The influence of different culture conditions on the production of aldehydes by diatoms

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by **Simon Dittami**
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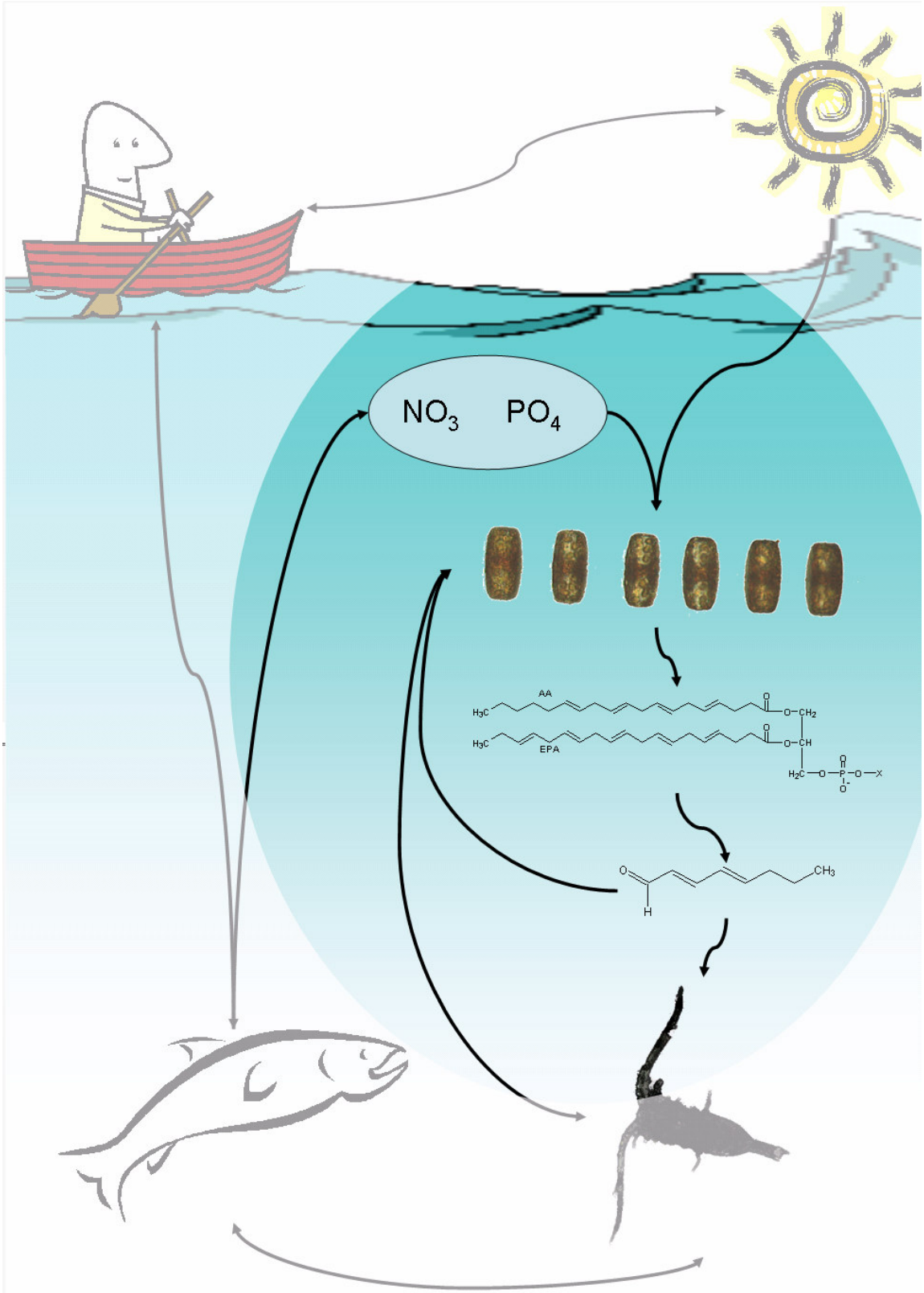
supervised by:

Prof. Dr. Heinz Brendelberger
Prof. Dr. Karen H. Wiltshire


NO3 PO4
AA
EPA

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Abstract

Diatoms are an important component in marine foodwebs; however their nutritional value for grazers has been frequently questioned in experiments using monoalgal diets. This diploma thesis examines the fatty acid composition of four different strains of *Thalassiosira rotula* under N and P limited conditions, in natural seawater, at different light intensities, at different cell densities, and with addition of low doses of octadienal. The same samples were further tested for the production of fatty acid derived polyunsaturated aldehydes (heptadienal, octadienal, octatrienal, decadienal, decatrienal) which are suspected to have negative effects on the survival of copepod offspring. Pigment content, quantum yield, chain-length and growth rate were also monitored.

The findings indicate that the production of aldehydes is strongly correlated with the ratio of examined unsaturated to saturated fatty acids. Phosphate- and nitrate-limited algae (including those cultured in natural sea water) as well as algae in the exponential growth phase contain less EPA and produce less aldehyde, while algae cultured in normal F/2 medium produced large quantities of aldehydes and contained more EPA in the stationary and death phase. Further, octadienal (0.02 µM), depending on the condition of the culture, led to an increase in aldehyde production and cellular EPA contents or even caused cell death. The other examined factors had no significant influence.

The possibility of aldehyde production as a mechanism of depriving grazers of essential fatty acids is discussed as well as a potential signaling role of aldehydes during plankton blooms.

Key words: Diatoms, *Thalassiosira rotula*, polyunsaturated aldehydes, octadienal, fatty acids, EPA, pigments, nutrient limitation, stress, signals.

1 Introduction

Over the last century our understanding of trophic interactions in marine environments has undergone many serious changes. From initial simple food-chain analyses, marine models have grown to be increasingly complex. They presently treat elaborate foodwebs with several different levels of interaction. Despite of the increase in complexity and detail, current models still are unable to reliably predict, or in some cases even to explain developments in marine trophic interactions. This is unfortunate because in times of rapid global change this kind of understanding is essential to ensure sustained usage of biological resources and to understand the long-term consequences of our current lifestyle.

Microalgae and in particular diatoms form the basis of the marine foodweb: "Photosynthesis by marine diatoms generates as much as 40% of the (…) organic carbon produced (…) in the sea," making them "responsible for ~20% of the global carbon fixation" (Armbrust et al. 2004). Fluctuations in their biological activity can have dramatic effects on the marine environment. Mann (1993) noticed that fish stocks varied synchronously, on a worldwide scale, despite different management strategies. He proposed that variation in global physical factors influencing microalgae may have had effects on all trophic levels of the foodweb. Merely looking at correlations with physical factors, however, often does not suffice: biological interactions have to be considered in order to asses the causes of global trends in the marine environment. Diatoms are excellent models in this sense. During diatom blooms a large proportion of the algae is not grazed and sinks to the seafloor unused. Indeed diatom blooms have been considered to be a consequence of the inability of grazers (mainly copepods) to follow the diatom populations quickly enough. In the 1990s, however, a number of publications demonstrated a negative effect of maternal diatom diets on the larval development of copepods. Some studies are listed in Table 1.

Table 1: Summary of studies assessing the negative effects of marine diatoms as sole food source.

Publication	**Grazer**	**Food**
Ianora & Poulet 1993	*Temora stylifera*	*Thalassiosira rotula*
Poulet et al. 1994	*Calanus helgolandicus*	*T. rotula*
Uye 1996	*Calanus pacificus*	*Thalassiosira weissflogii*
Ianora et al. 1996	*Acartia clausi*	*T. rotula*
Ban et al. 1997	16 copepod species	17 diatom species
Starr et al. 1999	*Calanus finmarchicus*	*Thalassiosira nordenskioldii*
		Naviula sp.
		Skeletonema costatum
		Chaetocerus debilis
Ianora et al. 2003	*Temora sp.*	*S. costatum*
	Acartia sp.	*T. rotula*
	Calanus sp.	
Ceballos & Ianora 2003	*Temora stylifera*	*T. rotula*
		T. weissflogii
		Phaeodactylum tricornutum
Ianora et al. 2004	*Calanus helgolandicus*	*S. costatum*

1.1 Effects of polyunsaturated aldehydes

These negative effects of diatoms on copepod reproduction have usually been attributed to the production of polyunsaturated aldehydes (PUAs). The production of aldehydes in diatoms is known in several species and strains (Miralto et al. 1999, Pohnert 2000, d'Ippolito et al. 2002a,b, Wichard et al. 2005b and others). Their toxicity has been assessed in several organisms such as copepods, sea urchins, diatoms, fungi, mollusks and even human carcinoma cells. Studies have usually looked at relatively high PUA concentrations, compared to the levels one would normally expect copepods to be exposed to during a bloom. Important studies are listed in Table 2, and in Section 2.3.3 there is an explanation for the use of only low doses of aldehyde octadienal (0.0025 µg/ml) in the present study.

Table 2: Studies on the toxic effects of aldehydes.

Publication	**Aldehyde**	**Concentration**	**Toxic for**
Miralto et al. 1999	Z,E,E-2,4,7-decatrienal Z,Z,E-2,4,7-decatrienal Z,Z-2,4-decadienal	0.5 µg/ml 0.5 µg/ml 11-17 µg/ml	sea urchin eggs copepod eggs human carcinoma cells
Casotti et al. 2001	Z,Z-decadienal	0.5 µg/ml	*T. weissflogii*
D'Ippolito et al. 2002a,b	Z,E-2,4-heptadienal Z,E-2,4-octadienal Z,E-2,4-octadienal Z,Z,E-2,4,7-decatrienal	~0.5 µg/ml mixed, as produced by *T. rotula* and *S. costatum*	*T. stylifera*
Pohnert et al. 2002b	Z,E-2,4-decadienal	1.1 µg/ml	sea urchin eggs
Adolph et al. 2003	E,E-2,4-decadienal E,E-2,4-octadienal E,Z-2,4-octadienal E,E-2,4-heptadienal E,Z-2,4-heptadienal	~1-10 µg/ml	sea urchin eggs
Ceballos & Ianora 2003	Z,Z-2,4-decadienal Z,Z-2,4-octadienal Z,Z-2,4-heptadienall	1-2 µg/ml ~2.5 µg/l ~2.5 µg/l	*T. stylifera*
Romano et al. 2003	Z,Z-decadienal	2-5 µg/ml	*C. helgolandicus* embryos, sea urchin eggs
Adolph et al. 2004	Z,E-2,4-octadienal E,Z-2,4-decadienal 2,4,7-decatrienal	agar diffusion assays: 50 µl of 1 mg/ml solution were applied	bacteria algae fungi mollusks sea urchin eggs copepods human cells
Ianora et al. 2004	Z,Z-decadienal	0.5-1.5 µg/ml	*C. helgolandicus* sea urchin eggs
Lewis et al. 2004	Z,Z-2,4-decadienal	0.005-0.1 µg/ml	*Nereis virens*

Casotti et al. 2005	Z,Z-2,4-decadienal	0.29 µg/ml	*T. weissflogii*
Pfaffenhöfer et al. 2005 (references therein)	not specified	10^-7 to 10^-5 µg/ml [1]	Polychaete larvae
Caldwell et al. 2005	E,Z/E,-2,4-decadienal	>=0.01 µg/ml >=0.01 µg/ml >=0.001 µg/ml >=0.1 µg/ml	*Asterias rubens* *Arenicola marina* *N. virens* *Psammechinus miliaris*
Vardi et al. 2006	Z,Z-2,4-decadienal	>>0.1 µg/ml	*T. weissflogii*

1.2 Pathway of aldehyde-production in diatoms

The biochemical pathway of PUA-production in *T. rotula* has been investigated in detail (Pohnert 2000, 2002a, 2004, Pohnert et al. 2002b, and Pohnert and Boland 2002c). It has proven to be similar to that found in higher plants (Siedow 1991, Hatanaka 1993, Matusi et al. 2000), e. g. for leaf odour production or as a stress induced defense reaction, and was previously discovered in the freshwater diatoms *Fragilaria sp. and Melosira varians* by Wendel and Jüttner (1996). The three steps that have been identified are exemplified in Figure 1. First polyunsaturated fatty acids (PUFAs) are liberated from phospholipids by the wound-activated phospholipase A2 (PLA_2) activity, followed by a lipoxygenase-mediated oxidation of the PUFA and cleavage of the hydroperoxy-fatty acid by the hydroperoxidlyase.

The PUAs produced vary according to different factors:

1. The position of the C-atom that is oxidized by the lipoxygenase. Pohnert (2000, 2002a, 2004) and Pohnert et al. (2002b) propose the oxidation of the 11th C-atom in *T. rotula*. With other diatoms such as the freshwater species *Gomphonema parvulum* (Pohnert et al. 2002b, Pohnert and Bouland 2002c, Pohnert 2004) or *Asterionella formosa* (Pohnert et al. 2002b) the oxidation of the 9th or 12th C-atom has been reported.
2. The fatty acids metabolized: As shown in Figure 1 eicosapentaenoic acid (EPA) is probably used for the production of

[1] probably a misprint as concentrations in the referenced paper are 1000fold higher

decatrienal while arachidonic acid (AA) is transformed to decadienal. The utilization of other PUFAs such as linoleic, linolenic, hexadecatrienoic acid or DHA for the production of other aldehydes such as hexenal or octadienal seems plausible and has been reported e. g. of leaves of higher plants (Hatanaka 1993).

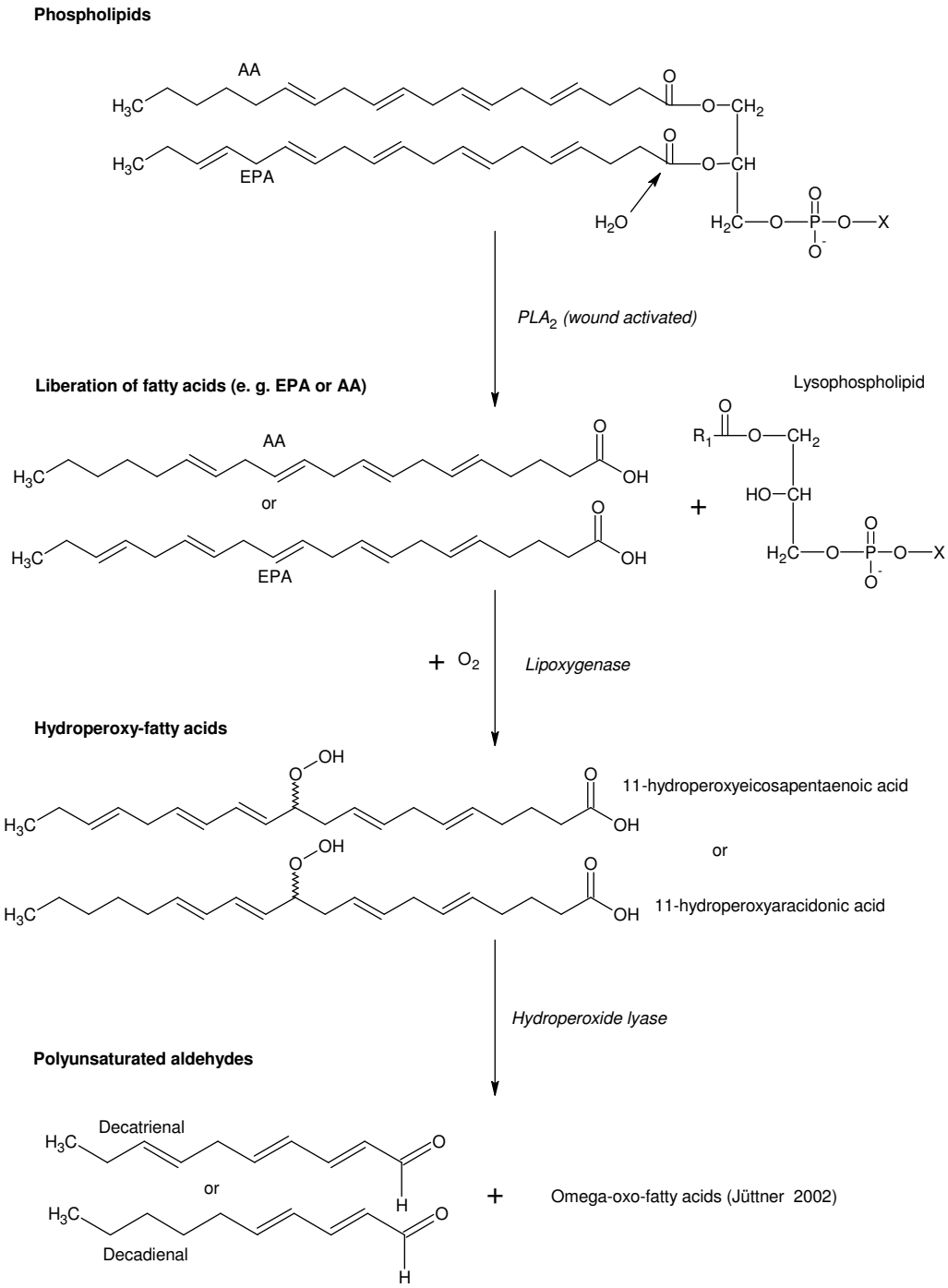

Figure 1: Pathway of aldehyde production in diatoms as described in Pohnert 2000, 2002a, 2004, Pohnert et al. 2002b, and Pohnert and Boland 2002c.

1.3 Open questions

Even though a great deal of information exists about the production of PUAs by diatoms, there are still many discrepancies and gaps in the information (see Pfaffenhöfer et al. 2005). The ecological significance of aldehyde production, for instance, is questionable, as it has only been shown to harm the second generation of copepods. The individual diatom, some of its clones, or even the "bloom community" do not seem to benefit, as it would normally be the case (Wolfe 2000.) Similarly, no negative effects of diatom blooms on the copepod population have been found in natural environments (Ban et al. 2000, Irigoien et al. 2002).

Another controversial topic is whether the toxicity is due to the aldehydes produced (e. g. Ianora et al. 2003) or caused by nutritional deficiency. Diatoms are considered to be high quality food (Brett & Müller-Navarra 1997). Nonetheless, when one puts all the facts together it appears that the production of aldehydes upon wounding might be the cause for a nutritional deficiency due to the lack of certain fatty acids (e. g. EPA). This effect would not have been detected in previous studies with the sampling and analysis techniques used.

It is also unclear which algae species or strains produce aldehydes under which conditions. Some have been reported to produce unsaturated aldehydes in one study and not in another. For example d'Ippolito et al. (2002b) detected several PUAs in *S. costatum*, whereas Wichard et al. were not able to find any in the same alga (2005b). The same is true for *T. rotula* strain CCMP 1018. It was initially believed not to produce aldehydes (G. Pohnert, K. Wiltshire, personal communication) but has recently been shown to produce low amounts of C-7 and C-8 PUAs (Wichard et al. 2005b). Other strains of *T. rotula* such as CCMP 1647 produce high quantities of several PUAs including decadienal and decatrienal (Wichard et al. 2005b). The production of algal volatile compounds (mainly PUAs) has been reported to be resource-mediated (Watson & Satchwill 2003) and dependent on the growth phase (Rashash et al. 1995) in several chrysophytes, but to my knowledge no experiments have been conducted to investigate this phenomenon with diatoms.

1.4 Study aims

The goal of this study was to examine factors that might be responsible for the differences in aldehyde production between the various studies, focusing on the diatom *T. rotula*. This species has already been thoroughly investigated. In this study, however it was determined to what degree nutrient availability and varying light regimes influence aldehyde production, fatty acid composition, and pigment content in various strains of *T. rotula*. Additionally we sought to determine whether or not changes in the fatty acid composition, the presence of bacteria in the medium, and the production of aldehydes were in any way correlated. The influence of low doses of the aldehyde octadienal (the signaling role of which has been proposed by Watson 2003, Casotti et al. 2005 and Vardi et al. 2006) on the aforementioned parameters was also tested and discussed.

All this information is intended to help understand how changing physical (light), chemical (nutrients, aldehydes), and biological (bacteria, grazers) parameters can affect the nutritional quality and toxicity of the key diatom species *T. rotula*. This would contribute to our knowledge of the dynamics of these factors and their ecological relevance for trophic interactions during plankton blooms.

2 Materials and methods

2.1 Experimental setup

Generally all experiments were set up similarly: The algae were cultured under defined conditions until they reached a particular growth phase and their fatty acid composition and aldehyde production were measured along with the other parameters described in 2.4. Figure 2 gives an overview of the various culture conditions and examined parameters. Four strains of the marine diatom *T. rotula* were cultured in three different nutrient conditions: medium with high phosphate and nitrate (NP), medium with low phosphate content (N) and medium with low nitrate content (P) (see 2.3.1). These two nutrients were chosen because they are key elements in almost all components of the cell and have been previously shown to influence the aldehyde production (Watson & Satchwill, 2003) and the fatty acid and pigment composition (Pistocchi et al. 2005) of other algae. Three replicates of each culture condition were made. The entire experiment was repeated with three different light conditions (see 2.3.2 for light conditions, and 3.3.1 for a table of treatments carried out successfully).

Additional experiments were carried out under "medium" light conditions: Two strains were kept in normal F/2 (NP) and were treated daily with low doses of octadienal in methanol or solely with methanol (see section 2.3.3). All four strains were also cultured in filtered seawater to estimate the effects of the rather unnatural environment F/2. The August and March strains were also successfully kept in larger 5 l flasks with F/2 medium based on seawater and harvested in different growth phases to assess the effects of different algal densities and growth phases. (Cultivation of the CCMP strains and cultivation using artificial seawater were not successful – see section 3.2.1). This will be referred to as "density-dependence experiment").

Each experiment, except the density-dependence experiment, was started by pipetting 5 ml of algal solution into each of the prepared culture flasks, which were then arranged randomly in a climate cabinet and rearranged daily.

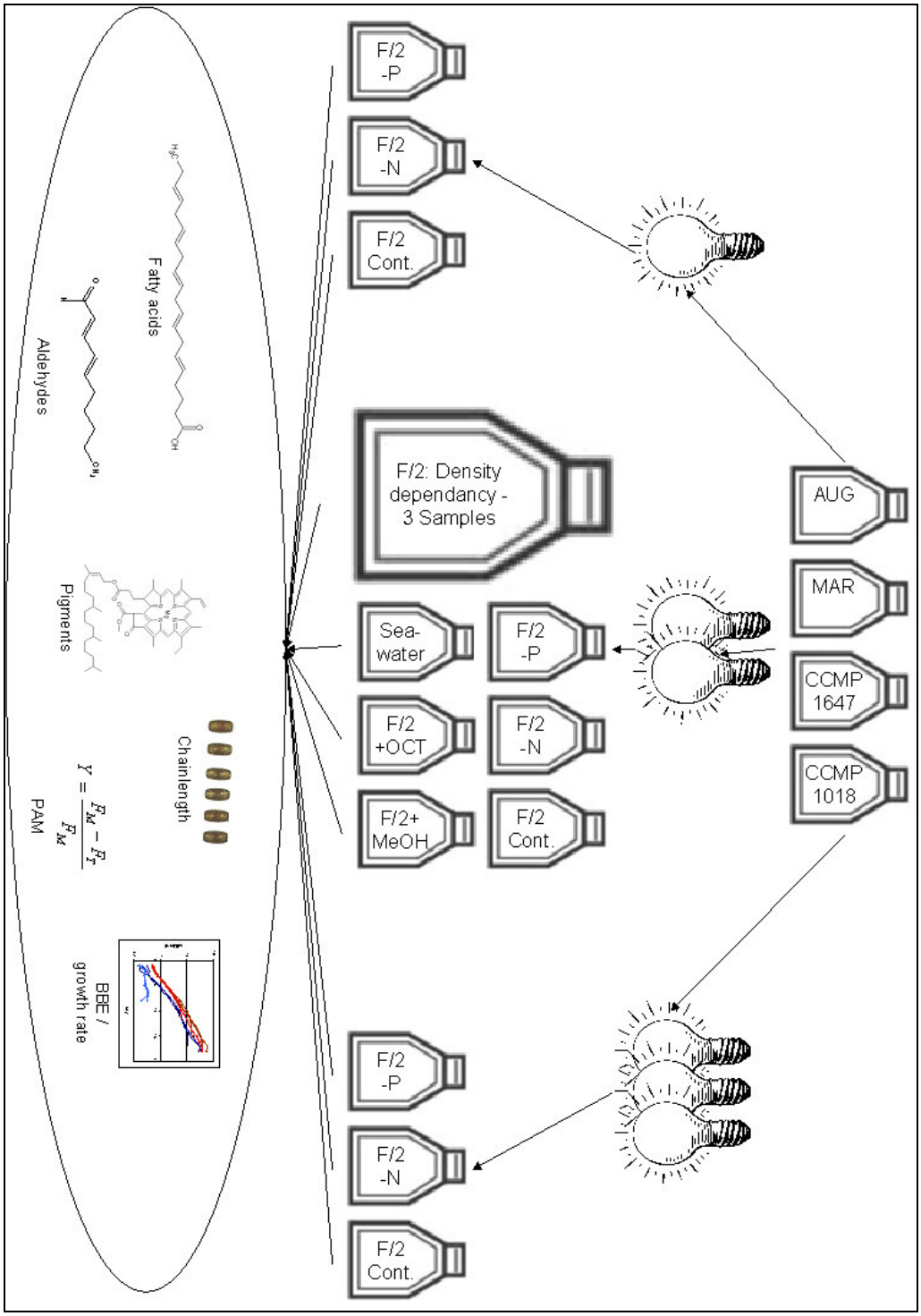

Figure 2: Overview of the experimental setup and the applied methods. CCMP 1018, CCMP 1647, MAR and AUG refer to the examined strains (see section 2.2). "-P" indicates phosphate limited, "-N" nitrate limited, and "Cont." normal F/2 medium treatments. "Oct" refers to treatments which were administered octadienal in methanol (only CCMP 1018 and 1647), "MeOH" to control treatments with only methanol (only CCMP 1018 and 1647).

The growth rate of each culture was monitored by daily measurements of chlorophyll fluorescence (2.4.3). A culture was harvested when its two-day average growth rate dropped below 10% of its maximum growth rate. In this way all cultures were harvested during approximately the same growth phase (see Fogg & Thake 1987 for a description of the various growth phases). This procedure was necessary because growth rate and maximum cell density depend strongly on the strain as well as the nutrient and light regime.

The density-dependence experiment was started by pipetting 15 ml of culture into five liters of fresh medium. The cultures were then partially harvested at different growth stages. Parts of each culture were harvested at three points in time:

- when the chlorophyll content of the algal solution (measured using chlorophyll fluorescence) exceeded 5 µg/l (exponential phase)
- when the two-day average growth rate dropped below 10% of its maximum growth rate (stationary phase)
- seven days after the second harvest (death phase)

2.2 Cultures

Four strains of the *T. rotula* were used for the experiments:

- CCMP 1018[2] (1018) – a strain of diatoms known to produce little or no PUAs (Wichard et al. 2005b), isolated from the Pacific ocean in 1968
- CCMP 1647[2] (1647) known to produce high amounts of PUA

as well as two strains isolated from the Helgoland Roads (see Figure 3)

- on March 11th 2004 (MAR) by A. Schwaderer
- and on August 29th 2005 (AUG) in the course of this work.

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[2] Both CCMP strains were purchased from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Maine, USA in August 2005

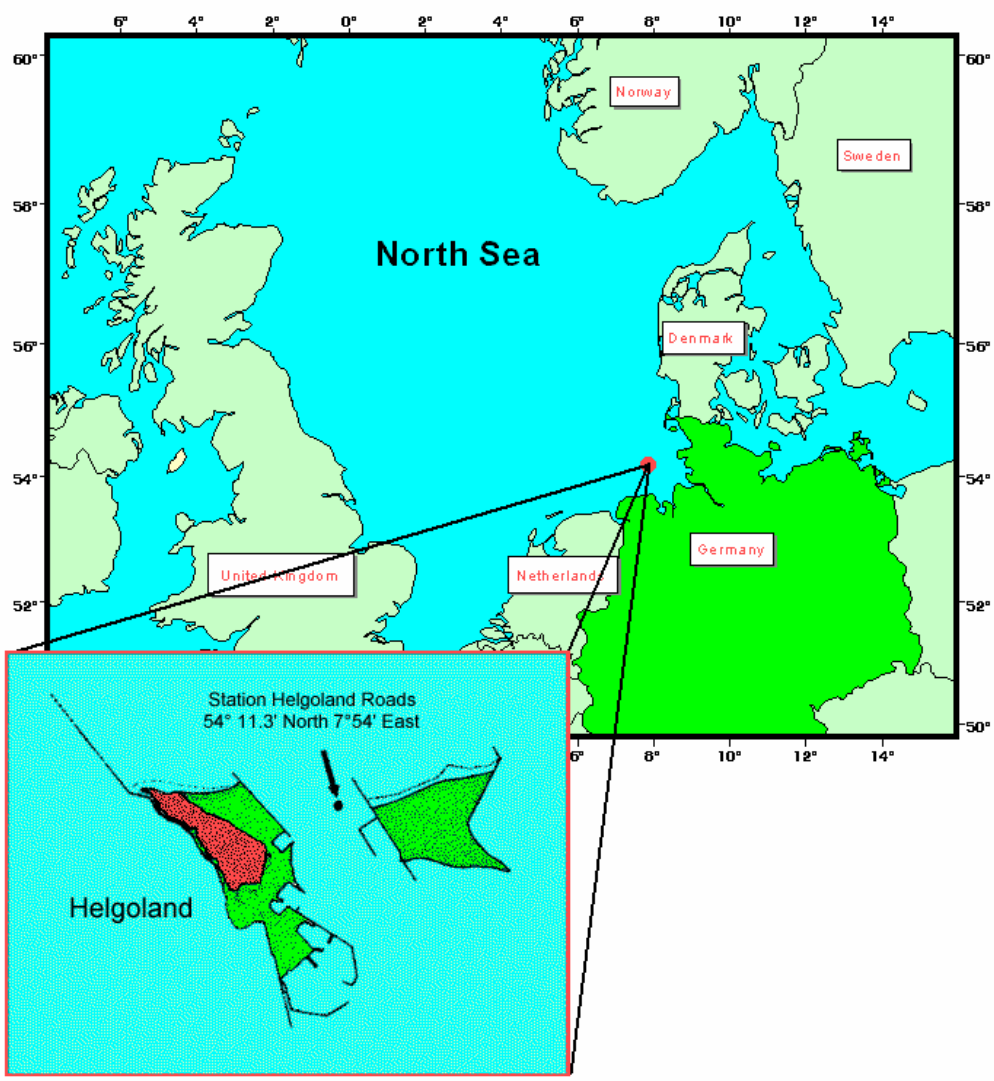

Figure 3: Geographical location of the Helgoland Roads (map taken from the homepage of the German Centre for Marine Biodiversity Research http://www.senckenberg.de/dzmb/plankton/station.html).

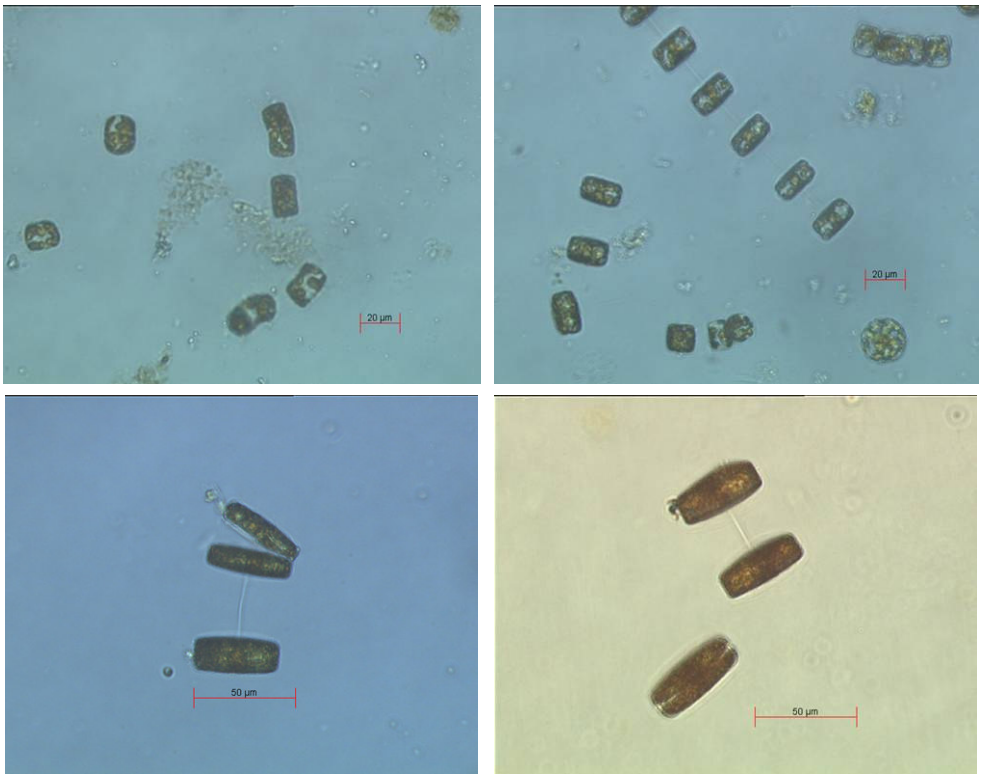

Figure 4: Photographs of the T. rotula strains. Top left: CCMP 1018, top right: CCMP 1647, bottom left: "March" strain, bottom right: "August" strain.

2.3 Culture conditions

2.3.1 Culture medium

The F/2 medium used for the experiments was produced with artificial seawater. 36.8 g guaranteed nutrient-free sea salt (hw Meersalz professional, Wiegandt GmbH, Krefeld, Germany) were dissolved in one liter of distilled water, resulting in a total salinity of about 31.5, measured twice using a Guideline Autosal salinometer and the UNESCO formula to calculate the salinity from the resistance (see Grasshoff 1976). Then F/2 nutrients (and silicate) were added according to Guillard and Ryther (1962) and Guillard (1975). In the nitrate- and phosphate-limited treatments 0.2% of the normal F/2 concentrations of nitrate or phosphate were added, resulting in concentrations similar to those found in the Helgoland Roads (see Figure 3). The F/2 media therefore contained nutrients and vitamins as described in Table 3.

Table 3: Nutrient concentration in the F/2 used for the experiments. P refers to the N-limited and N to the P-limited treatment. NP is normal F/2 medium. Values in italics do not differ from the NP treatment.

Nutrient	Conc. NP	Conc. P	Conc. N
$NaNO_3$	75 mg/l= 882.5 µM	0.15 mg/l = 1.7 µM	*75 mg/l= 882.5 µM*
Na_2HPO_4	5 mg/l = 35.22 µM	*5 mg/l = 35.22 µM*	0.01 mg/l=0.07 µM
Na_2SiF_6	10 mg/l= 78.09 µM	*10 mg/l= 78.09 µM*	*10 mg/l= 78.09 µM*
HCl	0.001%	*0.001%*	*0.001%*
$ZnSO_4$ x H_2O	0.015 mg/l	*0.015 mg/l*	*0.015 mg/l*
$CuSO_4$ x 5 H_2O	0.01 mg/l	*0.01 mg/l*	*0.01 mg/l*
$CoSO_4$ x 7 H_2O	0.012 mg/l	*0.012 mg/l*	*0.012 mg/l*
$MnSO_4$ x H_2O	0.2 mg/l	*0.2 mg/l*	*0.2 mg/l*
$FeCl_3$ x 6H_2O	0.5 mg/l	*0.5 mg/l*	*0.5 mg/l*
Na_2MoO_4 x 2H_2O	0.0065 mg/l	*0.0065 mg/l*	*0.0065 mg/l*
Na_2EDTA x 2 H_2O	5.0 mg/l	*5.0 mg/l*	*5.0 mg/l*
Vitamin B12	0.001 mg/l	*0.001 mg/l*	*0.001 mg/l*
Biotin	0.001 mg/l	*0.001 mg/l*	*0.001 mg/l*

After adding the nutrients the pH of the medium was adjusted to 7.5 by adding approximately 3 ml of hydrochloric acid (2%) to each liter of medium. In the final step the medium was sterilely filtered (0.2 µm filter, Schleicher & Schuell, Germany) into previously autoclaved (120 °C, 5 min) plastic flasks. 0.5 liter-portions were directly transferred into sterile cell culture flasks (175 cm², Corning Inc., NY, USA). The culture flasks were closed using sterile 0.2 µm vent caps, covered with aluminum foil to avoid evaporation through the vent cap, and stored at 15 °C until the beginning of the experiment. In the density-dependence experiment, the medium required, was filtered (0.2 µm) directly into previously autoclaved five liter 'Schott' bottles. The algae were added within 24 hours after filtration. Experiments using seawater (SW) were conducted with water taken from the Helgoland Roads (see Figure 3) on several days in September 2005 (salinity 31.8). After sterile filtration, the water was directly used for the experiments.

At the end of each experiment the remaining phosphate, nitrite, nitrate and silicate in the medium were measured, using wet chemical and photometric methods.

2.3.2 Light and temperature

During all experiments the algae were kept at 15°C in a 12h/12h light/dark cycle using a Rumed Type 1301 light culture cabinet (Rubart, Germany, see Figure 5 for light spectrum). The following light intensities were measured in the experimental setup with a Lambda Inst. LI-185 quantum meter:

L-: 3-4 µE/(m²*s) (=1 set of lights at 20%)

L0: 30-40 µE/(m²*s) (=2 sets of lights at 28%)

L+: 120-160 µE/(m²*s) (=2 sets of lights at 50%)

L0 was chosen according to Schöne (1972), who reported 2400 Lux (approximately 40 µE/(s*m²)) to be the optimal light intensity to culture *T. rotula* at 12 to 18 °C.

L+ was chosen according to Schnitzler et al. 2004, who reported light intensities of 150-200 µE/(m²*s) to induce increased photorespiration in *S. costatum*.

The density-dependence experiment was performed in a climate chamber (also at 15 °C and a 12h/12h light/dark cycle). Lighting was provided by OSRAM Lumilux De Luxe (L 58W/965 25X1) fluorescent tubes with the BIOLUX colour spectrum which is similar to natural daylight with a moderate UV content (see Figure 5 for light spectrum). The light intensity was adjusted to 20-30 µE/(m²*s).

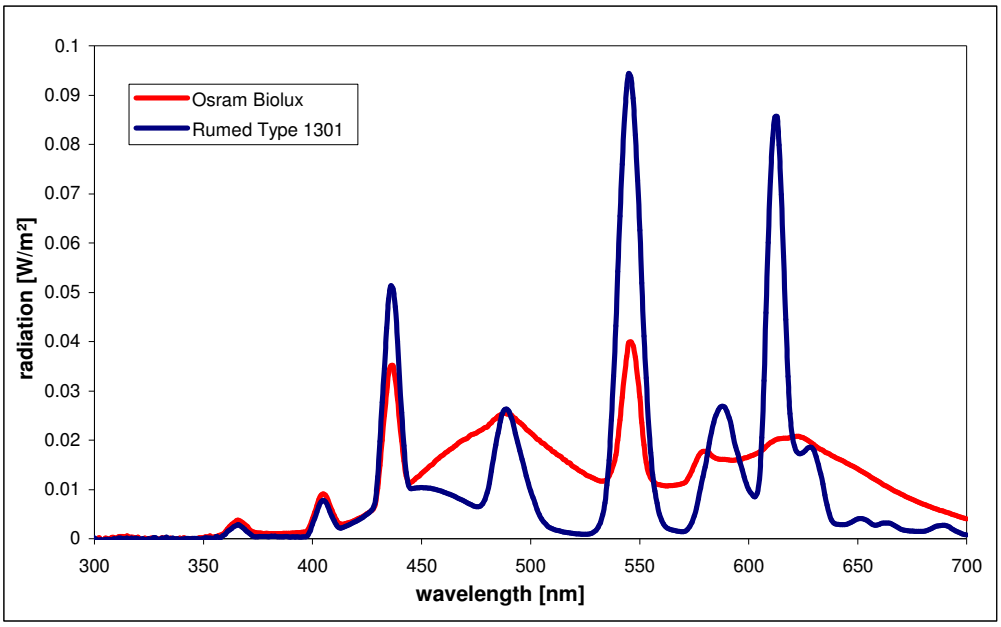

Figure 5: Light composition used for the experiments. The Osram Biolux lamps were used for the density-dependence experiment, while all other experiments were conducted using the Rumed Type 1301 climate cabinet. Both spectra were measured at about 20 µE/(m²s).

2.3.3 Aldehyde treatments

Due to time constraints and the limited number of samples that could be sent in for aldehyde analysis only one experiment with addition of aldehydes was performed. In a parallel experiment the diatom strains CCMP 1018 and CCMP 1647 were incubated in F/2 under L0-light and normal (NP) nutrient conditions. They were supplemented daily with 2 µl of a solution containing 1.25 µg of octadienal in methanol (OCT) or, in a control treatment, solely with 2 µl of methanol (MET). Unfortunately, with the CCMP 1018 strain, the applied quantity of methanol clearly had a negative effect on algal growth without octadienal (see section 3.1.1). Later, pure methanol as a solvent was replaced by a mixture of 10% methanol and 90% distilled water, and there was no more visible difference in growth between treatments that were administered methanol and treatments that were not. The final octadienal-concentration, however, remained 0.0025 µg OCT per ml and day. This is equivalent to a theoretical amount of PUA one would expect during a bloom of PUA-producing diatoms on the basis of the following assumptions:

1. The bloom contains 10^4 cells of PUA-producing algae per ml (numbers that have been reported by Ban et al. 1997).

2. Each cell of *T. rotula* produces 6.35 fmol of PUA upon disruption (Wichard et al. 2005b).
3. The average molecular weight of PUAs is that of decadienal: 152 g/mol.
4. 50% of all aldehydes produced are lost by sloppy feeding of the copepod grazers (Møller et al. 2003, Møller 2005). Once ingested aldehyde is assumed to bind to DNA or proteins and remain within the grazing organisms.
5. 50% of the diatoms are grazed per day.

Unfortunately, to my knowledge, no data on actual aldehyde concentrations and their half-lives in seawater are available thus far.

The OCT-solution (0.625 mg/ml) used in the experiments was produced by diluting 12.5 mg (=14.29 µl) of OCT (Sigma-Aldrich >=96%, predominantly trans, trans) with methanol (later 10% methanol in destilled water) to a final volume of 20 ml and filling the solution into ten 2 ml glass vials, which were each then sealed air-tight with a teflon-coated septum and used on three subsequent days of the experiment.

2.4 Analysis

2.4.1 Pigment quantification

High Performance Liquid Chromatography (HPLC) measurements were performed using a method based on Garrido et al. (2003), modified by B. Knefelkamp (in prep.). Pigments were extracted from the cells as described in Wiltshire et al. (2000) and pumped onto the column with a high pressure pump. With HPLC the major separating property is the polarity of the pigments. Highly polar pigments are more likely to be found in the polar mobile phase, whereas less polar ones remain longer on the column. Further the changing composition (and polarity) of the mobile phase (see Figure 7) allows even the more apolar pigments to leave the column. Upon leaving the column the pigments are detected with an appropriate detector and identified using the retention time, GC/MS analysis or, in this case, absorption spectra (see Figure 6 for a typical HPLC diagram, absorption spectra are recorded for each peak but not shown).

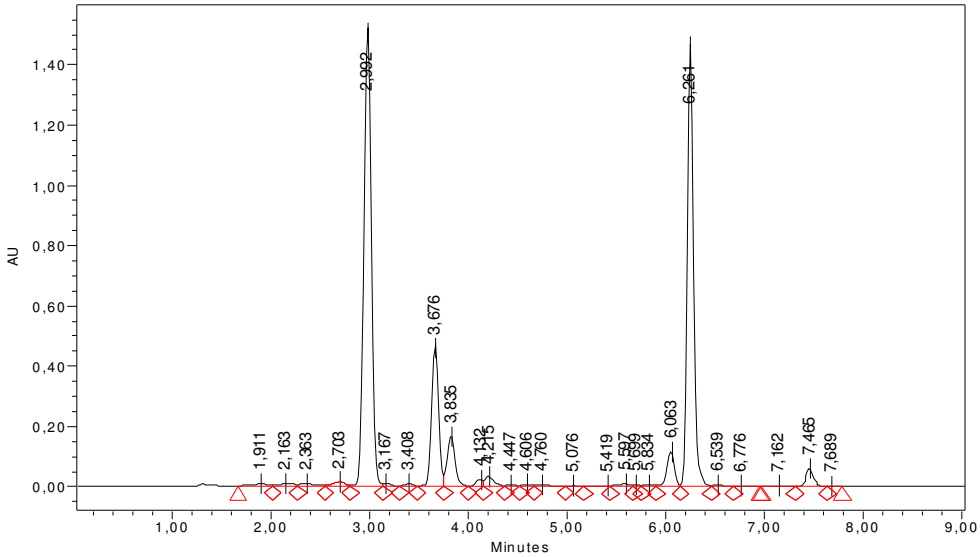

Figure 6: Example of an HPLC chromatogram of the T. rotula March strain during the death phase. The large peak at 2.992 min. represents fucoxanthin, the one at 6.261 min. chlorophyll a.

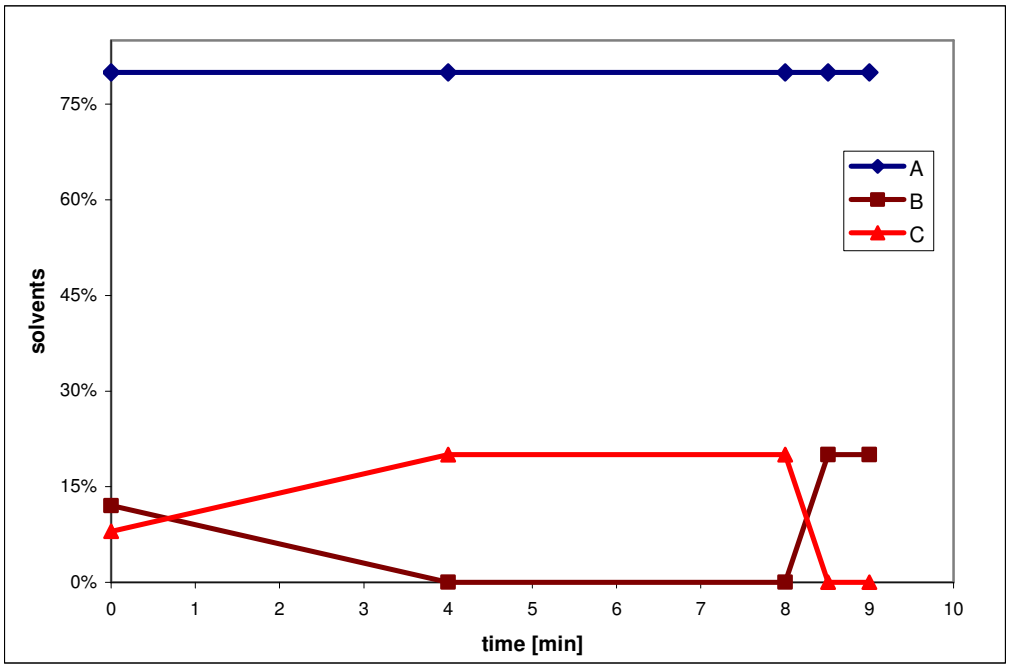

Figure 7: Solvents used for the HPLC analysis. Solvent A = methanol, solvent B = 25 mM pyridine solution in distilled water, adjusted to pH 5 with acetic acid, solvent C = acetone.

In this study the preparation of HPLC samples was conducted as follows: Depending on the algal density about 20 to 100 ml of each culture were filtered onto a 0.2 µm nylon filter (Whatman). The volumes used for HPLC-analysis of the density-dependence experiment were 500 ml during the

exponential phase and 100 ml during the stationary and death phase. The filter was then folded and put into a 15 ml Orange Scientific centrifuge tube, where it was covered with 2 ml of acetone (HPLC-grade, Lab-Scan, UK) and deep-frozen at -70°C for later analysis. After two to 100 days the sample was thawed, quartz sand was added and the sample was homogenized using a plastic-tipped rod. The sample was then placed in a dark ultrasound bath (Bandeline Sonorex Super RK 103H) on ice for 90 minutes for extractions. The extracts were filtered (0.2 µm) into glass vials using a 5 ml syringe and Spartan 30/0.2 RC filter units (Schleicher and Schuell, Germany).

10 µl of distilled water followed by 100 µl of the sample and again 10 µl of water were injected using a cooled autosampler (4°C). The flow rate was set to 1.3 ml/min, solvents were used as shown in Figure 7, and the column temperature was set to 25°C. Pigments were detected at 430 nm and 668 nm with a Waters 996 Photodiode Array (PDA) detector and identified by comparing the absorption spectra to that of commercially available pigment standards (Chlorophyll C3, Chlorophyll C2, Peridinin, 19-Butanoyloxyfucoxanthin, Fucoxanthin, 19-Hexanoxyfucoxanthin, Prasinoxanthin, Neoxanthin, Diadinoxanthin, Alloxanthin, Diatoxanthin, Zeaxanthin, Lutein, Chlorophll B, Echinenone, Chlorophyll a, beta-carotene) and the spectra described in Jeffrey et al. (1997).

2.4.2 PAM

The principle of pulse amplitude modulated fluorescence measurements, according to Maxwell and Johnson (2000), is based on the fact that every single photon that is absorbed by a chloroplast can be used for photosynthesis, re-emitted as light of a distinct but lower frequency than the exciting light (fluorescence) or dissipated as heat. Since the proportion of energy dissipated as heat is relatively constant, fluorescence can provide a measure of the current photosynthetic efficiency. Fluorescence (measured after excitation with the measuring light) of a dark-adapted cell (F0) is compared to that (Fm) of a cell exposed to very bright light (saturation pulse). The saturation pulse is assumed to "overload" Photosystem II (PSII) and temporarily close all reaction centers, forcing the entire energy to be emitted as fluorescence (and heat). The fluorescence signal during the saturation

pulse is therefore considered the maximum fluorescence (Fm, see Figure 8 for an example). The "(Quantum-)Yield" value is calculated as

$$Yield = \frac{F_m - F_0}{F_m} = \frac{F_v}{F_m} \quad (1)$$

and correlates excellently with the O_2-production and the efficiency of photosynthesis in plants (Björkman and Demming 1986). As photosynthesis is also tightly correlated to growth and reproduction, the PAM readings may serve as indicators of the plants' overall fitness.

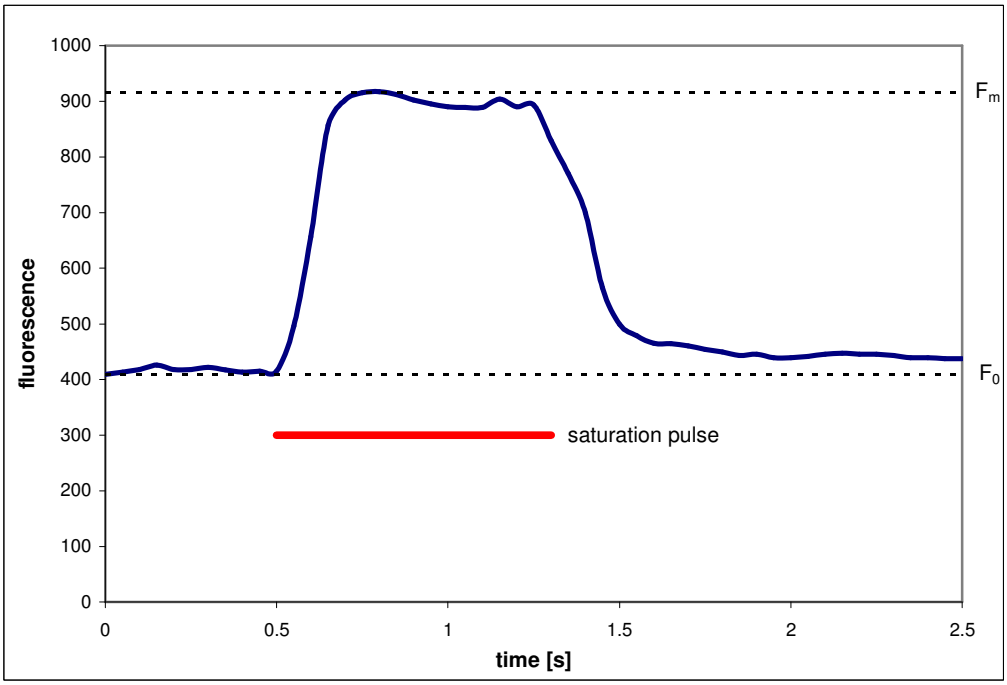

Figure 8: Typical PAM measurement. The blue line indicates the measured fluorescence, while the straight red line shows when the saturation pulse was given. Fluorescence is measured in relative units.

PAM measurements were performed every third day and immediately before the sample was harvested using a Walz PM-MF connected to a PAM-control unit. For each measurement 2 ml of each culture were dark-adapted for at least one minute and then measured using an actinic light intensity of two and a 0.8 second saturation pulse at a light intensity of twelve (given light intensities are relative units specific for the Walz PAM fluorometers, for a more detailed list of settings see Appendix). On harvesting days two samples were taken and measured three times each, on other days only one sample was measured only once. If necessary the sample was diluted with filtered seawater for the measurement.

2.4.3 BBE / multialgae fluorescence

The BBE Multialgae Fluorometer (as described in Beutler et al. 2002) is based on the same principle as PAM measurements. Again pigments are excited at certain wavelength and their fluorescence is recorded. The Multialgae Fluorometer, in contrast to the PAM, uses six different wavelengths (370, 450, 525, 570, 590, and 610 nm) to excite different antenna pigments and measure fluorescence. As algal groups differ strongly in their pigment composition and therefore in their fluorescence at different wavelengths (i. e. in their color), this information can be used to estimate the composition and quantity of main algal groups within a sample. This is done by mathematically fitting average fluorescence patterns of known groups to the recorded fluorescence. The Multialgae Fluorometer used in this study (bbe Cuvette Fluorometer, bbe Moldaenke GmbH, Kiel, Germany) can differentiate between green algae, blue algae, diatoms and cryptophytes. By measuring and correcting for "yellow substances", measurements are further improved. However, as there is a lot of variability in pigment composition between species of the same algal groups and even within species, the measured algal composition can only be seen as a rough guideline.

In this study BBE measurements were used as a quick fluorometric measure of the chlorophyll concentration in the samples. Chlorophyll, under similar culture conditions and for the same species, is considered a good marker for biomass (see Granberg and Harjula 1980 for constraints), and was therefore used to create growth curves on a daily basis. These, in turn, were the basis for deciding when the cultures were supposed to be harvested (see 2.1). Due to time constraints (one BBE measurement takes about three minutes), every sample was only measured once a day. The 25 ml used for analysis were put back into the original culture flasks after the measurement.

2.4.4 Counts

Cells were counted every second day according to the following procedure: The culture flask was gently turned upside-down twice to resuspend sedimented algae. 2 ml of medium were then carefully transferred into brown 2 ml Eppendorf caps using cut-off 1 ml pipette tips (to minimize fracture of cell chains). One drop of Lugol's solution was added, and the caps

were once more gently turned upside-down. Fixed samples were then stored at +2°C and counted later, using an Olympus CX41 microscope at 40x to 100x magnification (the latter only for the CCMP strains) and Sedgewick-Rafter-cells (Gratuculates Ltd., Tonbridge, UK). At least 400 cells were counted from each sample. The average weighted chain-length and standard deviation were calculated while counting, using a free Java program (see Appendix). The chain length was weighted, i. e. the average number of cells to which each cell is connected including the cell itself (see Lampert & Sommer 1999) was counted. For example a sample containing ten individual cells and one chain of ten cells has an average chain length of two cells but an average weighted chain length of 5.5 cells.

2.4.5 Fatty acids

Fatty acids were analyzed using gas chromatography (GC) and a method based on Wiltshire et al. 2000, modified according to M. Boersma and A. Malzahn (pers. comm.). The principle of GC is similar to that of HPLC: Substances are extracted from the cells (see below), evaporated and immediately injected into the column. In the case of GC, however, the mobile phase is usually helium, and the separating property is the strength of adsorption to the column, which in turn depends on the type of molecule, the column materials and the column temperature.

For this study fatty acid samples were prepared as follows: 200 ml of algal suspension were filtered onto precombusted Whatman GF/C filters at maximally 200 mbar. The filters were then deep-frozen at -80°C in 15 ml Orange scientific centrifuge tubes for about two months until the samples were freeze-dried and prepared for further GC-analysis.

In order to assess the dry weight of algae used for fatty acid analysis, the following weights (a, b, c) were measured. The total amount of salt (S) on the filter was calculated as indicated in (5) and (6) where "W" is the estimated amount of medium on the filter and "s" the salinity of the medium. The algal dry weight (DW) was then calculated according to (7).

$$a = empty_tube + clean_filter \quad (2)$$

$$b = tube + wet_filter + cells \quad (3)$$

$$c = tube + filter + cells \text{ (after freeze-drying)} \quad (4)$$

$$W = b - a \quad (5)$$

$$S = W \cdot s \quad (6)$$

$$\mathrm{DW} = \mathrm{c} - \mathrm{a} - \mathrm{S} \quad (7)$$

For analysis the freeze-dried filters were transferred to 10 ml glass centrifuge tubes (Schott, Germany) and covered with 4 ml of a dichloromethane (CH_2Cl_2)/methanol-mixture (2:1, V:V) containing 200 mg/l butylated hydrotoluene (BHT) to avoid auto-oxidation of unsaturated fatty acids. 10 µl of internal standard (saturated C13 and C23 fatty acid methyl esters, 0.25 µg/µl) were then added. After the sample was sonicated for 90 minutes in an ultrasound bath in ice-cold water, it was transferred to another fresh centrifuge tube where 2 ml of a 0.88%KCl solution were added to denaturize proteins and DNA (Wiltshire et al. 2000). The sample was then centrifuged at 4000 RPM and 0°C for 10 minutes, using a Sorvall refrigerated centrifuge (DuPont Instruments) and an SS-34 rotor. Two phases were then clearly visible: The upper phase (water) was removed along with most of the proteins between the two phases. The bottom phase (dichloromethane / methanol) containing the fatty acids was again transferred to a new centrifuge tube, and 3 ml of methanol/H_2SO_4 (93:3, V:V) were added. The samples were placed in a heat block at 70°C for one hour. This allowed the fatty acids and the methanol to react to fatty acid methyl esters (FAMEs). 2 ml of hexane were added and the sample was vortexed for about one minute, in order to transfer the FAMEs into the hexane, which, after renewed centrifugation, was then pipetted into a 1.5 ml glass vial and evaporated under a stream of nitrogen. This step was performed twice. The residue was then taken up in 100 µl of hexane. 1 µl of sample was analyzed using a Varian CP 3800 gas chromatograph. The samples were separated over a 30 m column (inside diameter 250 µm), the temperature of which was gradually increased from 60°C to 220°C. Substances that passed through the column were detected using a flame ionization detector (FID) (see Figure 9 for a typical chromatogram) and identified based on their retention time (RT). The following fatty acids were investigated in this study:

- eicosapentaenoic acid (EPA, 20:5n3, RT ≈ 52 min), the precursor of decatrienal (Pohnert et al. 2002b)

- arachidonic acid (AA, 20:4n6, RT ≈ 49 min), the precursor of decadienal (Pohnert & Bouland 2002c)
- alpha-linolenic acid (ALA, 18:3n3, RT ≈ 37 min); it can be converted to EPA and AA (Herbst 2004, references therein) and might be the precursor of octadienal
- docosahexaenoic acid (DHA, 22:6n3, RT ≈ 66 min); as an omega-3 fatty acid it may play an important role in nutrition
- saturated C16 and C18 fatty acids (16:0, RT ≈ 23 min; C18:0, RT ≈ 32.5 min); the most abundant saturated fatty acids in the sample.

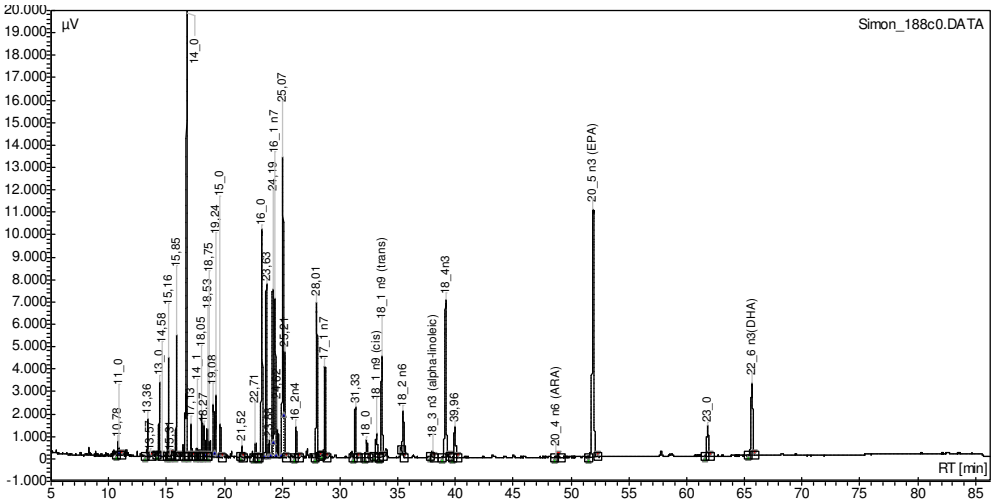

Figure 9: A typical chromatogram (in this case March algae during the death phase).

All identified peaks were checked manually to ensure that peak identification worked correctly. Non-recognized peaks were added manually, if present. In the case of AA, it was sometimes necessary to add methyl esters of AA (AAME) and to remeasure the sample in order to find out which of the two peaks close to the expected retention time of AAME was actually AAME.

2.4.6 Aldehydes

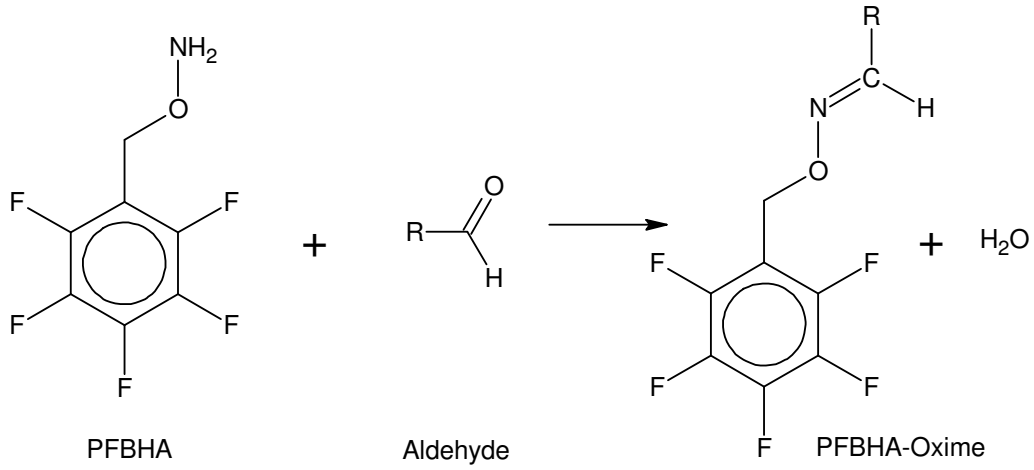

Figure 10: Aldehyde derivatization using O-(2,3,4,5,6-pentafluorbenzyl hydrolamine).

The analysis of aldehydes was performed according to the new method proposed by Wichard et al. 2005a. *O*-(2,3,4,5,6-pentafluorbenzyl) hyodroxylamine hydrochlorine (PFBHA·HCl) was added before wounding of the cells. Thus, the aldehydes were directly converted into *O*-pentafluorbenzyl-oxime derivates (see Figure 10) before reacting with proteins or DNA. *O*-Pentafluorbenzyl-oxime derivates were kindly identified and quantified after fragmentation with Electron Impact Ionization using GC/MS by Prof. Dr. G. Pohnert and T. Wichard from the Max-Planck Institute of Chemical Ecology in Jena, Germany. The preparation of samples sent to Jena was performed according to the following scheme (Wichard et al., 2005a; T. Wichard, personal communication):

1. 200 ml of cell culture (1.5 and 0.75 liters for lower density experiments) were gently concentrated onto Whatman nylon membrane filters (0.2 µm, max. 700 mbar), leaving a thin film of water on the filter.
2. The filter was rinsed with 1 ml 25 mM PFBHA·HCl in 100 mM Tris/HCl. The Tris/HCl buffer was produced by dissolving 3.0285 g of Tris (Sigma-Aldrich) in 200 ml distilled water, adjusting the pH to 7.8, and again adding distilled water to reach a total volume of 250 ml. 1 g of

PFBHA·HCl was then added to 160 ml of Tris-buffer, changing the pH to approximately 7.0.

3. The PFBHA·HCl solution containing the algae was then transferred into a 4 ml glass vial.
4. 5 µl of internal standard (1mM benzaldehyde in methanol) were added using a 5 µl micro-syringe (ILS, Germany). The internal standard was created by weighing 0.106 g = 101 µl benzaldehyde (Merck) and adding methanol (Merck, HPLC-Grade) to a total volume of 100 ml. 10 ml of the methanol / benzaldehyde mixture were taken and once more diluted with methanol to reach a total volume of 100 ml.
5. The sample was then cooled to approximately 4°C with ice and sonicated for 1 minute using a Sonoplus HD60 ultrasonic homogenizer (Bandelin electronic GmbH, Berlin, Germany).
6. The vial was sealed air-tight with a teflon septum and incubated at room temperature for 30 minutes.
7. 1 ml of n-hexane (Merck, GC-Grade) and 0.5 ml of methanol were added.
8. The sample was vortexed for 1 minute.
9. Six drops of concentrated sulfuric acid were added.
10. The sample was vortexed for one minute, again.
11. Both phases (a separation of phases was usually not distinct) were then transferred to two 2 ml Eppendorf caps and centrifuged for 90 sec. at 10^4 rpm in a minispin plus centrifuge (Eppendorf AG, Hamburg, Germany).
12. The hexane phase was then pipetted into a pasteur pipette, filled to ¼ with sodium sulfate, held in position by a small piece of a precombusted GF/C filter (Whatmann). The sample was then squeezed into a 1.5 ml glass vial (Agilent, Palo Alto, CA, USA) and the pipette rinsed with 1 ml of n-hexane.
13. The sample was kept at -20°C until it was evaporated to dryness under a stream of nitrogen a few weeks later.
14. The residue was then taken up in 50 µl hexane, transferred to 1.5 N8 glass vials with 200 µl high recovery inserts, sealed with a teflon coated septum (Macherey & Nagel) and sent to Prof. Dr. G. Pohnert and T. Wichard, MPI for chemical ecology, Jena, Germany for GS/MS analysis after a few weeks of storage.

2.4.7 dsDNA-quantification (bacteria)

DNA-quantification was performed using the Quant-iT™ PicoGreen dsDNA Reagent Kit (Invitrogen Molecular Probes, Eugene, USA). PicoGreen is a highly sensitive fluorescent DNA stain, facilitating quantification amounts of double-stranded DNA (dsDNA) as small as 0.2 ng (Molecular Probes 2005). PicoGreen that has bound to dsDNA shows fluorescence at 520 nm when excited by light of 480 nm wavelength.

To quantify the amount of double-stranded DNA (e. g. chloroplasts from lysed diatom cells and bacteria) in the algae culture medium, about 2 ml of sample were filtered over a Whatman GF/C filter. The filtrate containing the bacteria was then frozen at -20°C until the analysis was performed.

For the analysis 0.5 ml of PicoGreen reagent was diluted with 99.5 ml of 10 mM Tris/EDTA buffer. The Tris/EDTA (TE) buffer was produced by dissolving 1.21 g Tris (Sigma-Aldrich) and 292 mg EDTA (Merck) in 500 ml of distilled water. The pH was adjusted to 7.5 using 2% HCl, and distilled water was added to reach a total volume of 1 l. A series of standards with known dsDNA concentrations was created by diluting commercially available lambda-DNA standard (also available from Molecular Probes) to concentrations of 100, 50, 10, 1, and 0 ng/ml with TE-buffer. 1 ml of diluted PicoGreen reagent was added to 1 ml of sample or standard in a 2 ml Eppendorf cap. After incubation in the dark at room temperature for about two to five minutes 300 µl were transferred six times from each sample or standard onto a black 96-well well plate and measured with a Cary Eclipse spectrofluorometer (Varian, CA, USA) using the following settings: excitation wavelength 480 nm, emission wavelength 520 nm, PMT 1000 V, measuring time 0.5 sec. (see appendix for a more detailed list of settings). The linear regression of the measured standards was used to convert fluorescence values to actual DNA quantities.

In samples with mainly intact cells, it was assumed that most of the DNA in the medium was of bacterial origin. The DNA-quantities determined were therefore used as a rough estimate of quantity of bacteria in the medium. Unpublished results from experiments during the Trophodynamic Interactions course held in the Biological Station Helgoland, Germany in 2005 have shown that very low doses of decadienal have a strong effect on marine bacteria, and therefore this parameter was included in the experiment.

2.5 Statistical evaluation

The density-dependence experiment was analysed with Statistica using a repeated measures ANOVA (analysis of variance), with "strain" as an independent variable and time as within subjects factor. A Levene's test for homogeneity of variances as well as a Mauchly Sphericity test was used to ensure homogeneity of variances and that the data was non spherical.

The growth curves of the octadienal experiment were also compared using Statistica's repeated measures ANOVA function (see above). The fatty acid composition and the aldehyde production of the two treatments in this experiment were compared using an independent samples t-test (two sided). The homogeneity of variance was ensured using a Levene's test, and the data was shown to be normally distributed using a Kolmogorov-Smirnov-Test.

A fixed effects ANOVA (model I) was applied for the light and nutrient experiment, using fatty acid composition, individual fatty acid concentrations and aldehyde production as dependent variables and light, strain, and medium as independent variables. Where the data proved not to be normally distributed it was $\log_{10}$-transformed to meet this criterion. If the data still differed significantly from a normal distribution the ANOVA was perfomed anyway, as fixed effects ANOVAs are quite resistant to the violation of this criterion (Sachs, 2004). All tests were performed fully factorial, i. e. all possible interaction terms were considered in the model.

All data was plotted as mean values ± the standard deviation of the sample. In the light and nutrients experiment replicates that differed only in factors that were shown not to be significant, were sometimes averaged and plotted together. In this case the standard error was used instead of the standard deviation.

In a final step the other monitored variables (DNA concentration, chain-length, quantum yield, and maximum growth rate) were correlated (Pearson correlation, two-sided) with the fatty acid composition, the produced aldehydes, and the residuals from the performed ANOVAs. As several correlations were performed with the same dataset a Bonferroni correction was applied: The alpha-level (originally 0.05) was corrected to 0.0056 (as each factor was tested maximally nine times).

3 Results

3.1 Aldehyde experiment

3.1.1 Growth and fitness

As can be seen in Figure 11, very low concentrations of octadienal in methanol (Tukey HSD post hoc test, p=0.000429) but also of methanol (Tukey HSD post hoc test, p=0.0128) had significant negative effects on the growth of the CCMP 1018 strain of *T. rotula* (Repeated Measures ANOVA, $F_{2,10}$=9.957, p=0.00162, Mauchley Sphericity Test, p>0.05, Levene's test, p>0.05). This made the examination of the other parameters such as fatty acids and aldehydes impossible. With the CCMP 1647 strain effects were evident but not as pronounced and not statistically significant (Repeated Measures ANOVA, $F_{2,12}$=2.260, p=0.123, Mauchley Sphericity Test, p<0.01, Levene's test, p>0.05). The experiment with the CCMP 1018 strain was repeated with F/2 medium using filtered seawater. This had proven to be a better medium for both of the CCMP strains than artificial seawater. In filtered seawater there were no noticeable effects of octadienal on either the final cell counts or the growth rate. The final cell concentrations of each strain and medium are listed in Table 4. No effects of octadienal on either quantum-yield or chain-length were found (data not shown).

Table 4: Summary of final cell densities at harvesting time in each treatment (cells per ml ± standard deviation) and number of replicates that were successfully harvested.

Strain	Medium	Octadienal	Methanol
1018	ASW + F/2	n=0	n=0
1018	SW + F/2	15621 ± 5633, n=3	20332 ± 18645, n=3
1647	ASW + F/2	3530 ± 1276, n=3	2135 ± 261, n=3

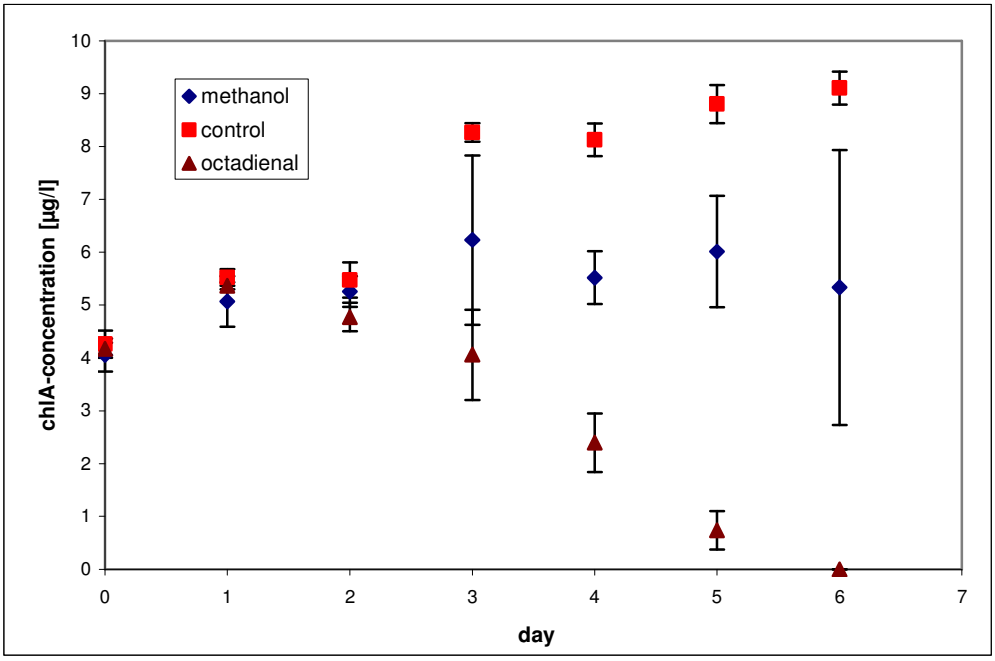

Figure 11: The effects of octadienal (0.0025 µg/ml) in methanol administered on day one, three, and five on the growth of the CCMP 1018 strain of T. rotula. The plot displays the average chlorophyll content of three replicates and the standard deviation. "Methanol" refers to the treatment without octadienal, while control refers to the cultures with neither methanol nor octadienal.

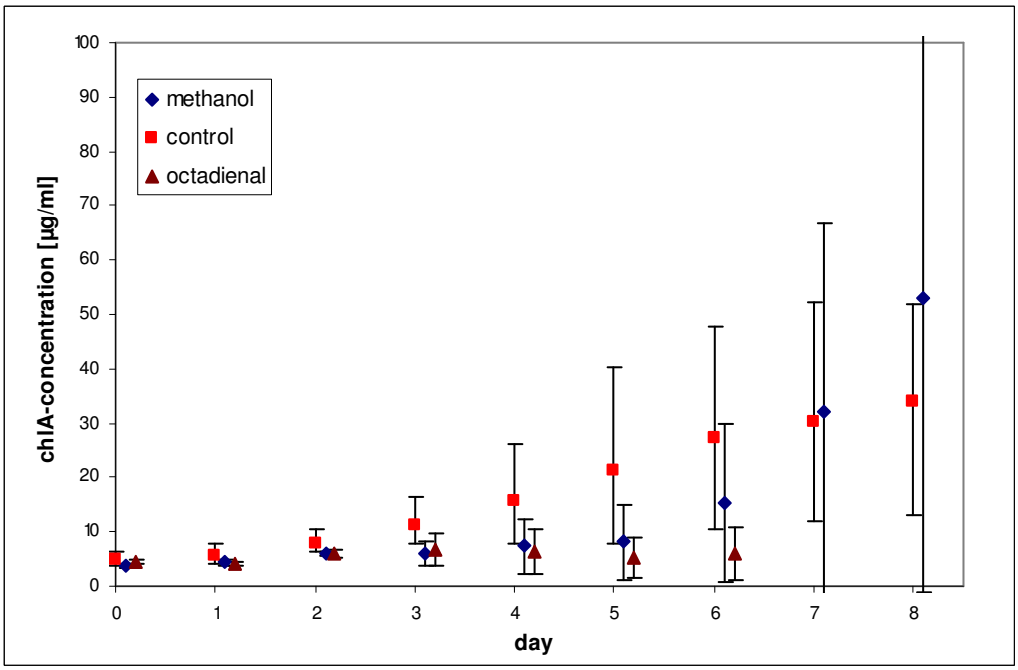

Figure 12: The effects of octadienal (0.0025 µg/ml) in methanol adminstered on uneven days on the growth of the CCMP 1647 strain of T. rotula. The plot displays the average chlorophyll content of three replicates and the standard deviation. "Methanol" refers to the treatment without octadienal, while control refers to the cultures with neither methanol nor octadienal.

3.1.2 Pigments

The octadienal-experiment only consisted of only six samples. A summary of all pigments found in the various strains of *T. rotula* can be found in the pigments section of the light and nutrient experiment (3.3.2). Nevertheless it should be mentioned that, as seen in Figure 13, the mean cellular content of chlorophyll a did not vary a lot among strains and treatments. All other examined pigments did not change in their proportions to chlorophyll a.

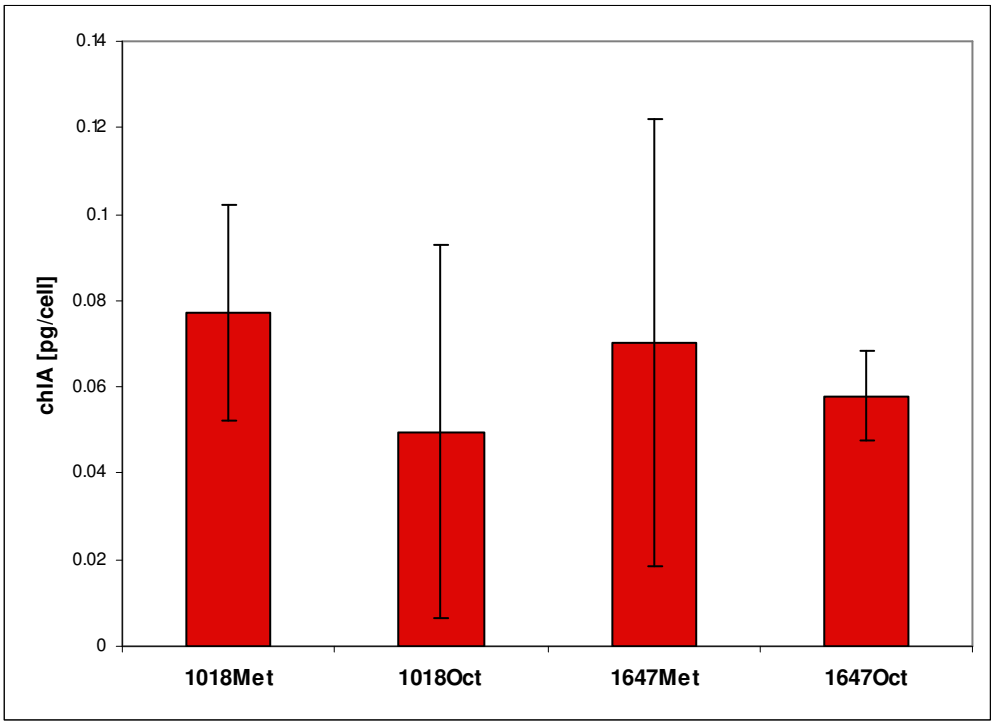

Figure 13: Chlorophyll a content of the various treatments in the octadienal experiment. The means of three replicates ± standard deviation are shown. "Oct" refers to treatments with octadienal; "Met" refers to control treatments with only methanol.

3.1.3 Fatty acid composition

As can be seen in Figure 14 octadienal did not seem to have a clear effect on the fatty acid composition of the CCMP 1018 strain cultured in normal SW+F/2 (2-sided t-test assuming homogenous variances, p=0.957).

With the CCMP 1647 strain cultured in ASW there appeared to be an effect: Algae treated with octadienal had a higher fatty acid content, however the effect was not statistically significant (2-sided t-test assuming homogenous variances: p=0.189). The increased ratio of unsaturated to saturated fatty acids in the CCMP 1647 algae treated with octadienal was close to significance (2-sided t-test assuming homogenous variances, p=0.0636, Levene's test, p>0.05, Kolmogorov-Smirnov-Test, p>0.05).

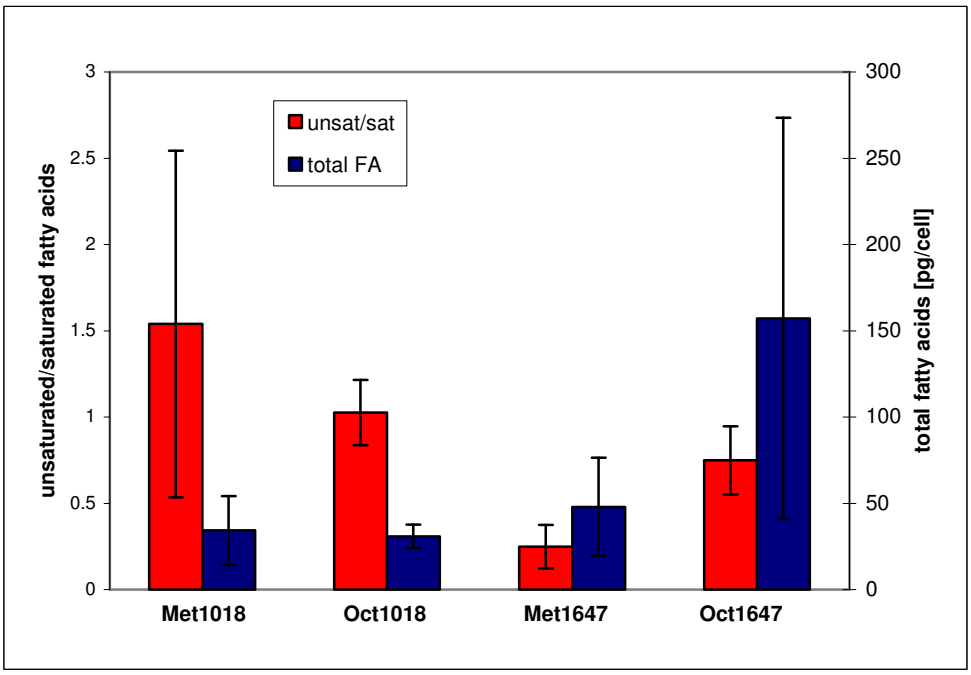

Figure 14: The effect of low doses of octadienal (final concentration 0.0025 µg/ml octadienal per day dissolved in methanol) on the ratio of saturated to unsaturated fatty acids (left) and the total fatty acid content (right) of the T. rotula strains CCMP 1018 and CCMP 1647. The means of three replicates (except Met1018, only two replicates) are plotted with the standard deviation. "Oct" refers to treatments with octadienal, "Met" to treatments with only methanol.

While the cellular amount of fatty acids doubled in the "octadienal" treatment, that of the unsaturated fatty acids AA, alpha-linoleic acid (ALA) and EPA was approximately five or more times higher than in the "methanol" treatment. The cellular content of DHA was the only parameter that decreased with octadienal (see Figure 15).

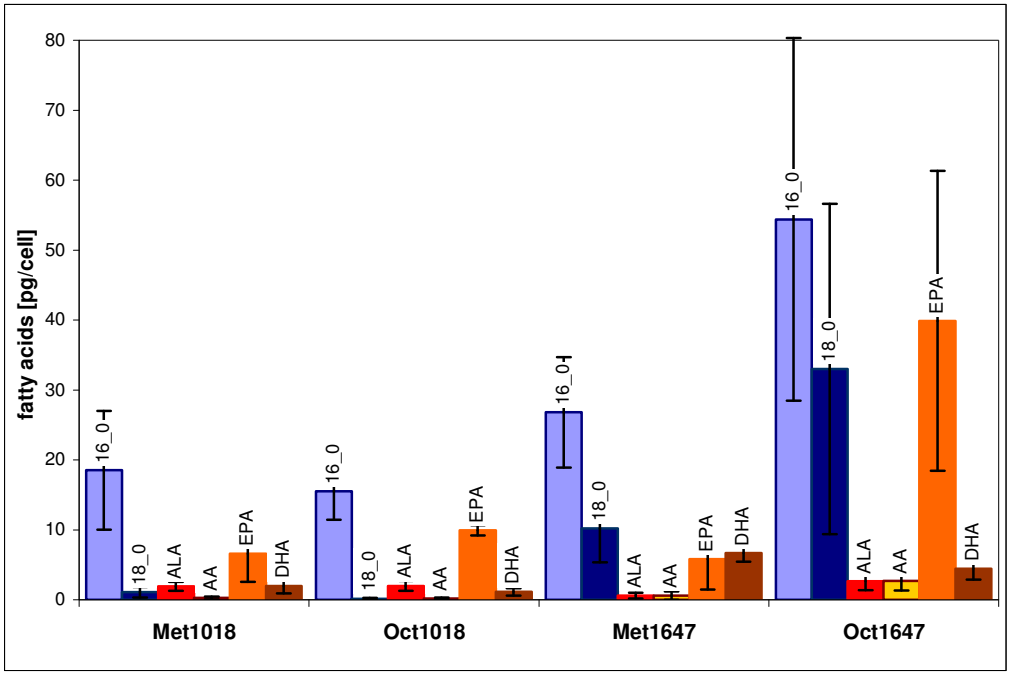

Figure 15: Fatty acid composition in the aldehyde treatments. The means of three replicates (except Met1018, only two replicates) and the standard deviation are shown. "Oct" refers to treatments with octadienal, "Met" to treatments with only methanol.

3.1.4 Aldehyde production

Due to low cell numbers in the CCMP 1647 aldehyde samples (see 3.1.1), the aldehyde analysis was not reliable for this strain. The growth rates of the CCMP 1018 strain in normal SW were not affected. As a result aldehyde analyses for this strain were successful. Although these samples did not show a strong change in fatty acid composition, the production of aldehydes was almost three times higher than in the controls (see Figure 16). Due to the small number of replicates (n=3) this observation could not be verified statistically (2-sided t-test assuming homogenous variances, $p=0.1308$, Levene's test, $p>0.05$, Kolmogorov-Smirnov-Test, $p>0.05$).

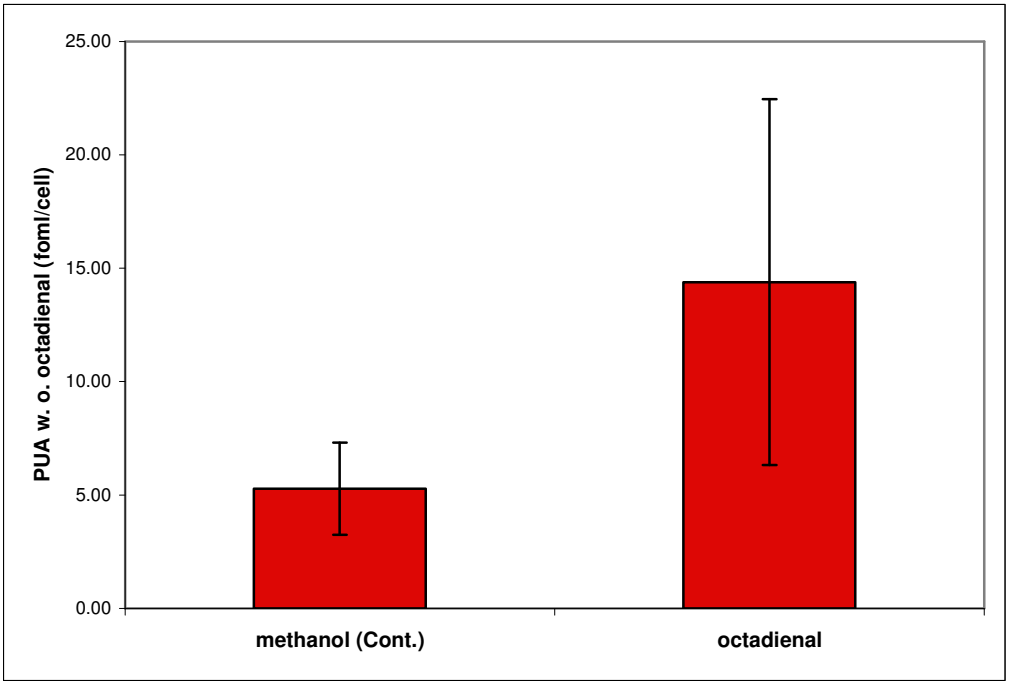

Figure 16: Difference in aldehyde production (all aldehydes except octadienal) in the CCMP 1018 strain with and without octadienal treatment. The means of three replicates ± standard deviation are shown.

3.2 Density dependence experiment

3.2.1 Growth and fitness

Table 5: Summary of final cell densities at harvesting time in each treatment (cells per ml ± standard deviation) and number of replicates that were successfully harvested.

Strain	Exponential phase	Stationary phase	Death phase
1018	1450, n=1	41350, n=1	31850, n=1
1647	n=0	n=0	n=0
MAR	499 ± 106, n=3	16300 ± 3950, n=3	10850 ± 368, n=3
AUG	419 ± 79, n=3	12956 ± 8416, n=3	7717 ± 536, n=3

As can be seen in Figure 17 both chain-length (Repeated Measures ANOVA, $F_{2,8}$=96.274, p=0.000003, Levene's test, p>0.05, Mauchly's Sphericity Test, p>0.05) and quantum yields (Repeated Measures ANOVA, $F_{2,8}$=183.685, p<0.000001, Levene's test, p>0.05, Mauchly's Sphericity Test, p=0.0123) declined from the exponential to the death phase. With respect to the quantum yields the data are assumed to have been spherical, and

therefore did not meet the prerequisites of a Repeated Measures ANOVA. With regard to chain-length there was a significant difference between the two strains examined (Tukey HSD post hoc test, p=0.00167). The mean weighted chain-length of algae isolated in August was higher than that of algae from March.

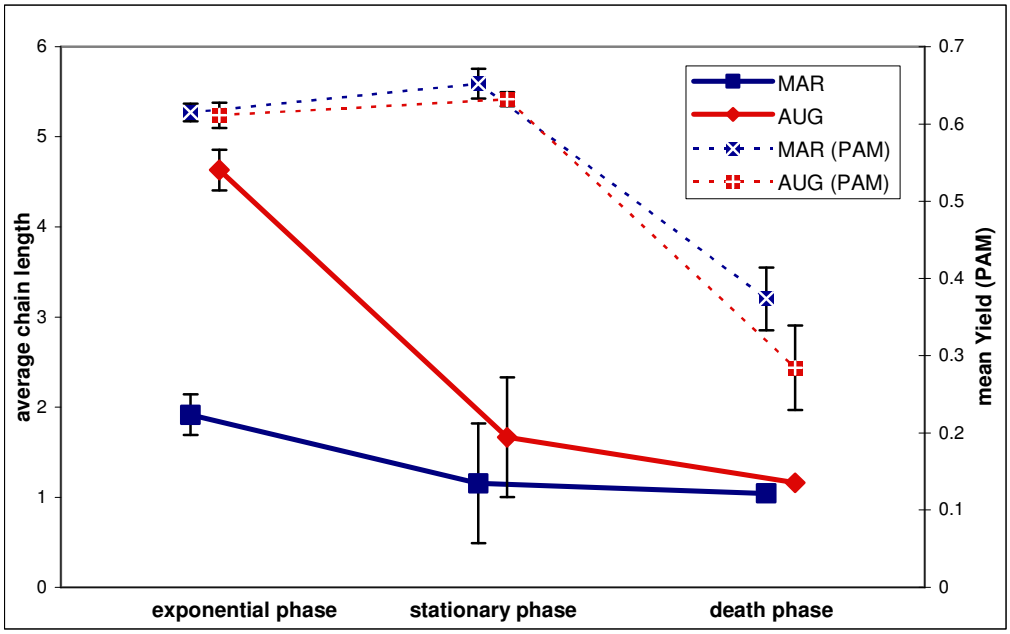

Figure 17: Average chain-lengths (weighted mean of three replicates) and quantum yields (three replicates, six measurements each) in different growth phases. The error bars represent the standard deviation.

3.2.2 Pigments

The total cellular content of chlorophyll a (see Figure 18) increased significantly from exponential phase to the death phase in both strains (Repeated Measures ANOVA, $F_{2,8}$=39.461, p=0.000073, Levene's test, p>0.05, Mauchly's Sphericity Test, p>0.05). At the same time the proportion of diadinoxanthin compared to chlorophyll a increased significantly from the stationary to the death phase (Repeated Measures ANOVA, $F_{2,8}$=157.658 p<0.000001, Levene's test, p>0.05, Mauchly's Sphericity Test, p>0.05). The diadinoxanthin-derived diatoxanthin decreased slightly in the stationary phase and later increased in the death phase. The other examined pigments did not change significantly in their proportions to chlorophyll a (see Table 6).

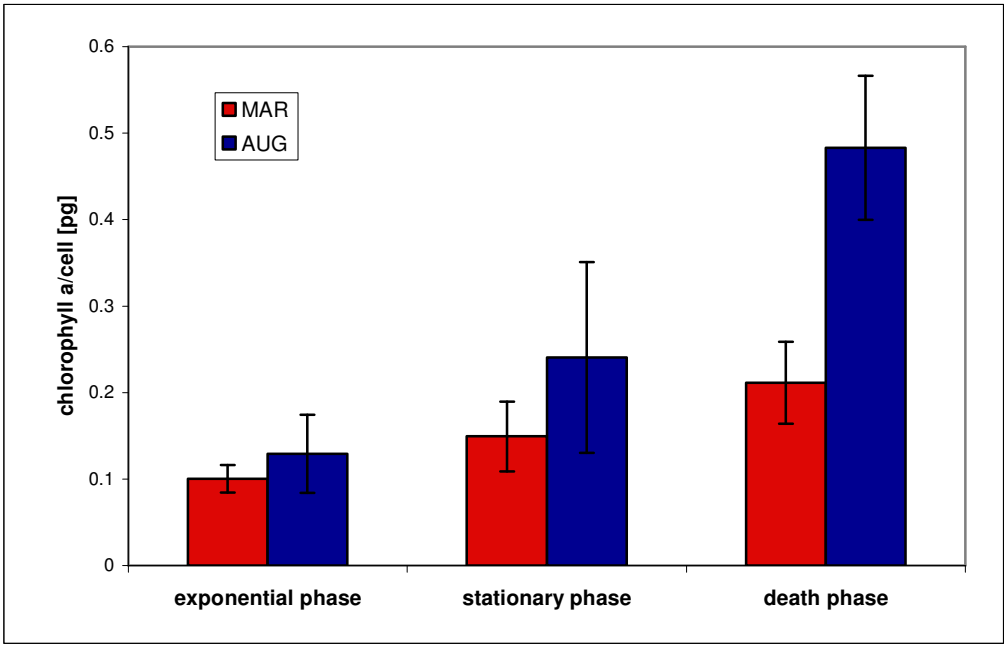

Figure 18: The change in the cellular chlorophyll a content in the various growth phases of the March and August strains of T. rotula. The means of three replicates ± the standard deviation are shown.

Table 6: Changes in the pigment composition relatively to chlorophyll a. The percentages were calculated by comparing the peak areas rather than the actual pigment concentration, as there were no calibration curves available for most of the pigments. The table shows mean values of six replicates of two strains (three MAR and three AUG) ± the standard deviation.

	exp. phase	**stat. phase**	**death phase**
Chlorophyll a	100% ± 0.0%	100% ± 0.0%	100% ± 0.0%
Fucoxanthin	118%± 22%	109% ± 9.2%	127% ± 8.0%
Diadinoxanthin	**6.2% ± 1.6%**	**4.6% ± 2.1%**	**28% ± 9.0%**
Ditatoxanthin	1.1% ± 1.7%	0.3% ± 0.3%	2.0% ± 0.4%
β-cartotene	4.6% ± 0.7%	4.9% ± 0.4%	4.5% ± 0.7%

3.2.3 Fatty acid composition

"Time" (phase) was shown to have a statistically significant effect on the fatty acid composition (Repeated Measures ANOVA, $F_{2,8}$=98.193, p=0.000002, Levene's test, p>0.05, Mauchly's Sphericity Test, p>0.05). The proportion of unsaturated fatty acids increased significantly in the stationary and death phase (see Figure 19). The factor "strain" did not have any

significant effects ($F_{1,4}$=0.208, p=0.672). A Tukey HSD post hoc test showed that differences in the fatty acid composition were significant among all phases. The total content of fatty acids per cell also increased significantly from the stationary to the death phase (see Figure 21), as was shown by a Repeated Measures ANOVA ($F_{2,8}$=6.019, p=0.0254; Levene's test, p>0.05, Mauchly's Sphericity Test, p>0.05). As seen in Figure 21 the shift in the ratio of unsaturated to saturated fatty acids was caused mainly by an increase in EPA (Repeated Measures ANOVA, $F_{2,8}$=62.03451, p=0.000013, Levene's test, p>0.05, Mauchly Sepericity Test, p>0.05). Other fatty acids did not show similar changes. 16_0, 18_0, and also DHA (Repeated measures ANOVA, $F_{2,8}$=32.202, p=0.000149, Levene's test, p>0.05, Mauchly's Sphericity Test, p=0.0149) even showed significant decreases. However, the given p-value for the change in DHA may not be reliable because the dataset was found to be spherical.

The changes in fatty acid composition coincided with changes in the nutrient and DNA concentrations of the medium: While silicate and phosphate dropped significantly in the stationary and death phase, nitrite and DNA-concentration and to a certain extent also nitrate increased (see Figure 20).

Measurements with the CCMP 1018 strain did show the same difference between exponential and stationary phase, but in the death phase the proportion of unsaturated fatty acids showed a strong decrease. Only one of the replicates reached reasonable cell densities and this one sample was strongly contaminated with flagellates (especially in the death phase). For this reason the data were not analyzed. Also due to problems with flagellates, it was not possible to cultivate the CCMP 1647 strain in large enough quantities to perform the density dependence experiment.

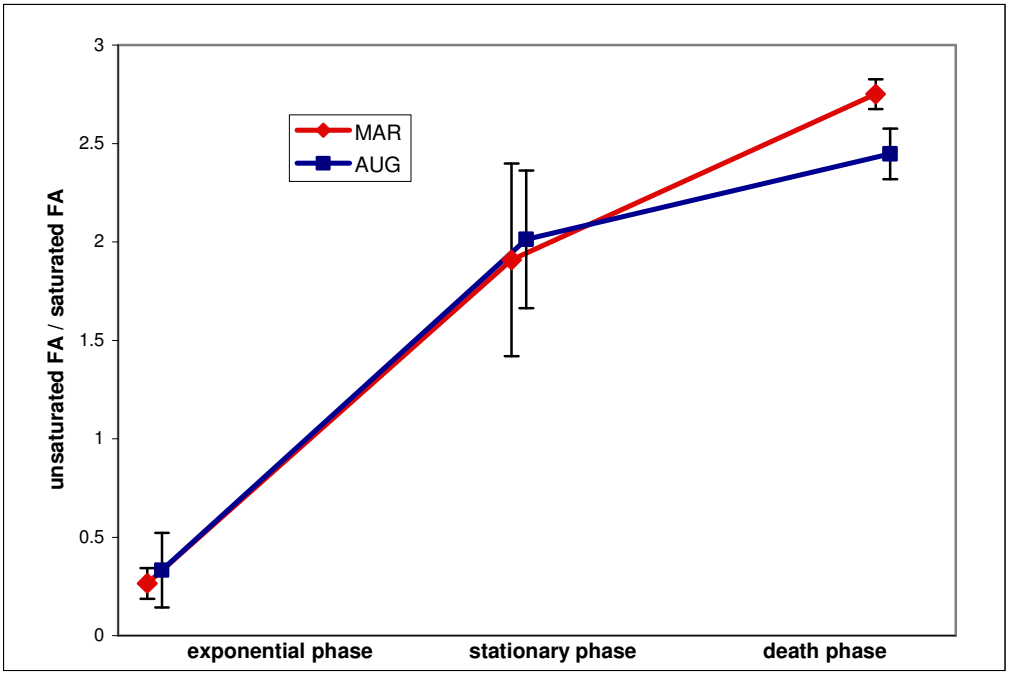

Figure 19: The effect of different growth phases on the ratio of unsaturated to saturated fatty acids in the August and March strain of T. rotula. The values for the two strains are the means of three replicates ± the standard deviation.

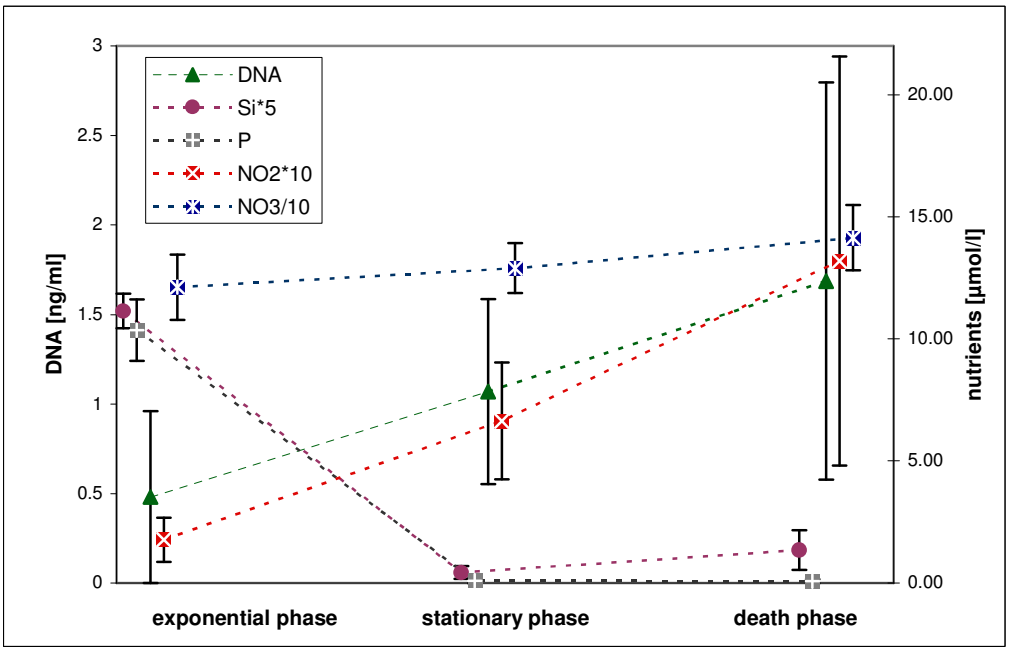

Figure 20: Nutrient regimes and DNA concentration in the medium during the various growth phases (means of six replicates ± standard deviation). All values were scaled as indicated in the legend and averaged for both strains as there were only minor differences between the strains.

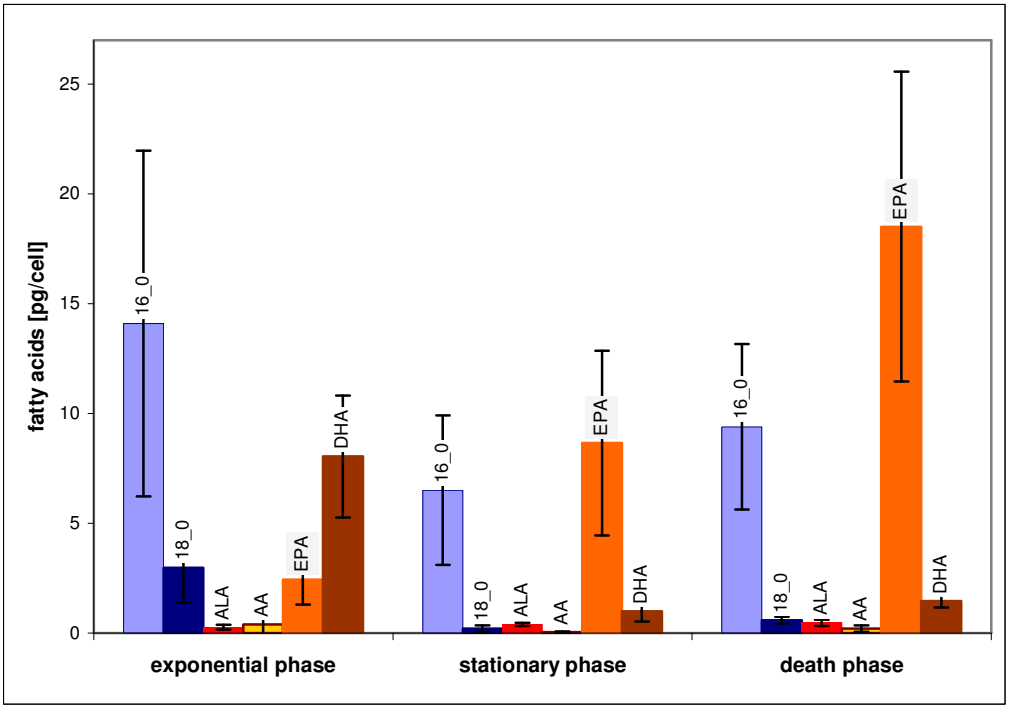

Figure 21: The cellular contents of fatty acids in different growth phases. Unsaturated fatty acids are plotted in red or brown, saturated fatty acids in blue. The bars are means of six replicates (three MAR and three AUG) ± the standard deviation.

3.2.4 Aldehyde production

Along with the change in fatty acid composition there was also a significant (Repeated Measures ANOVA, $F_{2,8}$=23.218, p=0.000466, Levene's test, p>0.05, Mauchly's Sphericity Test, p=0.0492) change in the production of polyunsaturated aldehydes (see Figure 22). In the exponential phase PUA production averaged about 1 fmol per cell, while in the death phase about 7 (MAR) or 23 fmol PUA per cell (AUG) were produced. There were no clear changes in the composition of produced PUAs. Decatrienal accounted for approximately 70% of the produced aldehydes, while heptadienal, octadienal and octatrienal accounted for about 10% each, unless the measured aldehyde concentrations were below the limit of detection as in the exponential phase.

Figure 22: PUA production of the March and August strains during the various growth phases (mean ± standard deviation).

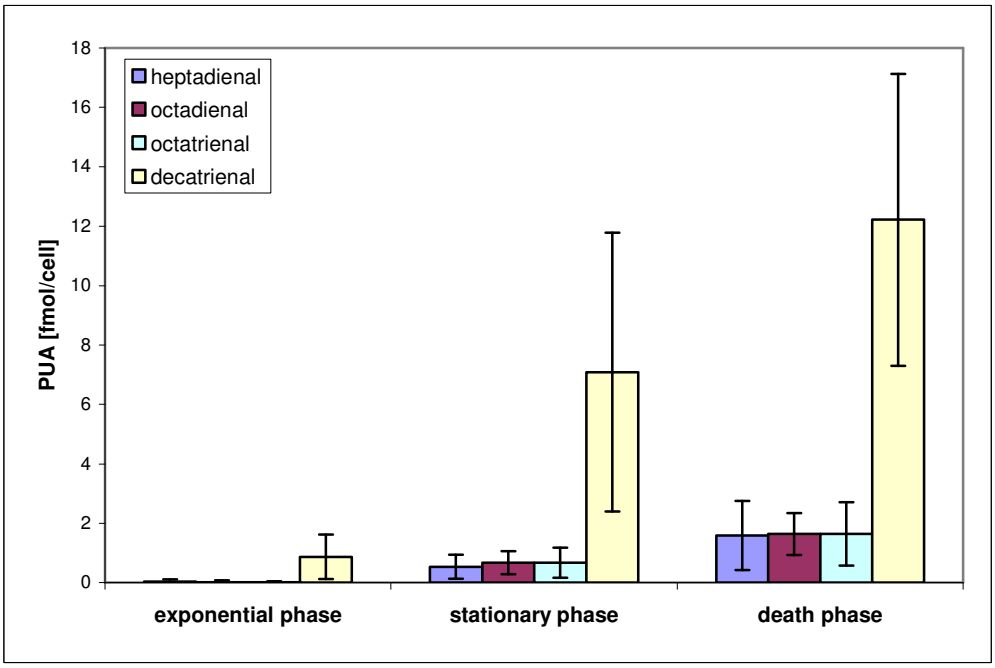

Figure 23: Changes in the composition of PUAs produced during the various growth phases (means of six replicates of the MAR and AUG strains ± standard deviation). Traces of decadienal were also found in all samples.

3.3 Nutrient and light experiment

3.3.1 Growth and fitness

There were strong differences in the ability of the various strains to grow under different nutrient-limited and light conditions. The CCMP strains, especially 1018, did not grow well in combinations of artificial seawater and high light intensities. The MAR and AUG strains grew very quickly under high light conditions although their yield values were significantly lower (ANOVA with yield as dependent and strain*light as independent variables, $F_{3,93}$=55.74, $p<0.000001$, Levene's test, $p>0.05$, Kolmogorov-Smirnov-Test, $p>0.05$, see Figure 24). A summary of final cell densities and numbers of successful replicates can be found in Table 7. At low light intensity, treatments with little phosphate (N) produced the lowest cell densities, while at high light intensities nitrite limitation (P) seemed to have a more severe effect on algal growth. There were no apparent changes in the chain-length related to nutrient or light regimes.

Table 7: Summary of final cell densities at harvesting time in each treatment (cells per ml ± standard deviation) and number of replicates that were successfully harvested.

		L-	L0	L+
1018	N	2149 ± 531, n=3	4000, n=1	n=0
	P	2792 ± 2294, n=3	1794 ± 217, n=3	n=0
	NP	7990 ± 834, n=3	1518 ± 157, n=3	n=0
	SW	-	3867 ± 18, n=3	-
1647	N	1053 ± 304, n=3	2058 ± 191, n=3	1201 ± 399, n=3
	P	1600 ± 372, n=2	4269 ± 1245, n=3	n=0
	NP	12623 ± 11297, n=3	41900 ± 24825, n=3	1008, n=1
	SW	-	3275 ± 1252, n=3	-
MAR	N	952 ± 34, n=3	1374 ± 841, n=3	7800 ± 1399, n=3
	P	2884 ± 250, n=3	986 ± 362, n=3	5353 ± 1145, n=3
	NP	10733 ± 2301, n=3	7200 ± 5678, n=3	9300 ± 2417, n=3
	SW	-	825 ± 274, n=3	-
AUG	N	247 ± 50, n=3	773 ± 23, n=3	5925 ± 1945, n=2
	P	1231 ± 1678, n=3	3347 ± 474, n=3	2819 ± 362, n=2
	NP	3154 ± 4825, n=3	5300 ± 1236, n=3	6282 ± 4770, n=3
	SW	-	268 ± 305, n=3	-

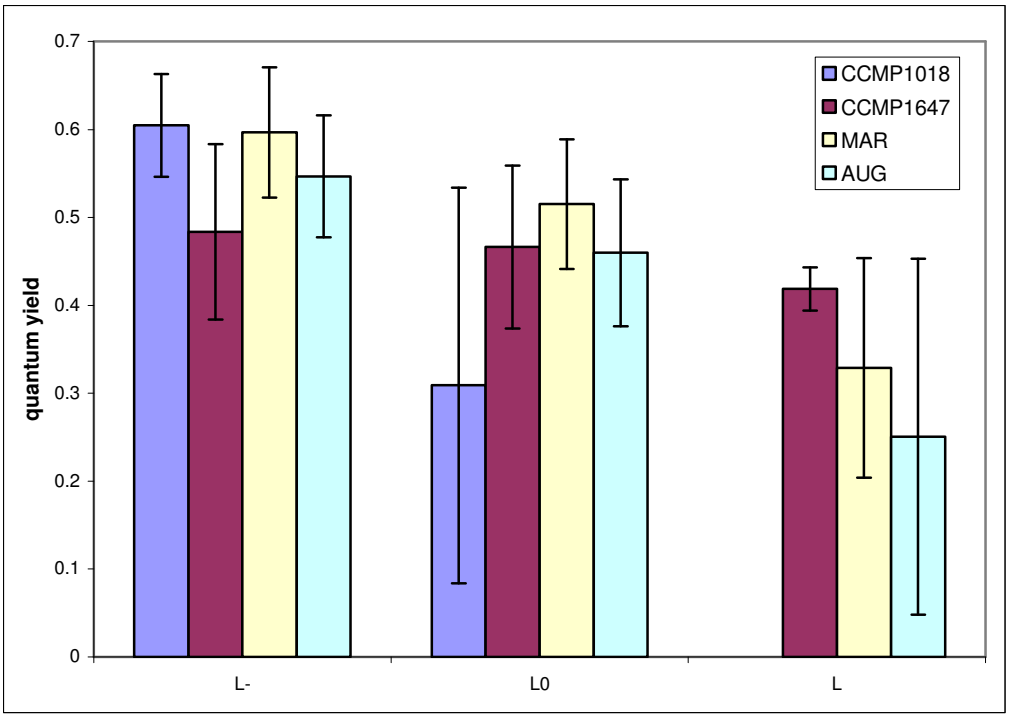

Figure 24: Quantum yield in various light intensities (mean values ± standard deviation – see Table 7 for the number of replicates of each strain and light combination).

3.3.2 Pigments

Many pigments have been identified in all strains of *T. routla*, but only those that were present in most samples and could be clearly identified (i. e. chlorophyll a, fucoxanthin, diadinoxanthin, diatoxanthin and ß-carotene) were analyzed. Nevertheless Table 8 contains a summary of all pigments identified in the various strains as well as light- and nutrient treatments.

Table 8: Summary of pigments found in the various strains and light- and nutrient treatments. The table lists the strains the pigment was found in. 1 = CCMP 1018, 2 = CCMP 1647, 3 = MAR, 4 = AUG, P = ASW+F/2 with low nitrate, N = ASW+F/2 with low phosphate, NP= normal F/2 medium based on artificial seawater, SW = natural seawater.

Pigment	Light	N	NP	P	SW
Chlorophyllide A	L-	2,4	1,2,3,4	2,3,4	----
	L0	2,3,4	2,3,4	2,3,4	1,2,3,4
	L+	2,3,4	2,3,4	3,4	----

Fucoxanthin	L-	1,2,3,4	1,2,3,4	1,2,3,4	----
	L0	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4
	L+	2,3,4	2,3,4	2,3,4	----
Neoxanthin	L-		1,2,3,4	3	----
	L0		1,2,3,4	1,3	1
	L+		3,4	3,4	----
Diadinoxanthin	L-	1,2,3,4	1,2,3,4	1,2,3,4	
	L0	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4
	L+	2,3,4	2,3,4	3,4	
Diatoxanthin	L-	1	1,2,3,4	1,2,3	----
	L0	1,2,3,4	1,2,3,4	1,2,3	1,2,3,4
	L+	2,3,4	2,3,4	3,4	----
Divinyl Chlorophyll a	L-		2,3,4	3	----
	L0		2,3,4		1
	L+		3,4		----
Chlorophyll a derivative	L-	1,2,3,4	1,2,3,4	1,2,3,4	----
	L0	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4
	L+	2,3,4	2,3,4	2,3,4	----
Chlorophyll a	L-	1,2,3,4	1,2,3,4	1,2,3,4	----
	L0	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4
	L+	2,3,4	2,3,4	2,3,4	----
ß-ε-Carotene	L-		3,4		----
	L0		2,3,4	3	1
	L+		3,4	3,4	----
Phaeophytin a	L-		1,2,3,4	3	----
	L0		2,3,4		1
	L+		3,4		----
ß-Carotene	L-	1,2,3,4	1,2,3,4	1,2,3,4	----
	L0	1,2,3,4	1,2,3,4	1,2,3	1,2,3,4
	L+	2,3,4	2,3,4	2,3,4	----

The cellular content of chlorophyll a was statistically examined with an ANOVA where medium, strain and light intensity were treated as independent variables. The residuals of the ANOVA were homogenous (Levene's test, $p>0.05$) but not normally distributed (Kolmogorov-Smirnov-Test, $p=0.000007$) and could not be normalized by transformation using the $\log_{10}$ or the square root function. The only factors with significant effects on the chlorophyll a

content of the cells were "strain" ($F_{3,73}$=10.94, p=0.000005) and "medium" ($F_{3.73}$=6.00, p=0.0010). As shown in Table 9 cells of the August strain had the highest content of chlorophyll a, followed by those of the March strain and the two CCMP strains. It was also evident, that in the August and March strains the highest cellular chlorophyll a concentrations occurred in normal F/2, while with the CCMP strains this treatment did not differ from the nutrient limited treatments. "Light" did not have any significant effect ($F_{1,73}$=0.1301, p=0.719322).

Table 9: Chlorophyll contents of the various strains under different nutrient treatments. (mean value ± standard error, see Table 7 for number of replicates).

Nutrients	1018	1647	MAR	AUG
N	0.023 ± 0.009	0.028 ± 0.007	0.066 ± 0.013	0.127 ± 0.040
NP	0.025 ± 0.008	0.026 ± 0.009	**0.243 ± 0.062**	**0.322 ± 0.091**
P	0.027 ± 0.006	0.021 ± 0.003	0.045 ± 0.013	0.111 ± 0.061
SW	0.032 ± 0.004	0.018 ± 0.003	0.083 ± 0.013	0.168 ± 0.106

Fucoxanthin, diadinoxanthin and β-carotene did not change significantly in their ratios to chlorophyll a, as was shown in another ANOVA (p>0.2 for all factors and all pigments). Only the proportion of diatoxanthin changed significantly (another ANOVA, Levene's test, p>0.05, Kolmogorov-Smirnov-Test, p>0.05) depending on "medium" ($F_{3,73}$=11.35, p=0.000006), "strain" ($F_{3,73}$=138.88, p<0.000001) "medium*light" ($F_{2,73}$=6.342, p=0.0029), "strain*light" ($F_{3,73}$=98.38, p<0.000001) and "strain*light*medium" ($F_{3,73}$=347.7, p<0.000001).

Generally CCMP 1018 produced higher amounts of diatoxanthin than the other strains. The CCMP strains (which were most sensitive to light) produced the highest amount of diatoxanthin at medium light intensities and did not grow well (1647) or at all (1018) at high light intensities. This was not the case in natural seawater, were only low levels of diatoxanthin were produced. The media in which the highest proportions of diatoxanthin occurred, were not phosphate limited (P and NP). The treatment that produced the higher proportions varied with the strain (see also Figure 25)

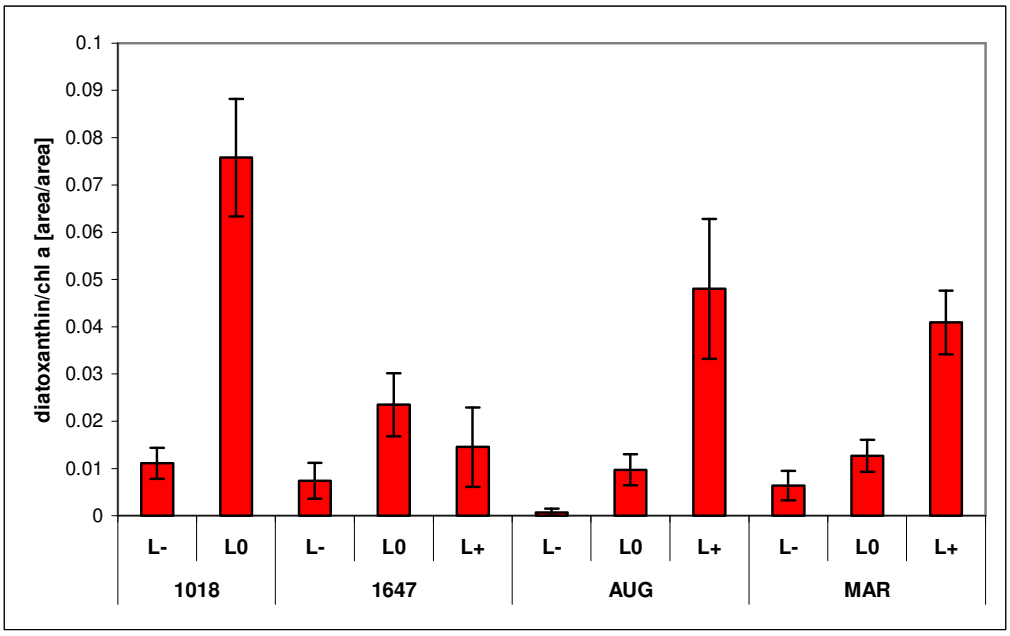

Figure 25: Ratio of diatoxanthin to chlorophyll a (area/area) in the various strains and light treatments (mean value ± standard error, see Table 7 for number of replicates).

3.3.3 Fatty acid composition

- **The ratio of saturated to unsaturated fatty acids**

In order to assess the factors that may influence the fatty acid composition an ANOVA was performed using the ratio of examined unsaturated to saturated fatty acids as dependent, and medium, strain and light intensity as independent variables. As the data were not normally distributed (Kolmogorov-Smirnov-Test, $p=0.0005$) they were $\log_{10}$-transformed to produce a normal distribution ($p>0.05$). The variances were considered homogenous (Levene's test, $p>0.05$). The results of the ANOVA are shown in Table 10. "Medium" had the strongest impact on the fatty acid composition (see Figure 26): Normal (non nutrient-limited) F/2-treatments had a higher ratio of examined unsaturated to saturated fatty acids. Furthermore, there were small but significant differences in the fatty acid compositions among the strains (see Figure 27). Also the interaction terms "light*medium" and "strain*light*medium" were significant (see 3.1, where nutrient limitation had different effects on final cell numbers in different light conditions). Light intensity alone did not significantly influence the ratio of

unsaturated to saturated fatty acids (see Figure 28). The interaction "medium*light*strain" may have been significant due to the increase of total fatty acids in the P treatment of the August strain at high light intensities. However, this data point consisted of only three values, only two of which were particularly high (and one of these two was contaminated with flagellates). For these reasons it will not be considered further.

Table 10: Result table of the ANOVA performed to assess the influence of light, medium and strain on the cellular ratio of examined unsaturated to saturated fatty acids.

	D. f.	Sum Sq.	Mean sq.	F value	Pr. (>F)
Strain	3	1.1748	0.3916	8.6972	5.24e-05
Medium	3	7.8262	2.6087	57.9392	<2.2e-16
Light	1	0.0997	0.0997	2.2137	0.1410995
Strain*medium	9	1.5971	0.1775	3.9411	0.0004004
Strain*light	3	0.338	0.1127	2.5026	0.0659515
Light*medium	2	0.8503	0.4251	9.4421	0.0002254
Strain*light*medium	6	0.9816	0.1636	3.6333	0.0032855
Residuals	73	3.2869	0.045		

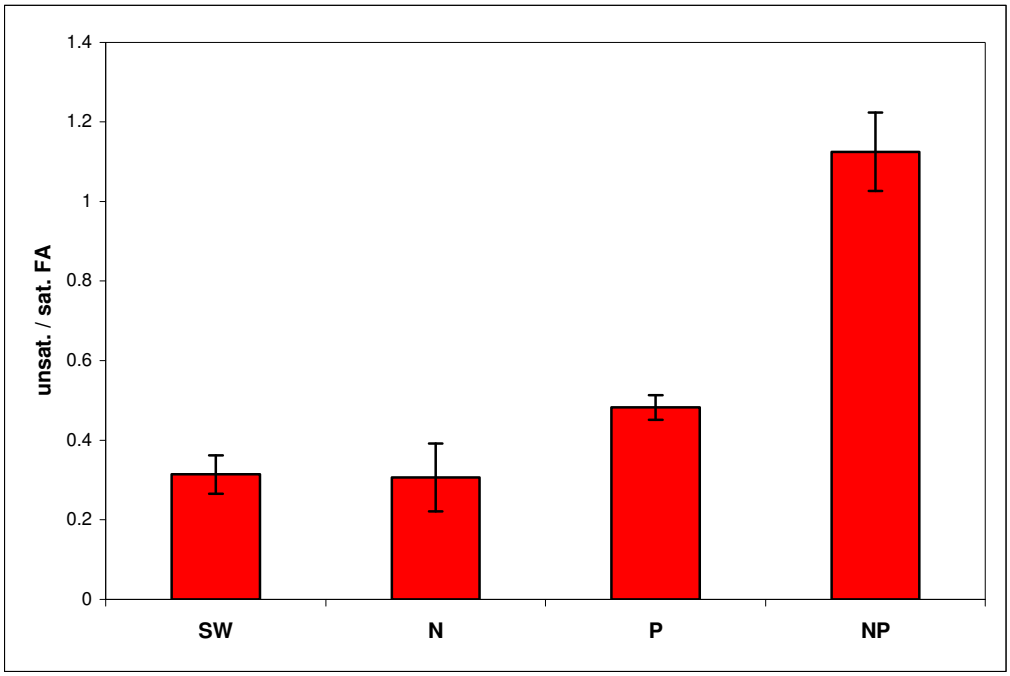

Figure 26: The effects of nutrients on the fatty acid composition (means of all strains ± standard error). N = F/2 based on ASW with low phosphate (n=30); NP = normal F/2 based on ASW (n=31); P = F/2 based on ASW with low nitrate (n=28); SW = filtered seawater without addition of nutrients (n=12).

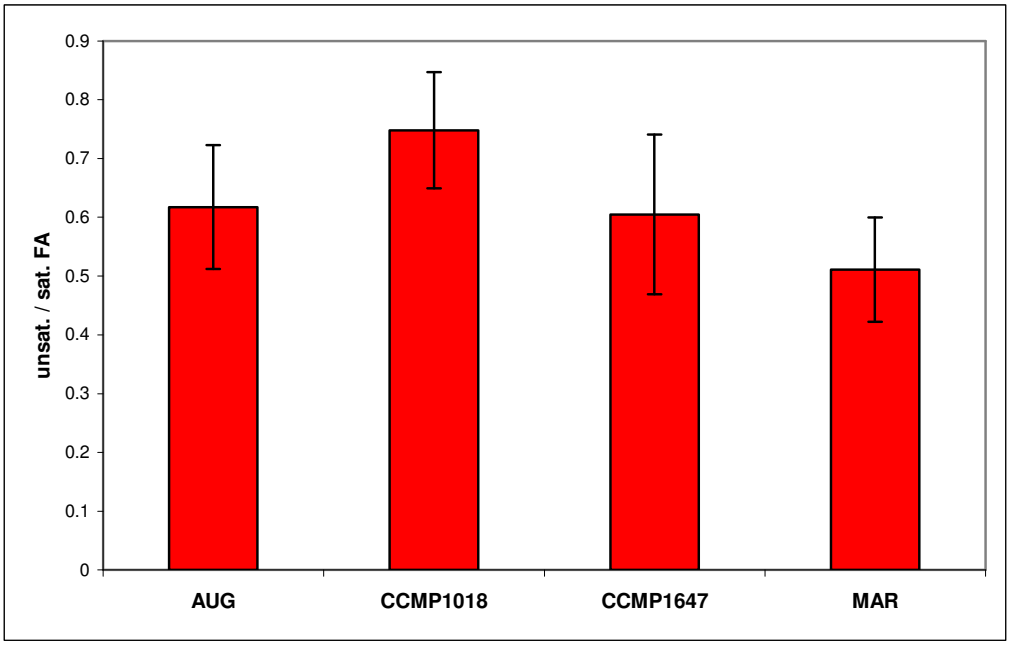

Figure 27: The effects of the factor strain on the average ratio of examined unsaturated to saturated fatty acids as mean ± standard error (AUG: n=28, CCMP1018: n=19, CCMP1647: n=24, MAR: n=30).

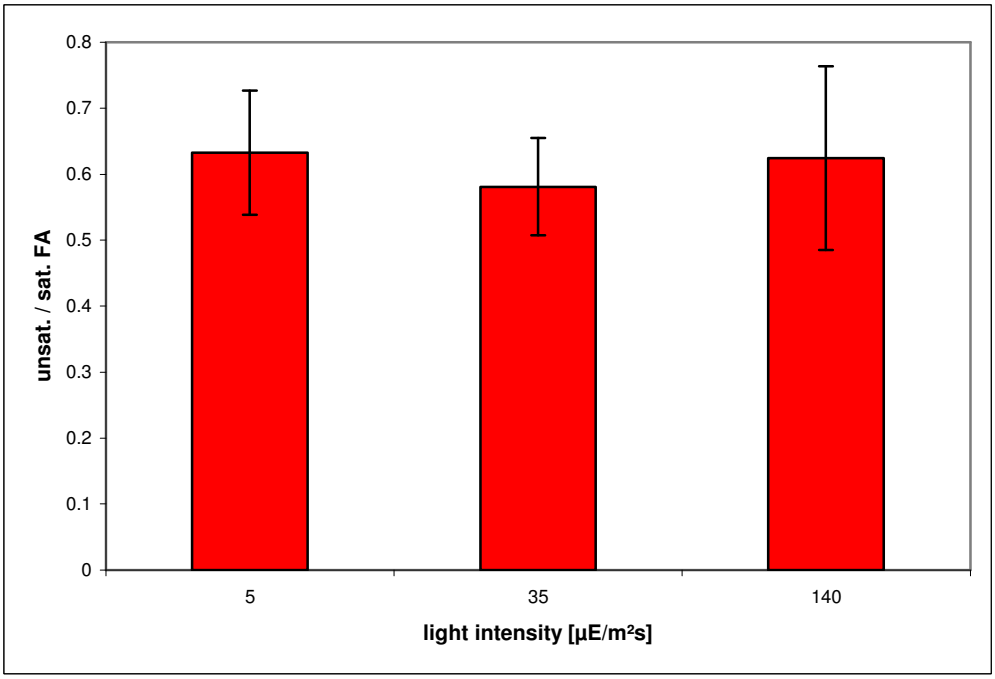

Figure 28: The effects of light intensity on the ratio of examined unsaturated to saturated fatty acids (means ± standard error; 5 µE/m²s: n=20, 35 µE/m²s: n=46, 140 µEm²s: n=20).

➢ The cellular content of fatty acids

The total amount of fatty acids per cell was not as variable as the fatty acid composition. It was tested in the same way as described above, with the total amount of fatty acids being used as the independent variable. Again the data differed significantly from a normal distribution (Kolmogorov-Smirnov-Test, p=0.000423) and were $\log_{10}$-transformed to match this prerequisite (p>0.05). The requirement of homogenous variances was not met (Levene's test, p=0.018), but the results can nonetheless be described here. As expected, the factor strain had a significant influence on the total content of fatty acids, as cell sizes varied a lot between the CCMP strains and those isolated from the Helgoland Roads. Further "medium" as well as the interaction terms "strain*medium" and "strain*medium*light" were significant, while light alone did not account for any variance (see Figure 28).

Table 11: Result table of the ANOVA performed to asses the influence of light, medium and strain on the cellular content of fatty acids.

	D. f.	Sum sq.	Mean sq.	F value	Pr. (>F)
Strain	3	7.5277	2.5092	25.999	1.51e-11
Medium	3	2.6441	0.8814	9.1321	3.31e-05
Light	1	0.0041	0.0041	0.0421	0.83798
Strain*medium	9	1.9749	0.2194	2.2736	0.02628
Strain*light	3	0.3264	0.1088	1.1272	0.34377
Medium*light	2	0.3361	0.168	1.741	0.18254
Strain*medium*light	6	1.4051	0.2342	2.4265	0.03405
Residuals	73	7.0454	0.0965		

The fatty acid composition in the various treatments and strains was highly variable (see Figure 29 - Figure 32). Still some general trends were found: In all strains except the CCMP 1018 strain, the seawater (SW) treatments had the highest cellular content of fatty acids, mainly due to an exceptionally high content of 16_0. Also in all strains except CCMP1018 there was a trend towards lower DHA-levels in the P and NP treatments compared to the N and SW treatments. 16_0 and 18_0 were always comparatively low in the NP treatment (compared, at least to the N treatment). EPA was comparatively constant, but the lowest levels were observed in the P-limited

treatment (N) for MAR and AUG strains, and in the NP treatment for the CCMP 1018 and 1647 strains.

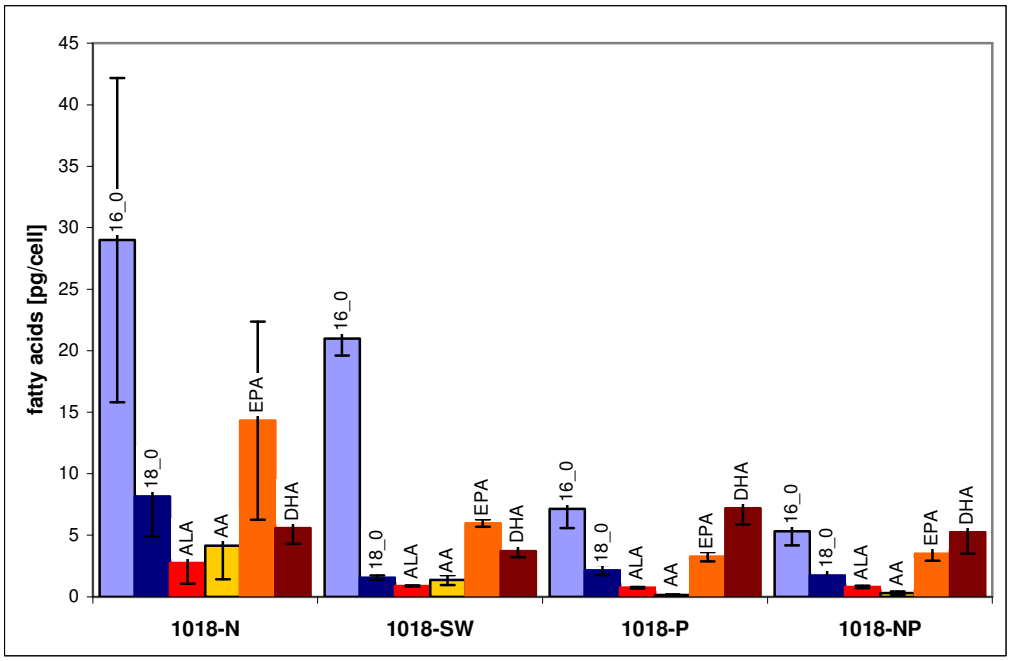

Figure 29: The fatty acid composition of the CCMP 1018 strain. The graph shows the means of all light treatments (which did not have significant effects on the fatty acid composition) ± standard error. The number of replicates for each nutrient treatment is given in Table 7. N = PO_4-limited F/2, P = NO_3-limited F/2, NP = normal F/2, and SW = seawater.

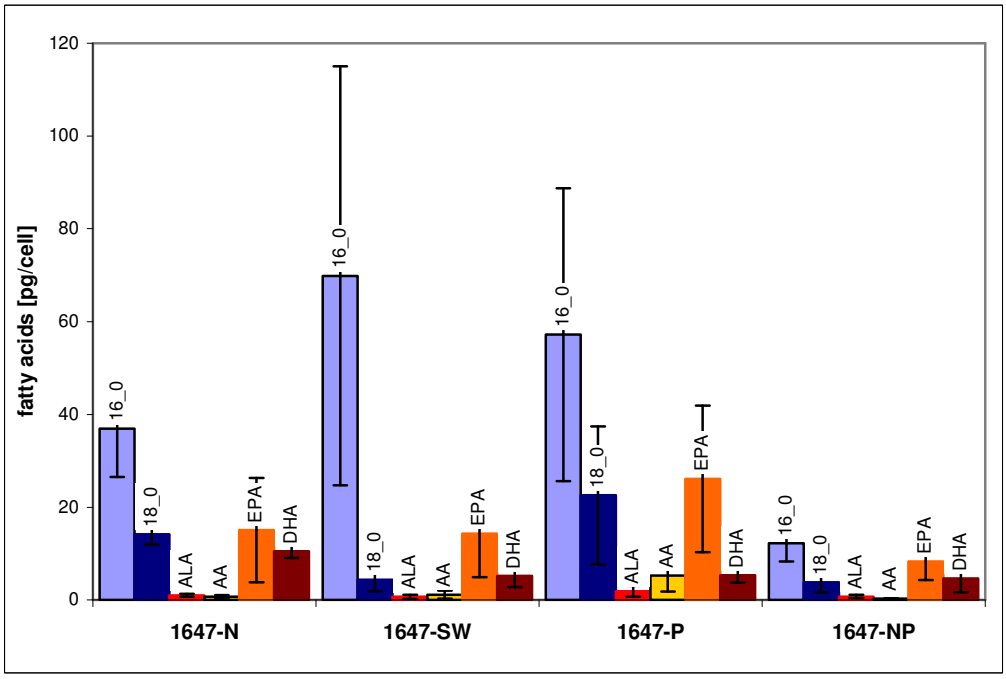

Figure 30: The fatty acid composition of the CCMP 1647 strain. See Figure 29 for further details.

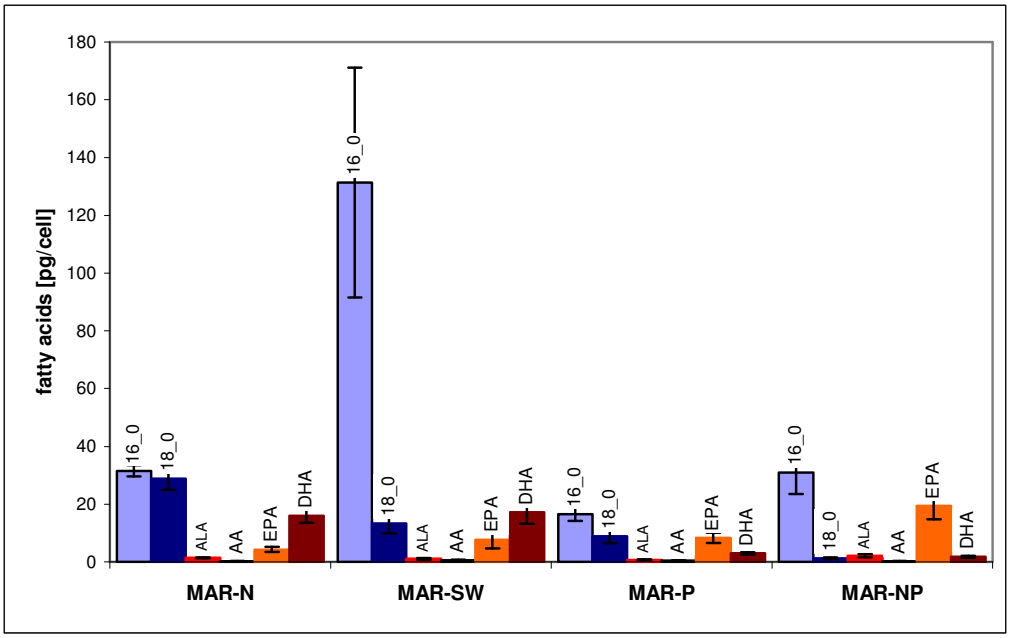

Figure 31: The fatty acid composition of the March strain. See Figure 29 for further details.

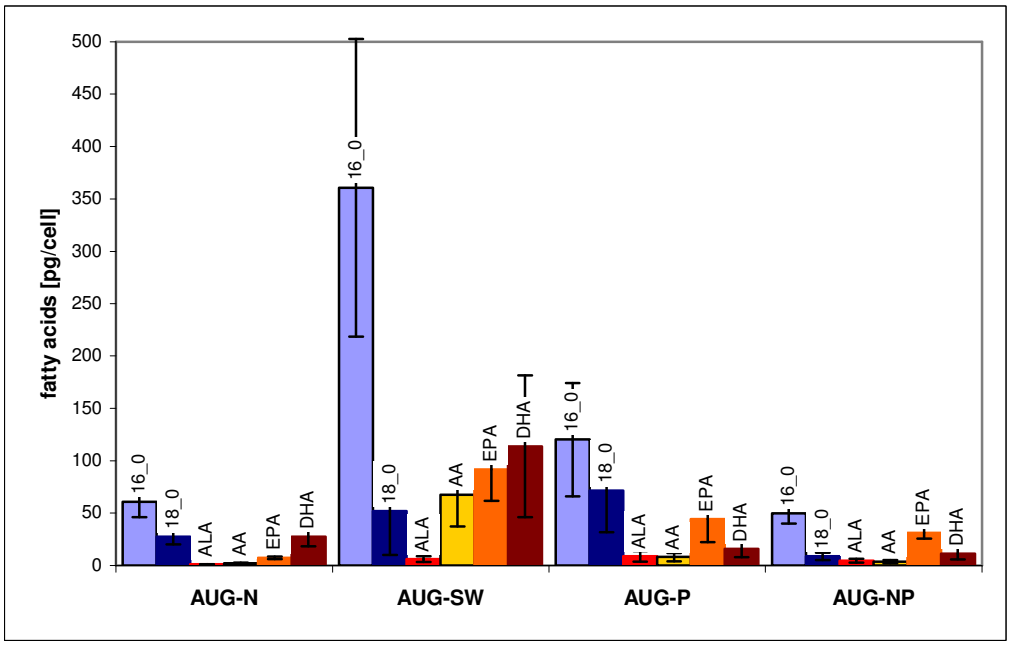

Figure 32: The fatty acid composition of the August strain. See Figure 29 for further details.

3.3.4 Aldehyde production

The production of aldehydes (PUAs) per cell was also analyzed by means of an ANOVA, although the data was not normally distributed

(Kolmogorv-Smirnov-Test, p=0.000003, see also Q-Q-Plot in Figure 33). Variances were considered to be homogenous (Levene's test, p>0.05).

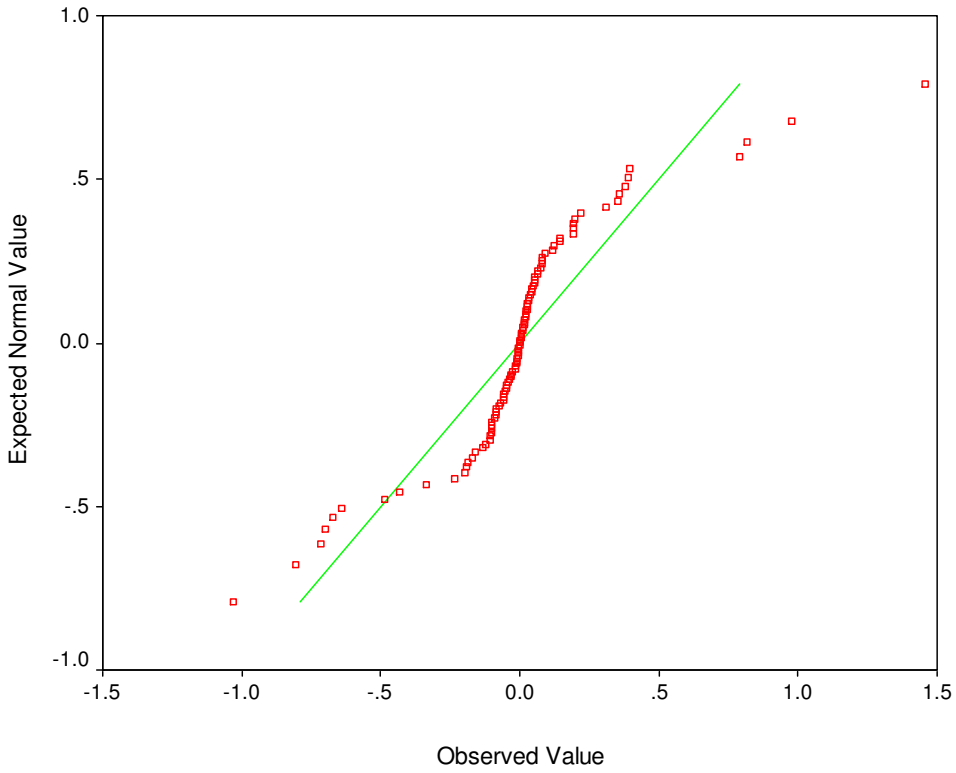

Figure 33: Q-Q plot for the residuals of the ANOVA relating the PUA-production per cell to light, strain and medium.

From the ANOVA it is evident that only the medium used for culturing had a significant influence on the total PUA production ($F_{3,49}$=8.9499, p=0.000078). As can be seen in Figure 34 algae cultured in normal F/2 (NP) produced higher quantities of all examined aldehydes than algae that were nutrient-limited.

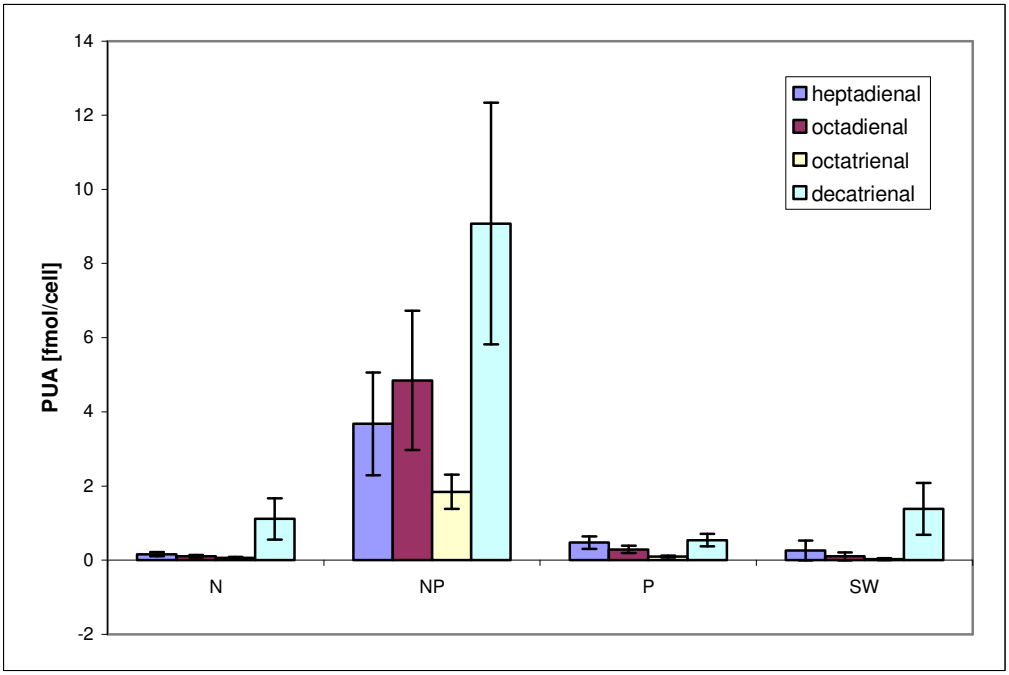

Figure 34: Average aldehyde production of all strains and light intensities in the various nutrient treatments (mean ± standard error). See Table 7 for numbers of replicates.

Even though "strain" as a factor is not significant ($F_{3,49}$=2.6181, p=0.0613) there is an obvious influence of this factor on the production of aldehydes. Table 12 summarizes the aldehyde production under normal light and non-limited nutrient conditions: while the CCMP 1018 did not produce any PUAs in this experiment, CCMP 1647 clones produced about 5 fmol PUA per cell. The March and August clones produced about 40 fmol PUA per cell. Interestingly, in this experiment March algae differed from August algae in the aldehyde composition: The former produced mainly decatrienal while the latter produced mainly octadienal. These trends were not as clear in nutrient-limited media, as the total amount of produced aldehydes was often below the detection limit.

Table 12: Summary of the aldehyde production of the examined strains in the stationary phase, at medium light (35 µE/m²s) and cultured in normal F/2 medium. The table lists the mean of three replicates ± standard deviation. All values are given as fmol produced PUA per cell.

	CCMP1018	**CCMP1647**	**MAR**	**AUG**
Heptadienal	0 ± 0	2.3 ± 2.1	1.1 ± 0.8	14.5 ± 9.2
Octadienal	0 ± 0	0.6 ± 0.5	5.3 ± 4.7	17.6 ± 13.9
Octatrienal	0 ± 0	1 ± 0.8	2.6 ± 2.1	4.3 ± 2.8
Decadienal	0 ± 0	traces	traces	traces
Decatrienal	0 ± 0	0.7 ± 0.5	31.2 ± 24.6	7.4 ± 5.3
Total PUA	**0 ± 0**	**4.6 ± 3.8**	**40.2 ± 32.1**	**43.7 ± 29.7**

Light did not have a significant effect on the aldehyde production ($F_{1,49}$=0.0039, p=0.953). Only the interaction term "light*strain" could be considered to have had some impact ($F_{2,49}$=2.3727, p=0.104). This was probably due to the fact that the March strain (due to the limited number of samples that were sent in for aldehyde analysis this strain was the only one to be examined under low light conditions) produced only very little aldehydes at low light conditions (about 1 fmol/cell on average). These sample were the first to be taken (about two months before the other samples), and therefore the effect may have only been an artifact.

3.4 Relationships between fitness, fatty acids, aldehydes, cell densities and other parameters

Aside from the variables explicitly manipulated, several parameters were monitored in order to find out whether they were in any way related to the production of aldehydes. Table 13 shows a matrix of all factors tested and their relation using Pearson correlations.

Table 13: Correlations among some of the parameters examined in the light and nutrient experiment. Only the 73 treatments with successful aldehyde determination were considered. P = significance two-tailed (Pearson), r = correlation coefficient, max. r = maximum growth rate. Values in bold letters are significant on an alpha level of 0.05 after applying a Bonferroni correction.

		Cells per ml	**Chain-length**	**Max. r**	**PUA**	**Unsaturated / saturated**	**Yield**	**DNA**
Cells per ml	r		-0.107	0.021	0.03	**0.512**	0.186	0.057
	p		0.366	0.859	0.799	**<.001**	0.115	0.634
Chain-length	r	-0.107		**-0.41**	0.078	-0.023	0.018	-0.102
	p	0.366		**<.001**	0.511	0.847	0.879	0.389
Max. r	r	0.021	**-0.41**		**0.337**	0.146	0.029	-0.227
	p	0.859	**<.001**		**0.004**	0.219	0.811	0.054
PUA	r	0.03	0.078	**0.337**		**0.445**	0.123	-0.083
	p	0.799	0.511	**0.004**		**<.001**	0.301	0.483
Unsat. / sat.	r	**0.512**	-0.023	0.146	**0.445**		0.158	-0.053
	p	**<.001**	0.847	0.219	**<.001**		0.182	0.658
Yield	r	0.186	0.018	0.029	0.123	0.158		-0.05
	p	0.115	0.879	0.811	0.301	0.182		0.672
DNA	r	0.057	-0.102	-0.227	-0.083	-0.053	-0.05	
	p	0.634	0.389	0.054	0.483	0.658	0.672	

The production of polyunsaturated aldehydes was positively correlated to the ratio of examined unsaturated to saturated fatty acids and to the maximum growth rate of a culture. The ratio of examined unsaturated to saturated fatty acids, in turn, was also positively correlated to the number of cells per ml. Interestingly the maximum growth rate was negatively correlated to the chain-length. DNA content in the medium and quantum yield were not significantly related to any of the other parameters. The correlations found here were in some cases not reflections of direct causal relationships. They were sometimes indirect effects of underlying factors manipulated during the experiment. For example nutrient-limited algae grew more slowly and produced less aldehyde. Therefore some correlations were calculated again with the residuals of the ANOVAs performed in 3.3.3 and 3.3.4 (see Table 14). As both ANOVAs were fully factorial, all of the variance that could be accounted for by the examined factors (light, medium and strain) was already

removed from the data. All observed correlations with this data were therefore associated with either a direct relationship between the observed factors, or another factor that was not taken into account in the ANOVA. The only significant correlation was a positive one between the residuals of the ratio of examined unsaturated to saturated fatty acids and the number of cells per ml.

Table 14: Correlations between the residuals of the ANOVAs performed in 3.3.3 and 3.3.4 and other factors monitored during the experiment (n=73 for PUA, n=102 for FA). P = significance two-tailed (Pearson), r = correlation coefficient, max. r = maximum growth rate, uns./sat. = ratio of examined unsaturated to saturated fatty acids. Values in bold letters are significant on an alpha-level of 0.05 after applying a Bonferroni correction.

Residuals		**Uns./sat**	**Yield**	**Max. r**	**Chain-length**	**Cells / ml**	**DNA**
PUA	r	0.061	-0.059	0.165	0.210	-0.111	-0.084
	p	0.611	0.622	0.164	0.074	0.352	0.482
Uns./sat.	r		0.0361	0.063	0.175	**0.343**	-0.018
	p		0.720	0.532	0.080	**<0.001**	0.859

Last but not least, the ratio of produced aldehyde to available fatty acids was examined. This was only done for EPA and the EPA derived aldehyde decatrienal (Pohnert 2000) as these substances were relatively abundant in the samples. Samples that did not contain any decatrienal because aldehydes were below the limit of detection were therefore not included in the analysis. The ratio of decatrienal to EPA was formed on the basis of molar concentration and not weight, as every EPA molecule yields one molecule of decatrienal (see Figure 1). In all samples examined the observed ratio of decatrienal to EPA ranged from 0 to 0.537 with a mean of 0.095 ± 0.0018 (standard error). An ANOVA revealed "medium" and "strain" as well "medium*light" and "medium*light*strain" as parameters with significant effects on the $\log_{10}$-tranformed ratio. There was, however a problem in that the dataset became unbalanced after zero-values were removed for the reasons described above. Nevertheless Figure 35 indicates how this ratio could vary depending on the nutrient conditions: While in N-limited conditions (P) on average only 2% of the cellular EPA was used for the production of decatrienal, this ratio increased to about 15% in P-limited conditions (N).

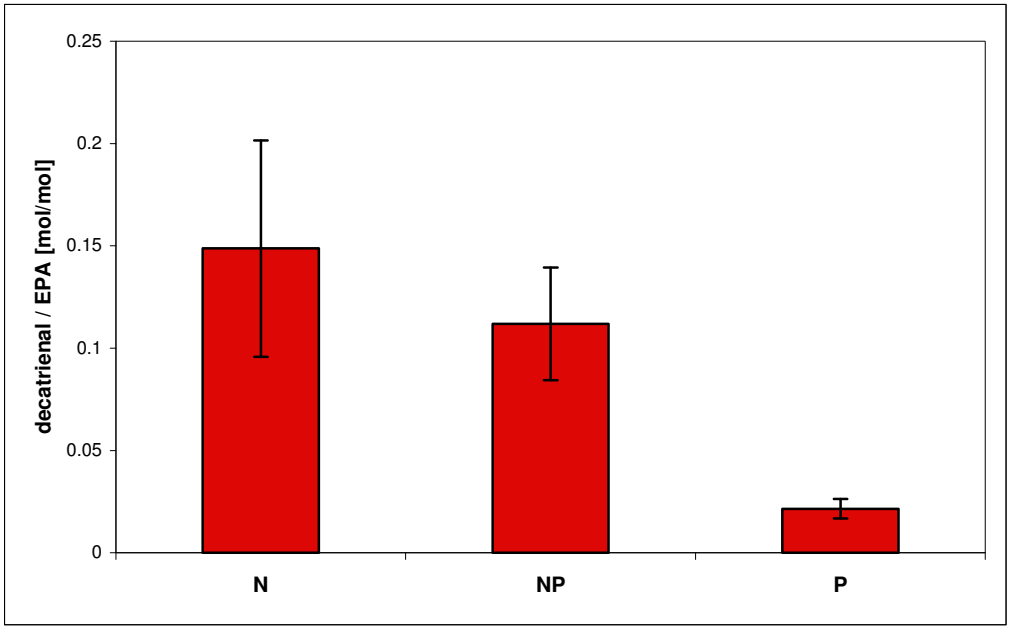

Figure 35: The ratio produced decatrienal to EPA under different nutrient conditions (mean ± standard error). N: P-limited F/2 (n=12), NP: normal F/2 (n=18), P: N-limited F/2 (n=16).

4 Discussion

The aim of this study was to determine if different culture conditions had an effect on the production of polyunsaturated aldehydes in *T. rotula*. This objective was reached and three important results were found:

1. Increased aldehyde-production was always correlated with the cellular content of EPA and a decrease in the content of saturated fatty acids and DHA.
2. Aldehyde-production was highly variable depending mainly on the cell density and the growth phase.
3. Aldehyde production could be induced in the CCMP 1018, which was known to produce only traces of aldehydes, by adding methanol and octadienal.

In addition, all results were somewhat similar to those previously published in the literature (Wichard et al 2005b for aldehydes, Dunstan et al. 1994 for fatty acids, and Jeffrey et al. 1997 for pigments: all typical pigments except chlorophyll c were detected), which can be taken as a validation of the methods. In the following section the investigated factors and their effects are treated individually. The general physiological and ecological interpretations of these findings follow in 4.2 and 4.3.

4.1 Culture factors affecting aldehyde production

The first topic to be discussed in this context is the observed differences between the strains.

4.1.1 Differences between strains

- **Adaptability of the various strains**

One of the interesting facts of this diploma thesis was related to day to day problems in culturing algae. The difficulties involved in this appeared to increase with the time a strain had already been in culture. The CCMP 1018 strain, for instance, had been in culture for 38 years and died quickly when exposed to light intensities of 140 µE/m²s. At medium light intensity (35 µE/m²s), there were no problems in natural seawater. When using artificial

seawater culturing was more successful with Osram Biolux fluorescence tubes (2.4.2) which had a more continuous spectrum. At 4 µE/m²s there were no culturing problems. Data on the pigment composition revealed that the proportion of diatoxanthin in the cells increased at medium light intensities, especially in the 1018 strain. Diatoxanthin is believed to be responsible for non-photochemical quenching and is formed by enzymatic deepoxidation of diadinoxanthin (Lavaud et al. 2004), when algae are exposed to high light intensities (Goericke and Welschmeyer 1992). This made sense, because at CCMP the algae have been usually cultured at low light intensities, using fluorescence tubes and natural seawater. They were only cultured at high light intensities in incubators with light bulbs that emit a continuous light spectrum (T. Riggens, CCMP, personal communication, Jan. 13th 2006). Still, it is not clear which components of natural seawater allowed the algae to endure higher (according to Schöne 1972, optimal) light intensities. In addition, there is no obvious reason why these substances would show an effect dependency on the light composition (Osram Biolux or Phillips lamps). Perhaps the increased UV-content of the Biolux lamps is necessary for the biosynthesis of some substances that are also present in natural seawater. It is also possible that the stressing factors synergize and when they all occur at the same time the algae simply do not grow anymore. However, the March and August strain that had been in culture for less than two years grew well in all conditions, although at high light intensities they also had increased levels of diatoxanthin.

There were also pronounced differences in cell size found in the cultures (see Figure 4). CCMP 1018 was smaller than CCMP 1647 (in culture for 12 years), which in turn was smaller than the March and August strains. Diatoms usually use cell division to proliferate, but their cell size decreases with every cleavage, as they always regenerate the smaller theca (Strasburger et al. 1991). When a minimum size is reached, cells can reproduce sexually and the cycle begins anew. Normally, sexual reproduction in diatoms occurs every two to 40 years (Edlund and Stoermer 1997 and references therein). The observed size differences in this study indicate that perhaps, under the applied culturing conditions sexual reproduction might not have taken place, resulting in smaller cells that gradually lost their ability to adapt to changing environments. In this context the fact that CCMP 1018 did not produce large amounts of aldehydes (in this study only traces of aldehydes were detectable

in all treatments except for the methanol and the octadienal treatment) is of particular interest, especially as in section 4.2.2 aldehydes are hypothesized signals that might even be responsible for the production of spores.

- **Seasonal variability**

A comparison of the March and August strain was intended to give us additional information about the seasonal variation in the aldehyde-production. Many diatom species have been shown to harbor several cryptic but physiologically different species, the succession of which is presumed to be a mechanism of adapting to changes in the environment (Medlin 1995). Assuming that aldehyde production is a grazer defense mechanism and that grazing pressure varies over the course of a year, one could expect different strains (genotypes or cryptic species) isolated at different times of the year to have different capabilities for production of aldehydes. In the present study only minor differences between the strains isolated in March and August were found. The fact that algae were only taken from two specific months is a problem because it in no way represents a seasonal analysis. Still the lack of a difference, if proven by more detailed studies, could be interpreted in two ways. It may indicate that aldehydes are not primarily related to grazer defense or that the cost of their (wound-activated) production is not very high and there is no evolutionary advantage of not producing aldehydes in times of low grazing pressure.

4.1.2 Bacteria (DNA)

Another factor that was suspected to be related to aldehyde production was the presence of bacteria. Although this factor was not manipulated during the experiment it was approximated for each sample by measuring the DNA concentration in the medium. In the density dependence experiment, the level of aldehyde production followed that of the DNA-concentration in the medium, but the light and nutrient experiment showed clearly that these two factors were not correlated. Therefore the hypothesis that aldehydes are a means of defense against bacteria seems improbable.

4.1.3 Stress

In contrast to the bacterial quantity, which may represent a form of stress, other "stressing factors" were directly manipulated in the different

treatments. Originally the level of stress for the algae was intended to be monitored by PAM-measurements. This method is well established for higher plants, but with phytoplankton its applicability is still questionable (see Parkhill et al. 2001). All non-contaminated, growing samples showed rather constant quantum yields of about 0.65, from the first day on until they were harvested in the early stationary phase. Only the samples harvested in the death phase and those under light stress showed lower PAM-values. In high light treatments (or medium light for CCMP 1018) this coincided with an increase in the ratio of diatoxanthin, which is an indication that the light intensities used were actually stressful for the examined algae.

An interesting observation related to stress and light regimes was, that in high light intensities, cultures reached higher cell densities in the media containing a lot of nitrate, while phosphate seemed to be a more limiting factor in low light conditions. Neeboda and Harrison (2004) suggested that the capability of nitrate uptake is inversely correlated to light intensity and growth rate, giving a possible explanation as to why lower nitrate concentrations might be more stressful at higher light intensities. On a level of aldehyde production and fatty acid composition this could explain why the interaction terms "light*medium" were significant in the ANOVAs.

The only difference in the aldehyde production which could be attributed to the factor "light" was in March strain between the low and the medium light intensity. However, it may have been caused by inefficient extraction and long storage time: The low light samples were the first to be prepared for aldehyde analysis. This explanation was also supported by the fatty acid data: There was no change in the ratio of unsaturated to saturated fatty acids, although this parameter correlated well with aldehyde production. Further there was no change in the diatoxanthin contents in these samples. Therefore it can be assumed, that the light intensities tested had only minor influences on the aldehyde production.

Besides quantum yield and diatoxantin-content other factors may complement the assessment of stress in algae. These include maximum growth rate, chain length and cellular chlorophyll a content. Still, none of these factors alone was sufficient to explain the differences in the aldehyde production found in the light and nutrients experiment. Currently efforts are being undertaken (K. Wiltshire, personal communication) to develop a collective parameter to describe the overall stress level of algae. This

parameter could comprise all of the aforementioned factors and provide a simple but more accurate measure of the alga's condition. It might also be more suitable to explain the observed differences in aldehyde production. Unfortunately, the development of such a parameter was beyond the scope of this study. However, it is possible that the growth phase could also provide a rough approximation of how high the stress-level was. Cultures growing exponentially may have been under milder stress than those in stationary or even death phase and therefore have shown less intense stress reactions. In the density dependence experiment, growth phase had a strong effect on the aldehyde production. As described by Pistocci et al. (2005) algae in the exponential phase contained larger proportions of saturated fatty acids and produced less aldehyde. Still, the light and nutrient experiment showed that growth phase alone cannot explain the results either. Here high aldehyde production and a high ratio of unsaturated fatty acids only occurred in the treatments with normal (non P- or N-limited) F/2 medium.

4.1.4 Nutrient limitation

Nutrient limitation can also be a source of stress. It is likely to have taken place in all cultures harvested in the early stationary or death phases. In the N and P treatments either phosphate or nitrate were limiting, in the NP treatment probably another nutrient e.g. silicate. This can be assumed, because non-limited cultures only showed about a ten-fold increase in cell density compared to the nutrient-limited cultures, although nitrate or phosphate were 100-fold more abundant. As already shown for chrysophytes by Watson and Satchwill (2003), aldehydes are mainly produced in treatments with high phosphate and nitrate (NP). At the same time cultures harvested in the exponential phase, when nitrate and phosphate levels were highest, produced little aldehyde. This suggests that nitrate and phosphate are not the primary causes of high or low aldehyde production. Limitation by a third nutrient, however, could explain the results of both experiments, as only the NP-treatment and the stationary and death phase of the density dependence experiment, which was also performed in F/2 medium, produced high quantities of aldehydes. This third nutrient might be silicate, another mineral or a vitamin. Further experiments would be needed to clarify this. However, there is still another factor that could explain the results: cell density. Treatments with normal (non P- or N-limited) F/2 medium reached higher cell

densities than the nutrient limited ones and the cell densities in the stationary and death phase were also a lot higher than those of the samples harvested in the exponential phase.

4.2 Possible explanations

How and why does cell density influence the production of aldehydes and the fatty acid composition? The most common explanations (shading and nitrate and phosphate limitation) have already been discussed above.

4.2.1 Confounding variables

Other options to be considered are that cell density could correlate with other confounding variables e. g. cell size, and even systematical counting errors at higher densities. The initial plan to control for such changes by determining the algal dry-weight of each sample as described in materials and methods, did not work well: The variance in the measurements was higher than the actual measured values. This may have been due to inaccuracies in estimating the amount of remaining salt on the filter. In future studies it would be recommendable to perform a C:H:N-analysis for each sample in order to have total carbon contents as a reference parameter. However, this would require larger culture volumes. Cellular biovolume was compared for some samples. There were no clear differences found, but a comprehensive analysis was not performed for reasons of time. To avoid effects of treatments that were only due to changes cell size and systematical counting errors, if possible, both absolute amounts and ratios, e. g. the ratio of examined unsaturated to saturated fatty acids, were documented. Even where this was not possible, for example with the production of polyunsaturated aldehydes, the observed difference between treatments were either much higher than those observed with other variables (e.g. the total content of fatty acids) or did not change significantly. This indicates that if confounding variables mentioned above had an effect on the results, it was probably much weaker than that of the different treatments.

4.2.2 A signaling role of aldehydes

- **The effects of the octadienal**

A more plausible explanation for the influence of cell density, in my opinion, was indicated by the results of the octadienal experiment. Although these results are not significant individually, they all point in the same direction and together provide a strong indication that octadienal might be used as an algal signal. There were three experiments performed with octadienal using different strain and medium combinations. The CCMP 1018 strain in natural seawater + F/2 nutrients (good culture medium) did not show any changes in growth rates, only small changes in the fatty acid composition (higher EPA levels and lower levels of saturated fatty acids), and a strong increase in aldehyde-production. The CCMP 1647 strain in artificial seawater + F/2 nutrients (suboptimal culture medium) showed a decrease in growth rate in the octadienal treatment, a clear shift in the fatty acid composition towards a higher ratio of unsaturated to saturated fatty acids, and cells were often deformed. Unfortunately, due to low cell numbers, the production of aldehydes was not measurable. The last experiment was performed with the CCMP 1018 strain in artificial seawater and F/2 nutrients. Here the algae did not grow well, even in the control treatments, and the addition of octadienal led to the death of all cells within a few days. However in this experiment methanol alone also had negative effects on the algal growth. This points to a constraint that needs to be kept in mind when interpreting these findings. It is the necessity to use a solvent when administering polyunsaturated aldehydes to the cultures. In the literature usually dimethylsulfoxide (DMSO) has been used for this purpose (e. g. Pohnert et al. 2002b). This substance has been shown to interact with other substances (e.g. antibiotics and also aldehydes), significantly increasing their effect (Keil 1967). For this study methanol was used as a solvent in a final concentration of 0.0004%. Ethanol concentrations of 0.1% have been considered harmless by Caldwell and Lewis (2005) and Vardi et al. (2006) even used final methanol concentrations of 1% without negative effects. Hence the applied methanol concentration would not have been expected to impact the diatoms. In this study, however, negative effects of the solvent on the algal growth occurred. Replacing pure methanol with 10%-methanol in distilled water (the final methanol concentration then was 0.00004%) solved this problem. Still it is possible that the described effects of

octadienal only occur in combination with the use of methanol as a solvent. Other solvents would need to be tested in order to resolve this issue.

Nevertheless, this experiment showed that even extremely low doses of octadienal (0.02 µM, see Table 2 for a list of concentrations used in the literature), at least in combination with methanol, can have a variety of effects on *T. rotula*, depending on the condition of the cells. They might:

1. increase the production of aldehydes,
2. cause a shift in the fatty acid ratio or even,
3. lead to cell death.

It is important to emphasize that all three of these effects were observed after administering the same dose of octadienal. This implies that the condition of an alga or its "stress level" play a key role in determining its response to aldehydes, but the measures currently applied are not capable of assessing this parameter.

- **Stress as an underlying factor**

The finding that even pure methanol induced aldehyde production (about 5 fmol per cell; without methanol only traces of aldehydes were produced) again shows the importance of comparing methanol to other solvents, but also points out the importance of stress as a possible underlying factor for changes in the aldehyde-production. In the literature it has been shown that algae are stressed by aldehydes (Casotti et al. 2005), and it is possible that this "stress" is responsible for the observed changes. On the other hand, by measuring calcium signals, Vardi et al. (2006) found out that *T. weissflogii* and *Phaeodactylum tricornutum* responded very specifically to Z/Z-decadienal, while similar substances such as E/Z-heptadienal and E/Z-octadienal (the substance tested in this study was Z/Z-octadienal) did not have any effects (Vardi et al., unpublished results). This would indicate that there might be specific receptors for certain aldehydes and the observed changes might not be a general stress response.

- **The role of aldehydes in the other experiments**

Irrespective of this, the concentrations of octadienal tested had effects on the production of aldehydes. This fact may also explain the results of the other experiments. Aldehydes are only released when a cell dies. Thus the "natural" aldehyde concentration in the medium is directly related to the number of dying cells. That in turn is related to the growth phase and also the cell density. At high cell densities the amount of aldehydes released might be

high enough to cause the effects observed without grazing. The applied octadienal concentration was calculated based on the assumption that about 500 cells were grazed per ml and day. In the NP treatments cell concentrations ranged from 5000 to over 40000 cells per ml. The assumption that in the stationary phase 500 cells per day die is therefore realistic. It is further possible that the release of low concentrations of aldehydes was responsible for the further increase in aldehyde production and the shift in the fatty acid ratio observed in the treatments with higher cell densities. This, in turn, would have increased aldehyde levels in the medium eventually, depending on the condition of the cells, leading to cell death.

- **Implications on natural plankton blooms**

The applied aldehyde concentrations were calculated to resemble realistic concentrations that would actually be encountered during diatom blooms (see 2.3.3). For this reason the ecological implications are of fundamental importance. When cells start dying (or being grazed) at higher cell densities, they release aldehydes. In unfavorable conditions, this would start a cascade of cell death and further release of aldehydes. The presence of such a condition-dependent suicide signal would be particularly beneficial for the algae if it were related, for instance, to the production of cysts. Both grazing pressure and unfavorable conditions could be avoided in this way. Unfortunately this was not examined in the course of this study. However, diatoms have been found to synchronize sexual reproduction and therefore the production of auxospores (Edlund and Stoermer 1997). This synchronization has so far been attributed to factors such as light and temperature (Potapova and Snoeijs 1997), but one could speculate that perhaps also chemical signals such as aldehydes might be involved.

- **Suggestions for further studies**

Even though these findings fit together, more experiments are required to validate them. Different aldehydes would need to be tested systematically in different but low concentrations, combined with other stressors, and using different solvents. Some efforts are currently being undertaken to try and establish a new method for easily monitoring calcium signals within cells using fluorescent calcium dyes in combination with blockers of xenotransporters. Once established, this may facilitate surveys of the signaling capacities of several substances at different concentrations. Other experiments could involve the screening of fatty acid composition and aldehyde production in

field samples during blooms. Lab experiments using filtered water from before, during, and after a *T. rotula* bloom might also produce interesting results, especially when considering the fact that in day to day lab experience medium based on filtered seawater sometimes turns out to be a poor culture medium, depending on the day the medium was filtered.

For all future studies that involve the determination of aldehyde production, some improvements in the extraction methods might help to produce clearer, less variable results. For instance, washing diatoms off a filter before the internal standard is added inevitably results in cell loss and an overall decrease in the documented cellular aldehyde production. A feasible alternative would be to sediment out cells with centrifugation. Another improvement would be to add the internal standard directly to the derivatization agent. This could reduce errors and help to improve the accuracy of future measurements.

4.3 Toxicity and nutritional quality

The last topic to be addressed is the toxic effects of diets consisting of aldehyde-producing diatoms on copepod eggs and larvae. The hypothesis mentioned in the introduction, that observed effects are due to nutrient deficiency, is a plausible alternative to that of "toxicity". Although diatoms are generally considered high quality food (Brett and Müller-Navarra 1997, DeMott and Müller-Navarra 1997, Armbrust et al. 2004), findings such as increased ingestion rates of "toxic" diatoms or the fact that "toxicity" decreases when copepods feed on non-axenic strains (Ianora et al. 2003) point towards nutrient limitation, even if these findings have not been interpreted in this way in the literature. Further, the effects on copepod embryos observed e. g. by Ianora et al. (2004, decreased growth rates, malformation in various larval stages and eventually death) are not only similar to those of fatty acid deficiency, but also reversible within a few days after a change in diet (Ianora et al. 2003).

This study was one of the first to assess both aldehyde production and fatty acid composition in all samples. For this reason it enables one to quantify the effects of aldehyde production on the availability of fatty acids in detail.

Samples for the fatty acids analysis were deep-frozen and freeze-dried immediately after filtration. As the production of aldehydes is wound-activated and depends on the activity of water-soluble enzymes (see 1.2), this procedure probably completely prevents the conversion of fatty acids to aldehydes. The method for assessing the production of aldehydes, on the other hand, was designed to maximize the aldehyde production (Wichard et al. 2005a) by breaking up the cells with ultrasound and incubating the sample for 30 minutes at room temperature. Although it is not clear how much aldehyde is actually produced when copepods graze on algae, it is not likely to be more than the amounts released in the course of the aldehyde preparation. Therefore, the molar ratio of an aldehyde to its precursor fatty acid is a good measure for the maximum percentage of fatty acids lost to the grazer by aldehyde production. Unfortunately, not all precursor fatty acids for all aldehydes are known and others (i. e. decadienal and its precursor arachidonic acid) were only present in trace amounts. Therefore this ratio was only calculated for decatrienal and EPA. It was shown to average at about 10% and hardly exceeded 25%, which leads to the conclusion that aldehyde production is not a means of depriving grazers of fatty acids. This is particularly true when one considers that those treatments that did produce higher quantities of aldehydes also had a significantly higher content of EPA. However, higher contents of EPA do not necessarily imply better food quality, as in the same treatments the cellular content of DHA decreased. St. John et al. (2001) found out that the growth of fish larvae, for instance, was negatively correlated to the EPA content and positively to that of DHA of their diet. Further, the ratio of DHA to EPA is also considered an important marker of food quality for copepods (Arendt et al. 2004) so that aldehyde-producing diatoms might still be a poorer source of food than those that do not.

The differences in the cellular content of polyunsaturated fatty acids have some economic relevance. As shown by A. Malzahn (in prep.) changes at the bottom of a food chain are detectable through several trophic levels. Knowing how to manipulate food quality at the bottom of the food chain e. g. by addition of low quantities of aldehydes or by altering nutrient availability and thereby increasing the amount of valuable ω-3 and ω-6 unsaturated fatty acids could not only make aquaculture more efficient but may ultimately be a means of increasing the quality of food for humans in a very easy and cost-efficient way.

It is also unclear whether the observed shift in the fatty acid composition is actually the cause of the parallel increase in aldehyde production. If this were the case one could hypothesize that a signaling effect as proposed above would be a possible evolutionary benefit of these changes. However, considering the low proportion of EPA that is actually transformed to aldehydes, one wonders why diatoms did not evolve a more efficient aldehyde production manner rather than producing higher amounts of polyunsaturated fatty acids. Perhaps "historic" reasons such as grazer deterrence could be the cause of this. Jüttner (2001) has shown EPA produced by benthic diatoms to deter and be toxic for grazers. It is possible that in former time this was also a means of grazer deterrence and increases in aldehyde production might have just been a secondary effect. Still aldehydes have taken on other functions in the course of evolution. One of these may have been toxicity for grazers, but this would need to be proven. The experiments conducted so far were immersion experiments that have only shown very high doses of aldehydes to have effects on grazers and might not be suitable to asses the actual toxicity of the substances (Caldwell et al. 2004). The findings of these experiments, together with the negative effects of monoalgal diets on copepod offspring have been considered sufficient evidence to label aldehyde production as an "insidious" (Miralto et al. 1999) mechanism of "birth control" (d'Ippolito et al. 2002b). The ecological benefit for algae as well as field studies that do not show such an effect are hardly considered.

4.4 Final remarks

The results of this study cannot disprove the hypothesis that aldehydes provide a grazer defense mechanism. They do, however, show that it is a highly variable process which depends on the environment as well as physiological changes within the cell. Although the chemical process of aldehyde production seems to be understood, its dynamics have so far only been studied in freshwater environments and are hardly understood. This study emphasizes the need to reevaluate some of the experiments performed with "toxic" or "non-toxic" strains of *T. rotula*. It further shows that alternative or also additional explanations for the ecological role of aldehydes besides grazer defense need to be taken into account.

I hope that by adding this information to the puzzle of trophodynamic interactions in marine environments I was not only able to clarify "the

influence of different culture conditions on the production of aldehydes by marine diatoms", but also to point out their importance on a larger scale. In the course of this work I have come to realize that, although phytoplankton is the basis of most life on earth, we have only just begun to understand it.

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9 Abbreviations and definitions

Species:

C. helgolandicus = *Calanus helgolandicus*

N. virens = *Nereis virens*

P. globosa = *Phaeocystis globosa*

P. minimum = *Procentrum minimum*

P. pouchetii = *Phaeocystis pouchetii*

P. tricornutum = *Phaeodactylum tricornutum*

S. costatum = *Skeletonema costatum*

T. longicornis = *Temora longicornis*

T. nordenskoeldii = *Thalassiosira nordenskoeldii*

T. rotula = *Thalassiosira rotula*

T. stylifera = *Temora stylifera*

T. weissflogii = *Thalassiosira weissflogii*

Culturing:

ASW = artificial seawater

CCMP = Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Maine, USA

FSW = filtered seawater

Chemistry:

AA = arachidonic (fatty) acid [20:4ω6] = (all-Z)-5,8,11,14 AA (IUPAC)

ALA = alpha-linolenic (fatty) acid [18:3ω3] = (all-Z)-9,12,15 ALA (IUPAC)

BHT = butylated hydrotoluene

DMSO = dimethylsulfoxide

DHA = docosahexaenoic (fatty) acid [22:6ω3] = (all-Z)-4,7,10,13,16,19 DHA (IUPAC)

dsDNA = doublestranded DNA

E = cis = same direction

EDTA = ethylenediaminetetraacetic acid

EPA = eicosapentaenoic (fatty) acid [20:5ω3] = (all-Z)-5,8,11,14,17 EPA (IUPAC)

FA = fatty acid

FAME = fatty acid methyl ester

MUFA = monounsaturated fatty acid

OCT = octadienal

PFBHA·HCl = *O*-(2,3,4,5,6-pentafluorbenzyl) hyodroxylamine hydrochlorine

PLA_2 = phospholipase A_2

PUA = polyunsaturated aldehyde

PUFA = polyunsaturated fatty acid

SAFA = saturated fatty acid

Tris = tris(hydroxymethyl)-aminomethane

X:YωZ: X = number of C-atoms, Y = number of double bonds, Z = first double bond (counting from methyl end and not the polar carbonyl group)

Z = trans = opposite direction

Methods:

ANOVA = analysis of variance

D. f. = degrees of freedom

EI = electron impact (ionization / fragmentation)

GC = gas chromatography

HPLC = high performace liquid chromatography

Mean sq. = mean squares

MS = mass spectrometry

PAM = pulse amplitude modulation

Pr. = probability (p)

RT = retention time

Sum sq. = sum of squares

Appendix

ChainCounter

ChainCounter is a free java-program written specifically for the purpose of counting cells and at the same time recording the chain-length in a sample. The program records the total number of cells and chains (including lengths), calculates the (weighted) mean chain-length and the standard deviation, and exports these values to a CSV (coma separated values) file. The program is free and runs on all systems (Mac, Windows, Unix, Linux, SunOS…) that have a current (>1.4) version of the Java Runtime Environment (JRE, see http://java.sun.com) installed. It is freely available from my homepage (http://dittami.gmxhome.de/chaincounter) and includes the source code, so that it can easily be adapted for other purposes.

GC-Reader

GC-Reader is another free java program designed to help put GC-chromatograms into tables. GC-Reader requires the export settings to be set to ASCI files with ";" as a separator and "," as a decimal separator. The GC-Reader opens all given files and looks for specific fatty acids or groups as specified in the file "known_acids.txt". The concentration for each fatty acid is then saved. The program's output is a new table containing all examined fatty acids of all samples (sample names are filenames). If a certain substance is not found in a sample either "0" or any other string can be filled into the table. GC-Reader allows you to extract information on the concentration of numerous or all fatty acids in several hundred samples with just a few clicks (assuming that all peaks have been previously identified either manually or automatically). Please be aware of the fact that this program was designed to work with the Varian GC 3800 on Helgoland only. As output formats of the GC might vary, the program may need to be adapted to work with other software versions. The program is free and runs on all systems (Mac, Windows, Unix, Linux, SunOS…) that have a current (>1.4) version of the Java Runtime Environment (JRE, see http://java.sun.com) installed. It is freely available from my homepage (http://dittami.gmxhome.de/gcReader) and includes the source code.

PAM settings

MI=2	LI=5
SI=12	ID=0:40
SW=0.8	IW=0:20
AI=3	TO=0.0
AW=0:15	TG=1.00
AF=1.00	LO=0
G=2	LG=1.00
D=1	M=A
FI=6	MLCAL=0
FW=0:05	ALCAL=0
EF=0.84	OBJFACT0=100
FO=0	OBJFACT1=600
CT=0:30	OBJFACT2=990
CI=1	OBJFACT3=810
LW=0:30	

Fluorospectrometer (PicoGreen) settings

Instrument Parameters	
Instrument	Cary Eclipse
Instrument Serial Number	el02075711
Data mode	Fluorescence
Ex. Wavelength (nm)	480.00
Em. Wavelength (nm)	520.00
Ex. Slit (nm)	10 Round
Em. Slit (nm)	5
Ave Time (sec)	0.5000
Excitation filter	Auto
Emission filter	Auto
PMT Voltage (V)	1000
Wellplate	ON

Plate format	Mel S
Read position	Well centre
Multi-zero	OFF
Auto-zero	OFF
Well	A1
Replicates	1
Sample averaging	OFF

Selbstständigkeitserklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit selbstständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Mit der Einstellung dieser Arbeit in die Universitätsbibliothek der Christian-Albrechts-Universität zu Kiel bin ich einverstanden.

Kiel, den 18.04.06 _____

(Unterschrift)