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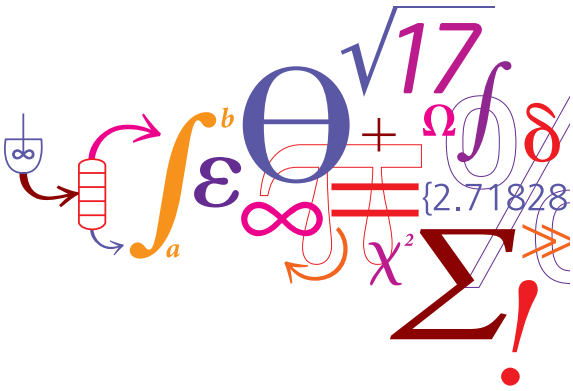

Claus Baggesen
Ph.D. Thesis
June 2014

DTU Chemical Engineering
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Preface

The work presented in this Ph.d. thesis was conducted fom November 2010 to June 2014 in the Microbial Engineering Research Group (MIC), Process, Dept. of Chemical and Biochemical Engineering, DTU. The thesis contains a summary, an introduction (Chapter 1) which contains theory and literature relevant for the project, and three manuscripts for publication (Chapters 2 – 4). The manuscript in Chapter 2 has been submitted to Journal of Applied Phycology and has been resubmitted after a first revision. The manuscript in Chapter 3 has been submitted to Algal Research. For these two manuscripts I have conducted all data processing and experimental work as well as writing the manuscripts. The manuscript in Chapter 4 is currently undergoing final scrutiny by the corresponding author before submission to Journal of Applied Phycology. For this manuscript I was involved in experimental work and contributed to manuscript revisions.

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Summary

Microalgae are nearly everywhere and they are responsible for a large part of the world's primary production.

Their ability to grow fast and to reach high cell densities makes them candidates for use in the production of biofuel. A key feature of many algae is the production of lipids as storage molecules. A variety of algae can produce large amounts of lipids and these easily be converted to biodiesel for use as transport fuel. Production of algal based biodiesel is however still limited mainly due production costs. Research is needed in order to lower the price of the final product.

In this study interactions between algae and bacteria have been investigated. Many previous investigations have revealed that algae when co-cultured with bacteria reach higher cell numbers and exhibit faster growth rates. Part of the study focuses on interactions between the green algae *Dunaliella salina* and three strains of bacteria, *Pelagibaca, Halomonas* and *Marinobacter*, isolated from the algal culture. Growing axenic *D. salina* in co-culture with the bacteria results in markedly higher cell densities. Another part of the study focuses on interactions between three strains of *Scenedesmus* like algae, isolated water samples obtained locally, and bacteria from one of the water samples. Interestingly the three closely related algae react very differently to being co-cultured with the bacteria. One algal strain is unaffected, one is promoted in growth, and one is negatively affected.

In the final part of the study the effect of hypo and hyper-saline shock on *D. salina* was investigated. Theoretically *D. salina* would produce lipids when stressed however no appearance of lipids was detected in the cells subjected to a hypo-saline shock and in the case of a hyper-saline shock lipids could be detected early on but disappeared within 48 h.

Resumé

Mikroalger findes næsten over alt, og de er ansvarlige for en stor del af verdens primærproduktion. Deres evne til at vokse hurtigt og nå høje celletætheder gør dem til gode kandidater til brug i produktionen af biobrændstof. Mange alger producerer lipider, som oplagsstof og en del af disse er i stand til at producere store mængder. Disse lipider kan let omdannes til biodiesel, der kan bruges som brændstof til transport. Produktionen af alge-baseret biodiesel er dog stadig begrænset,

hovedsageligt på grund af produktionsprisen. Videre forskning er nødvendig for at sænke prisen på det endelige produkt.

I dette studie er interaktionerne mellem alger og bakterier belyst. Mange tidligere undersøgelser har vist, at alger i co-kultur med bakterier udviser højere vækstrater og når højere celletætheder, end når de vokser axenisk. En del at studiet fokuserer på interaktionerne mellem grønalgen *Dunaliella salina* og tre bakteriestammer, *Pelagibaca, Halomonas* og *Marinobacter*, der er isoleret fra algekulturen. Når *D. salina* vokser sammen med bakterierne, er celletætheden markant højere end når *D. salina* vokser alene. En andel del af studiet fokuserer på interaktioner mellem tre *Scenedesmus* lignende algestammer og to bakterier isoleret sammen med den ene af algerne. Både alger og bakterier er isoleret fra lokale vandprøver. Overaskende reagerer de tre tæt beslægtede alger meget forskelligt på at vokse sammen med de to bakterier. Én stamme er stort set upåvirket, én stamme vokser bedre, og én vokser dårligere.

I den sidste del af studiet undersøges effekten af hypo og hyper-osmotisk salt-chok på *D. salina*. Teoretisk bør *D. salina* producere lipider, når den udsættes for stress, men ved hypo-osmotisk salt-chok ses ingen produktion af lipider. Ved hyper-osmotisk salt-chok ses tidligt en produktion af lipider, men efter 48 timer er disse forsvundet igen.

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Chapter 1

Introduction

1. Algae and biofuels

Photosynthetic microalgae (in the following referred to as algae) are living nearly everywhere. They grow in almost every kind of aquatic environment, from the arctic to the tropics, from pristine springs to heavily polluted wastewater ponds and from freshwater lakes to saturated saline pools. They also grow on land and some are even airborne. Algae are responsible for a great deal of the world's primary production and are as such a great candidate for use as a biofuel alternative to diminishing fossil fuel sources. Green algae (Chlorophyceae) such as *Dunaliella* and *Scenedesmus*, both thoroughly described in later chapters, often exhibit high growth rates and are able to grow to high densities, both prerequisites for a future production of affordable algal biofuel (Demirbas and Demirbas 2011). Some green algae produce high amounts of lipids, in some cases more than 50% of the cell content. These lipids can with minimal processing be converted to biodiesel and used as transport fuel. This is in contrast to e.g. bioethanol where pretreatment of biomass and fermentation processes are needed.

The main focus of this thesis deals with interactions between algae and bacteria. These interactions are important as it is known that many algae benefit from growing in co-culture with bacteria (Cole 1982). Algal /bacterial interactions can result in faster growth rates as well as higher cell densities. Increasing algal growth and lipid production are key factors in further research into algal based biofuel.

2. Potential of algae

As fossil fuel sources are diminishing the potential of using microalgae as an alternative source of energy has been explored. Some microalgae produce large amounts of storage products most often in the form of starch which can be fermented into ethanol. Another storage product of microalgae is di- and tri-acyl glycerols which can be transesterified into fatty acid methyl esters (FAME) and glycerol. After separating FAME's and glycerol the FAME's can be mixed with conventional diesel or used directly in a combustion engine depending on quality, i.e. number of double bonds, branching and length of the fatty acid chain. However all the fatty acid are combustible and

therefore focus in the production of biodiesel from algal biomass should be on the amount of lipids produced and not the quality and species (Knothe 2005).

There is no doubt that some microalgae can produce large amounts of lipids. Most algae like higher plants store the photosynthetically fixed CO2 as starch. A few algal species like Nanochloropsis store the fixed CO2 as lipids. Changes in the environment like nitrogen availability or high light can induce lipid production. In some cases up to 30 – 40 % of the dry weight (dw). It is difficult to assess how much algal biomass can be produced. Estimates vary from 3 $g*m^{-2}*day^{-1}$ in a commercial microalgal production facility located in Israel (Passell et al. 2013) up to 24 $g*m^{-2}*day^{-1}$ as an average of open pond cultivation of different microlagal species (Griffiths and Harrison 2009). Based on the commercial production capacity of 3 $g*m^{-2}*day^{-1}$ of algal biomass assuming a continuous production throughout the year a yield of 10.8 $tonnes*ha^{-1}$ of algal biomass can be obtained. This can be compared to the average annual yields of soy bean or canola of 2 $tonnes*ha^{-1}$. Assuming for microalgae, soy bean and canola a lipid content of 30% this will amount to 3.2 tonnes of microalgal oil and 0.6 tonnes of soy or canola oil. Calculations such as these have inspired the field of research on biodiesel based on microalgal biomass.

Life cycle assessment (LCA) of the commercial plant in Israel revaled a net energy return ratio (NER) (energy in /enegy out) on algal biodiesel of 33.44 compared to that of biodiesel production based on soy bean of 0.80 (Passell et al. 2013). LCA's studies by Lardon et al. (2009) report NER's ranging from 0.7 to 1.96. The difference in the calculated NER in the two studies originates in the 10 fold higher estimated algal biomass production used by Lardon et al. in their LCA calculations. Another LCA comparing production of microalgal biomass in raceway ponds to flat panel and tubular photobioreactors estimate NER's for oil production of 0.33, 0.6 and 14.3 respectively (Jorquera et al. 2010). However they excluded the energy needed for culture medium, oil extraction and subsequent production of biodiesel from the calculations. According to Khoo et al. (2011) 13% of the supplied energy is used in biomass production, 85 % in lipid extraction and 2 % in biodiesel production. In this study they find a NER of 4.4. Obviously excluding the energy consumption of lipid extraction from the calculations is subjective and the estimates of NER in different LCA analyses suggest that production of biodiesel based on microalgal biomass with the present technology is not energetically favorable. To alter this situation both a substantial increase in biomass production as well as an increase in lipid content of the microalgal cells is required in addition to optimized production processes.

Lundquist et al.(2010) examined the economics in five hypothetical cases of microalgal biomass production. In one case the emphasis is on wastewater treatment with biofuel as a byproduct vs. biofuel production with wastewater treatment as a byproduct. In a second case the production of oil plus biogas or biogas alone are compared. The third case compares production areas of either 100 or 400 ha. The fourth case compares year round biomass production to seasonal production due to low microalgal primary production in the winter. The four cases have different rate of biofuel and biomass production and was compared in the fifth case. The assumption for all five cases were 25 % lipid content of the algal biomass which were produced at 22 $g*m^{-2}*day^{-1}$. The conclusion of this comprehensive study of producing microalgal based biofuels is that it is not possible to produce microalgal biofuel cost competitively with fossil fuels or even other biofuel without increasing biomass and lipid production of the microalgae as well as improving the overall efficiency of the production processes.

The amount of microalgal biomass that can be produced in a volume of water essentially depends on three abiotic conditions. The amount of light available to drive photosynthesis and thereby initiate the growth of the microalgae, the amount of CO2 available for the photosynthesis and finally the amount of nutrients available for the metabolism. By growing microalgal cultures under conditions where these factors are changed it is possible to assess the potential for increasing microalgal biomass. In two studies by Chen et al. (2011) and Tang et al. (2011) the growth of *Dunaliella tertiolecta* is examined under varying conditions.

By growing *D. tertiolecta* in 650 ml culture flasks with different sources of nitrogen ($NaNO_3$ or NH_4Cl) supplied in different concentrations they find that 23 mM of $NaNO_3$ is the optimal concentration for biomass production as measured by the increase in optical density (O.D.) at 450 nm. Further increase of the NaNO3 concentration does not result in an increase in biomass.

By changing the light intensity the cell density, measured as cells $*ml^{-1}$, increased from ca. $10*10^6$ to $12*10^6$ after 8 days of growth when the light intensity was changed from 100 $\mu E*m^{-2}$ to 200 $\mu E*m^{-2}$. However a further increase to 350 $\mu E*m^{-2}$ had no effect on cell density. In relation to the light intensity it was also investigated if the length of the light dark cycle had any effect on final cell density and it was found that supplying the microalgal culture with constant light resulted in cultures reaching the highest cell densities around $8*10^6$. This was ca. 30 % higher than the cultures supplied with either 12 or 15 hours of light.

By bubbling cultures of *D. tertiolecta* with different air/CO_2 mixtures maximum cell densities around $6*10^6$ were reached with 2 and 4 % CO2. All other CO2 concentrations both higher and lover resulted in markedly lover final cell densities and supplying 100 % CO2 resulted in very little growth. In the all the experiments all factors except the one under scrutiny were kept in the optimal range. Therefore by optimizing abiotic factors it seems that the maximum cell density that can be reached with *D. tertiolecta* cultures would be around $12*10^6$ cells*ml^{-1}.

3. Lipid synthesis in algae

The fatty acid moieties of lipids are made in the chloroplast by activation of Malonyl-CoA made from Acetyl-CoA catalyzed by Acetyl-CoA carboxylase. Malonyl is then transferred from CoA to the acyl carrier protein (ACP), a protein cofactor. As a first step the Malonyl-ACP is condensed with Acetyl-CoA to form 3-ketobuteryl-ACP. In a series of reductions and dehydration reactions Buturyl-ACP is formed. Buturyl-ACP condenses with Malonyl-ACP to form 3-ketoacyl-ACP. This cycle continues until 16 or 18 carbon product is formed. The fatty acid is exported from the chloroplast to the endoplasmatic reticulum (ER) where an acyl-transferase transfers the acyl group to a glycerol backbone forming mono-, di- and tri-acyl-glycerols. Di- and tri-acyl-glycerols (DAG and TAG) are stored in lipid bodies in the cytosol (Ohlrogge and Browse 1995). In addition Fan et al. (2011) showed that TAG synthesis in *Chlamydomonas* is localized in the chloroplast (Fig 1). The series of observations leading to this conclusion is the following: 1: Lidid bodies are found in close association or within the chloroplast as shown by electron microscopy on nitrogen starved *Chlamydomonas* cells. 2: TAG accumulation requires fatty acid synthesis in the chloroplast demonstrated by inhibition of chloroplast localized fatty acid synthase (FAS) by cerulinin. This inhibition of TAG synthesis can be overcome by the addition oleic acid to the media. And TAG synthesis is dependent on both light and acetate. 3: Upon nitrogen starvation the fatty acid composition of lipids from the membranes in both the chloroplast and ER does not change. TAG synthesized during nitrogen starvation in Chlamydomonas predominantly have C:16 fatty acids at the sn-2 position of the glycerol backbone as analysed by a lipase release assay of the fatty acids. This strongly suggests that TAG is made in the chloroplast as chloroplast membrane lipids are enriched in C:16 while ER lipids are enriched in C:18 fatty acids.

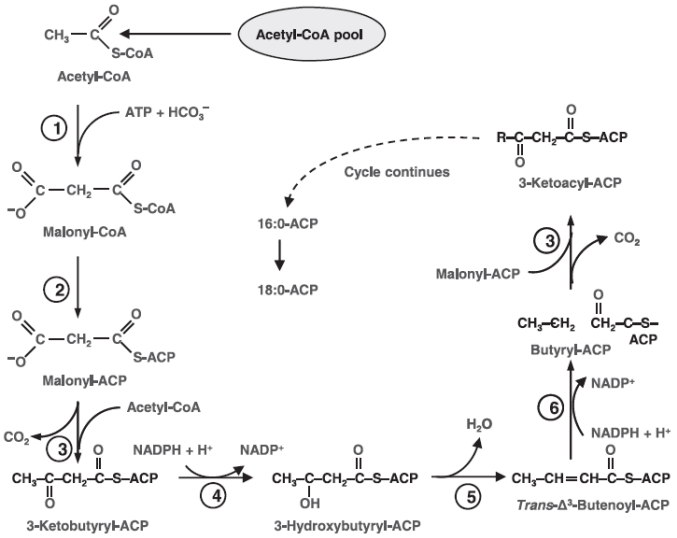

Fig. 1 Fatty acid de novo synthesis pathway in chloroplasts. From Hu et al. 2008

In *Chlamydomonas* and other microalgae the positioning of TAG in lipid bodies is induced by nitrogen limitation and the increase in TAG can be as high as 15 fold (Wang et al. 2009). In addition *Chlamydomonas* mainly synthesize starch during nitrogen limitation thus starchless mutants of *Chlamydomonas* is used to analyze lipid deposition and remobilization. During nitrogen stress starch is made in 5 fold higher quantitites than TAG in *C. reinhardtii*. Interestingly in the starchless mutants the level of TAG synthesis during nitrogen stress is comparable to the level in wildtype cells. The accumulated starch and TAG is readily remobilized when nitrogen is resupplied and the cells are kept in the dark (Siaut et al. 2011). These observations clearly indicate that starch and TAG served as storage compounds that enables the microalgal cell to recover from unfavorable conditions. When stressed for nitrogen the fatty acid composition changes in *Chlamydomonas*. The TAG synthesized contains considerably more 16:0 and 18:1 than the lipid from non-stressed cells where the most abundant fatty acids are 16:0, 16:4 and 18:3. Thus nitrogen stress both results in a change in lipid composition in favor of more TAG as well as a change in the fatty acid composition of the lipid (Fig 2) (Siaut et al. 2011). In addition TAG synthesis is coupled to the cell cycle of *Chlamydomonas reinhardtii*. Yao et al. (2012) found that temperature sensitive (ts) mutants arrested in cell cycle accumulated TAG upon shifting the cultures to the restrictive temperature. Basically

the ts-mutants displayed two lipid phenotypes. One phenotype accumulated more lipids and the other phenotype shifted the lipid distribution from polar to neutral as analyzed by lipid extraction.

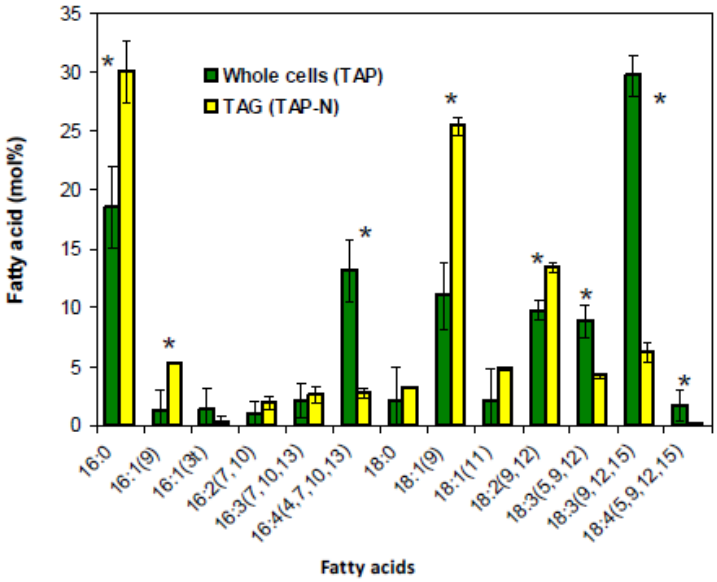

Fig. 2 Comparison of the fatty acid composition of the TAG fraction and total lipids. The TAG fraction was isolated from cells cultivated in TAP-N for 2 days while total lipids were from cells grown in TAP medium. From Siaut et al. 2011

4. Interactions between algae and bacteria

4.1. General bacteria-algal interactions

In aquatic systems a part of the food web is the interaction between phytoplankton and bacteria. In a classical sense the phytoplankton photosynthesizes and produce more phytoplankton. The plankton exude dissolved organic matter (DOM) and dissolved organic carbon (DOC) during growth and when the cells die they either sink to the bottom where they are digested or the cells lyse and are digested directly in the watercolumn by bacteria. The bacteria in return remineralize the nutrients, in particular phosphorus and nitrogen, incorporated in the algal DOM and and thus enables further phytoplankton growth (Fig. 3).

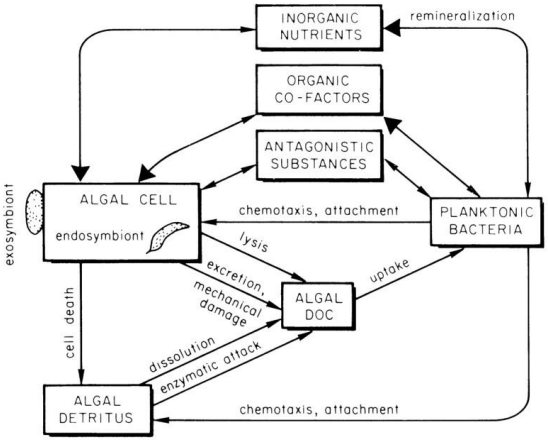

Fig. 3 A simplified diagram of some of the interactions that occur between algae and bacteria. Omitted from this diagram are a host of indirect interactions such as bacterial metabolism of chelators or allelopathic substances. The arrows with the large heads denote the predominant direction of a process. From Cole 1982

However the interaction between phytoplankton and bacteria seem to be more specific than just engaging in the overall ecology of the aquatic environment. As an example Danger et al. (2007) analysed the growth of both axenic and non-axenic *Scenedesmus ubliquus* cultures in full media and in phosphate limited media. In full media the presence of bacteria had no influence on *Scenedesmus* growth whereas in phosphate limited media a significant decrease in the growth of scenedesmus in the presence of bacteria was observed. This suggests that *Scenedesmus* and the bacteria compete for the limited source phosphate. It reasonable to assume that competition for other limiting nutrients takes place between phytoplankton and bacteria.

4.1.1. Dissolved organic carbon (matter)

Autotrophic phytoplankton fix carbon in the form of CO2 and in the calvin cycle of the photosynthesis organic carbon molecules are produced which is used for algal growth. Some of this carbon is eventually leaked into the water as dissolved organic matter (DOM) which is utilized by bacteria for growth. A specific source of carbon for the bacteria comes in the form of glycolate. Glycolate is also produced in the phytoplankton plastids when excess oxygen, produced during photosynthesis, forces the cells to enter photorespiration. The glycolate produced is released by the cells and certain bacteria which possess the glycolate utilization gene glcD are able to incorporate glycolate and use it for growth. Leboulanger et al. (1997) measured diel glycolate

concencentrations, by HPLC, and found that the concentration was correlated with phytoplankton production. High amounts of glycolate were measured during the day and low amounts during the night suggesting rapid bacterial uptake. In another study the genetic response to these diel variations of glycolate concentration was measured (Lau et al. 2007). It was shown that transcription of glcD was correlated with daily glycolate variations. By sequencing the glcD gene they were also able to follow the composition of the glycolate utilizing bacterial community during a phytoplankton bloom and found that some bacteria were only present during the onset of the bloom while others were present the whole time indicating indicating that the interactions are only opportunistic. Another source of carbon for the bacteria comes in the form of extracellular polymeric substances (EPS) which is exuded by especially diatoms. Studies reveal that as the EPS is released by the diatoms into the biofilm which they inhabit and the abundance of γ-proteobacteria increases suggesting that this response is specific within the bacterial community (Haynes et al. 2007). This specificity between diatom and bacteria is further substantiated in experiments done by Bruckner et al. (2011). They find that the secretion of EPS as well as the growth of several diatom species isolated from biofilms in Lake Constance is affected by the bacteria present. By co-culturing the diatoms with specific strains of bacteria, or by adding cell free bacterial growth media they measure differences in growth rate, depending on which bacteria is present, as well as differences in the amount of secreted EPS. A similar specific interaction between the pelagic diatom *Thalasisosira weissflogii* and associated bacteria is found by Gärdes et al. (2011). Bacteria that attach to the diatoms affect the synthesis of transparent exopolymer particles exuded (TEP) by the diatoms. TEP causes the diatoms to aggregate and form marine snow. The synthesis of TEP requires photosynthetically active diatom cells as well as interaction with specific bacteria. The interaction between *T. weisflogii* and the bactaria *Marinobacter* is mediated by chemotaxis (Sonnenschein et al. 2012).

4.2. Specific interactions: Iron and Siderophores

4.2.1. Iron in plants/phytoplankton

Iron is essential for all aerobic organisms like phytoplankton. It plays an important part in the photosynthetic apparatus, mitochondrial redox processes and in nitrate assimilation (Allen et al. 2008) and regulates light harvesting and photochemical conversion processes in algae like *Dunaliella* and *Phaeodactylum tricornutum* (Greene et al. 1992, Greene et al. 1991). In particular photosystem I (PSI) and ferredoxins which accepts electrons from PSI can be affected by iron

limitation (Richier et al. 2012, Jordan et al. 2001). Iron limitation induces chlorosis by reducing Chl a synthesis leading to inhibition of the formation of PSI- light harvesting complex (PSI-LHCl) resulting in to further reduction in photosynthetic capacity (Yadavalli et al. 2012) (Fig. 4).

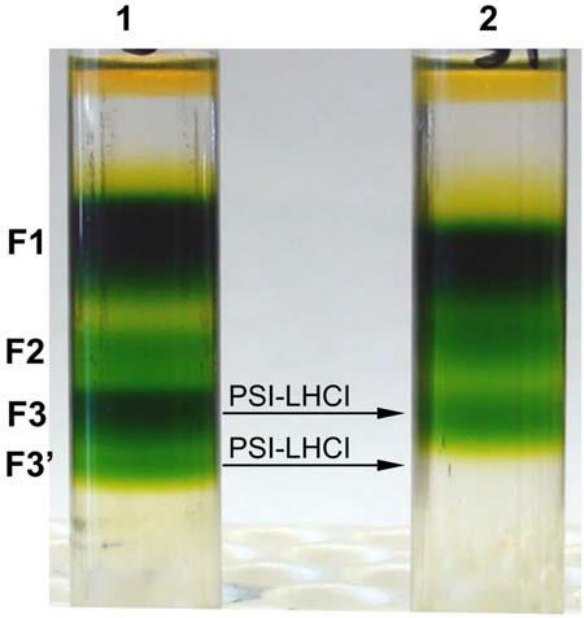

Fig. 4 Fractions of PSI-LHCI supercomplexes from solubilized thylakoid membranes by sucrose density centrifugation. F1 (LHCII), F2 (PSI-LHCI and PSII), F3 and F3' (Both contains PSI-LHCI supercomplexes). **Lane 1**- Control PSI-LHCI supercomplexes, and **Lane 2**-PSI-LHCI supercomplexes isolated from Fe deficient culture. From Yadavalli et al. 2012

Analyses of algal genomes reveal several putative uptake systems for Fe(II). One system found in *Chlamydomonas* and *P. tricornutum* is members of the ZIP family of zinc transporters composed of eight transmembrane regions. In both *Chlamydomonas* and *P. tricornutum* the transcript for the ZIP transporter is up-regulated under iron deficiency (Blaby-Haas and Merchant 2012, Allen et al. 2008). The ZIP family transport zinc and iron passively. In addition the NRAMP (natural resistance-associated macrophage protein) metal ion transporter family has been recognized in the *Chlamydomonas* and the *T. pseudonana* genome. In *T. pseudonana* the NRAMP is up-regulated during iron deficiency The NRAMP transporters contain 10 – 11 transmembrane domains. Interestingly the NRAMP is absent from the *P. tricornutum* genome (Kustka et al. 2007). The NRAMP family also transports manganese in a proton gradient dependent fashion.

In *T. pseudonana* Fe(III) is assimilated into cells by the aid of a multi-copper ferroxidase and two iron permeases. The ferroxidase oxidizes Fe(II) to Fe(III) which can enter the cell via the permeases. Transcripts are up-regulated during iron limitation (Morrissey and Bowler 2012, Kustka et al. 2007).

4.2.2. Iron in the marine environment

In the marine environment iron is often a limited resource, an estimated 30 – 40% of the world ocean have limiting concentrations of iron (Wells et al. 1995, Moore et al. 2012). Iron concentration in the world ocean is < 0.2 nM and on average 0.07 nM (Johnson et al. 1997). It has been demonstrated that the iron concentration can be as low as 20 – 30 pM, a concentration that will not support a large phytoplankton production (Martin et al. 1991). An extensive number of iron fertilization experiments show that iron is limiting to ocean phytoplankton growth and that addition of iron increases phytoplankton growth (Boyd et al. 2007). John Martin (1988) was among the first to point out that iron was a limiting factor for primary growth in the ocean and what is now known at the Iron Hypothesis speculates that adding wast amounts of soluble iron to the ocean will increase phytoplankton growth (Martin 1990). The phytoplankton will act as a CO2 sink and possible decrease atmospheric CO2 levels. However the idea of adding iron to the ocean in as scale that is large enough to decrease CO2 levels is not universally accepted. Among others Aumont & Bopp (2006) and Buesseler et al. (2008) point out several disadvantages. Most importantly is that the CO2 sequestering observed in mesoscale experiments is not sufficient to even sequester one third of the CO2 released to the atmosphere due to human activity. Also a broader assessment of the ecological impact of iron fertilization is needed.

4.2.3. Siderophores

In order to deal with iron limitation many microorganisms produce siderophores, iron chelating compunds. In the marine environment where the iron concentration as noted above is very often limiting to growth many bacteria produce siderophores. The siderophores produced by marine bacteria appear in two major forms. One containing citric acid and the other containing β-hydroxyaspartic acid (Fig. 5). Many of the siderophores are photoreactive when chelated to Fe(III) which under natural sunlight will be spilt form the siderophores-Fe(III) complex and released as Fe(II) (Amin et al. 2009, Butler 2005). Both free iron as well chelated iron can be assimilated by prokaryotes. The most common iron transporters are Fe(III) ATP-binding cassette transporters,

Fe(II) is assimilated via generalized metal 2+ transporters as the NRAMP family (Hopkinson and Barbeau 2012).

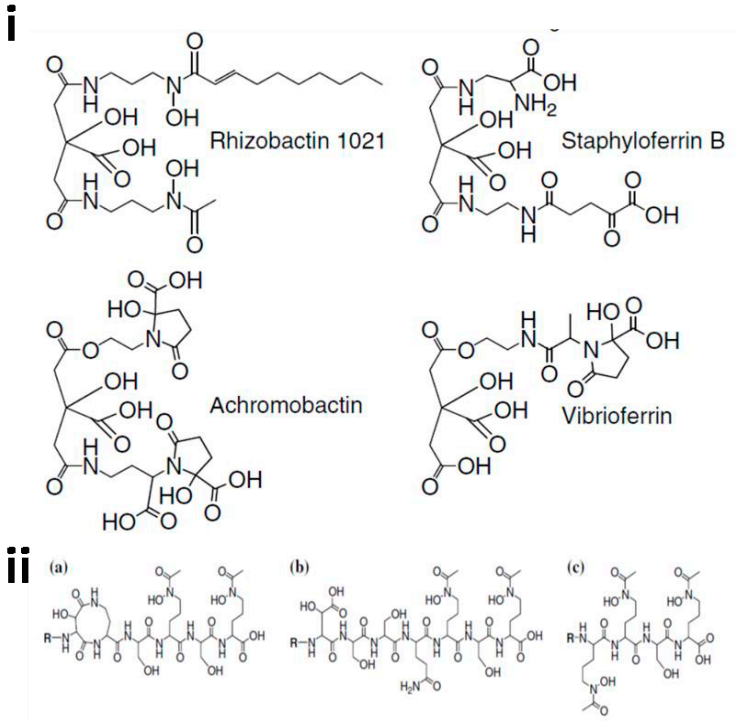

Fig. 5 Siderophores. **i)**Citric-acid-containing siderophores. **ii)** β- hydroxyaspartic acid containing siderophores. Modified from from Butler 2005

4.2.4. Algal/bacterial interactions

In nature very few siderophore have been isolated and identified (Gledhill and Buck 2012) but some investigations reveal a possible connection between bacterially produced siderophores and algal growth.

Keshtacher Liebson et al. (1995) showed that *Dunaliella bardawil* benefits from growing in association with bacteria under iron limiting conditions. They demonstrate that growth of *Dunaliella bardawil* is not affected by the presence of the bacteria when iron is abundant. In this case $2x10^{-6}$ M. However as the iron concentration in reduced the presence of bacteria in the algal

culture has a significant effect on the final cell numbers in the algal culture. At $5x10^{-6}$ M Fe the cell numbers in the xenic culture is almost 2-fold higher than in the axenic algal culture after two weeks of growth. When the iron content is further reduced, $5x10^{-6}$ M Fe, the effect of the bacteria on algal growth wears off (Fig. 6). They further demonstrate that an isolate of the halophile *Halomonas*, isolated from the *D. bardawil* culture, is benficial to the growth of *D. bardawil* under iron limited conditions. The effect of co-culturing with *Halomonas* compared to axenic culturing is very similar to the results from the xenic/axenic experiment. Furthermore they show that *Halomonas*, when grown in co-culture with *D. bardawil*, release a siderophores and speculate that this siderophores is involved in the growth enhancement of *D. bardawil*.

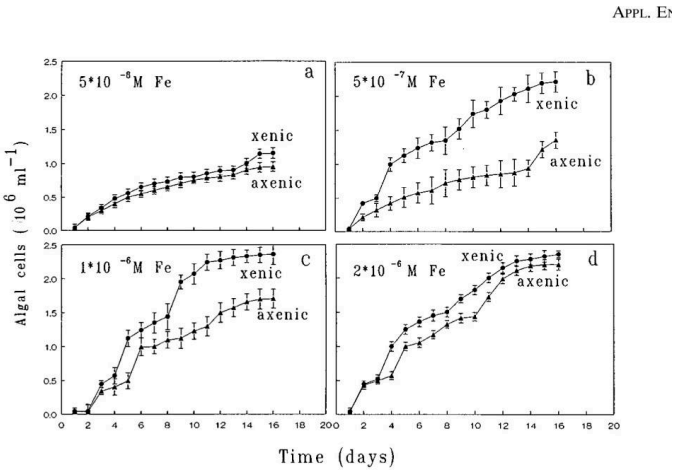

Fig. 6 Effects of Fe concentration and bacterial coculture on growth of *D. bardawil* under axenic and xenic conditions. From Keshtacher-Liebson et al. 1995

Amin et al. (2009) then demonstrated that several isolates of the bacterium *Marinobacter* produce the photoactive siderophore vibrioferrin, a member of the citric-acid siderophore class. The vibrioferrin producers are restricted to two subclades in their 16s rRNA phylogeny and all producers of vibrioferrin are associated with algae. In iron uptake experiments, where Fe(III) and vibrioferrin is added to the growth media, with the vibrioferrin producing *Marinobacter* DG879 as well as the dinoflagellate *Scrippsiella trochoidea* they demonstrate that iron uptake in both the bacterium and the algae is promoted in the presence of sunlight. Thereby they show that vibrioferrin, which releases the chelated iron molecule under photolysis, is involved in making iron available to both bacteria and algae. In a growth experiment where *Marinobacter* DG879 is added

to a culture of *S. trochoidea* and compared to an axenic culture they then showed that growth rate as well as the final cell numbers of *S. trochoidea* are greater in the co-culture (Fig. 7).

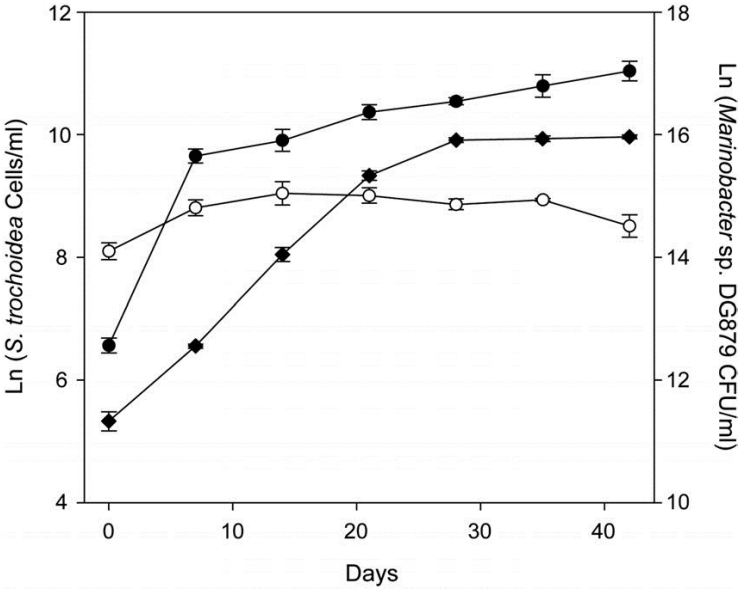

Fig. 7 Growth pattern of a binary culture of the bloom-forming *S. trochoidea* and an associated VF-producing *Marinobacter* strain. Growth of *S. trochoidea* (filled diamonds) in the presence of Marinobacter sp. (closed circles) is shown alongside *Marinobacter* sp. growing alone (open circles). From Amin et al. 2009

Over the course of a bloom of *Lingulodinium polyedrum* at the Californian pacific coast Yarimizu et al.(2014) investigated the presence of siderophore producing bacteria with the aid of quantitative-PCR. Primers for these genes were designed in a previous study to quantify the presence of siderophore producing bacteria in the North-Atlantic between Iceland and Scotland (Gärdes et al. 2013). By comparing the number of vibrioferrin and petrobaction producing bacteria, detected by presence of the genes psvB and asbE, to the total bacterial numbers they make several conclusions. First the most abundant siderophore gene is asbE involved in petrobactin synthesis. In contrast Yarimizu study (2014) found that vibrioferrin producing bacteria are the more abundant. The total number of bacteria, as detected by 16s rRNA primers, as well as the number of siderophore producers increase during the *L. polyedrum* bloom and the propotion of siderophore producers increases in the population of bacteria. They suggest that the siderophore biosynthetic capacity of

the bacteria could provide a source of available iron for the dinoflagellate during the bloom. However no direct evidence for the involvement of iron in the bloom was provided

4.3. Specific interactions: Vitamins

4.3.1. Vitamins and vitamin auxotrophy in algae

In phytoplankton, as in other organisms, vitamins play an important part in the metabolism. Both vitamin B_{12} (cobalamin) and B_1 (thiamine) have been shown to be important for phytoplankton growth (Fig 8) (Tang et al. 2010, Croft et al. 2005). Tang et al (2010) investigated 27 species of phytoplankton involved in Harmful Algal Blooms (HAB's) by comparing the growth in vitamin free and complete media. The growth media contained antibiotics to prevent bacterial growth. A culture that ceased to grow in absence of vitamins and resumed growth when the vitamin was added back to the media was judged to be vitamin dependent. The outcome of these analyses was that 26 species required B12, 20 species required B1 and 10 species required B7 (biotin).

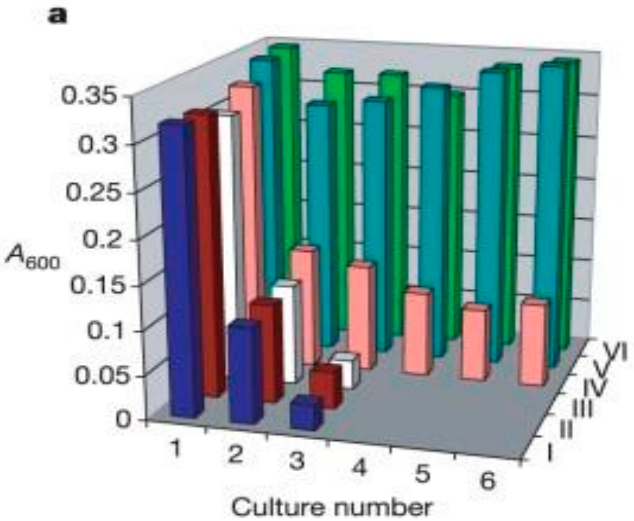

Fig. 8 Effect of vitamin B_{12} deprivation on the green alga *L. rostrata*. a, Growth of *L. rostrata* in Jaworski's medium for six subcultures. I, no supplements; II, 10 mM methionine; III, 1 µM folate; IV , 1 µM folate and 10 mM methionine; V, 10 µg l^{-1} vitamin B_{12} , 1 µM folate and 10 mM methionine; VI, 10 µg l^{-1} vitamin B_{12} . The values represent the optical density of the cultures measured at 600 nm (A600) after 7 days in continuous light. From Croft et al. 2005

Croft et al. (2005) compiled from literature a survey of 326 species of phytoplankton of which 171 species were reported to be B12 auxotrophs. In addition representatives a number of species from each phylum were retested in minimal growth media for B12 dependency. I each case the B12 requirement were as reported in the literature. In a later review Croft et al. (2006), in addition to the B12 requirement, also investigated the requirement for B1 and B7. From literature they found that out of 306 species 61 required B1 and 14 B7. All the species that required biotin also required thiamine and/or cobalamin.

Thus it appears that a substantial number of phytoplankton species requires B vitamins for growth

In the algal metabolism B1 most importantly acts as a co-factor for some of the enzymes in the citric acid cycle. It is also involved in the biosynthesis of branched-chaain amino acids (valine, isoleucine and leucine) (Bertrand and Allen 2012). All enzymes involved in the biosynthesis of B1 in phytoplankton have not been characterized. Therefore it is difficult to establish whether B1 auxotrophy is due to the lack of a single enzyme /pathway or the absence of the genes for the entire pathway. For instance Tang et al. (2010) observed that two strains of *S. trochoidea* sampled from the same location were either B1 prototroph or auxotroph.

Vitamin B12 is a co-factor in the methionine pathway. In phytoplankton two pathways for the synthesis of methionine exist. A B12 dependent methionine synthase (METH) and a B12 independent methionine synthase (METE). It appears that METE is absent from algae that are B12 dependent for growth and that METE is present in algae that are B12 independent. Higher plants have no apparent B12 dependent enzymes (Helliwell et al. 2011). By comparing 15 sequenced algal genomes and B12 dependent growth a strong correlation between the absence of a functional METE gene and B12 auxotrophy was found (Helliwell et al. 2011).

4.3.2. Vitamin experiments

Vitamin B12 in exclusively synthesized by prokaryotic organisms (Warren et al. 2002) and thus provide the vitamin to B12 dependent phytoplankton. Experiments have shown that only some B12 producing bacteria are able to support phytoplankton growth. Kazamia et al. (2012) added to an axenic culture of *Lobomonas rostrata* three different rhizobial strains. Only one of these, *Mesorhizobium loti*, was able to sustain algal growth in an extended sub-culture system (Fig. 9). Co-culture growth experiments in which the ratio of algae to bacteria was varied from 10^{-6} to 10^{3} showed a degree of regulation since the ratio in all co-cultures stabilized around 10^{-2} to 10^{-1}. If B12

was added to such a stabilized co-culture the ratio dropped to 10^{-5} while addition of glycerol increased the ratio to 1. This suggests that the interaction between algae and bacteria is facultative. Growth experiments with the bacteria growing inside a dialysis-tube and the algae outside showed that the effect of the bacteria on algal growth was due to a small diffusible molecule. The effect of the bacteria on alga growth can be mimicked by the addition of vitamin B12 to media suggesting that the diffusible molecule could be B12. This was substantiated by quantifying METE gene expression in *Chlamydomonas*. In the presence of B12 or a B12 producing *M. loti* strain hardly any METE expression could be detected. In the absence of B12 or non-growing bacteria the METE expression was detected. In a later paper a mathematical model was developed describing the dependence between the algae and bacteria. The model propose that *M. loti* regulates its level of B12 synthesis resulting in a mutualistic interaction with the algae (Grant et al. 2014)

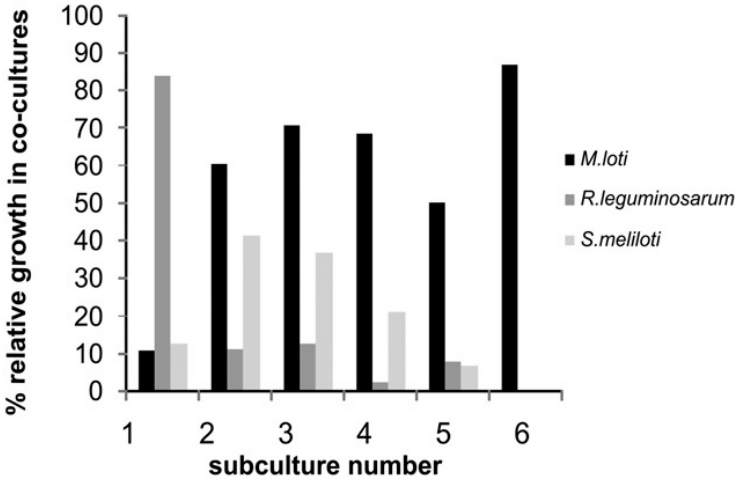

Fig. 9 Growth of *Lobomonas rostrata* in co-culture with rhizobial strains. The B12-dependent green alga *L. rostrata* was maintained in co-cultures in autotrophic medium with three different sequenced rhizobial strains: *M. loti* MAFF303099 (black bars), *R. leguminosarum* bv viciae 3841 (dark grey), and *S. meliloti* RM 1021 (light grey). After six subcultures only the cultures with *M. loti* had viable algal cells, and in fact could be propagated indefinitely. From Kazamia et al. 2012

Another insight into the symbiosis between phytoplankton and vitamin producing bacteria is provided by Xie et al. (2013). They find that the METE gene expression in *Chlamydomonas reinhardtii* is reduced by heat stress and thus if no B12 is available the cell will ultimately die (Fig.

10). However when the heat stressed *C. reinhardtii* culture is supplied with B12, either from adding B12 to the growth media or by co-culturing with B12 producing bacteria the METH expression is activated. In conclusion they argue that by retaining both the METH and METE synthases *C. reinhardtii* has as an advantage over species that only has one of the genes.

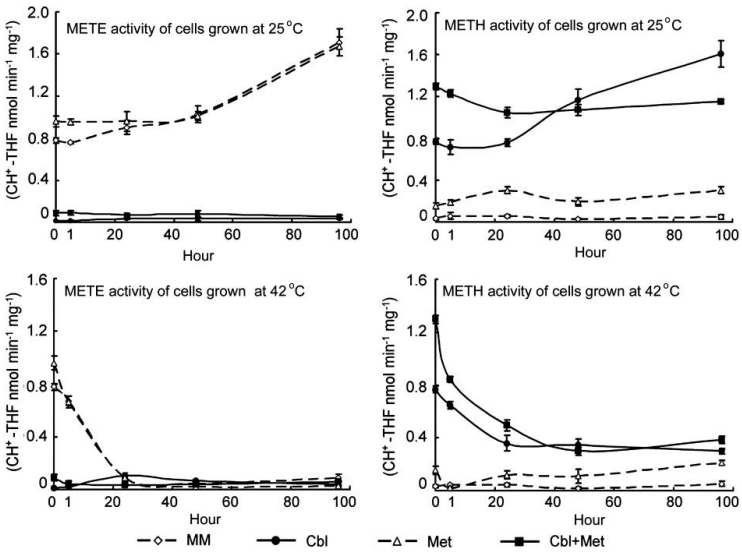

Fig. 10 Effect of temperature on *C. reinhardtii* methionine synthase activity. METE and METH activity of *C. reinhardtii* cells grown in the absence (MM) or presence of cyanocobalamin (Cbl; 1 mg l^{-1}), methionine (Met; 0.2 mg ml^{-1}) or both amendments (Cbl + Met).Cultures were grown for 3 days at 25 °C before a 42 °C temperature upshift, except the controls were maintained at 25 °C. Crude cell extracts were used in the methionine synthase activity assays and all assays were conducted at 37 °C for 45 min. From Xie et al. 2013

4.3.3. *Vitamins in the sea*

While small scale experiments like those describe above clearly show that the growth and fitness of some phytoplankton species can be improved by vitamin producing bacteria it not entirely clear or agreed upon how important these interactions are on a larger scale. The concentration of B-vitamins in a large costal region of the pacific was measured at various depths and found to be very low. Sañudo-Wilhelmy et al. (2012) therefore speculate that this concentration is so low that it may regulate the composition and growth of phytoplankton in the sea. The opposite view of on this comes from Droop (2007) who by extensively review od the available literature reached the conclusion that the phytoplankton requirement for vitamins are overestimated and that the amount

of vitamins in the sea are hight enough to support phytoplankton growth. He the therefore contests the findings by Croft et al. (2005) that 25% of all primary production takes place as a result of a symbiosis between phytoplankton an B12 producing bacteria.

4.4. Specific interaction: Roseobacters

Investigations of marine bacterioplankton communities often show a substancial presence of bacteria from the roseobactor-clade. Bacteria in the roseobacter-clade are often associated with blooms of marine phytoplankton (Buchan et al. 2005). The reason behind this association could be that roseobacters are able to degrade the algal metabolite dimethylsulfoniopropionate (DMSP) (Yoch 2002). DMSP is used by roseobacters as a carbon and sulfur source. DMSP is a precursor of dimethyl sulfide (DMS) which is an important greenhouse gas. Therefore it is important elucidate the biosynthesis of DMSP by the algae and its breakdown by the marine bacteria. DMSP is produced by a variety of algae but most importantly in the prymnesiophytesis and the dinophytes. DMSP concentration in phytoplankton cells increases with increasing salt concentration in the media (Kirst 1990) suggesting that DMSP acts as an osmolyte. Sunda et al (2002) provide evidence that DMSP and its breakdown products scavenge reactive oxygen species and thus may also act as an antioxidant. However little is still known about the regulation of the pathways involved in DMSP synthesis. Kettles et al. (2014) analysed by enzyme activity, gene expression and proteome analysis the assimilation of sulfur during increased salinity, increased light and nitrogen starvation, conditions known to induce DMSP synthesis. Interestingly increased sulfur assimilation is not required for DMSP synthesis (Fig. 11).

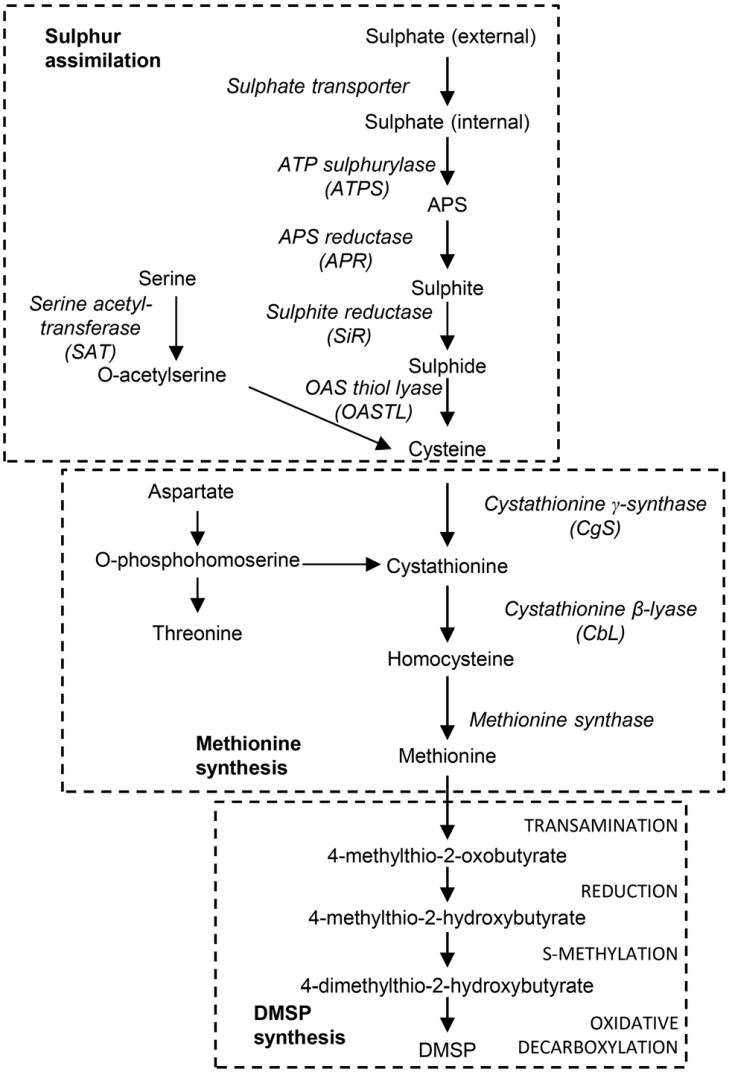

Fig. 11 A proposed pathway of sulphate assimilation and DMSP biosynthesis in algae. Enzymes between the methionine and DMSP have not yet been identified. Putative reactions are shown in capitals. From Kettles et al. 2014

Chemotaxis allows roseobacters to colonize the surface of phytoplankton where it sheds its flagella to become immotile. Upon coloninzing the surface the rosoebacter has acces to both organic material and DMSP. In return the bacteria produce growth promoting compounds like auxins, siderophores and vitamins (Seyedsayamdost et al. 2011). One key element in the tight association between phytoplankton and roseobacters is the bacterial synthesis of tropodithietic acid (TDA).

TDA induces the sessile stage of the roseobacters,which facilitates the colonization of the phytoplankton, and it also acts as an antibiotic that keeps other bacteria away from the host (Geng et al. 2008). As an example the bacteria *Silibacter* sp. TM1040 is able to form large lumps of cells when grown without shaking while its mainly single cells if the culture is shaken. Silibacter also produces a compound that is able to inhibit the growth of a number of marine bacteria (Bruhn et al. 2007). Later studies of the same *Silibacter* strain showed that the antibiotic compound is TDA (Geng et al. 2008). Geng and Belas (2010) founds that TDA acts both as a quorum-signaling molecule and an autoinducer and thus stimulates further colonization of the algal cells. Interestingly it has been shown that as the algal bloom ages and the cells start to break down the colonizing roseobacters starts to produce a variety of algaecides, collectively known as roseobacticides, that causes further breakdown of the algal cells and thus disrupting the mutualistic symbiosis. When the algal cells start to die the algal lignin breaks down and the breakdown products triggers the production of the roseobacticides. In a dose-response assay Seyedsayamdost et al. (2011) found that the production of roseobacticises varied with the species as well as concentration of the different algal lignin breakdown products. The breakdown product ρ-coumaric acid (ρCA) triggers the production of Roseobacter Motility Inducer (RMI), a roseobacticide, which causes loss of motility as well as cell enlargement of the algal cells but as the name implies it also induces the production of flagella associated proteins in the bacterial cells and thus restores motility in the sessile bacterial cells which lets the bacteria colonize new phytoplankton (Fig. 12) (Sule and Belas 2013).

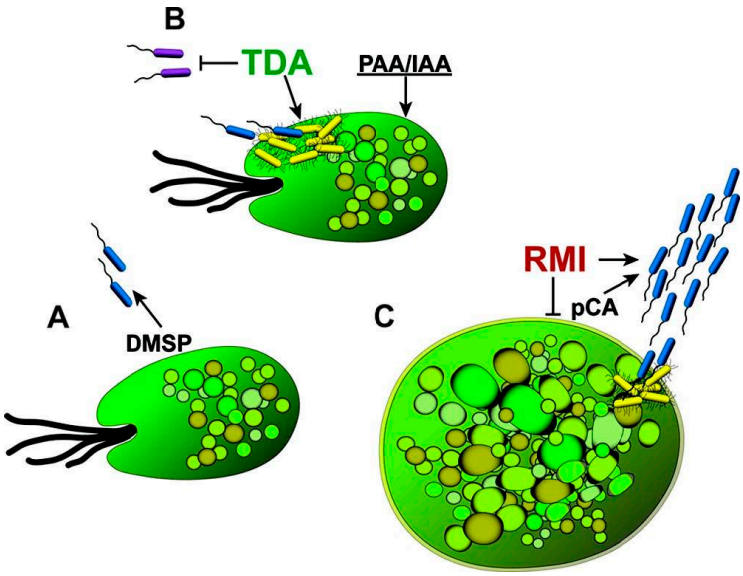

Fig. 12 RMI (Roseobacter Motility Inducer) and TDA quorum signaling molecule (tropodithietic acid): the lingua franca of the roseobacter-algal symbiosis? Shown is a model describing the roles of TDA and RMI in the roseobacter-algal symbiosis. (**A**) Roseobacters in their motile phase (blue bacteria) swim toward algae, directed by chemotaxis to attractants such as DMSP. DMSP is metabolized by the bacteria, which use its sulfur for synthesis of bioactive compounds, including TDA (and most likely roseobacticides). Proximity to the host and TDA itself induces the sessile phase (yellow bacteria). (**B**) TDA acts as a quorum signal to induce its own synthesis and initiate sessile phase in other roseobacters. TDA also acts as an antibiotic, inhibiting nonroseobacters (purple bacteria). As the roseobacter biofilm develops and matures on the algal surface, PAA, IAA, and other algal growth-promoting compounds are synthesized by the bacteria. (**C**) As the symbiosis matures, algal cell death increases, resulting in an increase in lignin and its breakdown products, including pCA, which induces RMI synthesis. RMI flips the biphasic switch toward the motile phase, breaking the symbiosis, which is further harmed by the algicidal action of RMI. The swim-or-stick switch is reset, permitting the roseobacters to renew its symbiosis with a fresh algal host. From Sule and Belas 2013

On a further note it has been shown that TDA is biosynthesized from phenylacetic acid (PAA) (Geng et al. 2008) and PAA is a known growth promoting hormone in higher plants (Wightman and Lighty 1982). As one example in the literature of a plant hormones effect on microalgal growth *Chlorella vulgaris* was immobilized and grown in alginate beads together with *Azospirillum* bacteria which synthesize indoelacetic acid (IAA) or with IAA deficient *Azospirillum* mutants. These analyses showed a significant effect of IAA on *Chlorella vulgaris* growth (de-Bashan et al. 2008). However there is little evidence that PAA, IAA and other plant hormone-like molecules play an important part in the relationship between bacteria and aquatic phytoplankton.

5. Conclusions

It is important to note that the experiments performed during this Ph.d. study were lab-scale model experiments. Taking conclusions from the following chapters and applying them to full scale production plants may not be possible.

However from the experiments conducted it can be concluded that bacteria do benefit the growth of *Dunaliella salina* under certain conditions (Chapter 2). Experiments to further elucidate the exact mechanisms behind this algal /bacterial interactions could of course be interesting but it the co-culture were to be tested on a larger scale it might be sufficient to know what bacteria are beneficial.

Interestingly the experiments with the locally isolated (Chapter 3) show that closely related algal strains react very differently to being co-cultured with bacteria. If local algal strains should be utilized in biomass /biofuel production this shows the importance of selecting the right algal strain as well as conducting experiments to see how this strain is affected by endemic bacteria.

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Chapter 2

Halomonas, *Marinobacter* and *Pelagibaca*, a novel symbiont, improve growth of *Dunaliella salina* under iron limited conditions

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Abstract

Many studies have shown that algae benefit from growing symbiotically with bacteria. Understanding of the mechanisms behind this co-existence may contribute to optimize algal biomass formation for production of healthcare, pharmaceuticals, biodiesel and other microalgae based products. The present work is a study on interactions between a strain of *Dunaliella salina* obtained from a culture collection and three marine bacteria isolated from the culture. By sequence analysis of 16s rRNA encoding genes three bacterial genera were identified: *Pelagibaca, Halomonas and Marinobacter*. Of the three genera *Pelagibaca* is previously undescribed as a growth promoting symbiont whereas the other two, *Halomonas* and *Marinobacter* are commonly found in association with microalgae. Growth experiments showed that all three bacterial strains promoted growth of *D. salina* under iron limited conditions. Physical contact between algae and bacteria was not required for the growth promoting effect. All three bacterial strains produced siderophores, molecules that facilitate iron uptake, which may explain the growth promoting effect.

Keywords

Microalga; *Dunaliella salina*; *Halomonas*; *Marinobacter*; *Pelagibaca*; Symbiosis; Growth promotion

1. Introduction

Microalgal biomass has a vast area of applications, from biodiesel and phycoremediation to animal feed and healthcare products (Spolaore et al. 2006, Milledge 2011, Delrue et al. 2012). Some of the products, such as β-carotene and astaxanthin (Fon Sing et al. 2013) are commercially available while others are still being researched. To bring these products out of the lab it is crucial that, besides demand, that production cost is kept low. One part of this is the ability to produce large amounts of biomass with the smallest amount of resources (nutrients, water, cooling/heating etc.) possible. When growing algae in outdoor raceway ponds, which have lower operational costs than photobioreactors (Delrue et al. 2012, Jorquera et al. 2010), it is impossible to keep the cultures axenic. It is however known that many algae benefit from living symbiotically with bacteria (Cole 1982). Algal bacterial interactions can lead to increasing growth rates of the algae as well as maximizing final cell numbers, i.e. biomass (Keshtacher-Liebson et al. 1995, Amin et al. 2009, Croft et al. 2005). Not all of the mechanisms behind this growth promoting effect of bacteria are known. Vitamins (Croft et al. 2005, Kazamia et al. 2012) and siderophores (Amin et al. 2009) are two components produced by the bacteria that promote algal growth. Vitamin B12 (Croft et al. 2006, Xie et al. 2013, Sañudo-Wilhelmy et al. 2014) is a cofactor in methionine synthesis while siderophores are iron chelating molecules which facilitate iron uptake in both the bacteria as well as the algae (Soria-Dengg et al. 2001, Amin et al. 2012). Bacterial symbionts that enhance either growth rates or final cell numbers could become very important in large scale production of microalgal biomass as these will both improve fitness of the culture as well as lower nutrient requirement and thereby overall production cost. Another benefit could be that a healthy balanced population of bacteria and microalgae may be better protected against unwanted microbial contaminants.

The aim of this study was to find bacteria that effected the growth of *Dunaliella salina*, a chlorophyte which holds promise as a potential feedstock for biodiesel production and which is already used in the commercial production of β-carotene (Fon Sing et al. 2013). We isolated three different bacteria from a *D. salina* culture and purified the algae. We then tested the effect of the

individual bacteria on algal growth in lab scale model experiments and propose siderophores as a possible mechanism leading to the enhancing effect of the bacteria on algal growth.

2. Materials and methods

2.1. Strain, isolation and media

Dunaliella salina, strain CCAP 19/30 was obtained from *Culture Collection of Algae and Protozoans*, Scotland. This isolate was non-axenic and contained a variety of bacteria. In the following this isolate is designated as the *primary culture*. To obtain an axenic microalgal culture the primary culture were grown in SG medium (Sager and Granick 1953) with 3% NaCl (SG 3%).. Cell from the exponential phase were harvested by centrifugation at 700 g for 5 min. The pellet was resuspended in fresh SG 3%. This was repeated 3 times. The next day antibiotics were added to the culture to obtain the following concentrations: Penicillin 200 $mg*l^{-1}$, Streptomycin 100 $mg*l^{-1}$, Chloramphenicol 20 $mg*l^{-1}$. After 7 days of growth the culture was visually checked for bacteria by phase contrast microscopy. The culture was diluted and plated on SG +3% agar plates with the same concentrations of antibiotics as previously mentioned. When colonies of *D. salina* cells appeared they were picked, resuspended in SG 3%, visually checked for bacteria and again plated on antibiotic containing media. Colonies appearing on these plates were picked and resuspended in SG 3% media. They were again visually checked for bacteria by microscopy but also with DAPI staining and fluorescence microscopy using the protocol of Croft (2005). Axenity was also validated by *PCR* (See below for *PCR* details). The axenic cultures were kept for further use in liquid SG 3% media.

Bacteria from the primary *D. salina* 19/30 culture were cultivated on modified Marine Agar (Zobell 1941) plates (MAR). Modifications were a higher NaCl concentration, 24 $g*l^{-1}$ instead of 19.45 $g*l^{-1}$, and using $FePO_4$ 1 $g*l^{-1}$ instead of ferric citrate. To obtain individual isolates of bacteria, colonies were picked and continuously restreaked onto new plates. When the bacterial isolates were deemed pure, colonies were picked and suspended in the modified MAR media, agar omitted, containing 20% glycerol and kept at -80 °C.

2.2. Identification, PCR and sequencing

To identify the isolated bacteria, genomic DNA was extracted with the Genomic Mini kit (A&A Biotechnology, Gdynia, Poland). Afterwards the 16s rRNA encoding genes was amplified by *PCR* and subsequently sequenced (Eurofins MWG Operon, Ebersberg, Germany). Gel electrophoresis

was done in 1% agarose gels using Gelred to stain the DNA. GeneRuler DNA ladder mix (Thermo Scientific, Waltham, MA, United States) was used as size marker. The *PCR* reactions were with the primers fD1 (5'-GAGTTTGATCCTGGCTCAG- 3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') (Weisburg et al. 1991) based on *E. coli* positions 8–27 and 1512-1492 respectively and the following cycle: initialization: 95 °C 4 min, 30 x (denaturation 95 °C 30 sec, annealing 58 °C 30 sec, elongation 72 °C 2 min) and final elongation 72 °C 7 min. The primers were also used for sequencing. To identify the bacteria the obtained sequences were compared to known sequences in Genbank using the BLAST algorithm (Altschul et al. 1990). The most probable identity of the isolated bacteria was then determined by the known organism with the highest homology in the BLAST analysis.

2.3. Growth experiments

The algal or mixed algal-bacterial cultures were grown in T25 flasks (SARSTEDT AG & Co, Nümbrecht, Germany) containing 7.5 ml of culture at 22 °C under 140 µmole*m^{-2}*s^{-1} on an orbital shaker (80 rpm) in a 14/10 light/dark cycle. Algal cells were counted on a Cell Lab Quanta SC Flow Cytometer (Beckman Coulter, Brea, CA, United States). To make the mixed cultures 20 µl of bacterial culture, grown in liquid MAR, were added to 7.5 ml of axenic algal culture. CFU counts of the three bacteria were done to enumerate the number of bacteria added to the algal cultures.

Bacteria were also grown in a Visking Dialysis Membrane (MEDICELL INT. LTD, London, UK) with a 12 – 14 kDa cut-off. The tube was prepared according to the manufacturer and autoclaved before use.

All culturing experiments were done in triplicate.

2.4. Chromeazurol S (CAS) assay

A modified version of the universal CAS assay (Schwyn and Neilands 1987) was performed according to Pérez-Miranda *et al*. (Pérez-Miranda et al. 2007) to test if the isolated bacteria produced siderophores. For this an aqueous solution of peptone and yeast extract was depleted for iron by extracting with 3% 8-hydroxyquinoline in chloroform before being used in the preparation of the modified MAR medium. $FePO_4$ was also omitted from the media to remove any trace iron that could interfere with the assay. Bacteria were grown on the iron depleted MAR plates for 2 days

at 25 °C. A 0.7 % agar overlay containing the CAS was then applied to the plates and the de-colorations was observed.

2.5. TEM (Transmission Electron Microscopy)

In order to determine if bacteria were physically associated with *D. salina*, either as endosymbionts or attached to the surface of the algae, transmission electron micrographs were made of microalgae cultures. The algal cultures were mixed 1:1 with 4% glutaraldehyde in 0.2 M cacodylate buffer containing 4% M sucrose. After 1 h at 4 °C the cells were rinsed 3*30 min in cacodylate buffer with decreasing sucrose content. After rinsing the cells were post-fixed for 1 h in 2% osmium tetroxide in 0.2 M cacodylate buffer. Before dehydration the material was rinsed in buffer. Each dehydration step lasted 20 min at 4 °C in the following EtOH concentrations: 30, 50, 70, 90, and 96%. Dehydration was completed in 2 changes in absolute EtOH with molecular sieves (100%) each lasting 20 min. Following two rinses in propylene oxide the material was transferred to a 1:1 mixture of Spurr's embedding mixture (Sigma-Aldrich, St. Louis, MO, US) and propylene oxide and left uncovered overnight. This was followed by 6 h in a fresh Spurr mixture before embedding in another change of Spurr. The resin was polymerized overnight at 70 °C. Sectioning was carried out on a Reichert Ultracut E ultramicrotome (Leica Microsystems, Wetzlar, Germany) using a diamond knife. The sections were collected on slot grids (Rowley and Moran 1975) stained in uranyl acetate and lead citrate. The grids were examined in a JEM-1010 electron microscope (Jeol Ltd., Tokyo, Japan), fitted with a Gatan 792 digital camera (Gatan Inc., Pleasanton, CA, US) at the Biological Institute, University of Copenhagen.

3. Results/Discussion

3.1. Isolation of algae and bacteria

Many algal cultures obtained from culture collections are not axenic. The algal isolate often contains one or more species of bacteria. Many of these bacteria, at least those described, are often of the same genera as those found in natural algal environments. This leads to the assumption that they were co-collected with the algae.

Bacteria are present in large number in the *D. salina* CCAP 19/30 culture as observed under the light microscope. An axenic isolate of *D. salina* was obtained from the primary culture by repeating re-streaking as described in materials and methods. Axenity was also verified with PCR control reactions on DNA from the purified algal culture that revealed no bacterial DNA.

To isolate the bacteria an aliquot of the culture was spread on MAR plates and three different types of colonies appeared. Other bacteria could in principle be present in very low numbers and would therefore not be detected on these plates. Thus a selection was already applied and it may be that not all the different species of bacteria present in the primary culture was detected in this study.

3.2. Identification of Bacteria

From the three isolates of bacteria the 16s rRNA encoding genes were amplified and sequenced. The three types of bacteria were identified as *Halomonas* sp., *Marinobacter* sp. and *Pelagibaca* sp. by comparing them to known sequences in Genbank using the BLAST algorithm. *Pelagibaca* sp. showed 81% sequence identity with both *Halomonas* sp and *Marinobacter* sp. *Halomonas* sp. and *Marinobacter* sp. were 89% identical. The sequences are available in Genbank at accession numbers KJ573106, KJ573107 and KJ573108.

3.3. Growth of Dunaliella salina with or without bacteria

3.3.1. *Primary vs. axenic cultures*

When grown in standard SG 3% media ($FeCl_3$: $3.7*10^{-5}$ M) the final cell number of a primary and an axenic culture were the same (data not provided). Keshtacher-Liebson et al. (1995) showed that in media with limiting iron concentrations *Halomonas* sp. promoted the growth of *Dunaliella bardawil*. This observation led us to analyze the growth of *D. salina* in SG 3% media with varying low iron concentrations. The $FeCl_3$ concentrations were 2 µM, 1 µM, 0.37 µM and 0.037 µM. Initial *D. salina* cell concentration were 80.000 cells $*ml^{-1}$. When grown in iron limited media there was a 2-fold difference in cell densities after 5 days (Fig. 1). The primary cultures continued to grow for a long time and though the growth eventually slowed down stationary phase was not reached until day 19. Final cell numbers reached $50*10^6$ cells $*ml^{-1}$. Axenic cultures reached maximum cell concentration of $2.5*10^6$ cells $*ml^{-1}$ after 5 days and quickly died. After day 9 it was no longer possible to determine cell numbers in the axenic cultures as they had died off. Based on this experiment the iron concentration of the SG 3% media was chosen to be $2*10^{-6}$ M and all further experiments were done with this concentration. Similar positive effect on the growth of axenic cultures has been reported. Park et al. (2008) shows that adding bacteria to axenic cultures of *Chlorella ellisoidea* results in higher final cell numbers.

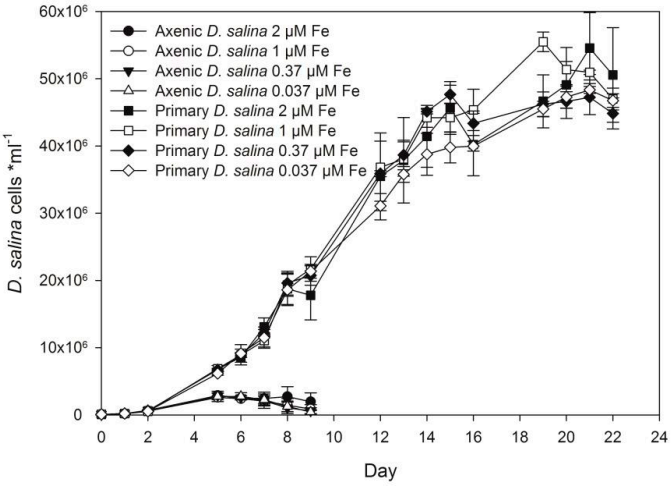

Fig. 1 Bacterial enhanced growth of *D. salina* under iron limited conditions. Growth of axenic and primary *D. salina* cultures in SG +3% with different $FeCl_3$ concentrations. Circles and triangles = axenic cultures, squares and diamonds = primary cultures. Error bars represent standard deviation. All cultures were grown in triplicate

3.3.2. *Growth of Dunaliella salina in co-culture with Halomonas, Marinobacter and Pelagibaca*

The growth of axenic and primary *D. salina* cultures was compared to the growth of *D. salina* in co-culture with either one of the three bacterial isolates *Halomonas* sp., *Marinobacter* sp. and *Pelagibaca* sp.

For this, axenic cultures were inoculated with a fixed volume (20 µl) of outgrown bacterial culture. As the volume of bacterial media added to the axenic algal cultures was very small the influence of this media on the algal growth was deemed negligible. I.e. the amount of Fe added to the algal cultures were only $9*10^{-11}$ moles. This raised the molarity of the cultures by less than 0.02 µM which as seen in Fig. 1 has no effect. The amounts of bacteria added as determined by CFU counts were. *Halomonas* sp.: $7*10^6$, *Marinobacter* sp.: $96*10^6$, *Pelagibaca* sp.: $480*10^6$. *Halomonas* sp. however grew in chains and the CFU counts will not reflect the actual number of cells. Marinobacter sp. and *Pelagibaca* sp. were both very small cells. Axenic *D. salina* co-cultured with a bacterium reached 6-fold higher cell densities than when grown without bacteria (Fig. 2). The axenic culture entered stationary growth phase after one week with maximum cell densities around

6 *10^6 cells *ml^{-1}. Co-cultures and primary cultures entered stationary growth phase after about 2 weeks and reached a maximum cell density around 30 *10^6 cells *ml^{-1}. The flow cytometer was also used to measure the size of the cells. The surface area of the cells varied between 180 μm^2 and 260 μm^2 with no correlation between cell size and culture conditions.

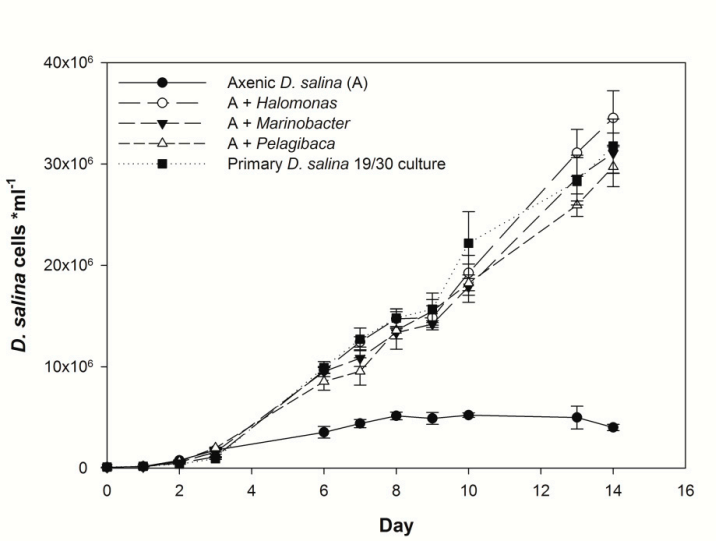

Fig. 2 Effect of co-culturing *D. salina* with a single bacterial isolate. *Halomonas* sp., *Marinobacter* sp. *and Pelagibaca* sp. shows similar growth stimulating effect. Filled circles = axenic cultures (A), open circles = A inoculated with *Halomonas* sp., filled triangles = A inoculated with *Marinobacter* sp., open triangles = A inoculated with *Pelagibaca* sp. and filled squares = primary cultures. Error bars represent standard deviation. All cultures were grown in triplicate. $FeCl_3$: 2µM

The growth stimulating effect of *Pelagibaca* sp. on *D. salina* was similar to that of *Halomonas* sp. and *Marinobacter* sp. The effects were depending on the size of the bacterial inoculum (Fig. 3). With a 20 µl inoculum the final cell densities was similar to that of the primary cultures. With a 2 µl inoculum final densities reached 20 *10^6 cells *ml^{-1} and with a 0.2 µl inoculum the final cell densities were similar to that of the axenic cultures. Thus the presence of bacteria promoted algal growth however in a dose dependent manner.

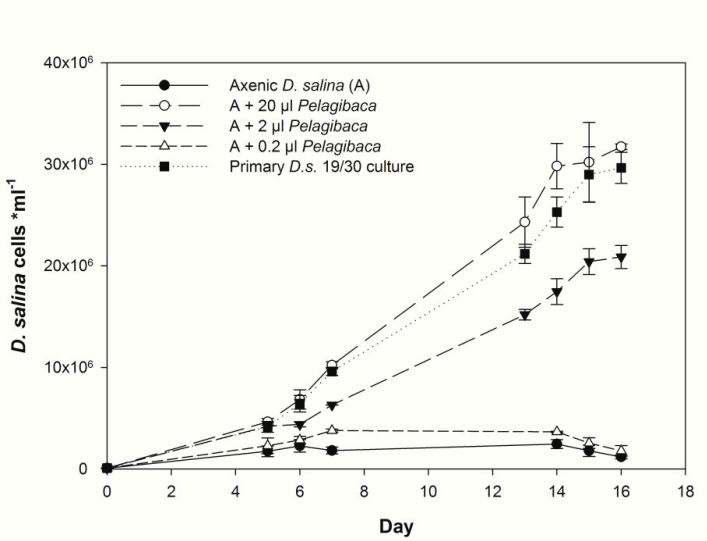

Fig. 3 Growth stimulating effect of *Pelagibaca* sp. on *D. salina* is dependent on bacterial inoculum. Filled circles = axenic cultures (A), open circles = A + 20 µl *Pelagibaca* sp. culture. Filled triangles = A + 2 µl *Pelagibaca* sp., open triangles = A + 0.2 µl *Pelagibaca* sp. and filled squares = primary cultures. Error bars represent standard deviation. All cultures were grown in triplicate. $FeCl_3$: $2*10^{-6}$ M

The growth stimulating effect of *Halomonas* sp. on *Dunaliella* sp. has previously been observed (Keshtacher-Liebson et al. 1995) and Le Chevanton et al. (2013) found that *Alteromonas* sp. and *Muricauda* sp. has a positive effect on maximal biomass increase of *Dunaliella* sp. due to organic nitrogen remineralization. *Marinobacter* sp. has been reported to enhance the growth of the dinoflagellate *Scrippsiella trochoidea* (Amin et al. 2009). Our finding that *Pelagibaca* also stimulated algal growth has not previously been reported. *Pelagibaca* sp. is however phylogenetically rooted in the order *Rhodobacterales* which is abundant in the marine environment and often associated with algal blooms (Cho and Giovannoni 2006, Hahnke et al. 2013).

The primary culture has been under selection pressure in the laboratory since its collection in 1976. This selection pressure is quite different from selection pressure in the natural environment. It is reasonable to assume that an equilibrium between *D. salina* and other microorganisms present. The microorganisms will either have no or positive impact on *D. salina* growth. Therefore it is not surprising that the three bacteria isolated have a positive effect on the growth of *D. salina*.

In order to test whether physical interactions between algae and bacteria were required for the growth promoting effect, bacterial cells were placed in a dialysis tube. One ml of SG 3% containing 20 µl of bacterial culture in a dialysis tube was immersed into axenic *D. salina* cultures and the growth of the algae was measured. Final cell numbers were 6-fold higher than in axenic cultures without bacteria in the dialysis bag (Fig. 4). These growth experiments showed that the growth stimulating effect of the bacteria is due to a diffusible factor.

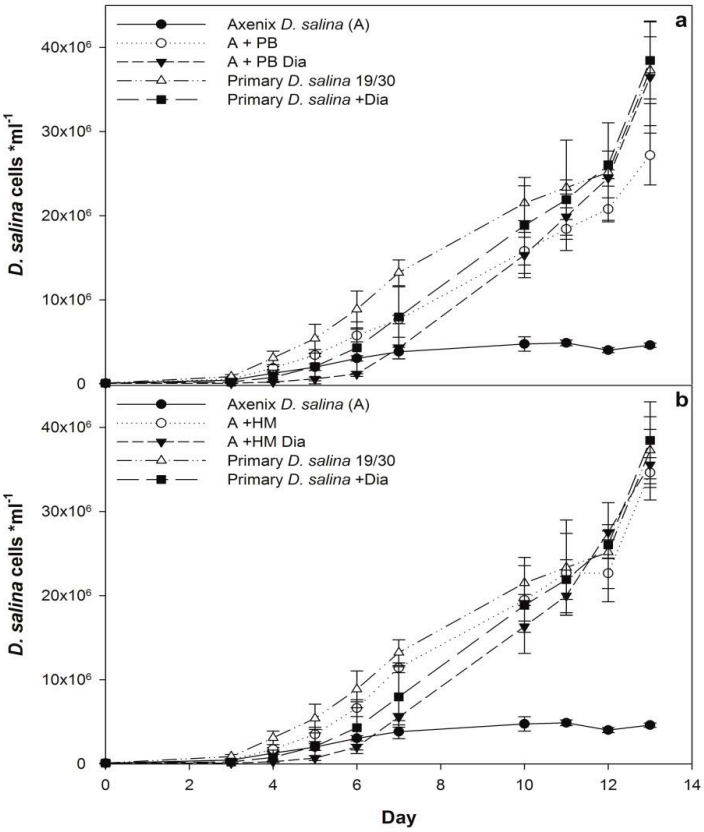

Fig. 4 Diffusible factors are responsible for the algal growth enhancing effect of bacteria. Bacteria and algae are separated by a dialysis tube. **a)** *D. salina* + *Pelagibaca* sp. Filled circles = axenic cultures (A), open circles = A inoculated with *Pelagibaca* sp., filled triangles = A + *Pelagibaca* sp. in dialysis tubes, open triangles = primary *D. salina* culture and filled squares = primary *D. salina* + dialysis bag **b)** D. salina + *Halomonas* sp. Filled circles = axenic cultures (A), open circles = A inoculated with *Halomonas* sp., filled triangles = A + *Halomonas* sp. in dialysis tubes, open triangles = primary *D. salina* culture and filled squares = primary *D. salina* + dialysis bag. Error bars represent standard deviation. All cultures were grown in triplicate. $FeCl_3$: $2*10^{-6}$ M

The lack of direct physical interactions between algal and bacterial cells was further corroborated by TEM analyses as well as DAPI staining of primary algal cultures that showed that bacterial cells were only present in the vicinity of the algae cells. Bacteria either attached to or inside the *D. salina* cells were never observed. TEM image provided (Fig. 5).

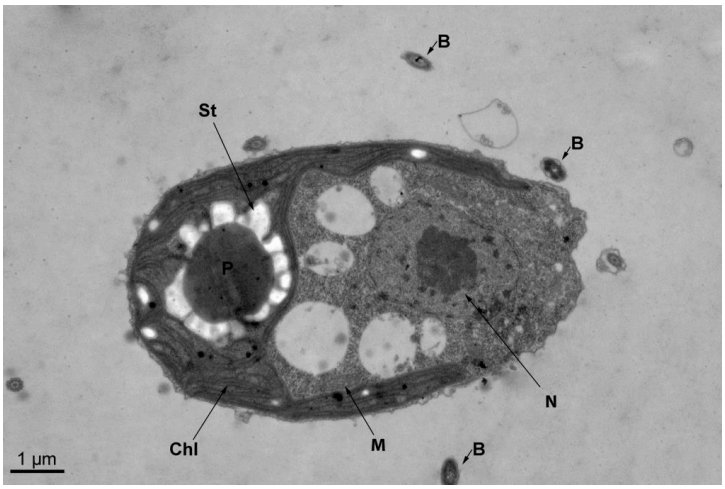

Fig. 5 TEM micrograph of a *D. salina* cell surrounded by bacterial cells. Note no attached or endosymbiotic bacteria. B: bacteria, Chl: chloroplast, M: mitochondrion, N: nucleus, P: pyrenoid; St: starch grain

At the end of the experiments the absence of bacteria in the axenic cultures was verified by PCR. As can be seen in Fig. 6 no 16s rRNA was amplified in the axenic cultures whereas clear bands are visible in the primary culture.

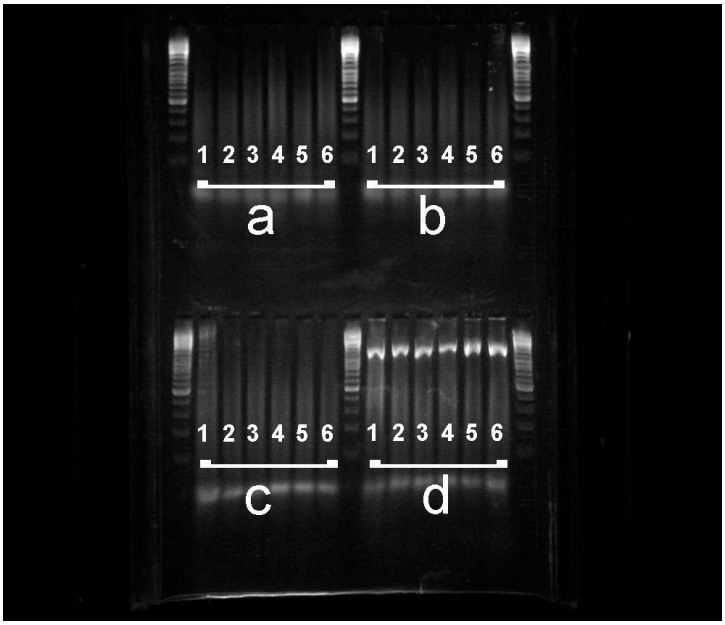

Fig. 6 Gel electrophoresis of PCR products amplified from 16s rRNA encoding genes on axenic and primary cultures. **a, b & c)** DNA extracted from the triplicate axenic algal cultures. 1 & 2 DNA extracted from cell pellet, 3 & 4 DNA extracted from supernatant. 5 & 6 DNA extracted from growing axenic culture. **d)** DNA extracted from primary culture. Numbers as above. Only the primary culture **(d)** contained bacterial 16s rRNA

3.4. Test for presence of siderophores

As the growth promoting effect was only observed under iron limiting conditions and was due to a diffusible factor we expected iron chelating siderophores produced by the bacteria to be the cause. This is in agreement with previously reported results (Keshtacher-Liebson et al. 1999, Amin et al. 2009) and both the genera *Halomonas* and *Marinobacter* contain species that produce siderophores (Vraspir et al. 2011, Vraspir and Butler 2009). Siderophores facilitate iron uptake in both algae and bacteria. In natural environments iron is a growth limiting factor (Soria-Dengg et al. 2001).

To analyze if the three isolated bacteria produced siderophores the bacteria were grown on plates containing iron depleted MAR medium. The CAS assay (Schwyn and Neilands 1987) was performed with a CAS overlay according to Pérez-Miranda *et al*. (2007). As a positive control *E. coli* which is a known producer of siderophores (Neilands 1993) yielded a de-colorization of the overlay while an overlay without bacteria did not change color (Fig. 7). The three different bacteria *Halomonas* sp., *Marinobacter* sp. and *Pelagibacca* sp. also produced a clear change in coloration of

the overlay. The de-coloration was visible after an hour and became even clearer within 24h as the bacteria continued to grow. As both *Halomonas* and *Marinobacter* are known to produce siderophores and the de-coloration on the *Pelagibaca* plates was similar in appearance to the de-coloration on the *Halomonas* and *Marinobacter* plates we find it likely that the three bacteria isolated from the primary *D. salina* culture each produced siderophores under Fe limiting conditions.

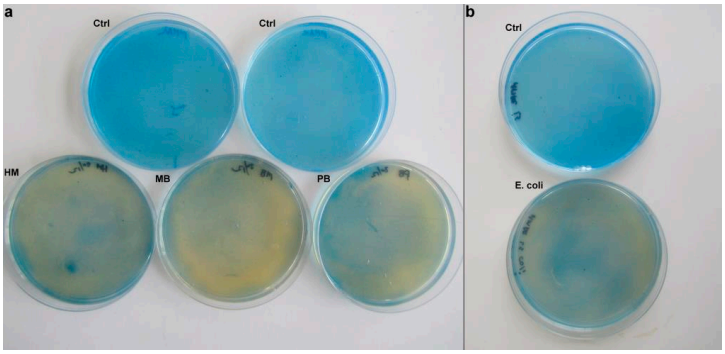

Fig. 7 The three isolated bacteria all produce siderophores under iron limited conditions. **a)** O-CAS assay performed with HM = *Halomonas* sp., MB = *Marinobacter* sp. and PB = *Pelagibaca* sp. Ctrl plates are plates without bacteria + overlay. **b)** Positive control with *E. coli*

The dose-dependent effect of the *Pelagibaca* sp. inoculum (Fig. 3) could be explained by either low siderophore production or that the siderophore produced is not as effective in chelating iron as the ones produced by the other bacteria *Halomonas* and *Marinobacter*.

4. Conclusion

The primary culture of *D. salina* hosted at least three different bacteria. All promoted algal growth under iron limited conditions. *Halomonas* and *Marinobacter* have previously been described as promoting algal growth. This work in addition showed that *Pelagibaca* sp. has a similar growth promoting effect on *D. salina as Halomonas* and *Marinobacter*. This is a novel observation. The growth promoting effect is not dependent on a physical association between the algae and bacteria but is caused by a diffusible factor made by the bacteria. We cannot pinpoint the mechanism behind the growth promoting effect however we are certain that vitamin B12 is not the effector. Axenic *D. salina* grows in SG 3% media which does not contain B12 furthermore it has been reported that *D. salina* does not require B12 for growth (Provasoli and Carlucci 1974). Based on siderophore

analysis we suggest that *Pelagibaca* sp. promoted growth of *D. salina* as a result of siderophore mediated Fe uptake in the algae.

5. Acknowledgements

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Chapter 3

Bacterial influence on the growth of three *Scenedesmus*-like algal isolates

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Keywords

Scenedesmus, *Acutodesmus*, *Pseudoclavibacter*, *Pseudomonas*, Growth, Stimulation

Abstract

Several investigations show some bacteria have a beneficial effect on the growth of algae. Investigations of this symbiosis may help to optimize the growth of algal biomass for use in biofuels, health food, animal feed and several other algal biomass based products. This study examines the growth of three different algal strains each isolated from fresh water samples from three locations in southern Scandinavia. Growth was analyzed either as axenic algal cultures or in co-culture with bacteria isolated from the sample from one of the location. The algal end bacterial strains were identified by sequence analysis of the 18s and 16s rRNA encoding genes, respectively. All three algae were identified as belonging in the Scenedesmaceae family. The two isolated bacteria were identified as *Pseudoclavibacter* sp and *Pseudomonas* sp. Even though the three algae are closely related the effect of the bacteria on algal growth varies. One algal strain is promoted in growth, one is negatively affected and one is unaffected by the presence of the bacteria.

1. Introduction

The use of microalgae as a feedstock for the production of biofuels, protein rich feed for animals and products for the healthcare industry is of growing interest as reviewed by Spolaore et al. (2006) and Fon sing et al. (2013). Many of these products are of great value and production cost can easily be covered. Biofuels from microalgal biomass are, on the other hand, products that are too expensive compared to traditional fossil fuels and other biofuels and thus an effort to maximize production and lower production cost is needed to make biofuels from algal biomass an economically relevant alternative (Lundquist et al. 2010). Another application of microalgae is in wastewater treatment and coupling this to the production of biomass would be one way of improving the production economy of algal derived biofuels (Olguín 2012). There are two obvious approaches to optimizing the production of algal biomass. One is to induce faster growth rates and the other to grow more biomass per volume. It is well known that co-culturing algae with bacteria can be beneficial to the algae and improve growth rates as well as increase final cell number in the culture (Cole 1982, Park et al. 2008). Finding the right bacteria to co-inhabit the algal cultures could be an important part of optimizing large scale culturing of algal biomass. Other important aspects of producing microalgal biomass are the design of the production facilities and the strict control of the running cost of the growth facility especially when growing algal biomass for products like biofuels. Outdoor raceway ponds are currently the most attractive way of growing large amounts of algal biomass (Jorquera et al. 2010). These ponds are, since they are not closed systems, susceptible to contamination by bacteria and other organisms. A way of minimizing deleterious effects of this contamination could be to introduce growth promoting bacteria to the algal culture as a healthy balanced co-culture of algae and bacteria may be less susceptible to contamination by other harmful organisms. An important aspect of growing algal biomass in open pond systems is that the algae species preferable should be of local origin. A reason for this is that the algae are adapted to the pertaining variations in temperature, light and day length. Furthermore, introduction of microorganisms form foreign geographic locations may threaten local biodiversity and ecology.

The aim of this study was to isolate local Scandinavian bacteria and algal species with potential for biomass production and to investigate the impact of bacterial presence on algal growth. We studied three strains of Scenedesmaceae and two strains of bacteria from water samples and analyzed the effect of bacteria on the growth of the algae.

2. Material and Methods

2.1. Algae and bacterial isolates, media and growth conditions

Algae and bacteria were isolated from water samples taken from various locations in Denmark and Sweden. For the exact locations see table 1. For algal isolates water samples were diluted and spread on agar plates containing TEM media (Gorman and Levine 1965). Axenic algal cultures were obtained by continuous re-streaking. When no bacteria could be detected by microscope and no bacterial colonies were seen on agar plates the cultures were deemed axenic. Preliminary identification of the algae was done by microscopy were the isolates were also checked for the presence bacteria.

To isolate pure strains of bacteria water samples were spread on LB agar plates (Bertani 1951). Colonies were picked and restreaked until the isolates were deemed pure. The pure colonies were picked and suspended in LB media containing 20% glycerol and kept at -80 °C for further use.

Table 1 *Sampling locations and isolate ID's.*

Location	Description	Coordinates	Algae	Bacteria
1	Pond near Heinge, Skåne, Sweden.	55°41'10.7"N, 13°54'42.5"E	A2	
2	Pond near Risø, Roskilde, Denmark.	55°41'13.57''N, 12°6'02.91''E	A5	B5_1, B5_2
3	Frederiksborg Castle Lake, Hillerød, Denmark.	55°55'58.44''N, 12°18'12.23''E	A7	

2.2. Identification, PCR and DNA sequencing

Genomic DNA was extracted using phenol as described by Cheng and Jiang (2006). Afterwards the 18s/16s rRNA encoding genes was amplified by *PCR* and subsequently sequenced. The *PCR* reactions amplifying 18s rRNA were performed with the primers ss5 (5'-GGTTGATCCTGCCAGTAGTCATATGCTTG- 3') and ss3 (5'-GATCCTTCCGCAGGTTCACCTACGGAAACC-3') (Rowan and Powers 1992) based on *Prorocentrum micans* positions 4-33 and 1764-1794, respectively. The *PCR* reactions amplifying 16sRDNA were with the primers fD1 (5'-GAGTTTGATCCTGGCTCAG- 3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') (Weisburg et al. 1991) based on *E. coli* positions 8–27 and

1512-1492, respectively. The *PCR* reactions were with the following cycles: initialization: 95 °C 5 min, 30 x (denaturation 94 °C 15 sec, annealing 1 min (55 °C for the algae and 58 °C for the bacteria), elongation 72 °C 2 min) and final elongation 72 °C 10 min using the HotStar HiFidelity Polymerase Kit (QIAGEN Sciences, Germantown, MD, United states). The primers were also used for sequencing. Nucleotide sequencing was performed by Eurofins MWG Operon, Ebersberg, Germany. The obtained sequences were compared to known sequences in the Genbank database (National Center for Biotechnology Information) and the most probable identity of the isolates was determined by the closest BLAST (Altschul et al. 1990) hit. Both algal and bacterial sequences are available in Genbank at the following accession numbers. A2_2: KJ855304, A5_1: KJ855305, A7_1: KJ855306, B5_1: KJ855302, B5_2: KJ855303.

2.3. Growth experiments

The algal growth experiments were done in TEM media. The start concentration of the algae was aimed at 80.000 cells*ml^{-1}. Bacteria were grown in LB media at 22 °C on an orbital shaker at 80 rpm for two days and 10 µl of bacterial culture was inoculated into 7.5 ml of algal culture. With a small inoculum of this size the possible diluting effect of the LB media on the growth of the algae was considered negligible. The algal cultures were grown in T25 flasks (SARSTEDT AG & Co, Nümbrecht, Germany) containing 7.5 ml of TEM medium at 22 °C with 140 µmole*m-2*s-1 of light on an orbital shaker (80 rpm) in a 14/10 light/dark cycle. The algal cells were counted in a Cell Lab Quanta SC Flow Cytometer (Beckman Coulter, Brea, CA, United States).

All growth experiments were done in triplicate

3. Results

3.1. Algal and bacterial isolates

The fresh water samples taken from the three locations contained a variety of different bacteria and microalgae as judged by microscopy. It was not the intension to perform a complete identification or isolation of these organisms. The intension was to isolate microalgae that could be relevant for lipid production as well as bacteria living in their communities. Choosing only one media for isolation of algae and one media for the isolation of bacteria excluded many of these organisms from isolation. However, several types of algal colonies were detected on solid media but some of these algae aggregated to larger lumps when grown in liquid media. They were therefore impossible

to count and quantify and was not analyzed further. Only algae that grew well on both solid and subsequently in liquid media were used in the liquid growth experiments.

From two locations in Denmark and one in Sweden (Table 1) three algal strains all visibly resembling *Scenedesmus* spp. (Fig. 1) were isolated. Two strains of bacteria were isolated from location 2.

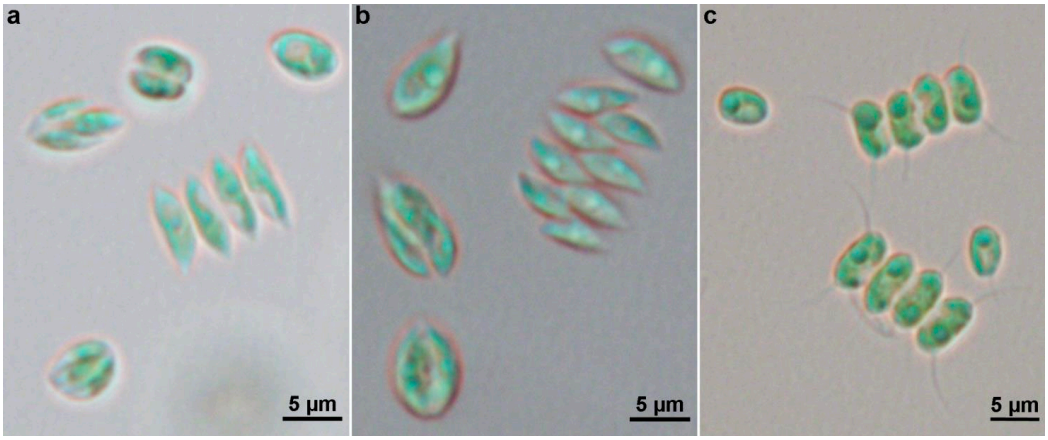

Fig. 18 Three Scenedesmus like isolates. **a**) A2 isolated from a pond near Heinge, Sweden. Loc. 1 **b**) A5 isolated from a pond near Risø, Denmark. Loc. 2 **c**) A7 isolated form Frederiksborg Castle Lake, Hillerød, Denmark. Loc. 3

Viewed under the light microscope the three algal isolates are morphological different. Algal isolate A2 comprised single cells and coenobia comprising up to 4 cells (Figure 1 a). Algal isolate A5show similarities with A2, but A5 have single cells and coenobia comprising up to 8 cells (Figure 1 b) Algal isolate A7 is different with a more ovoid single cells and coenobia mostly comprising 4 cells and the cells are with spines (Figure 1 c). Based on these observations the three isolates were anticipated to be different.

3.2. Identification of alga and bacteria

DNA was prepared from each isolated algal strain and a fragment from the 18s rDNA was amplified by PCR using conserved primers, sequenced and compared to known sequences in the Genbank database. The 3 isolates were identified as belonging in the Scenedesmaceae family. The highest scoring hits in all three BLAST comparisons were *Scenedesmus* and *Acutodesmus* but it was not possible with this approach to identify the three algal isolates further. According to Hegewald and Wolf (2003) the genera Scenedesmus & Acutodesmus are closely related.

DNA was also isolated from the 2 bacterial isolates from location 2 and a fragments from 16s rDNA were amplified by PCR using conserved primers and sequenced. The bacterial species were attempted identified by comparing them to known sequences in Genbank using the BLAST algorithm. The most probable identity of the isolates was determined. B5_1 was identified as *Pseudoclavibacter* sp. and B5_2 was identified as *Pseudomonas* sp.

3.3. Growth of algal isolates inoculated with bacteria

Growth of axenic algal cultures was compared to the growth of algal cultures inoculated with the bacterial isolates.

The algal isolate A5 reached 1.5-fold higher cell density after 7 days when inoculated with bacteria than when grown axenically (Fig 2). The growth promoting effect was obvious, irrespective of bacterial strain (B5_1 or B5_2) added. At 7 days post-inoculation the axenic cultures entered stationary phase reaching a cell density of ca. $10*10^{-6}$ cells*ml^{-1}. The cultures inoculated with bacteria continued to grow and reached approximately $18*10^{-6}$ cells*ml^{-1} in cell density after 2 weeks.

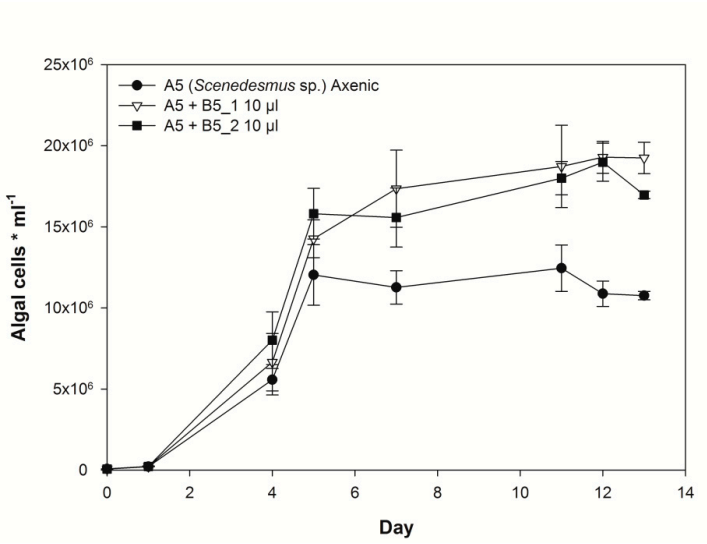

Fig. 2 Bacteria B5_1 and B5_2 promote the growth of algae A5 isolated from Loc. 2 (Table 1). When inoculated with the bacteria B5_1 (▽) and B5_2 (■) A5 reach 2-fold higher cell densities than when grown axenically (●). Error bars represent standard deviation. N = 3

In contrast, growth of the algal isolate A2 from location 1 was only limited affected by inoculation with the bacteria isolated from location 2, B5_1 & B5_2 (Fig. 3). Both axenic cultures and cultures inoculated with bacteria reached cell densities between 20 and $24*10^{-6}$ cells*ml^{-1} within 10 days. Thus growth of algae A2 is not influenced by the addition of bacteria. All cultures reached stationary phase after growth for 5 days.

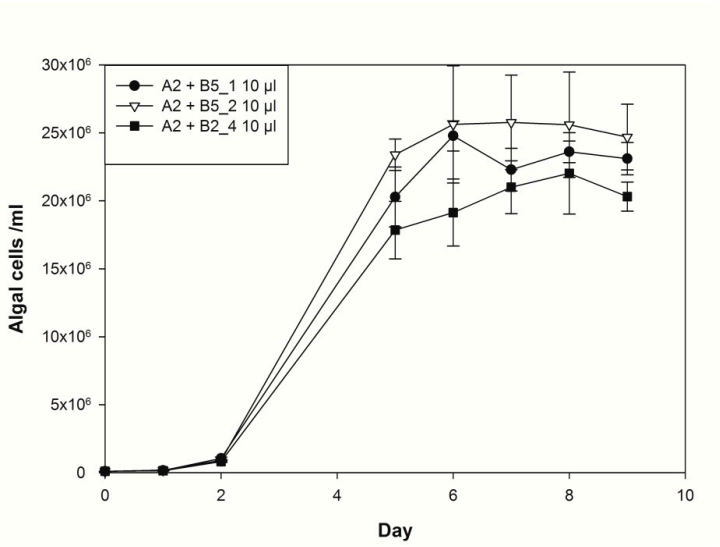

Fig. 3 Inoculation with bacteria B5_1 and B5_2 does not affect growth of algae A2 from Loc. 1. The axenic A2 cultures (●) reach cell densities that are comparable to the cultures inoculated with the bacteria B5_1 (▽) and B5_2 (■). Error bars represent standard deviation. N = 3

The third isolate, A7, from location 3, showed a different growth response when inoculated with the two bacteria isolated from location 2. The axenic cultures of A7 reached cell densities ca. 2-fold higher than when the culture was inoculated with bacteria B5_1 or B5_2 (Fig. 4). Thus the addition of bacteria had a detrimental influence on the growth of A7. All cultures reached stationary phase after 4 days of growth and axenic growth resulted in cell densities around $7.5*10^{-6}$ cells*ml^{-1}.

4. Discussion

We have isolated three Scenedesmaceae strains, A2, A5 and A7, from three different freshwater samples. The algae are different with respect to morphology (Fig1) and with respect to their growth

phenotypes in the presence or absence of two bacteria isolated from location 2. The two bacteria had quite different effects of the growth of the three Scenedesmaceae isolates. The growth of A5 from location 2 was augmented in the presence, while the growth of the A7 from location 3 was suppressed by both of the bacteria. The growth of the A2 from location 1 was unaffected by the presence of either bacterial isolates. It is unlikely that the algal starter cultures contained bacteria that were not detected when axenity was determined. However with the size of the inoculation and no detectable "endemic" bacteria in the algal cultures we find it plausible that the added bacteria are the cause of the seen effect.

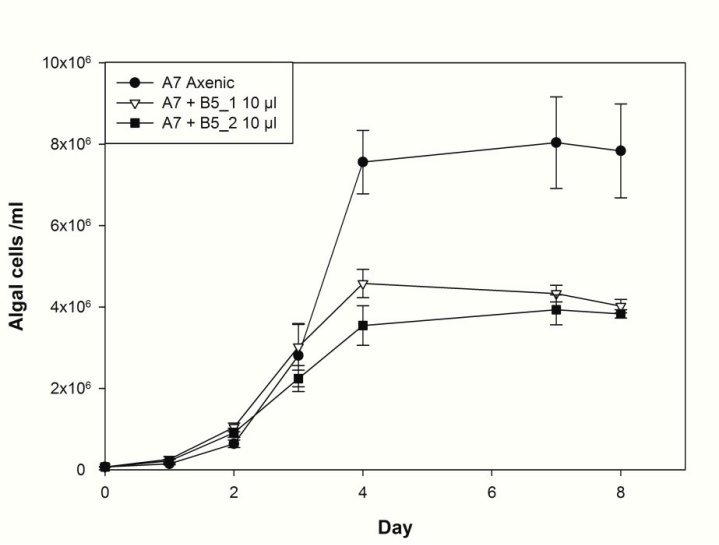

Fig. 4 Bacteria B5_1 and B5_2 repress growth of A7 from Loc. 3. Axenic A7 cultures (●) reach 2-fold higher cell concentration than the cultures inoculated the bacteria B5_1 (▽) and B5_2 (■) both isolated from Loc. 2. Error bars represent standard deviation. N = 3

The two isolated bacteria are, based on BLAST hit identification, *Pseudoclavibacter* sp. and *Pseudomonas* sp. Even though the bacteria have a similar effect on algae they are co-cultured with the two genera are not related. *Pseudoclavivater* is a gram-positive Actinobacteria whereas *Pseudomonas* is a gram-negative Proteobacteria. *Pseudomonas* is known as a growth enhancing symbiont in algal cultures from several studies. Mouget et al. (1995) speculate that the positive effect of *Pseudomonas* on the growth of *Scenedesmus bicellularis* is due to removal of

photosynthetic oxygen. *Pseudoclavibacter* has been found in sewage sludge (Srinivasan et al. 2012) and could thus be an important associate of algae grown in wastewater.

Vitamins produced by bacteria has a positive influence on the growth of some microalgae (Croft et al. 2005) and, among others, *Dunaliella bardawil* can utilize siderophores produced by bacteria to acquire iron and reach higher cell numbers than *Dunaliella bardawil* cultures grown without bacteria (Keshtacher-Liebson et al. 1995). It is also known that many bacteria produce compounds which are deleterious to the growth of green algae (Kodama et al. 2006). Interestingly our findings show that three apparently closely related algae species react very differently to inoculation with the same bacteria. It would be interesting to explore the reasons behind both the growth promoting and suppressing effects of these bacteria.

A question to ask is whether the effect is caused by a small molecule. This may be answered by separating algae and bacteria by a dialysis membrane and determine the effect (Kazamia et al. 2012). Also it could give a hint of possible physical interactions needed for the growth promoting or demoting effects and of possible molecules involved. Another question to ask would be whether a cell free filtrate of a bacterial culture would have a similar effect on the growth of the algal cultures as the bacterial inoculum. This could reveal if the presence of the algae triggers bacterial synthesis of growth promoting or demoting substances.

The different responses of the three Scenedesmaceae to growth with bacteria show the importance of investigating the relationship between algal and bacterial growth. One strain of algae may grow fast and to high densities but if exposed to certain bacteria growth will be compromised. Attempting to produce algal biomass outdoors, either purely for biomass or in dual purpose systems with wastewater treatment and biomass production, will be dependent on the knowledge on how the algal growth is affected by the bacteria that may enter the culture. If the algae show a positive growth response when co-cultured with an added bacterial culture it could be beneficial to inoculate the culture with the growth promoting bacteria.

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Chapter 4

Lipid production in *Dunaliella salina* during saline stress and adaptation

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Abbreviations: NR, Nile red;

Key words: Neutral lipids, polar lipids, osmotic stress, adaptation

Abstract

The salt tolerant green microalgae *Dunaliella salina* was exposed to hyper-saline shock by transfer cells from growth media containing 9% NaCl to media containing 12% or 15% NaCl. Cells only slowly adapted and grew significantly slower than the original 9% NaCl culture. Fluorescence microscopy and flow cytometry with Nile Red as fluorescence stain to visualize neutral lipid revealed the transient appearance of neutral lipids. Neutral lipid could be detected as early as 2 hours after the shift to higher salt concentration but disappeared 48 h after the transfer. However the appearance of neutral lipid in hyper-saline shocked cells was not paralleled in amounts of extractable lipids as analyzed by GC-MS of the fatty acid methyl esters. In contrast cells transferred from media containing 3% NaCl to 9% NaCl quickly adapted to the higher salinity but did not show the transient appearance of neutral lipids as a result of the salt shock. At later stages in batch cultures, when nitrogen and other nutrients were exhausted, these *D. salina* cells gradually accumulated neutral lipids as visualized by Nile Red staining and quantified by lipid extraction.

Cells subjected to hypo-saline shock by transfer from growth media containing 9% NaCl to media with 1.5%, 3% or 6% NaCl quickly adapted and then grew faster than the original 9% NaCl culture. Transient appearance of neutral lipids was not detected in these cells. Cells grown in media containing in 1.5% and 3% NaCl were rather uniform in sizes below 10 µm while cells at higher salt concentrations, 6%, 9% 12% or 15% NaCl, were larger and very heterogeneous in size.

1. Introduction

Dunaliella salina is a salt tolerant unicellular green algae capable of surviving sudden changes in the salt concentration in the environment, conditions that are common in pools along the sea-coast. The algae can survive both hypo- and hyper-osmotic shock. Hypo-osmotic shock is accompanied by a rapid swelling of the algae cell including many of the organelles like the chloroplast, the nucleus and vacuoles (Maeda & Thompson, 1986). In contrast hyper-osmotic shock induces a rapid decrease in cellular volume and in the volume of organelles. The decrease in cellular volume is produced by in-vagination of the plasmalemma without loss of surface area while the nucleus, chloroplast and mitochondria lose membrane surface area through membrane fusion with the endoplasmic reticulum (Einspahr *et al.*, 1988). These changes are important for the ability of *D. salina* to survive and adapt to wide range of changes in salt concentrations of the environment. Thus during osmotic shock *D. salina* experiences a number of cellular changes but one of outmost importance is the induction of compatible solutes like glycerol during hyper-saline shock as a measure against reduction in cell volume and for the protection of enzymes and cellular structures while the amount of glycerol is diminished upon hypo-osmotic shock (Chen *et al.*, 2009) (Chen *et al.*, 2011). The synthesis of glycerol is controlled by the activity of glycerol-3-phospahte dehydrogenase, the committed step in glycerol biosynthesis (Chen & Jiang, 2009)(Chen *et al.*, 2012). By expressed-sequence-tag profiling of hyper-saline shocked *D.* cells, cDNA coding for enzymes in glycerol synthesis, as e.g. glycerol-3-phosphate dehydrogenase, is upregulated. (Alkayal *et al.*, 2010). Also a rapid increase in phosphatidylcholine turnover and an increase in amount of lyso-phophatidylcholine and lyso-phosphatidate (LPA) and fatty acids suggests activation of lipases (PLA2) during the rapid response to hyper-osmotic shock (Einspahr *et al.*, 1988) (Ha & Thompson, 1991) (Ha & Thompson, 1992).

Pigment content also increased as a result of increased light intensity, increased salt concentration or nitrogen depletions. The increase in β-carotene content possibly is a chlorophyll protecting measure (Ben-Amotz & Avron, 1983).

An important property of β -carotene synthesis is that it is somewhat connected to triacylglycerol synthesis. Inhibition of the fatty acid synthase with cerulenin results in a decrease in triacylglycerol (TAG) synthesis in addition to an inhibition of the production of β -carotene under conditions that normally favor β -carotene synthesis (Rabbani *et al.*, 1998). Transcripts for phytoene synthase and phytoene desaturase increased substantially when *D.* cells were shifted to high light or high salt under nutrient deplete conditions. The presence of nutrients inhibited this transcriptional response (Coesel *et al.*, 2008). Oleic acid, a fatty acid mainly associated with tri- and diacylglycerols, correlated with the amount of β -carotene as a result of high light stress (Mendoza *et al.*, 1999) (Lamers *et al.*, 2010). The neutral lipids are known to be accumulated in lipid bodies of the chloroplast and in the cytosol (Davidi *et al.*, 2012). The correlation between β -carotene accumulation and the concomitant accumulation of oleic acid was further analysed in a turbidostat study that concluded that oleic acid may be a key component of lipid-globule-localized triacylglycerol (TAG) and β –carotene (Lamers *et al.*, 2012).

Lipids are mainly synthesized when nutrient like nitrogen are limited and cell growth ceases. The accumulation of lipid under such nutrient limiting conditions can be rather impressive and neutral lipid as di- and tri- acylglycerols (DAG, TAG) accumulate as a consequence of the inhibition of photosynthesis and cell growth (Davidi *et al.*, 2012) (Jiang *et al.*, 2012) (Gao et al 2013). Thus, nutrient limitation appears to be the main regulatory factor controlling lipid and carotenoid accumulation.

Limited analyses are available on the accumulation of lipids and the fatty acid composition as a function of salt concentration in the growth media. Takagi *et al.*, (2006) found an increase in lipid content as salt was increased from 0.5M to 1 M in the growth media (Takagi *et al.*, 2006) while the lipid composition of the plasma membrane was constant when the NaCl concentration in the media changed rom from 0.85M to 3.4M (Peeler et al 1989). Possibly major changes in plasma membrane composition are not needed to conserve membrane integrity following osmotic shock.

Photosynthesis is needed for neutral lipid biosynthesis as evidenced by the observations that lipid accumulation is inhibited in the dark or in cells treated with dichlorophenyl-dimethyl urea (DCMU), a herbicide that blocks electron transport from photosystem II to plastoquinone (Wang *et al.*, 2011)

In steady state DAG and TAG are predominantly made *de novo* from glycerol-3-phosphate via glycerol-phosphate-acyltransferase and lyso-PA acyltransferase forming PA that upon

dephosphorylation forms DAG (Arisz and Munnik_J lipid Res_2011). PDAT phospholipid:diacylglycerol-acetyl-transferase is involved in membrane lipid turnover while synthesizing triacylglycerol in *Chlamydomonas reinhardtii*. Knockout mutants of PDAT constructed by RNAi technology have increased concentration of chloroplast membrane lipids as momoglactosyldiacylglycerol (MGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) while triacylglycerol levels are impaired (Yoon *et al.*, 2012). Thus TAG is appears both essential during growth under optimal conditions and during stress conditions where turnover of membrane lipids and concomitant production of TAG is prevalent. TAG may then serve as carbon and energy reserve for the algal cell to be activated when growth resumes.

D. salina has a potential also to be applied in biotechnogical attempts to produce microalgal based biodiesel due to its ability to synthesize lipid and to grow in salt water, an environment that does not compete with agricultural practices. An equally important property is the absence of a rigid cell wall allowing less complicated and less costly lysis- and extraction schemes to be applied for the recovery of the oil.

Therefore we have analyzed in batch cultures the long- and short term effects of changes in salt concentration of the growth media on lipid productivity of *D. salina*. Lipid was estimated by fluorescence following staining with Nile Red and by lipid extraction subsequent fatty acid methyl ester analyses using GC-MS.

2. Materials and methods

2.1. Strains and culture conditions

Dunaliella salina wild type strain CCAP 19/30 [Teodoresco, 1905] was obtained from *Culture Collection of Algae and Protozoa*, (SAMS Research Services Ltd, Dunstaffinage Marine Laboratory, OBAN, Scotland). Growth conditions were cycles of 9/15 hours of dark/light at 150 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetically active radiations with shaking and 2% CO_2. *D. salina* cells were grown in Brackish water medium =1/2Swes (http://sagdb.uni-goettingen.de/culture_media/06%20Brackish%20water%20medium.pdf) by replacing seawater with indicated amount of NaCl.

2.2. Fluorescence microscopy

Nile red (NR), 9-diethylamino-5H-benzo(α)phenoxazine-5-one (Sigma-Aldrich), was used to reveal the presence of neutral lipid droplets [Pinkart et al, 1998]. Cells were stained in a 0.1 µg ml^{-1} solution of NR and incubated in the dark at room temperature for 10 min. NR fluorescence was observed with a fluorescent microscope (Olympus DP21) equipped with a 40x objective using a 475 nm band pass excitation filter and a 580 nm long pass barrier filter (Olympus). Images were recorded with a digital camera (Olympus DP21).

2.3. Flow cytometry

Cells stained with NR were analyzed using a high resolution flow cytometer (Counter Cell Lab Quanta™ SC and MPL, Beckman Coulter), equipped with a diode laser and HBO mercury lamp. This instrument allows simultaneous monitoring of the electronic volume (EV) as a sizing parameter, side scatter (SS) as a granularity differentiating parameter, three independent fluorescence parameters and provides absolute counts. The flow cytometer was interfaced with a Coulter computer software system for six parameter data acquisition and analysis. Fluorescence detection was carried out with the laser at 488 nm and 22 mW. Fluorescence emission was analyzed separately at two spectral settings by employing a 600 nm long pass barrier filter to split the incoming emitted light and using a 575 nm band pass filter (NR fluorescence), or a 670 nm long pass filter (red chlorophyll auto-fluorescence) as the emission filters in front of the respective fluorescence detectors. Analyses were done at room temperature and cells were automatically re-suspended just before counting.

2.4. Lipid analyses

Total cellular lipid was extracted from *D. salina* cells by a modified Bligh and Dyer method [1959]. Cells (5-10 $x10^6$) were harvested by centrifugation at 1500 g for 5 min. Cells were re-suspended in 5 ml methanol:chloroform (4:1, v/v) containing 25 ppm butylated hydroxytoluene (BHT) and incubated for 30 min at 55 degree with shaking at 1000 rpm. The mixture was centrifuged at 10000 g for 10 min and the supernatant was transferred to a glass tube with Teflon-lined screw cap. Cells were re-extracted with methanol:chloroform (4:1, v/v), centrifuged and the supernatant was combined with the previous extracts. Appropriate amounts of the odd fatty acid heptadecanoin (C17:0) (Sigma-Aldrich) was added as an internal standard. Alternatively cells were extracted using the same solvents in a DIONEX ASE 350 Accelerated Solvent Extractor. Aminopropylsilica

columns (Sigma-Aldrich) were used to separate polar lipid from neutral lipid according to the protocol of Pinkart [1998]. Lipid extracts were dried by evaporation at 50 ^{o}C under a stream of N_2.

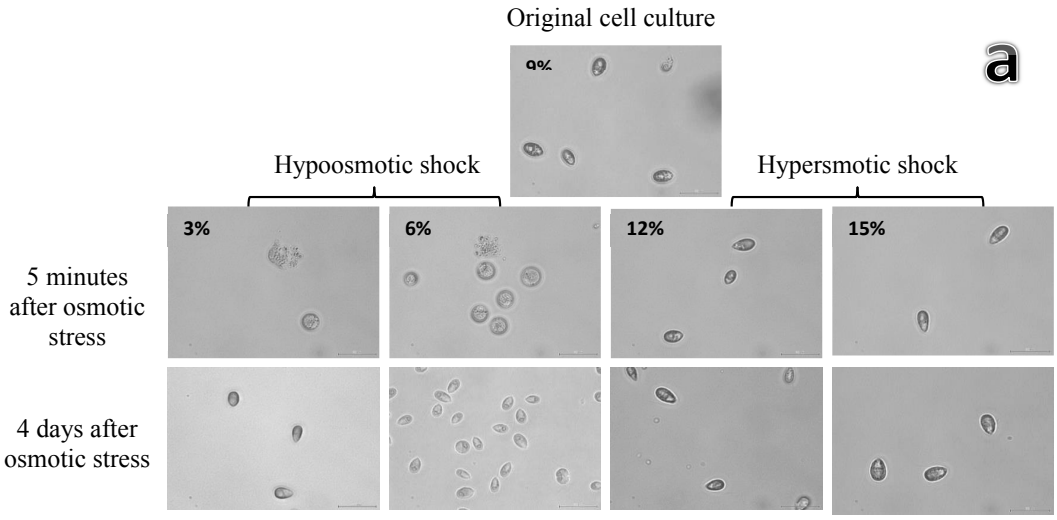
Fatty acid methyl esters (FAMEs) were prepared using 1.25M HCL (in methanol) as an acid catalyst for GC-MS analysis. Methyl pentadecanoate (Sigma-Aldrich) was used as internal standard. FAMEs were analyzed by GC-MS using a Hewlett Packard HP 6890 gas chromatograph interfaced to a HP5973 Mass Selective Detector (Agilent, Denmark). Samples (1 µl) were injected in split mode (1:20) using a HP 7683 autosampler (Agilent, Denmark). The source and rod temperatures were 230°C and 150°C, respectively. The FAMEs were separated using a 0.32 mm i.d. x 30 m WCOT fused silica column coated with VF-23ms at a thickness of 0.25 µm (Analytical, Denmark). The carrier gas was He at a flow rate of 1.2 ml/min. Separation of a wide range of FAMEs was achieved using a temperature program from 70°C to 250 °C at 10°C/min. The applied ionization energy was 70 eV. Full mass spectra were recorded every 0.3 s (mass range *m/z* 40 – *m/z* 450). FAMEs were identified by retention time, fragmentation patterns using NIST search engine version 2.0 f. (Agilent, Denmark), and comparison with purified FAME standard

3. Results

3.1. Adapted cultures

Dunaliella salina has an impressive capacity to grow in different salt concentrations. Using batch cultures we aimed to identify conditions under which salt could induce increase in amount of lipids in the cells. Upon hypo-saline shock conditions, achieved by transfer from growth media containing 9% NaCl to media with 3% or 6% NaCl, cells transiently and rapidly (within minutes) increased in sizes and slowly over hours acquired their original size and volume (Figure 1A). Upon hyper-saline shock by transfer from 9% NaCl to 12% or 15% NaCl cells transiently decreased in size (within minutes) and then slowly within hours regained the original size (Figure 1A). Cells that were adapted to and grown for 4 days in media containing 3, 6 or 9% NaCl displayed a great variation in cell sizes and in cell volume (Figure 1B and Table 1). Cell diameter and cell volume differ significantly from cells grown in 1.5% NaCl to cells in 9% NaCl. Cells in low concentration of NaCl were smaller and more uniform in size than those grown in media with higher concentrations of NaCl. In particular the media with 6% and 9% NaCl resulted in a very large variation in the volumes of individual cells. This property reflects that *Dunaliella salina* has a potential to regulate the cell volume thereby allowing the cells to tolerate large variations in the salt concentration of the growth media. Batch cultures of adapted cells also showed a variation in the growth rate, resulting

in a 2-3 fold higher cell yield in a 3 or 6% NaCl media compared to a 12 or 15% NaCl media (Figure 2A). The time required to reach maximal cell density also differed. Cells in media with 3% and 6% NaCl grew faster and reached maximal cell density 8-10 days after start of the culture while cells grown in 12% and 15% NaCl require 22-24 days to reach maximal density. Cells grown in media with 9% NaCl reached maximal density after 13-15 days. Lipids were extracted at day 14 from the



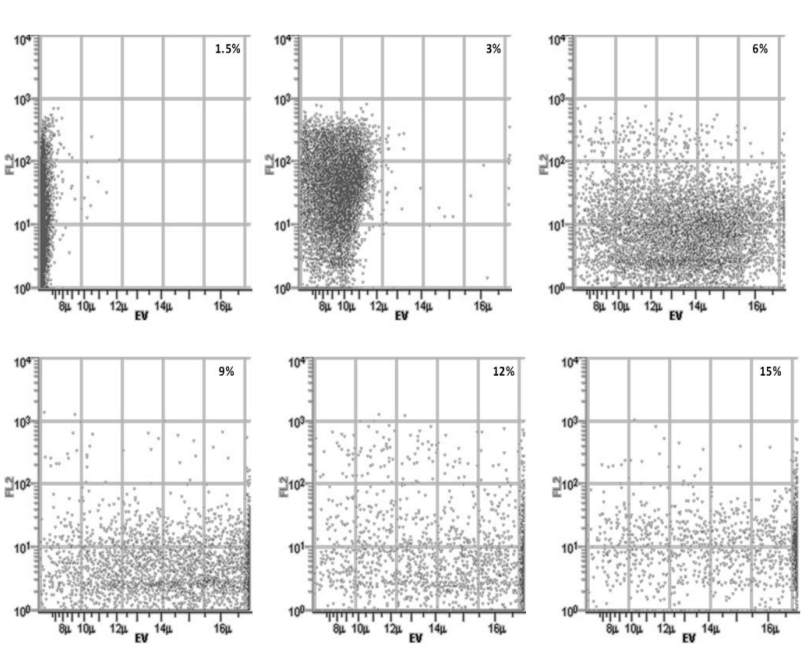


Figure 1 Morphology of *Dunaliella salina* after NaCl stress. Cells were grown in ½SWES medium containing 9% NaCl and then transferred to ½SWES medium containing 3%, 6%, 9%, 12% and 15% NaCl. (**a**): Cells were analyzed by microscopy after incubation for 5 minutes and 4 days. (**b**): Cell size (EV) and Nile Red fluorescence (Fl2) of *D. salina* grown in ½SWES medium containing different amounts of NaCl. Cells were analyzed after 4 days of incubation by flow cytometry.

Table 1. Average cell sizes of *Dunaliella salina* grown in different salinity of Swess.

Salinity	**22°C**[b]	
	Cell diam (μm)	Cell volume
1.5%	3.96±0.01	33±0.62
3%	9.60±0.33	432±2.1
6%	13.22±0.06	1210±14.8
9%	14.88±0.03	1230±16.3

[a.] Cells for measurements were harvested after 4 days incubation in Swess medium containing indicated percentage of NaCl.

[b.] The number of the cells assessed by flow cytometer is over 5000 and more than 90% of the representatives were evaluated

[c.] Cell diameter and cell volume data are reported as the mean value ± standard deviation from three independent biological replicates

cultures grown in 3%, 6%, 9%, 12 % NaCl and separated into polar and neutral lipid fractions. The amount of lipid was determined following trans-esterification and GC-MS analyses of the corresponding fatty acid methyl esters. Cells grown in media with 3%, 6%, 9% NaCl contained approximately equal amounts of lipid while cells grown in media with 12% contained app. 1.5x times more. The neutral lipid fractions were approximately equal in amounts (Figure 2B). Thus the fatty acid composition were similar indicating that increasing the concentration of salt in the medium did not significantly influence the overall fatty acid composition of the cells (Figure 2C). However the slow growth of cells in media with 12% NaCl compromised the lipid productivity compared to the productivity of those grown in media with 3%, 6%, 9 % NaCl. Therefore, both the maximal cell density as well as the time required to reach maximal density is important parameters for evaluating the potential of a microalgae for lipid production.

3.2. Saline-shock from 9% NaCl

Next *Dunaliella salina* cells grown to mid logarithmic phase were transferred to media with different NaCl concentrations. Cell growth was monitored by cell counting and flow cytometry of Nile Red stained cells. Cells experiencing hypo-saline shock by transfer from media with 9% NaCl to either 3% or 6% NaCl quickly adapted and then grew slightly faster than the original 9% NaCl culture. In contrast cells experiencing hyper-saline shock by transfer from 9% NaCl to 12% or 15% NaCl only slowly adapted and grew slower than the original 9% culture (Figure 3A). The doubling

time in days for cells in media containing 9% NaCl was1.12 while the doubling times were 0.95, 0.89, 2.07 and 2.46 for cells shifted from 9% NaCl to 3%, 6%, 12% and 15% NaCl, respectively.

Thus cells transferred from media with 9% NaCl to medium with 15% NaCl slowly grew but contained amount of neutral lipids that could be detected by NR fluorescence one to two days after the transfer (Fig 3 B). Cells transferred to medium containing 15% NaCl contained more NR fluorescent neutral lipid than the cells transferred to media containing 12% NaCl one to two days after the transfer. Neutral lipids were not detected one to two days after the transfer to media containing 9%, 6% or 3% NaCl (Fig 3 B). Thus only cells transferred from 9% NaCl to higher NaCl concentrations stimulated the transient accumulation of neutral lipids.

The transient appearance of yellow fluorescent neutral lipids as detected both with fluorescence microscopy and by flow cytometry in cells shifted from media with 9% to 15% NaCl prompted us to attempt to quantify the amount of lipid by extraction, trans-esterification and subsequent FAME analyses by GC-MS in a separate experiment. A transient increase in neutral lipid as detected by Nile Red fluorescence was again apparent

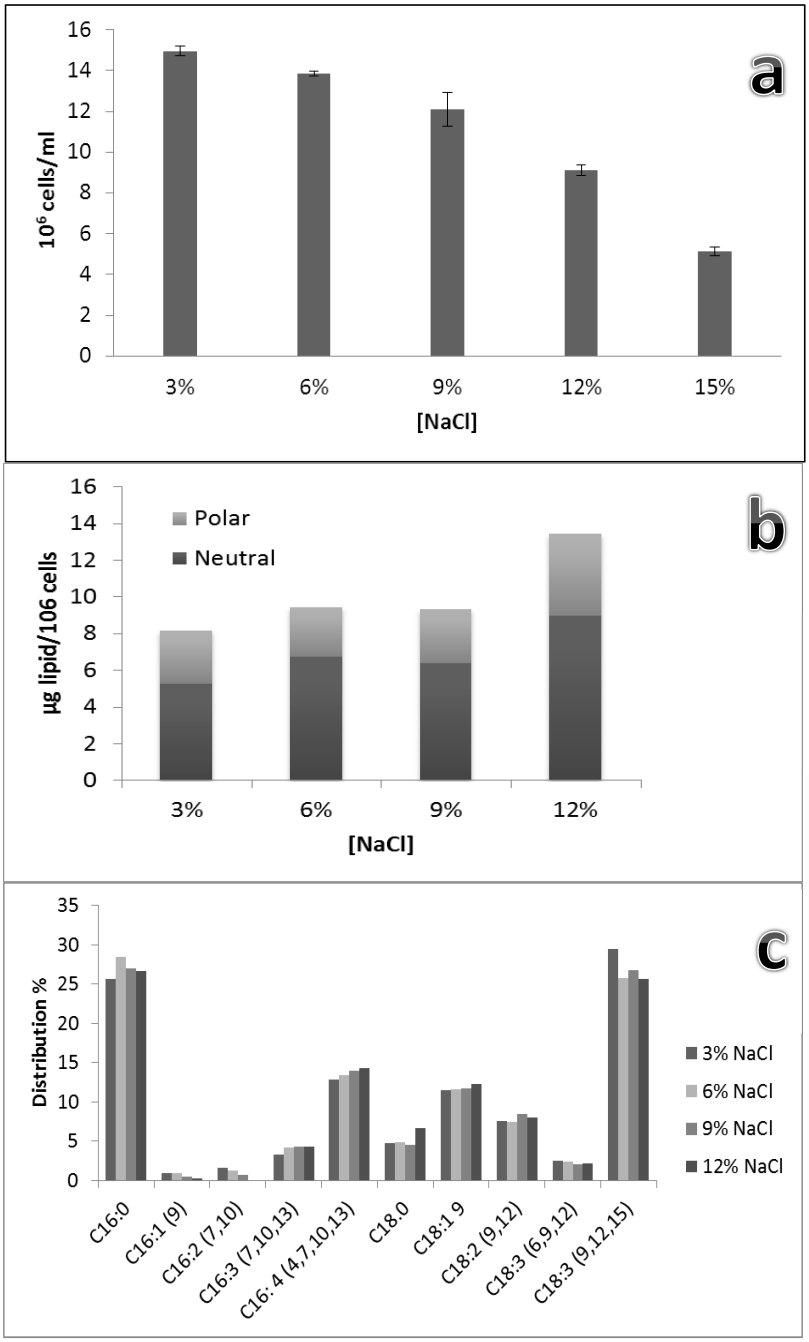


Figure 2 *D. salina* grown in ½SWES media containing different amounts of NaCl. (**a**): Maximal cell density of *D. Salina*. Cell numbers were cells were determined after incubation of 5-11 days. (**b**): Lipid analysis after incubation of 14 days. Lipids were extracted and separated into polar and neutral lipidsand their fatty acid methyl esters were quantified by GC-MS. (**c**): Fatty acid composition of total lipids. The positions of double bonds in unsaturated fatty acids are given in brackets.

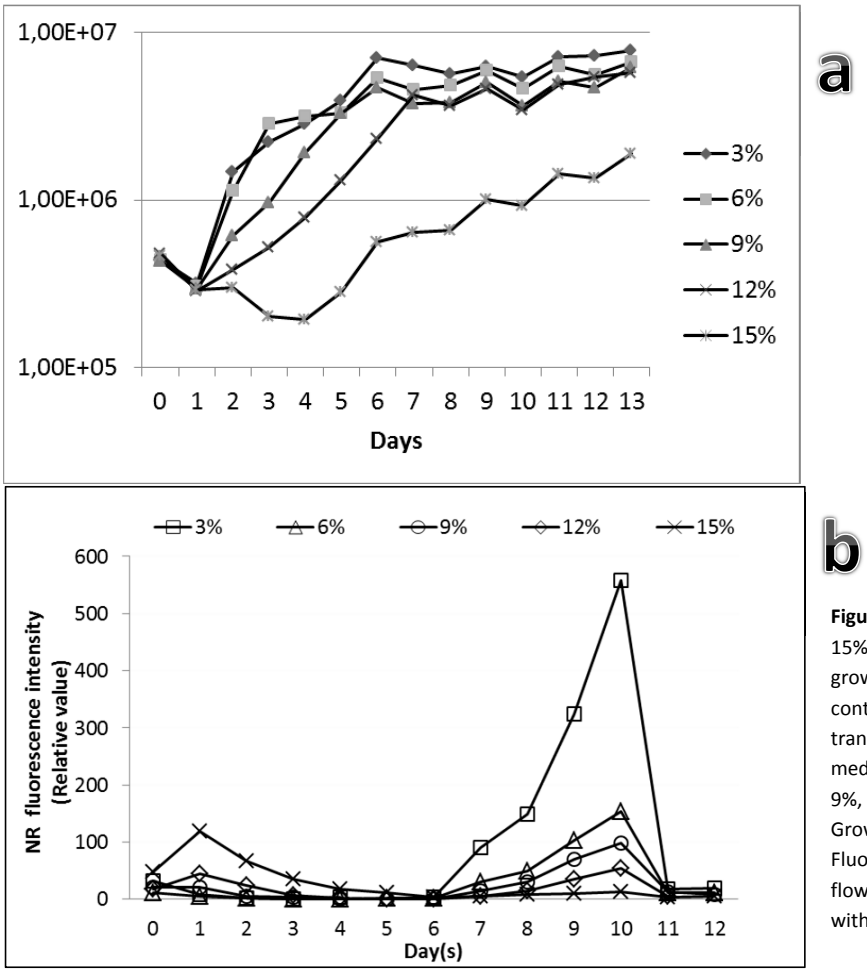


Figure 3 *D. salina* after 9% to 15% NaCl stress. Cells were grown in ½SWES medium containing 9% NaCl and then transferred to ½SWES medium containing 3%, 6%, 9%, 12% and 15% NaCl. (**a**): Growth curves and (**b**): Fluorescence as detected by flow cytometry after staining with Nile Red

(Figure 4A). Cells from media with 15% NaCl cells did not grow within the 48 h of the experiment while cells transferred to media with 9% NaCl did. However, the appearance of neutral lipid in hyper-saline shocked cells was not reflected in amounts of extractable lipid (Figure 4B). Neutral lipids comprised app. 15- 20% of the total lipid and slightly more in the cells transferred to media with 15% NaCl. This may indicate that the appearance of neutral lipid merely reflects a possible transformation of e.g. polar lipid to neutral lipids only detectable by the sensitive analyses with Nile Red fluorescence. Lipid analyses did suggest a two fold increase in the amount of lipid in cells shifted to 15% NaCl compared to cells kept in 9% NaCl after 48h. Both neutral and polar lipid

classes appeared to be increased. However the fatty acid composition was not changed. The C16:0 and C18:3 were the major fatty acids of the total lipid fraction reflecting that the polar lipid fraction was the main lipid fraction (not shown). The apparent increase in extractable lipid per cell at

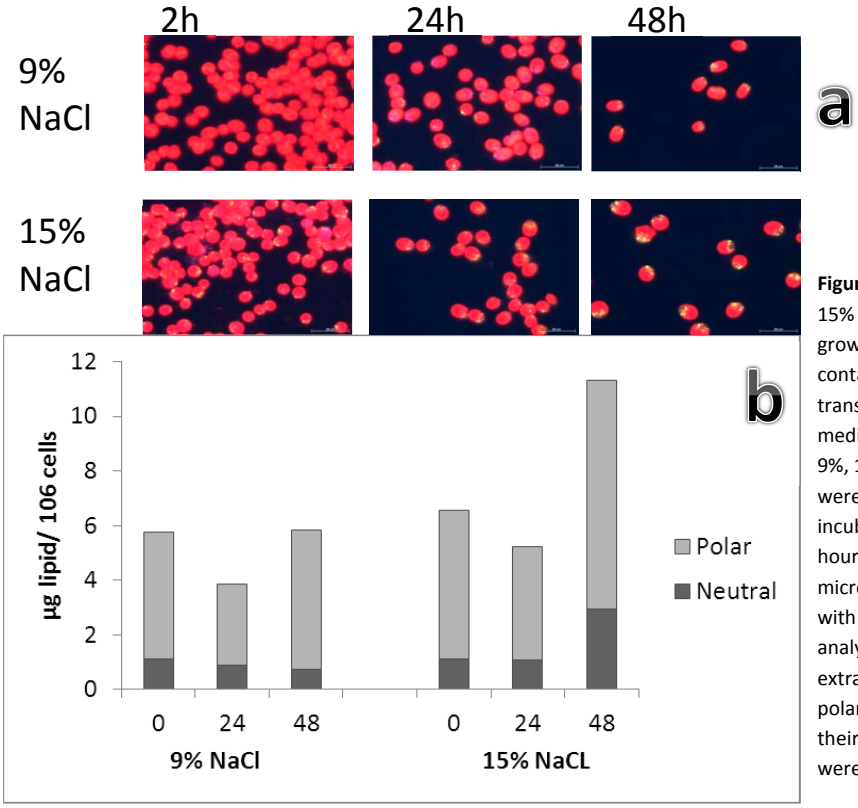


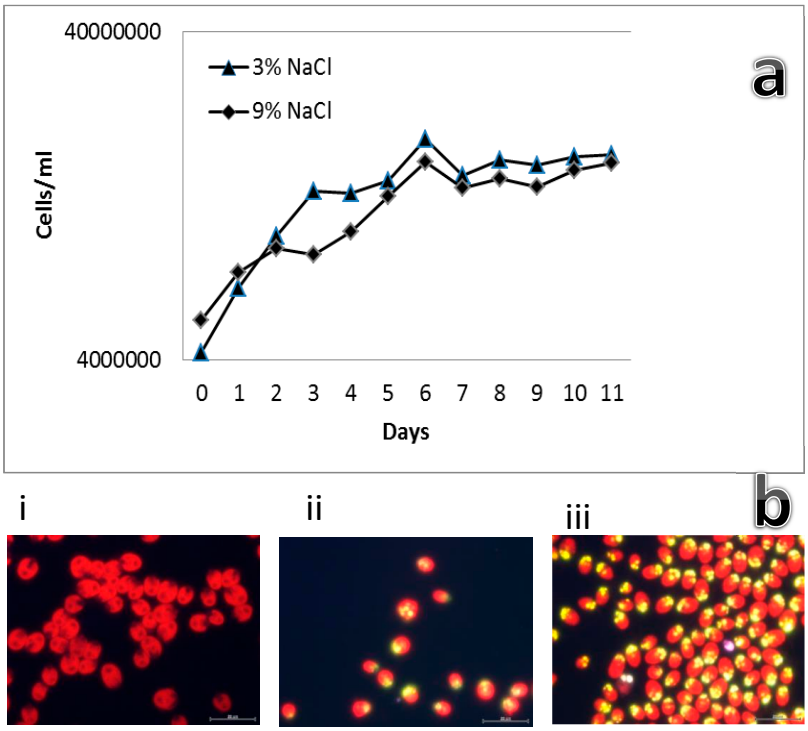
Figure 4 *D. salina* after 9% to 15% NaCl stress. Cells were grown in ½SWES medium containing 9% NaCl and then transferred to ½SWES medium containing 3%, 6%, 9%, 12% and 15% NaCl. Cells were analyzed after incubation for 2, 24and 48 hours. (**a**): Fluorescence microscopy after staining with Nile Red and (**b**): Lipid analysis. Lipids were extracted and separated into polar and neutral lipids and their fatty acid methyl esters were quantified by GC-MS.

48 h may simply reflect the fact that the cells in media with 15% NaCl are larger than those in media with 9% NaCl or that they did not divide. Thus the lipid accumulation may be a result of a block in cell division. In *Chlamydomonas* a genetic block in cell division results in an increased accumulation of neutral lipids (Yao et al). Thus the transient appearance of neutral lipid cannot constitute an appreciable factor in designing strategies to increase lipid productivities of *Dunaliella salina.*

3.3. Saline-shock from 3% NaCl

To examine if the transient induction of lipids also were detectable at conditions that is much closer to the saline conditions of sea water experiments were performed with cells cultivated in media with 3% NaCl. Cells were grown to mid log phase and transferred to media with 3% or 9% NaCl. The cells were then analyzed by

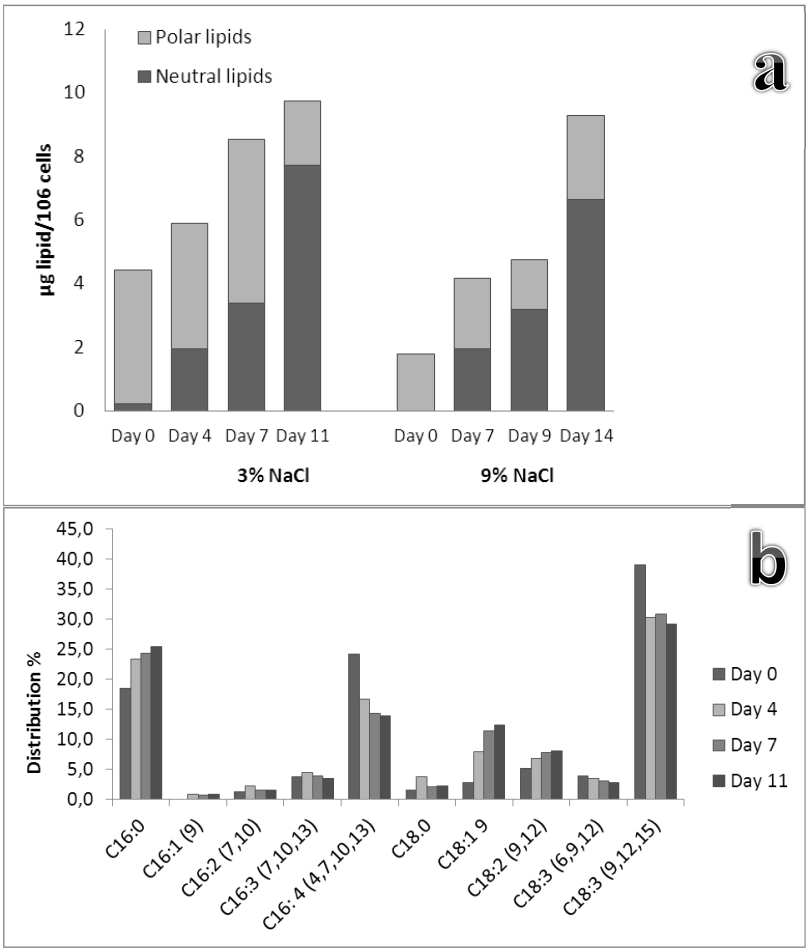
NR fluorescence and by lipid extraction. The 3% and the 9% cultures grew equally well without any significant lag phase (Figure 5A). Under these circumstances the cultures entered stationary phase after 6-7- days. However a transient increase in neutral lipid was not detected within the first few days after transfer to media with 9% NaCl. Neutral lipids only started to accumulate as the cells entered the stationary phase and



Figur 5 *D. salina* after 3% to 9% NaCl stress. Cells were grown in ½SWES medium containing 3% NaCl and transferred to ½SWES medium containing 3% and 9% NaCl. (**a**): Growth curves and (**b**): Nile Red fluorescence microscopy. (i): Just before the transfer, (ii): After growth in 3% NaCl for 11days (iii): After growth in 9% NaCl for 14 days.

growth ceased due to the exhaustion of nutrients in the batch culture. The accumulation of neutral lipids was evident after the cultures reached stationary phase at day 11 and day 14 in the 3% NaCl and 9% NaCl cultures, respectively (Figure 5B).

Lipid was extracted at different time points during the course of the growth experiment and separated in polar and neutral lipid. As expected from the fluorescence analyses neutral lipid accumulated as the batch culture matured and nutrients in the media were exhausted (Fig 6A). The prevalence of neutral lipids at later stages of the cultures was also reflected in the change in the fatty acid composition of the lipids. Neutral lipids reached ca. 70% of the total lipid at the end of growth. Accordingly C16:0, C18:1 and C18:2 increased in abundance. Fatty acids 16:0, C18:3 and C16:4 however were the most abundant fatty acids during the entire time course (Figure 6B). The neutral lipids peaked in the culture with 3% NaCl at day 11 while the culture with 9% NaCl peaked 3 days later, reflecting the slightly slower growth of *Dunaliella salina* in media with 9% NaCl. Thus, a hyper-saline shift increasing the NaCl concentration from 3% to 9% did not result in compromised growth or induced a transient increase in the amounts of neutral lipids.



Figur 6 Lipid analyses during growth of *D. salina* in media after 3% to 9% NaCl stress. The cells were grown in ½SWES medium containing 3% NaCl and transferred to ½SWES medium containing 3% and 9% NaCl. Cells were harvested at the indicate time points. (**a**): Lipids were extracted and separated into polar and neutral lipids and their fatty acid methyl esters quantified by GC-MS. (**b**): Fatty acid composition of total lipids of cells grown in media with 3% NaCl. Cells grown in media with 9% NaCl had a similar composition

4. Discussion

Transient and rapid response to changes in the osmolarity and/or salinity of the growth media is known to cause a number of changes in *Dunaliellea sp.* As a first response to hyperosmotic shock glycerol is synthesized via a pathway involving glycerol-3-phosphate dehydrogenase (G3PDH) as the key enzyme converting dihydroxyacetone phosphate to glycerol-3-phosphate. The activity of G3PDH is stimulated when hyperosmotic stress is applied resulting in a transient accumulation of *de novo* synthesized glycerol as a compatible solute which role is to maintain cell volume and protect enzymes against inactivation [Chen,Lu & Jiang 2012]. Also Glycerol-3-phosphate may

serve as a precursor for the synthesis of phosphatadic acid by the action of acyltransferases. PA is an intermediate in lipid synthesis and as a lipid messenger in signaling reaction. Lyso-PA and Lyso-PC is increased in *Dunaliella salina* by salt stress while in *Chlamydomonas* PA and Lyso-PA are increased [Einspahr,Maeda & Thompson 1988] [Testerink & Munnik 2011] . In steady state PA is predominantly made *de novo* from glycerol-3-phosphate via glycerol-phosphate- acyltransferase and Lyso-PA acyltransferase while PA during salt stress arises from PPI by the action of PLC to form DAG that in turn is converted to PA by diacylglycerol kinase. During stress PA is also converted to LPA by PLA_2 [Arisz & Munnik 2011]

These salt stress-induced changes in signaling lipids occur within minutes after the application of the environmental stress while the transient appearance of neutral lipid reported here occur after hours. It is tempting to speculate that this is a late consequence of the immediate lipid messenger response to salt stress. Neutral lipid can indeed play a major contribution in controlling the development of vital organs in plants. This was recently demonstrated by the identification of a nonspecific phospholipase C5 (NPC5). The product of this lipase reaction, diacyl glycerol (DAG), controlled the development of lateral root formation in *Arabidopsis thalianana [Peters,Kim,Devaiah,Li & Wang 2014]* . NPC5 expression is induced by salt. Deletion of the gene for the lipase leads to reduced formation of lateral roots whereas overexpression of the gene increases lateral root formation. Addition of DAG restores lateral root formation in plants deleted for the gene encoding NPC5. Thus the transient accumulation of neutral lipids in *Dunaliella salina* may have similar significance. Mutants of Arabidopsis with defects in diacylglycerol acyltransferase, the last step in TAG synthesis, are osmo-sensitive during seed germination [Lu & Hills 2002] indicating that neutral lipids act as important component in the ability of seeds to withstand osmotic challenges. In wheat embryos an increased synthesis of TAG resulted from an increase in manitol or sucrose added to the growth medium (Rodriquez-Sotres and Black Planta 1994) and *Brassica napus* microspore-derived cultures synthesized TAG as a consequences of excessive amounts of sucrose in the media [Weselake,Pomeroy,Furukawa,Golden,Little & Laroche 1993] . The latter effect was subsequently shown to be due to an increased DAGT activity resulting in a transient increase in TAG up to 12 hour after transfer from media containing 6% sucrose to media with 14% sucrose. The subsequent decrease in TAG after 12 hours was inversely related to an increase in a TAG lipase [Nykiforuk et al. 2002] . By using NR and fluorescence to detect neutral lipids it became clear that only osmotic upshift from 9% NaCl to 15% NaCl induced neutral lipid appearance. Even though *Dunaliella salina* has a pronounced capacity to recover from and

survive large variations in salt concentration, it is evident that even though *D. salina* grow well in 9% salt it does not have the capacity to fully recover a shift to 15% NaCl and cell division is accordingly slowed down. However this salt shock is not lethal. In contrast cells shifted from 3% to 9% quickly recovered and resumed growth. Shifting cells from 3% to 9% did not result in neutral lipid emergence. Transient appearance of neutral lipid could be dependent on the inhibition of cell division cells are experiencing at the shift from 9% to 15%. Yao et al showed that a genetic block in cell division resulted in accumulation of neutral lipids in *Chlamydomonas*. In addition the effect of nutrient limitation, primarily nitrogen limitation, slows down cell division and photosynthesis and as a secondary result triggers green microalgae to accumulate neutral storage lipids. This effect is of course also seen in batch cultures when the cells are in stationary phase and depletes the medium for e.g. nitrogen (Figure).

In addition to the effects on the osmotic conditions, NaCl also have a salt effect by itself. *Dunaliella sp.* can tolerate large variations in salt concentration in the media and therefore possess an effective way to exclude salt by means of e.g. a Na^+ extrusion coupled to a NAD(P)H redox system in the plasma membrane ([Katz & Pick 2001] . To what effect the observed long term consequences are linked to the osmotic conditions created by NaCl or to the effect of NaCl itself remains to be elucidated.

In conclusion we have analyzed the long- and short term effects on lipid productivity by changes in the salt concentration of the growth media for *Dunaliella salina*. One expectation that a sudden change in salinity could result in increased lipid productivity was not met. As cell growth slowed down as result of the stress, the increase in amounts of lipid per cell did not justify the conclusion (Figure) underlining the main observation of numerous studies that cell growth and lipid productivity are inversely linked.

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6. References

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