochattonella* has since then been held responsible for several fish mortality events in the North Sea, Skagerrak and Kattegat (Edvardsen et al. 2007, Riisberg and Edvardsen 2008). *Pseudochattonella* has been observed blooming in the Gulf of Gdansk, Baltic Sea (Łotocka 2009) and was also observed in the Oslofjord in October 2008 (Berge et al. 2009).

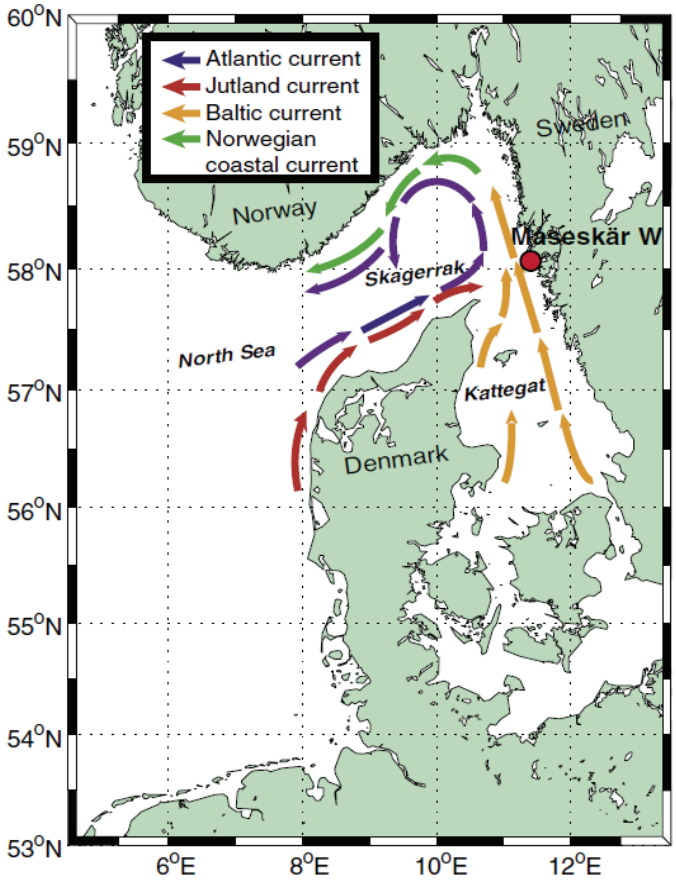

Figure 1 The currents in Skagerrak. Blooms of *Pseudochattonella* follows this path. From Anderson et al. 2006.

The blooms in 2001, 2006, 2007 and 2011 occurred early, overlapping the spring bloom of diatoms (Edvardsen et al. 2007, Riisberg and Edvardsen 2008, SMHI; AlgAware, IMR; Algeinfo). Interestingly, blooms in 1998, 2000, 2002 and 2004 occurred later in the season, April – May (Lu and Göbel 2000, Aure et al. 2001, Backe-Hansen et al. 2001, Riisberg and Edvardsen 2008). Previous knowledge about *Pseudochattonella* was from *P. verruculosa* known to form recurrent harmful algal blooms (HAB) in Japan (Imai et al 1998, Hallegraeff and Hara 2003, Hosoi-Tanabe et al. 2007). Effects of temperature and salinity on the growth responses, performed on *P. verruculosa* (Yamaguchi et al. 1997) and *P. farcimen* (Skjelbred and Naustvoll 2006) indicated a difference in optimal temperature preferences between these species.

1.2 Habitat

The pelagic environment in Skagerrak is influenced by inflow of Atlantic and Jutland Coastal Water where high salinity water mix with freshwater from large rivers (figure 1); and Kattegat is influenced by brackish water from Baltic Sea mixed with Skagerrak water and freshwater from surrounding rivers (Aure et al. 1998). The surface temperature, transparency and chlorophyll a concentration have increased while nutrient concentrations have decreased in the last decades (McQuatters-Gollop et al. 2007, McQuatters-Gollop and Vermaat 2010). A regime shift for plankton and fish was observed for the North Sea in the mid-1980s mainly caused by switch in the winter North Atlantic Oscillation (NAO) from negative to positive phase (Aebischer et al. 1990, Beaugrand 2004, Beaugrand et al. 2008) shown in figure 2. Similar regime shift was also observed in the central Baltic Sea (Alheit et al. 2005) and Mediterranean Sea (Conversi et al. 2010). Data from the Continuous Plankton Recorder (CPR) visualised an increased phytoplankton colour for the North Sea from the mid-1980s (Reid et al. 1998) and there were also some indications of changes in phytoplankton community (Dickinson et al. 1992, Reid et al. 1992). How these changes influence the occurrence of *Pseudochattonella* is unknown.

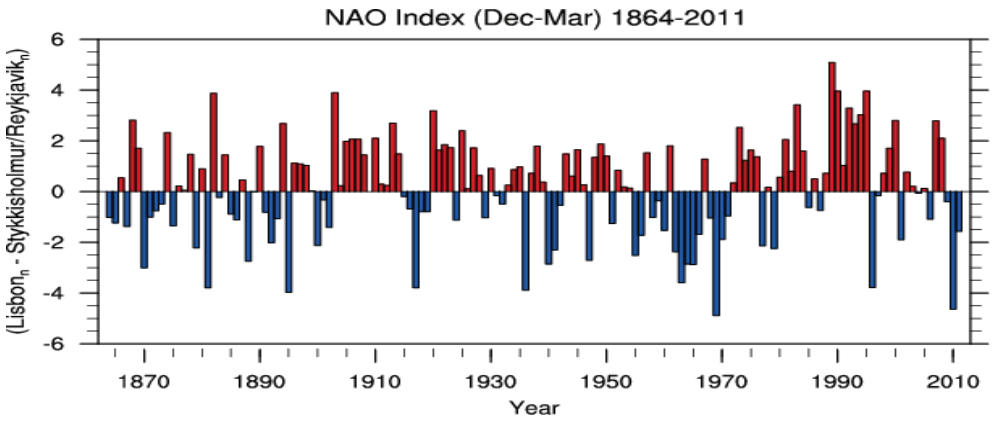

Figure 2 Winter index of NAO from 1864 to 2011 is based on the difference of normalized sea level pressure between Lisbon and Reykjavik.
From http://www.cgd.ucar.edu/cas/jhurrell/nao.stat.winter.html

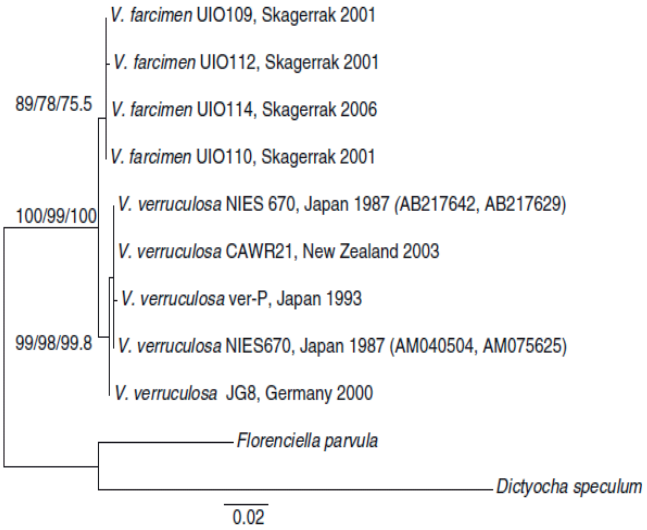

Figure 3 Evolutionary relationship in the genus *Pseudochattonella* (*Verrucophora*; Eikrem et al. 2009) based on mitochondrial and plastid sequence data. The genus contains two species, *P. farcimen* and *P. verruculosa*.
From Riisberg and Edvardsen 2008.

1.3 Taxonomy

Pseudochattonella was previously classified in the genus *Chattonella*; class Raphidophyceae, but genetic analyses revealed a closer relationship to the class Dictyochophyceae (Bowers et al. 2006). Further works confirmed the taxonomic status of *Pseudochattonella* (Edvardsen et al. 2007, Hosoi-Tanabe et al. 2007). *P. verruculosa* (Y. Hara et Chihara) Hosoi-Tanabe, Honda, Fukaya, Inagaki et Sako was first described as *Chattonella verruculosa* Hara et Chihara (Hosoi-Tanabe et al. 2007). Pigment analysis from the bloom in 1998 gave an indication that actually a Dictyochophyceae taxon was involved (Backe-Hansen et al. 2001). Strains isolated from the Skagerrak bloom in 2001 and from the Skagerrak in 2006 were found to belong to a new species, *P. farcimen* (Eikrem, Edvardsen et Throndsen) Eikrem, Edvardsen et Throndsen which differ both genetically and morphologically from *P. verruculosa* and the genus contains currently two species (figure 3 from Riisberg and Edvardsen 2008). The closest relative is a newly described species, *Florenciella parvula* Eikrem (Eikrem et al. 2004). While *P. verruculosa* possibly is an introduced species from the Pacific Ocean, *P. farcimen* currently seems to be an endemic species for the North Sea, Skagerrak and Kattegat (Riisberg and Edvardsen 2008). However this species might also occur in other geographical regions. Probes are developed to discriminate the two *Pseudochattonella* species (Dittami et al., in prep) and a library of expressed sequence tags (EST) has been analysed (Dittami et al. 2012).

1.4 Life cycle

There is not much information available for life cycles in the class Dictyochophyceae; one study suggested a possible life cycle for *Dictyocha speculum* (Henriksen et al. 1993). Also in old stock cultures of *Pseudochattonella* cells without flagella have been observed at the bottom of culture flasks, but examination of cells stained with acridine orange gave no information whether these cells were multinucleate (Unpublished data); the nucleus in *P. farcimen* is very branched (Riisberg and Edvardsen 2008). No differences in DNA content have been observed in extremely small cells compared to normal sized cells so far; this might be a more ecological trait than part of a life cycle.

1.5 Aim and outline of the thesis

The actual ichthyotoxic mechanism in *Pseudochattonella* is not understood (Hallegraeff and Hara 2003) and since *Pseudochattonella* belongs to the class Dictyochophyceae and is not, as first anticipated, closely related to *Chattonella* spp. (class Raphidophyceae) (Edvardsen et al. 2007, Hosoi-Tanabe et al. 2007); the ichthyotoxic mechanism may well be different from that of *Chattonella* species. In the cytotoxicity assays two different colorimetric methods were used to test how algal extracts influence the metabolic activity in two different cell lines from fish, actually cells from embryo and liver. More exact, how the mitochondrial enzymatic reduction of a compound to a measurable product was affected. In the third cytotoxicity assay a colorimetric method was used to test membrane integrity in liver cells, a compound converted by non-specific esterases from a non-polar non-fluorescent substrate to a polar fluorescent product in cytosol. Brine shrimp were exposed to living algal cells to examine whether the *Pseudochattonella* are toxic also to invertebrate organisms. Finally two different species of fish, cod and salmon, were exposed to algal cells for testing acute mortality since blooms of this genus kills fish.

An assay was to set up to find out more specific information about the optimal environmental conditions for these taxa; they are living in a part of the ocean with considerably environmental changes during seasons. Three environmental factors considered to be important were used in the assay; temperature, salinity and light. The assays were performed in microplates which gives the possibility to run large number of independent experimental units per assay. Maximum growth rates and optimal environmental conditions were estimated for several strains of both species.

DNA contents were estimated by a method of staining nuclei with DNA specific dyes and for analysis of the samples a flow cytometer was used. As internal standard for DNA content Chicken Red Blood Cells (CRBC) were utilized.

2. METHODS

2.1 Algal culture conditions

Stock cultures of *Pseudochattonella* were grown in IMR ½ medium (Eppley et al. 1967), supplemented with 10 nM selenium (Edvardsen et al. 1990, Imai et al. 1996) with salinity of 25 psu, at 4 and 12 ºC. The different salinity treatments were made by adjusting with distilled H_2O before nutrient addition. Illumination was provided by a combination of cool white L 18 W / 840 and warm white L 18 W / 830 fluorescent lights from OSRAM at a 14 :10 light : dark cycle, and a photon flux rate of approximately 50 µmol photons m^{-2} s^{-1}.

2.2 Cytotoxicity assay (MTS) with chinook salmon embryo cells

Embryo cells from chinook salmon were used and algal extracts exposed to the cells at three different concentrations. After 24 h of exposure the cells were incubated with MTS. The test followed the protocol of CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega Corporation, USA) which is composed of a tetrazolium compound (MTS) and an electron coupling agent (PMS); whose function is to transfer electrons from NADH or NADPH to MTS in mitochondria resulting in formation of formazan (figure 4). The concentration of formazan is therefore an indicator of mitochondrial activity and was measured in an absorbance platereader at 490 nm after 24 and 44 h of incubation. The viability of the cells were determined on basis of the absorbance of cells exposed in L 15 medium (negative control), and a background value from wells without cells as minimum value of measured absorbance.

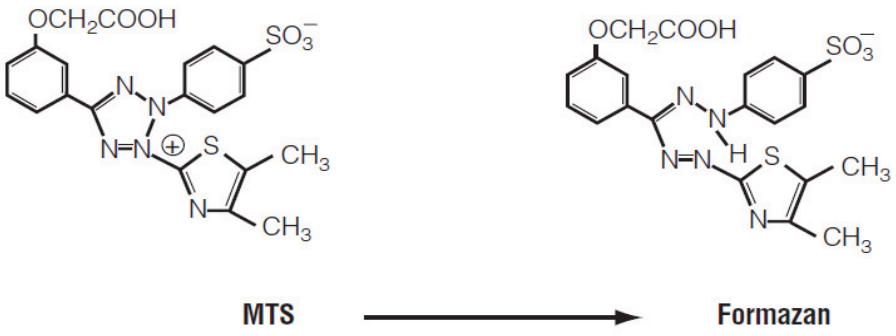

Figure 4 MTS is reduced to formazan by encymatic activity in mitochondria. Formazan absorbs light with a peak at 490 nm.

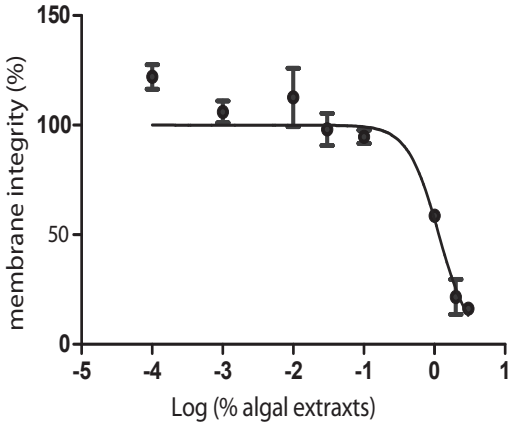

Figure 5 Non-linear relationship between concentration of algal extracts and the membrane integrity of the cells.

2.3 Cytotoxicity assay (AB and CFDA-AM) with rainbow trout primary hepatocytes

Hepatocytes from rainbow trout were isolated using the perfusion method of Tollefsen et al. (2003). Hepatocytes were cultured as primary cells and exposed essentially as described by Tollefsen et al. (2008). Following cell isolation, primary hepatocytes were exposed to algal extracts and the concentrations of the fluorescent metabolites of AB and CFDA-AM were measured simultaneously in a fluorescence plate reader using the wavelength pairs of 530–590 nm and 485–530 nm (excitation–emission), respectively. The viability of the cells was determined on basis of the fluorescence of cells exposed to the solvent control DMSO (no effect) and the maximum toxicity obtained for $CuSO_4$ (10 mM). Strains of the dictyophyte *Pseudopedinella pyriformis* and the diatom *Skeletonema* sp. were used for comparison. Toxic effects use to be non-linear (figure 5). The algal extracts causing 50% effect (EC_{50}) was calculated using a non-linear Hill (logistic) equation.

2.4 Fish bioassays

To study acute toxic effects of *Pseudochattonella* to cod and salmon, *P. farcimen* strain UIO113 and *P. verruculosa* strain NIES670 were prepared in containers at three concentrations to which 10 fish were added per container. The presumably non-toxic dictyophyte *P. pyriformis* was used as a comparison. The water was aerated to avoid oxygen depletion. Natural seawater was used as negative control. After 24 h the experiments were ended by killing the fish with chlorobutanol and preserved in 4 % formaldehyde for subsequent histopathological examinations. The fish exposure experiments were performed at the facilities of Norwegian Institute for Water Research (NIVA) at Solbergstrand, Norway, from where the fish also originated. Fixed samples from the fish were subjected to standardized histological techniques and stained with haematoxylin and eosin. Pathological lesions were noted for secondary lamellas in fish gills. The lesions were grouped as

hypertrophy (swelling of cells), edema (fluid pockets between the epithelium and the basal membrane), detachment of epithelium and necrosis (lysis of nuclei and cells).

2.5 Brine shrimp bioassay

Toxicity test performed with nauplii of brine shrimp followed the protocol of Edvardsen (1993). Dried cysts were incubated for 24 h and hatched nauplii were separated from the non-hatched cysts and incubated for another 24 h. 10 stage II nauplii and 1 ml algal culture were added to each well of a multi-well culture plate. Toxicity of *P. farcimen* and *P. verruculosa* cultures in exponential or stationary phase was tested with maximal concentration of $0.08 - 9.80 \cdot 10^5$ cells ml^{-1}. The nauplii were exposed at room temperature in darkness or under continuous illumination for 24 h. Seawater (salinity of 25 psu) and dense, non-diluted cultures of *P. pyriformis* and *Skeletonema* sp. were used as negative controls. Sodium dodecyl sulphate, SDS, in a range from 5 – 32 mg l^{-1} was used as positive control. The LC_{50}- value for SDS was in the range 9.6 ± 4.8 mg l^{-1} for *Artemia*.

2.6 Assay for estimating growth and loss rates

The growth experiments were carried out in culture plates with white or black walls, all with optical bottom. Each plate was covered with a gray-scale gradient. Irradiance was measured for six replicates (white microplates) and four replicates (black microplates) at three depths of the wells through the bottom of the plates. The experiments were constructed as 3-way factorial designs with temperatures ranging from 4 to 21 ºC, 5 salinities (15, 20, 25, 30 and 35 psu) and light levels ranging from 5.3 - 96.7 µmol photons m^{-2} s^{-1}; 14:10 hour light: dark cycle. Daily measurements of *in vivo* fluorescence (IVF) were used as a non-destructive proxy for population size. IVF readings were made in a microplate reader with excitation at 460 nm and emission at 680 nm. All experiments were ended before the algae reached the stationary phase.

Specific growth rate (μ; day^{-1}) in each experimental unit was calculated as the slope of a linear regression for log-transformed IVF against time. Data from at least four consecutive days after day two were used in the analysis. All experiments were terminated before reaching stationary phase. This first level of data reduction gave a single specific growth rate estimate for each experimental unit. In the second level of data reduction, a light response curve was fitted to all pairs of specific growth rate (μ; d^{-1}) and irradiance (E; µmol photons m^{-2} s^{-1}) for each combination of temperature and salinity. The light response curve (figure 6) was based on a Poisson single-hit model (Dubinsky et al. 1986, Baumert 1996):

$$\mu = \mu_{\max}\left(1 - \exp\left(-\frac{E - E_0}{E_k - E_0}\right)\right)$$

This model has three parameters (μ_{max} = the light-saturated maximal growth rate, E_0 = the compensation light level where growth becomes zero, and E_k = the saturating light level where the initial slope of the curve extrapolates to the asymptotic level), which are treated as random effects across the temperature and salinity treatment levels (Pinheiro and Bates 2009). Finally, the fitted values for the light response parameters μ_{max} and E_0 were analyzed as functions of temperature and salinity by generalized additive models (GAMs; Wood 2006). A GAM model was first fitted to show the overall temperature and salinity response pattern across all strains. GAM models for each strain were then used to estimate parameter values and their associated standard errors at optimal growth conditions (maximal μ_{max} and minimal E_0). The locations of temperature and salinity optima were estimated by resampling with replacement within each strain/species and calculating the μ_{max}- or 1 / E_0-weighted mean temperature and salinity of each bootstrap sample. This procedure yielded a cloud of bootstrapped optima which can be visualized as 95% confidence ellipses in temperature, salinity space. Loss rates were estimated as the negative of the net, specific growth rate extrapolated to zero light. The statistical computing environment R (www.r-project.org) was used for all analyses.

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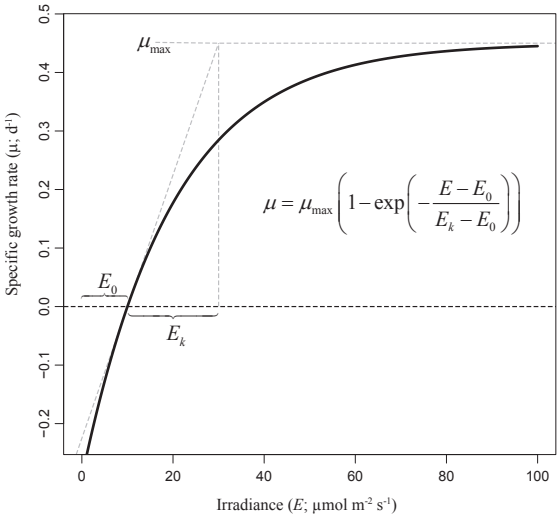

Figure 6 The model used to estimate maximum growth rates

2.7 Flow cytometric estimation of DNA content

Live cells of *Pseudochattonella* were harvested approximately 2 h after the start of the light period, from which nuclei were released and isolated with a HEPES buffer described by Marie et al. (2000). RNAse was added and the mixture was incubated for 30 min at 37 ºC. The nuclei were then stained with propidium iodide (PI) or SYBR Green I for 20 min at room temperature in darkness. Fluorescent beads with diameter 0.5 µm and Chicken Red Blood Cells (CRBC) were used as internal standards. The samples were analysed in a FACSCalibur flow cytometer with an argon laser providing excitation light of488 nm and the standard filter setup for emission measurements. Calculation of DNA content followed the formula by Galbraith et al. (1997):

$$(\text{Sample DNA content}) = (G1_{\text{sample}} / G1_{\text{standard}}) * (\text{Standard DNA content})$$

$G1_{\text{sample}}$ and $G1_{\text{standard}}$ are the linear-scale fluorescence peak values of sample and standard, respectively. The standard DNA content used was CRBC = 3.01 pg DNA cell $^{-1}$ (Johnston et al. 1999).

The amounts of nuclear DNA per nucleus (pg) were converted into genome size (Mbp) according to the following relationship applied by Doležel et al. (2003):

$$\text{genome size (Mbp)} = 978 \cdot \text{DNA content (pg)}$$

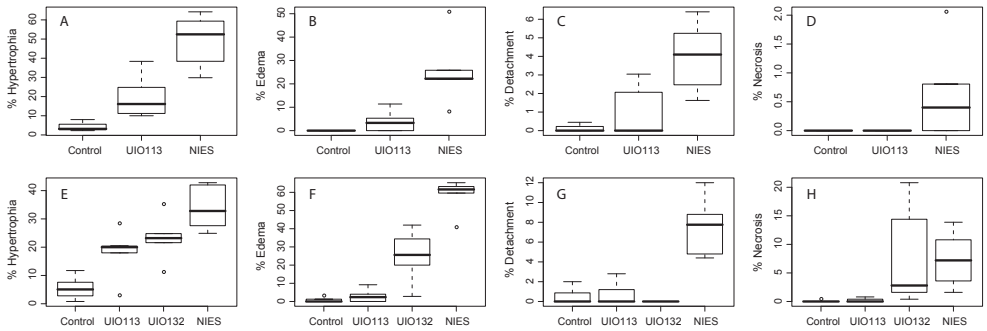

Figure 7 Box plots of percentages injured secondary lamellae per specimen, grouped by lesion type and treatment for cod (A-D) and salmon (E-H). Note different scale on Y – axis.

3. MAIN RESULTS

3.1 Ichthyotoxicity of *Pseudochattonella* (paper I)

Pseudochattonella is a potentially ichthyotoxic flagellate observed in the North Sea, Kattegat and Skagerrak. Six different bioassays using fish cells, fish and brine shrimp nauplii were assessed as test systems for investigating toxic mechanisms of *P. farcimen, P. verruculosa* and *Pseudopedinella pyriformis*. Metabolic activity in embryo cells from chinook salmon (CHSE-214) was measured with a colorimetric method using MTS. The metabolic activity was inhibited by two strains, for one strain increased metabolic activity was recorded whereas two strains did not affected metabolic activity when embryo cells were exposed to extracts from strains of *P. farcimen*. Metabolic activity increased after exposure to both strains of *P. verruculosa.* No change in metabolic activity was observed for cells exposed to *P. pyriformis*. Primary hepatocyte cells from rainbow trout showed metabolic inhibition using AB and loss of membrane integrity using CFDA-AM when exposed to DMSO extracts both from *Pseudochattonella* spp. and from the putatively non-toxic heterokonts *P. pyriformis* and *Skeletonema* sp. The same algae extracted with H_2O showed no adverse effects in the bioassays used. No acute mortality was observed after 24 h exposure of cod fry and salmon smolts to *Pseudochattonella* and *P. pyriformis*. However, histological examinations of fish gills showed strong effects on secondary lamellae (hypertrophy, edema, detachment and necrotic cells, figure 7). No acute toxicity of brine shrimp larvae could be detected after 24 h exposure to any of the *Pseudochattonella* strains.

This study showed that higher concentrations of both *Pseudochattonella* and *P. pyriformis* affect cell metabolism and can cause damage to gills. The toxicity of *Pseudochattonella* varied by species, strain and bioassay system, and was most clearly demonstrated when salmon smolts or cod fry were exposed to bloom concentrations of *P. verruculosa* strain from Japan.

3.2 Optimal environmental conditions for *Pseudochattonella* (paper II and III)

The development of cost effective algal-based products depends on screening experiments for selecting suitable strains/species for a specific use, or optimal growth conditions for the yield of a specific end product. Therefore, a miniaturized and low-cost assay for algal growth, loss rates and estimation of compensation light was tested and optimized. Combinations of microplates with white and black walls were utilized. The laser prints used to create light gradients did not affect the spectral quality of the light inside the wells. There was no vertical light attenuation observed in the white microplates while the slope for vertical light attenuation in the black microplates was steeper for higher surface irradiance into the wells than in them received less irradiance. More light was absorbed by black walls, so the total amounts of irradiance were lower in black microplates compared to white microplates in which the walls reflected light back into the medium. More light were absorbed in empty wells of white microplates than in wells filled with water, while the opposite was observed for black microplates; more light were absorbed in wells filled with water than in empty wells. Linear relationships were observed for log IVF as function of time over the range of time used in the experiments and positive responses measured as growth rates (μ; day $^{-1}$) was observed for increasing irradiance. To confirm the use of

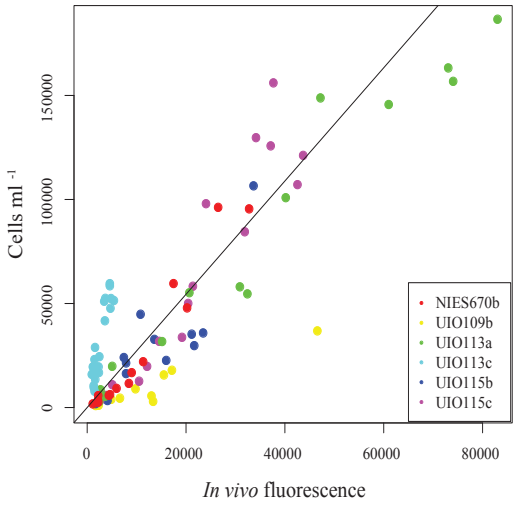

Figure 8 Measured IVF and estimated cell concentration calculated by flow cytometery from the same wells. R^2 is 0.77.

IVF readings in the platereader for estimating cell concentration, samples were prepared at the end of the experiment and cell concentrations were calculated by flow cytometer. The regression line showed a linear trend and R^2 was estimated to 0.77 (figure 8).

The assay was used for examining five strains of *P. farcimen* and two strains of *P. verruculosa* to compare the light response of specific growth rates over a range of temperatures and salinities to get more knowledge of the ecology for this ichthyotoxic genus. Optimal environmental conditions for the genus are shown in figure 9A using data from all strains in a compiled dataset. Maximum growth rates observed for each of the strains were in the range 0.41 - 0.61 day $^{-1}$ (figure 9B). Optimal environmental conditions for achieving maximum growth rates for each strain of *Pseudochattonella* were estimated by re-sampling with replacement and calculating the μ_{max} for each sample by bootstrapping 10,000 times (figure 9C). Lower temperature optima were observed in the *P. farcimen* (9 - 15 ºC) than *P. verruculosa* (12 - 20 ºC). *P. farcimen* also preferred somewhat lower salinity

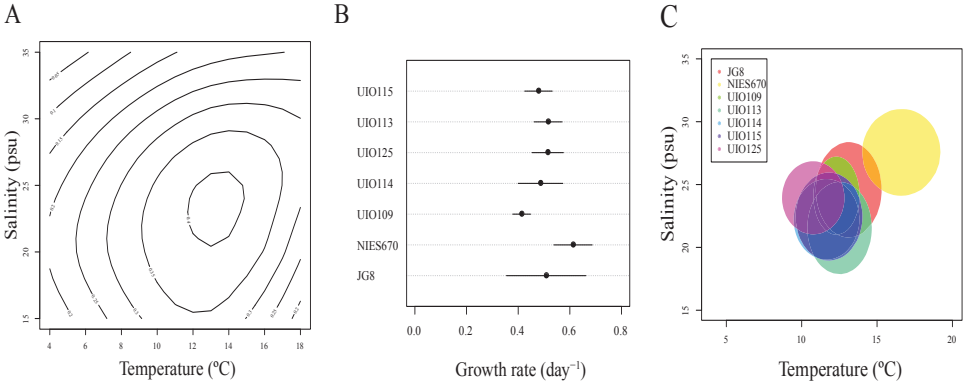

Figure 9 Maximum growth rates for compiled data from all strains of *Pseudochattonella* (A). Maximum growth rates shown for each strain (B). Maximum growth rates for each strain of *Pseudochattonella* estimated by resampling with replacement and calculating the μ_{max} for each bootstrap sample (bootstrapped 10,000x). The *P. verruculosa* strain NIES670 clearly prefers higher temperature and salinity than *P. farcimen* strains (C).

(18 - 26 psu) than *P. verruculosa* (20 - 32 psu). All strains showed a positive response to light, especially at optimal temperatures and salinity conditions. Lowest maintenance costs were observed for intermediary salinity due to costs of osmotic regulations and at lower temperatures, 6 - 12 ºC (figure 10). The estimated loss accounted for 5.4 - 59.7 % of daytime primary production. Lowest compensation light, the irradiance necessary to achieve positive growth rates for the strains was estimated to be ranging from 5 - 15 µmol photons m^{-2} s^{-1}. Optimal temperatures for growth are some higher than optimal temperatures for light conditions (figure 11). This might be because enzymatic kinetic of growth is temperature dependent while the assimilation part of photosynthesis is independent of temperature. Saturation light for maximum growth rates ranged from 18 - 52 µmol photons m^{-2} s^{-1} for the optimal conditions of temperature and salinity (figure 12A, B). The regression line for compensation and saturation light values showed a linear trend with R^2 at 0.99 (figure 12C).

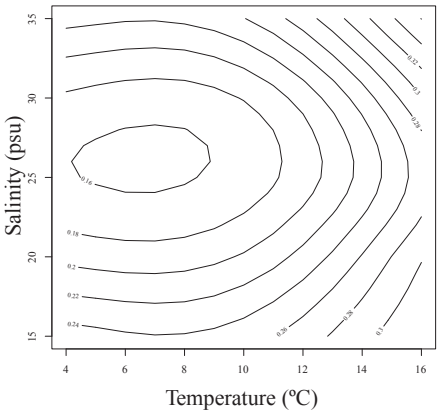

Figure 10 Loss rates were lowest at intermediary salinities and increased with temperature for both species of *Pseudochattonella.*

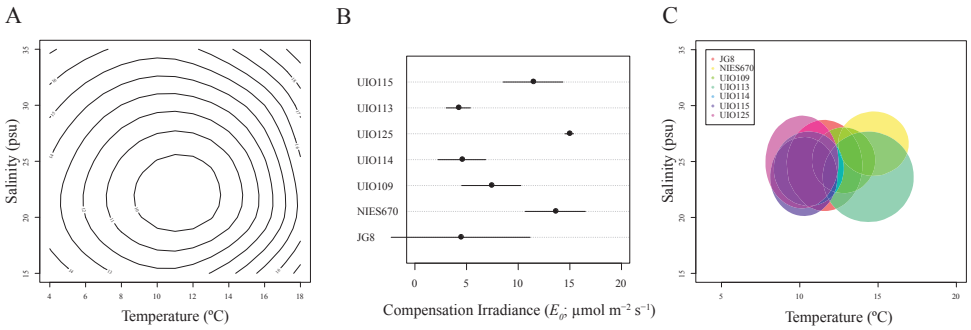

Figure 11 Compensation light for compiled data from all strains of *Pseudochattonella* (A). Compensation light for each strain (B). Compensation light for each strain of of *Pseudochattonella* estimated by resampling with replacement and calculating the l_0 for each bootstrap sample (bootstrapped 10,000x; C).

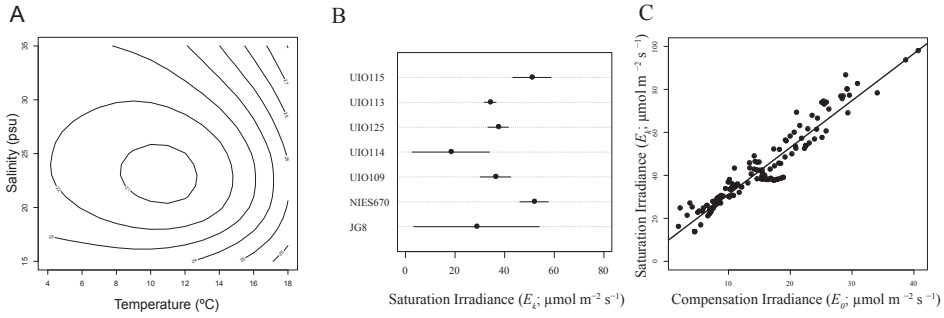

Figure 12 Saturation light for compiled data from all strains of *Pseudochattonella* (A). Saturation light for each strain (B). Saturation light and compensation light showed a linear trend (C).

3.3 DNA content in *Pseudochattonella* (paper IV)

DNA content were estimated in thirteen strains of *P. farcimen* and two strains of *P. verruculosa* using two fluorescence dyes, propidium iodide (PI) and SYBR green I (figure 13). The yield was different for the two dyes. Mean DNA content estimated in *P. farcimen* was 0.46 ± 0.11 pg cell $^{-1}$ (453 Mbp) for nuclei stained with PI and 0.60 ± 0.11 pg cell $^{-1}$ (590 Mbp) for nuclei stained with SYBR green I. Mean DNA content in *P. verruculosa* strain JG8 was estimated to 0.77 pg cell $^{-1}$ (750 Mbp) for nuclei stained with PI and 1.42 pg cell $^{-1}$ (1393 Mbp) for nuclei stained with SYBR green I. DNA contents in *P. verruculosa* strain NIES670 stained with SYBR green I was 0.85 pg cell $^{-1}$ (833 Mbp), a ratio of 1.7 between the two strains. Most cells were in the first peak (1C-value) assumed to represent the G_1 phase in the cell cycle, with only a minor fraction in the second peak (2C-value) assumed to represent the G_2+M (mitosis) phases of the cell cycle (figure 14). No alternating generations in life cycle were observed in our study.

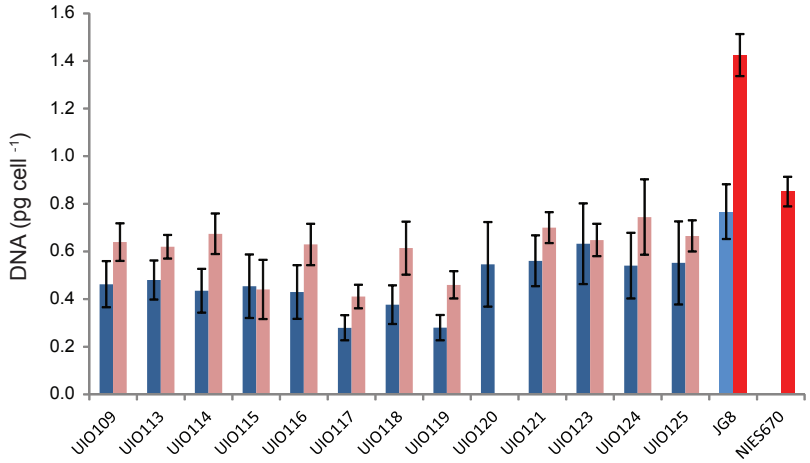

Figure 13 DNA content for the strains of *P. farcimen* and *P. verruculosa* (JG8 and NIES670) using PI (dark blue for *P. farcimen* and light blue for *P. verruculosa)*SYBR Green I (pink for *P. farcimen* and red for *P. verruculosa*).

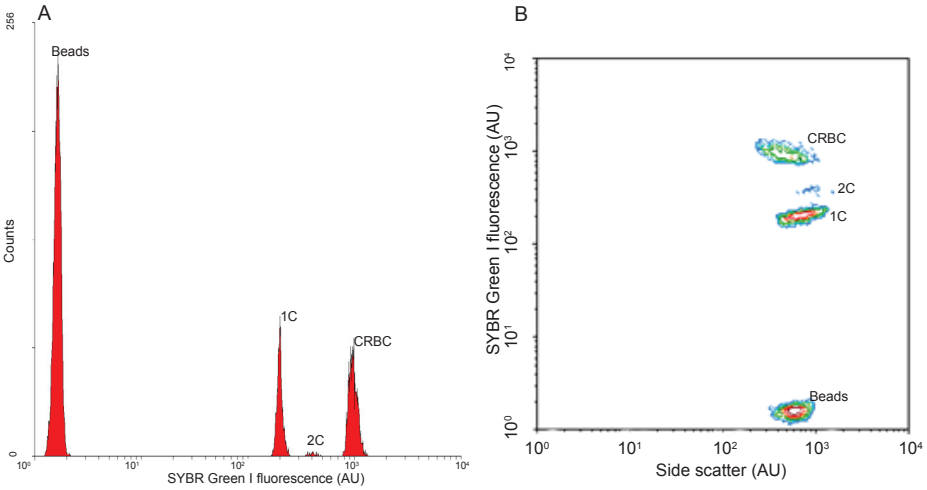

Figure 14 Histogram showing the 1C and 2C peaks of DNA content in *P. farcimen* strain UIO113. Additional peaks for internal standards, beads and CRBC are shown **(A)**. Contour plot of nuclei from *P. farcimen* strain UIO109 stained with SYBR Green I with signal from side scatter plottet against SYBR green I fluorescence (AU) on y-axis **(B)**.

4. DISCUSSION

Pseudochattonella is potentially ichthyotoxic flagellates preferring brackish water. *P. verruculosa* affected fish gills more than *P. farcimen* in our study, but the massive fish mortality in 2001 was probably caused by the latter species according to cells isolated during the bloom (Riisberg and Edvardsen 2008). From Japan and New Zealand we know that *P. verruculosa* is ichthyotoxic (Hosoi-Tanabe et al. 2007, MacKenzie et al. 2011). No acute toxicity to *Artemia* larvae were observed in our study or in undiluted seawater from the bloom in 2001 (Edvardsen et al. 2007). The assay with hepatocytes worked very well for specific compounds (Tollefsen et al. 2008), but were too sensitive for the algal extracts used in our studies.

The use of both black and white microplates in the growth assays showed how important the colour of walls is in such experiments (Diehl et al. 2002, Kemp et al. 2009). However there might be possible to run the whole experiment in white microplates with an extended light gradient. Growth rates were in the range of 0.4 - 0.6 day $^{-1}$ which gives a maximum of approximately one cell division a day (actually ln (2) ≈ 0.69 day $^{-1}$).

Compensation lights necessary to achieve positive growth rates were estimated ranging from 5 - 15 µmol m^{-2} s^{-1}, which is in accordance with previous work (Lewitus and Kana 1995). Saturation light was estimated to range from 18 - 52 µmol m^{-2} s^{-1} which is also similar to other studies (Zondervan et al. 2002, Aydin et al. 2009). Of the two known species, *P. farcimen* is cryophilic while *P. verruculosa* prefer more temperate water. However, stock cultures of *P. verruculosa* could be maintained at 4 ºC for several months (Personal observations). The loss rates increased with temperatures; minimizing losses are as crucial for maintenance as growth (Tilzer and Dubinsky 1987).

The differences in yield for the two dyes used might be caused by different binding properties and differences in structures of the genomes and proteins connected to DNA molecules (Doležel et al. 1992, Veldhuis et al. 1997, Greilhuber 2008). Salinity of the buffer used might influence the signal from PI, the estimates from SYBR Green I stained cells might be more correct (Marie and Edvardsen, unpublished data). The DNA content of *Pseudochattonella* strains were in the range seen among other members of Ochrophyta (Veldhuis et al. 1997, Connolly et al. 2008). The DNA content in *P. farcimen* was smaller than *P. verruculosa*; the DNA content in strain JG8 was approximately twice of *P. farcimen* strains and also higher than the DNA content in NIES670 strain. This indicates that there might be different ploidy level in these two strains, but copy numbers of whole genome clusters and horizontal gene transfer might also have occurred as have been shown for other algae (McGrath and Katz 2004, Bowler et al. 2008, von Dassow et al. 2008, Koester et al. 2010). No alternating generations in life cycle were observed in our study, but Henriksen et al 1993 suggested a possible life history for the distantly related *Dictyocha speculum*.

Pseudochattonella also forms immotile cells attached to the bottom of wells in older cultures (Personal observations).

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Appendix; paper I-IV

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A high-throughput method for measuring growth and loss rates in microalgal cultures

Birger Skjelbred[a b], Bente Edvardsen[a], Tom Andersen[a]

[a]University of Oslo, Department of Biology, P.O. Box 1066 Blindern, NO-0316 Oslo, Norway

[b]Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, NO-0349 Oslo, Norway

Corresponding author: birger.skjelbred@niva.no

Phone: +47 98227781

Faks: +47 22 18 52 00

Abstract

A miniaturized and low-cost assay for algal growth and loss rates, and estimation of compensation light was developed and optimized. Microalgal cultures were grown in white 96-well microplates to estimate specific growth rates at 6 temperatures, 5 salinities, and 8 light levels. Data from black 24-well microplates at 6 temperatures, 5 salinities and 5 light conditions were used in addition to estimate loss rates and compensation light. Absorption and reflection of light were different in the white and black microplates. Growth rates were estimated from daily *in vivo* fluorescence (IVF) measurements using a microplate reader fitted with a fluorometer. To validate the microplate algal growth assay, IVF was compared with cell counting by flow cytometry. Maximal growth rate for the test alga *Pseudochattonella farcimen* (Heterokonta) was estimated to 0.52 ± 0.05 day $^{-1}$ at optimal temperatures ranging from 9 - 14 °C and salinities 18 - 26 psu. Lowest value of compensation light as photosynthetic photon flux density (PPFD) was 4.2 ± 1.2 µmol photons m^{-2} s^{-1} and lowest saturation light, 34.1 ± 3.7 µmol m^{-2} s^{-1}, was observed in the temperature range 5 - 11 °C and salinity range 23 - 28 psu. Minimum loss rate was obtained at temperatures 5 - 8 °C and salinities 26 - 31 psu. Blooms of *P. farcimen* have been recorded in nature under conditions similar to those minimizing loss rates rather than maximizing growth rates in this study. The microalgal assay described here allows for a large number of conditions to be tested, and accurate optimal conditions for growth and loss rates to be obtained.

Keywords Phytoplankton; microplates; growth rates; light; salinity; temperature; loss rates; compensation light; saturating light

Introduction

Most of our knowledge about photosynthesis and the photosynthetic apparatus have been obtained from experiments with algal cultures (Govindjee and Krogmann 2005). Ecological aspects of aquatic microorganisms like carbon assimilation, nutrient demands, photosynthetic rates, temperature dependent growth (Eppley 1972; Geider 1987; Goldman and Carpenter 1974; Goldman et al. 1972; Turpin 1991), as well as assessments of the impact of future environmental conditions (Fu et al. 2010), are acquired from algal bioassays. Phytoplankton accounts for about half of the global primary production making this group important for the carbon cycle of the world (Falkowski et al. 1998). Algal cultures will be crucial in the development of sustainable fuels (Christi 2007; Gouveia and Oliveira 2009), bioactive metabolites for medical use (Singh et al. 2005; Skulberg 2004) and essential compounds for human and animal nutrition, like proteins and fatty acids (Becker 2004; Borowitzka 1997; Harwood and Guschina 2009; Patil et al. 2007; Rosenberg et al. 2008). The development of cost effective algal-based products depends on screening experiments, to select suitable strains/species for a specific use, and find optimal growth conditions for the yield of a specific end product.

Culture flasks are still used in most algal assays, but microplate assays have several advantages (Blaise et al. 1986) and microplates can be an appropriate alternative to flasks (Pavlić et al. 2006; Rojíčková et al 1997; St. Laurent et al. 1992). Microplate-based assays are also regularly used in toxicity assays (Blaise and Vasseur 2005; Gregor et al. 2008; Slabbert and Venter 1999), although McDonald et al. (2008) cautions compounds from plastic ware typically used in life science assays can contaminate samples and bias toxicity assay results.

In vivo chlorophyll fluorescence of phytoplankton has for a long time been used for non-destructive estimation of biomass *in situ* and growth rates in microalgal cultures (Brand and Guillard 1981; Büchel and Wilhelm 1993; Huot and Babin 2011; Lorentzen 1966). IVF in combination with microplates has been used for estimating growth in filamentous algae (Karsten et al. 1996) but has also been considered suitable for planktonic species (Fai et al. 2007).

The costs of producing the light harvesting apparatus must be lower than the benefit of absorbing quanta in photosynthetic organisms (Raven 1984). The energy gains from photosynthesis have to exceed respiration loss to achieve positive growth (Reynolds 2006; Talling 1957). In photosynthesis, light energy absorbed by the light-harvesting complexes is transferred via chlorophyll *a* to the photosystem (PS II and PS I) reaction centres. The induced PSII–PSI electron transport from water, as the primary electron donor, is coupled with the formation of a proton gradient which provides the reducing energy for CO_2 fixation and other biochemical processes in plants and algae (Rohacek and Bartak 1999). Fluorescence is the re-emission of photon energy as an electron returns from an excited singlet state to the ground state in a chlorophyll molecule (Cosgrove and Borowitzka 2011). The observed fluorescence comes only from PSII, while PSI has virtually no fluorescence at room temperature (Falkowski and Raven 2007).

The photosynthetically active radiation (PAR) flux necessary to balance respiration and give zero net photosynthesis is defined as the compensation point which is often found to be around 1% of average surface irradiance (Falkowski and Raven 2007). The compensation point is taxon specific, as we know different taxa show preference for different depths or light conditions (Reynolds 2006). Respiration includes all reactions which produce CO_2, as well as the catabolic processes related to them which do not themselves produce CO_2 (Raven and Beardall 2003).

In this study we have developed microplate based assay where an algal strain can be exposed to all combinations of three treatment factors (light, temperature and salinity) simultaneously. The test alga used in this demonstration is a putative ichthyotoxic flagellate, *Pseudochattonella farcimen* (Edvardsen et al. 2007; Skjelbred et al. 2011). However, the assay can also be used to find optimal growth conditions for other microalgae, and test other treatment factors.

Material and methods

The test alga used in this work was a strain (UIO113) of *Pseudochattonella farcimen* Eikrem, Edvardsen et Throndsen isolated from Norwegian coastal waters (Skagerrak) in March 2001 by Lars Naustvoll (Edvardsen et al. 2007). Stock cultures of *P. farcimen* were grown in IMR ½ medium (Eppley et al. 1967), supplemented with 10 nM selenium (Edvardsen et al. 1990; Imai et al. 1996) with salinity of 25 psu, at 12°C, and at irradiance approx. 50 µmol m^{-2} s^{-1} PPFD, and a 14h:10h light: dark (L:D) cycle. The salinity of the medium was adjusted with distilled H_2O before additions of nutrients, and measured with a S/Mill refractometer (ATAGO CO Ltd, Japan).

The experiments were constructed as 3-way factorial designs with 6 temperatures (4 to 21 °C), 5 salinities (15, 20, 25, 30 and 35 psu) and 8 or 5 irradiances ranging from 5.3 - 96.7 µmol photons m^{-2} s^{-1}, and a 14h:10h L:D cycle. Illumination was provided by Fluora® L 18 W / 77 (OSRAM, Germany). Three growth experiments were carried out in 96-well culture plates with white walls and optical bottom (Nunc AS, Denmark). Each treatment combination was performed in 4 replicates, giving a total of 960 experimental units per experiment. An experiment used to estimate loss rates were carried out in 24-well culture plates with black walls and optical glass bottom (Greiner Bio-one, Germany). Each treatment was performed with 4 replicates, with totally 600 experimental units per experiment. The culture plates were sealed (Sealing tape; Nunc AS, Denmark) to avoid evaporation, but aloud transmission of gases. Each plate was covered with a gray-scale gradient foil made in Adobe Photoshop, printed on transparency, and laminated. Irradiance was measured in 6 (white microplates) or 4 (black microplates) replicate wells at three depths of the wells through the bottom of the plates using optical fiber (Ocean Optics, FL, USA) and a Li-1000 DataLogger (LI-COR, Lincoln, Nebraska, USA) equipped with a US-SQS/L submersible spherical micro quantum sensor (Heinz Walz GmbH, Effeltrich, Germany). A rig was constructed to place the quantum sensor at reproducible vertical and radial positions within the microwell geometry (Fig. 1). To keep stable temperature during the experiments, the culture plates were placed upon a water bath (Fig. 2), and temperature was recorded continuously every 5th min during the experiment with temperature recorders

(LogTag Recorders Ltd, Hong Kong).

Daily measurements of *in vivo* fluorescence (IVF) were measured and used as a non-destructive proxy for population size. IVF readings were made with a FLx 800 microplate reader (BioTek, Inc. Vermont, USA) with excitation / emission at 460 nm / 680 nm and filter bandwidths of 40 and 30 nm, respectively. All experiments were ended before the algae reached the stationary phase. Subsamples for flow cytometry (Becton Dickinson FACSCalibur, San Jose, CA, USA) were taken at the end of the experiment and fixed in 0.5 % glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) and 0.5 % paraformaldehyde (Merck KGaA, Damstadt, Germany), final concentrations. TrueCount beads (Becton Dickinson, San Jose, CA, USA) and 0.5 µm latex beads (Polysciences Inc, USA) were used as internal standards for converting flow cytometry counts to absolute cell concentrations.

Specific growth rate μ (day^{-1}) in each experimental unit was calculated as the slope of a linear regression for log-transformed *in vivo* fluorescence against time. Data from at least four consecutive days after day two were used in the analysis. All experiments were terminated before reaching stationary phase. This first level of data reduction gave a single specific growth rate estimate for each experimental unit. In the second level of data reduction, a light response curve was fitted to all pairs of specific growth rate (μ; d^{-1}) and irradiance (E; µmol photons m^{-2} s^{-1}) for each combination of temperature and salinity. The light response curve was based on a Poisson single-hit model (Dubinsky et al. 1986, Baumert 1996):

$$\mu = \mu_{\max}\left(1-\exp\left(-\frac{E-E_0}{E_k-E_0}\right)\right)$$

This model has three parameters (μ_{max} = the light-saturated maximal growth rate, E_0 = the compensation light level where growth becomes zero, and E_k = the saturating light level where the initial slope of the curve extrapolates to the asymptotic level), which are treated as random effects across the temperature and salinity treatment levels (Pinheiro and Bates

2009). Finally, the fitted values for the light response parameters μ_{max} and E_0 were analyzed as functions of temperature and salinity by generalized additive models (GAMs; Wood 2006). A GAM model was first fitted to show the overall temperature and salinity response pattern across all strains. GAM models for each strain were then used to estimate parameter values and their associated standard errors at optimal growth conditions (maximal μ_{max} and minimal E_0). The locations of temperature and salinity optima were estimated by resampling with replacement within each strain/species and calculating the μ_{max} - or 1 / E_0 -weighted mean temperature and salinity of each bootstrap sample. This procedure yielded a cloud of bootstrapped optima which can be visualized as 95% confidence ellipses in temperature, salinity space. Loss rates (L_0) were estimated as the negative of net, specific growth rate, extrapolated to zero light. The specific growth rates were calculated in an ordinary growth experiment (environmental conditions; 25 psu, 13 °C and 97 µmol photons m^{-2} s^{-1}) using 50 ml culture flasks (Nunc AS, Denmark) and 100 ml Erlenmeyer flasks with three replicates and counted by Utermöhl method (Utermöhl 1958) for validation. The statistical computing environment R (www.r-project.org) was used for all analyses.

Results

The distribution of PPFD was the same for shaded wells, except that the irradiance was lower; the laser printed foil used to create light gradients did not affect the spectral quality of the light inside the wells (Fig. 3). Light spectra in wells with maximum irradiance compared to minimum amount of PPFD available showed a high correlation (R^2 = 0.97). There was no vertical light attenuation observed in the white microplates (Fig. 4a) measured with the equipment shown in Fig. 1. The slope for vertical light attenuation in the black microplates was steeper for higher surface irradiance than in those receiving lower surface irradiance (Fig. 4b). More light was absorbed by black walls, so the total amounts of PPFD were lower in black microplates compared to white microplates in which the walls reflected light back into the medium. More light were absorbed in empty wells (filled circles; Fig. 5a) of white microplates than in wells filled with water (open circles), while

the opposite was observed for black microplates (Fig. 5b); more light were absorbed in wells filled with water than in empty wells. Each line represents a mean of the replicates for the 3 depths measured. The temperature treatments were stable during the experiment with standard deviations <0.5 °C within each temperature level. There was a consistent 1 °C temperature difference between light and dark periods in all temperature treatments.

Linear relationships were observed for log IVF as a function of time over the range of time used in the experiments (3 - 7 days after inoculation, Fig. 6a). Positive growth responses to increased irradiance were observed until light saturation occurred (Fig. 6b). Low positive growth rates at 4 °C were achieved for all salinities except 35 psu. Increasing temperature from just 4 to 6 °C caused enhanced growth rate. In the temperature range 11 - 16 °C growth occurred at all salinities. No positive growth rates occurred at 20 °C. Optimal salinity and temperature conditions using a compiled dataset of one black and three white microplates were estimated to be 18 - 26 psu and 9 - 14 °C with maximum specific growth rates at 0.52 ± 0.05 day^{-1} (Fig. 7a). The specific growth rates estimated were 0.69 ± 0.04 day^{-1} in Erlenmeyer flasks and 0.64 ± 0.02 day^{-1} in culture flasks; the specific growth rate for these environmental conditions (25 psu, 13 °C and 97 µmol photons m^{-2} s^{-1}) as mean of three experiments using microplates were 0.61 ± 0.08 day^{-1}. Predicted maximal values for µ from the Poisson model compared with observed values are shown in Fig. 7b. The growth rates increased with PAR for the light gradient used, except at suboptimal conditions of temperature and salinity. Lowest compensation light occurred at a mean photon flux rate of 4.2 ± 1.2 µmol photons m^{-2} s^{-1} in the temperature range 5 - 11 °C and salinities 23 - 28 psu (Fig. 8a). Saturation light for maximum growth rates were 34.1 ± 3.7 µmol photons m^{-2} s^{-1} in the white microplates for the optimal conditions of temperature and salinity (Fig. 8b). The regression line for compensation and saturation light values had a linear trend with R^2 at 0.99 (Fig. 8c).

Lowest loss rates were observed at intermediate salinities (Fig. 9a) and increased with temperature (Fig. 9b). The lowest growth rate needed for maintenance to compensate for loss rates was achieved at temperatures in the range 5 - 8 °C and salinities 26 - 31 psu

(Fig. 10a). When temperatures were close to 20 °C, the loss rates were too high to achieve positive growth and the yields were low and decreasing. The correlation between predicted values by the model fits the observed values of maintenance loss rates from the data well with a R^2 at 0.72 (Fig. 10b).

To validate the use of IVF readings in the platereader for estimating growth rate, samples for flow cytometry were prepared at the end of the experiment. A plot with estimation of cell concentrations from flow cytometry, and *in vivo* fluorescence had a R^2 of 0.95 in white microplates and 0.83 in black microplates (Fig. 11).

Discussion

Chlorophyll a mainly absorbs light in the blue and red parts of PPFD (Falkowski and Raven 2007) so the light source was well suited for photosynthesis, as also indicated by the manufacturer's specifications (www.osram.com). However the distribution of PPFD from our light source is not comparable with irradiance from real sunlight (Kemp et al. 2009; McCree 1972).

Light attenuation was different for the white and black microplates shown in Fig. 4. There was no measurable attenuation of light in the white plates for the three depths measured in the wells, indicating that absorption was low and reflection high in this material. Most of light attenuation in the black wells was due to absorption of the walls, while the effect of the water itself was negligible. The importance of wall material color on light reflection and absorption has also been observed in mesocosm tanks for outdoor experiments (Kemp et al. 2009). In an outdoor field experiment enclosures were covered with opaque, white or black plastic (Diehl et al. 2002). Both experiments concluded that black walls increased light attenuation. The difference between empty wells and wells filled with water might be due to different light refraction in air and liquid, a greater proportion of light was refracted and hit the walls in wells filled with water. In microplates filled with water the black walls absorbed more of the light, the opposite were observed for white walls; the light was reflected from

the walls (Fig. 5).

The study of physiological responses of organisms in laboratory occasionally reveals a discrepancy between the environmental conditions the organisms appear to be best adapted for in the laboratory and the environmental conditions of their habitat in nature (Brand 1984). While *Pseudochattonella* may bloom in the Skagerrak at very low temperatures (Edvardsen et al. 2007), the growth optimum of this alga was observed at higher temperatures in the laboratory for this study (Fig. 7a). Growth primarily depends on light and temperature; the more these parameters approach optimal demands, the broader is the salinity range tolerated (Kirst 1990). This is also observed in our experiments; the highest growth rates were observed at temperatures from 9 - 14 °C for PPFD of approximately 34 µmol m^{-2} s^{-1} and in this range positive growth rates were observed for all salinities.

The optimal temperatures for growth shown in Fig. 7 are somewhat higher than optimal temperatures for optimal light conditions shown in Fig. 8. This might be because some processes, like light absorption, excitation energy transfer and photochemistry are temperature independent (Raven and Geider 1988) while enzyme activities are temperature dependent (Geider and Osborne 1989). The well fitted regression line for compensation and saturation light indicates that excess carbon is respired. In most cases, photosynthetic activity of microalgae become light saturated within 200 µmol m^{-2} s^{-1} (Masojídek et al. 2011). In this study *P. farcimen* achieved maximum growth at saturating irradiance of 34.1 ± 3.7 µmol m^{-2} s^{-1}. The L:D cycles in the experiment were set to 14:10. In addition to photon flux, qualities of PPFD, length of light and dark periods are important factors for net photosynthesis. Mixing depth and the rate of vertical mixing influences the amount of PPFD available and therefore primary productivity in photosynthetic organisms living in the ocean (Huisman et al. 1999; Van Ruth et al. 2010). There are costs related to production of the light harvesting apparatus (Raven 1984). Enzyme activities and the possibilities for higher growth rates, but also respiration increases with higher temperature (Reynolds 2006), as showed in Fig. 9b. This gives cold-water adapted species utilizing low light conditions an advantage in the use of nutrients available in early spring. In our experiments

maintenance costs were lowest at the lower temperatures, so it might not be necessary to have such a high cell division rate to achieve a higher biomass in early spring. Growth showed a sigmoid curve while respiration increased linearly with temperature in a study with the diatom *Leptocylindrus danicus* (Verity 1982). The costs were lower at optimal salinity (Fig. 9a), since less energy was necessary for osmotic regulation, by inorganic and organic compounds (Bisson and Kirst 1995; Kirst 1990). The lowest growth rates needed for maintenance to compensate for loss rates in *P. farcimen* were observed at the temperatures in the range 5 - 8 °C and salinities 26 - 31 psu (Fig. 10); this may explain why this species grows at such low temperatures in its natural environment, as long as the irradiance is above the compensation point (Raven and Geider 1988). It should be noticed that the validity of these compensation point estimates apply to the spectral composition and photoperiod used in our assays, while they may be different under other environmental conditions (Hobson and Guest 1983). Algae are capable of adaptation to changes in light conditions (Falkowski and LaRoche 1991; Richardson et al. 1983). Accessory pigments in *Pseudochattonella,* like carotenoids and chlorophyll *c,* contribute to optimal harvesting and utilizing of the available light (Dubinsky et al. 1986; Edvardsen et al. 2007).

The microalgal assay described here allows for a large number of conditions to be tested, and more accurate optimal conditions for growth and loss rates to be obtained. This information is important for the understanding of an organism's adaptations and requirements to its environment, but also for optimisation of the growth conditions during commercial algal production.

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Fig. 1 Equipment for measuring light irradiance (PPFD) in the microplates.

Fig. 2 Growth experiment set up. The microplates were covered with a light gradient foil and placed upon a water bath to keep stable temperature. A temperature logger measured temperature with 5 min intervals during the experiment.

Fig. 3 Light quality available in wells of white plates with lowest irradiance on x - axis and highest irradiance on y - axis, adjusted R^2 is 0.97.

Fig. 4 Light attenuation by depth in the microplates for different irradiances at the surface level. Note different values on the y– axes. Log values for light attenuation in **(a)** white microplates for the eight light conditions, **(b)** black microplates for the six light conditions.

Fig. 5 Light in microplates measured at three depths in empty wells (filled circles; whole lines) and wells filled with water (open circles; dashed lines). Each line represents a mean for each of the 3 depths measured. **(a)** White microplates. **(b)** Black microplates.

Fig. 6 (a) Algal abundance (as log *in vivo* fluorescence) over time (day) at variable irradiance and temperature at salinity 25 psu. Increasing light (µmol photons m^{-2} s^{-1}) upward the rows and increasing temperature (°C) from left to right in columns. **(b)** Specific growth rate (μ; day $^{-1}$) as a function of irradiance, temperature and salinity from three independent growth experiments using white microplates and one experiment using black microplates, with data compiled. Irradiance measured as PPFD, µmol photons m^{-2} s^{-1}. Increasing temperatures (°C) upward the rows and increasing salinity (psu) from left to right in columns. Only the growth rates for values of $\mu > 0$ are included. No positive values were obtained at 20 °C.

Fig. 7 (a) Maximum specific growth rates (μ; day $^{-1}$) from three independent experiments in white microplates with variable combinations of salinity and temperature. **(b)** Plot showing predicted μ-values from the Poisson model (x-axis) and observed μ-values (y-axis) from one of the experiments using white microplates.

Fig. 8 (a) Compensation light for combinations of salinity and temperature. **(b)** Saturation light for combinations of salinity and temperature. **(c)** Correlation between compensation light value and saturation light value for the different combinations of salinity and temperature, adjusted R^2 is 0.99.

Fig. 9 Loss rates for *Pseudochattonella farcimen* in the black microplates. **(a)** Loss rates as a function of salinity. Intermediate salinities have the lowest loss rates. **(b)** Loss rates as a function of temperature. Loss rates increases with temperature.

Fig. 10 (a) Specific growth rates (d^{-1}) necessary for maintenance of growth at different conditions of temperature and salinity. **(b)** Predicted values of loss rates from the model used plotted against the observed values from the data, adjusted R^2 is 0.72.

Fig. 11 Comparison of algal abundance estimated by *in vivo* fluorescence (IVF; AU) and flow cytometric cell counts (cells ml^{-1}) in **(a)** white microplates, adjusted R^2 is 0.95, (IVF was mean of three wells) **(b)** black microplates, adjusted R^2 is 0.83.

Figure 1

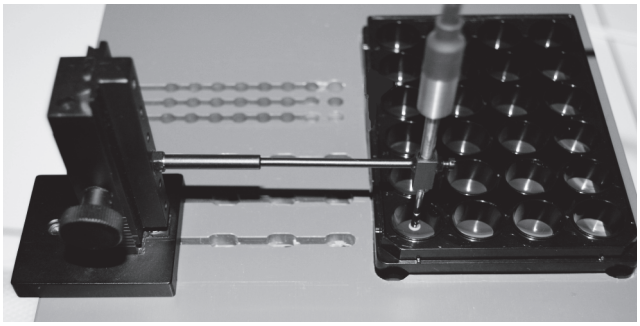


Figure 2

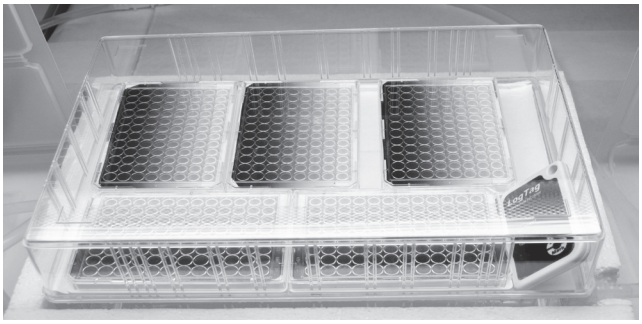


Figure 3

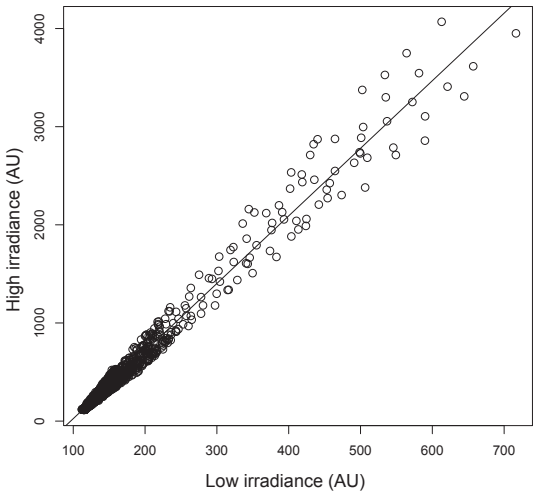

High irradiance (AU)
0
1000
2000
3000
4000
100
200
300
400
500
600
700
Low irradiance (AU)

Figure 4

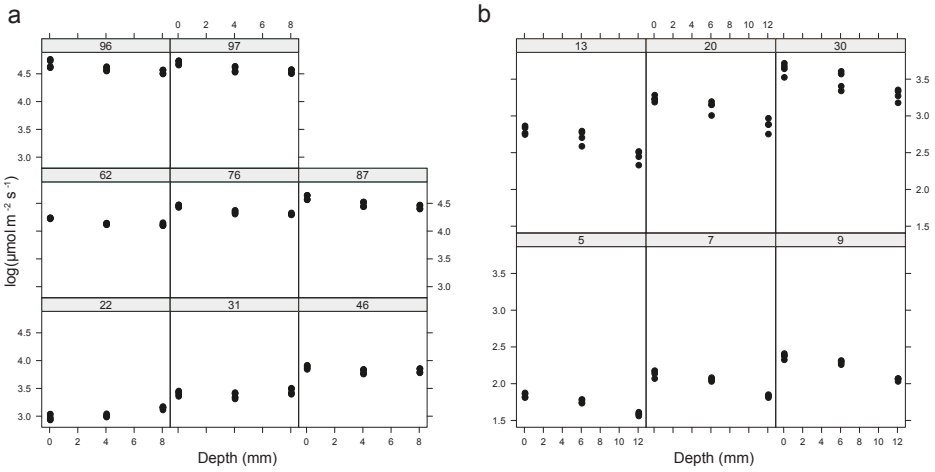

a
96
97
62
76
87
22
31
46
log(µmol m-2 s-1)
Depth (mm)
b
13
20
30
5
7
9
Depth (mm)

Figure 5

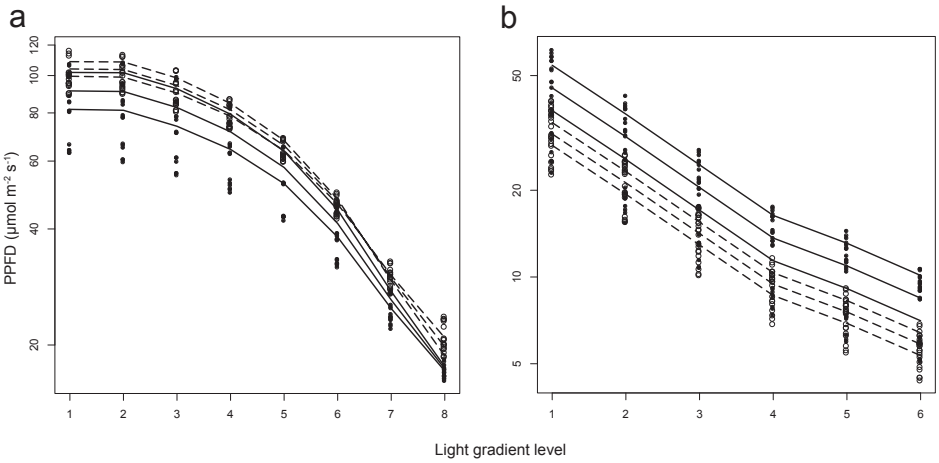


Figure 6

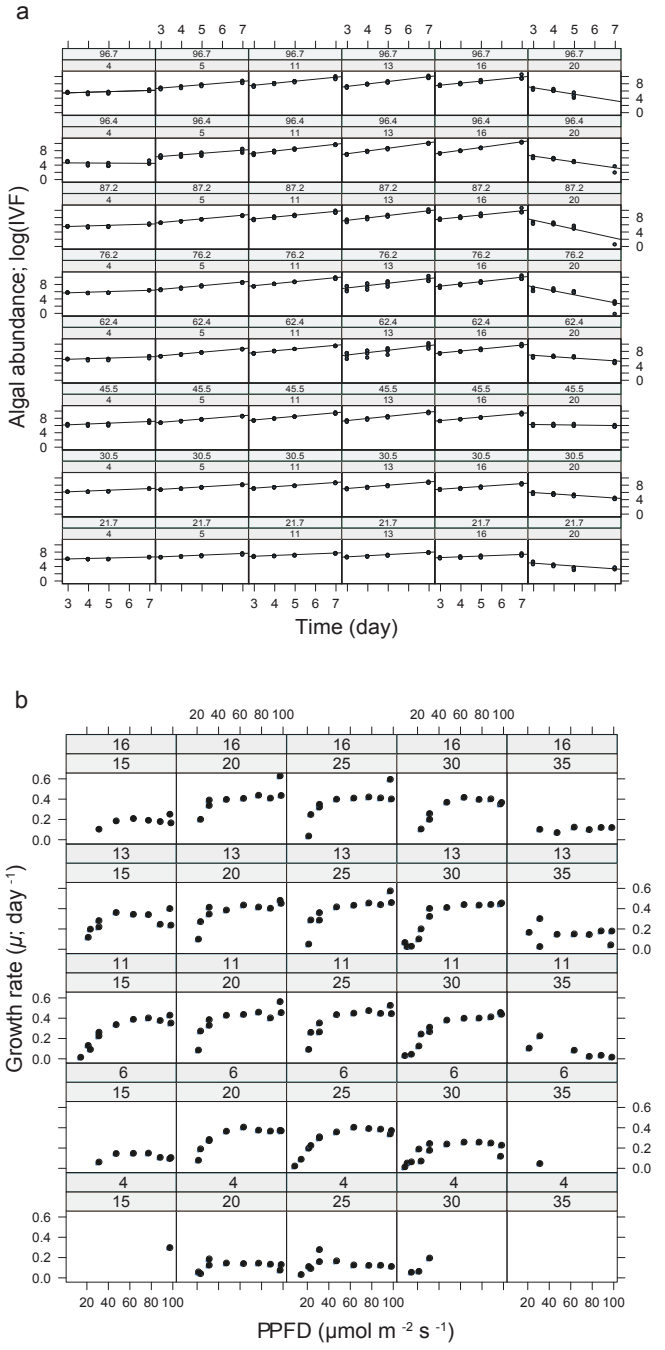

a
Algal abundance; log(lVF)
Time (day)
b
Growth rate (μ; day -1)
PPFD (μmol m -2 s -1)

Figure 7

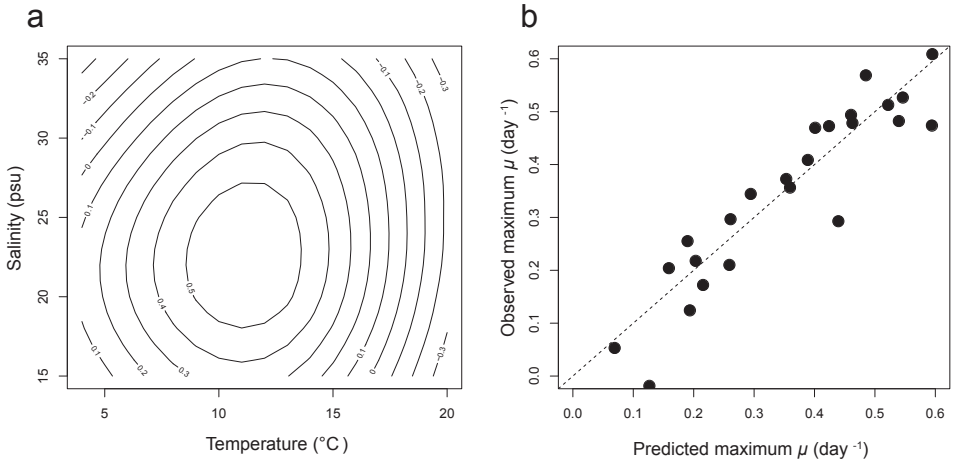

a
b
Salinity (psu)
Temperature (°C)
Observed maximum μ (day -1)
Predicted maximum μ (day -1)

Figure 8

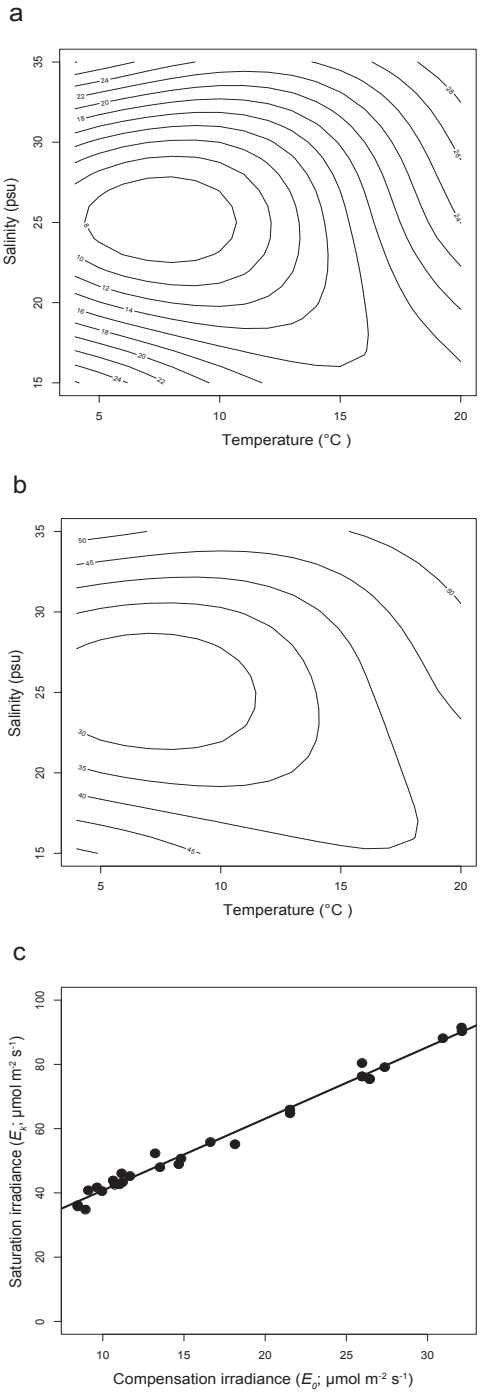

a
Salinity (psu)
Temperature (°C)
b
Salinity (psu)
Temperature (°C)
c
Saturation irradiance (E_k; µmol m^{-2} s^{-1})
Compensation irradiance (E_o; µmol m^{-2} s^{-1})

Figure 9

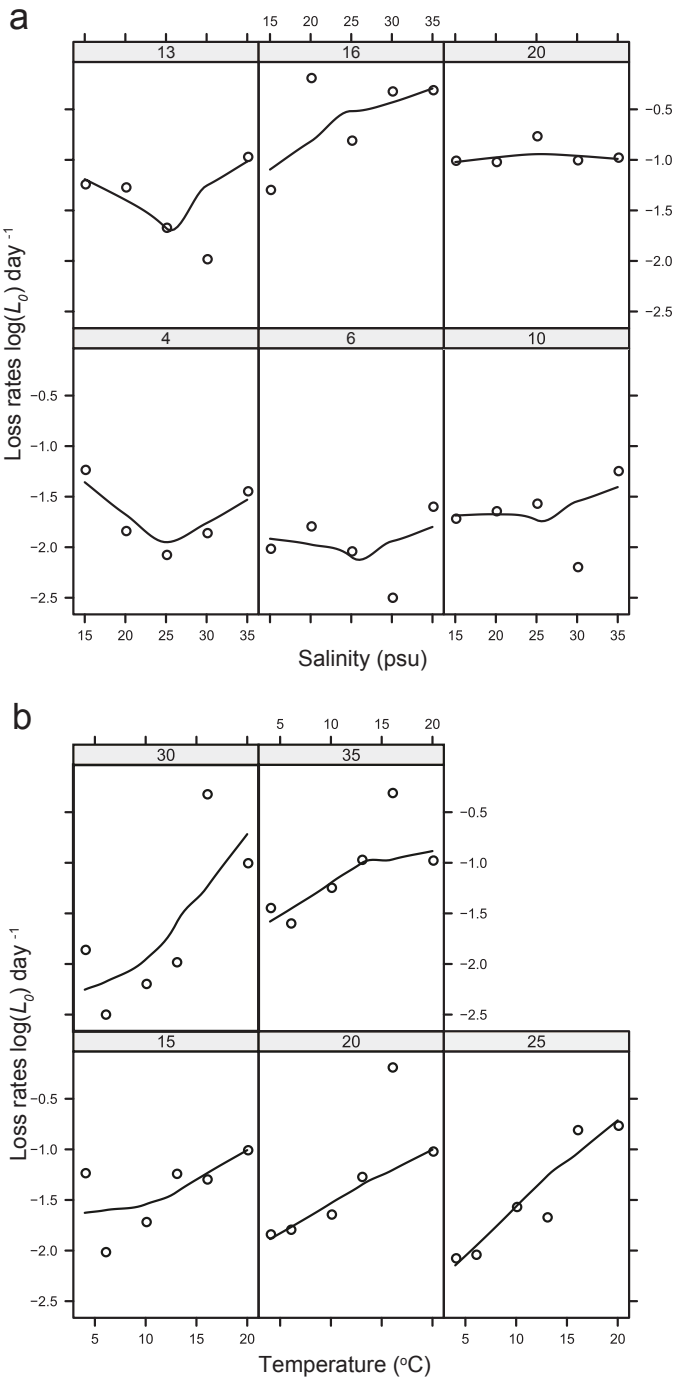

a
13
16
20
4
6
10
Loss rates log(L_0) day^{-1}
Salinity (psu)
b
30
35
15
20
25
Loss rates log(L_0) day^{-1}
Temperature (°C)

Figure 10

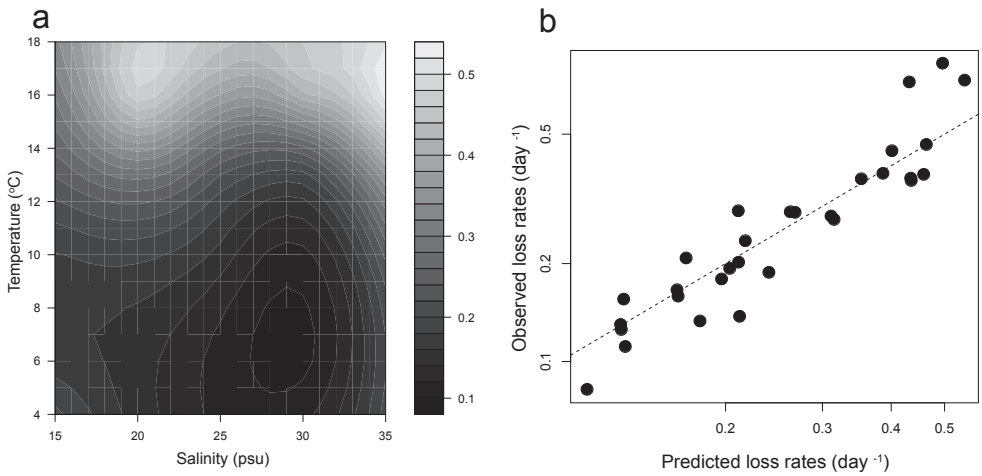
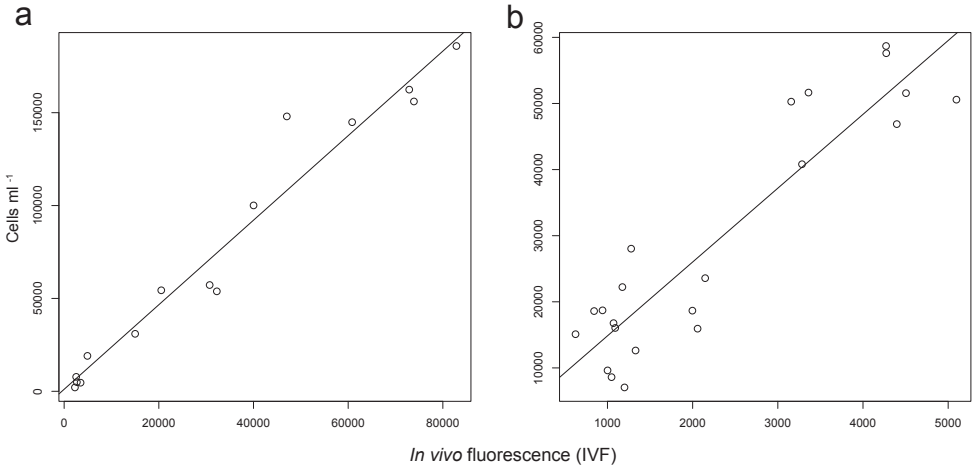

a
Temperature (°C)
Salinity (psu)
b
Observed loss rates (day -1)
Predicted loss rates (day -1)

Figure 11


a
b
Cells ml -1
0
50000
100000
150000
0
20000
40000
60000
80000
10000
20000
30000
40000
50000
60000
1000
2000
3000
4000
5000
In vivo fluorescence (IVF)

III

IV