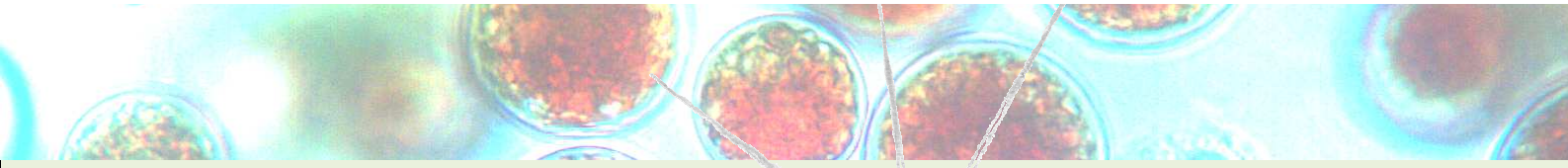
MICROALGAL APPLICATIONS

ECOLOGICAL OPTIMIZATION OF BIOMASS AND LIPID PRODUCTION BY MICROALGAE



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SUMMARY

Microalgae have higher growth rates and higher lipid content than terrestrial plants and the yield per unit area is even higher by several orders of magnitude. Furthermore, the production of microalgae does not compete for fertile land for food production. Therefore, microalgae are in the focus of research for biodiesel production, nutritional supplements and aquaculture approaches. However, after almost half a century of research the full promise of microalgae as a feedstock for biofuel production has remained largely unfulfilled. My research was motivated by the obvious gaps in the application of ecological pros of microalgae.

DIVERSITY-PRODUCTIVITY RELATIONSHIPS: THE ROLE OF DIVERSITY FOR MICROALGAL LIPID PRODUCTION

The relationship between diversity and productivity within terrestrial and algal primary producers has been well documented in ecology. However, the importance of diversity for lipid production for biofuel remains limited. Hence, I set out to investigate, experimentally, whether diversity may also affect lipid production in microalgae. Microalgae from all major algal groups were grown in a large number of treatments differing in their diversity level. Additionally, I compared the growth and lipid production of laboratory communities with the lipid production of natural lake and pond phytoplankton communities along a diversity gradient. This comparison showed that the lipid production of selected laboratory monocultures was not significantly higher than that of natural phytoplankton communities.

The lipid production in general increased with increasing diversity in both natural and laboratory microalgal communities. The underlying reason for the observed 'diversity-productivity' relationship seems to be resource use complementarity. Additionally, a very important observation was that diversity also influences the specific lipid production of each microalgae in the high diverse communities.

DIVERSITY- LIGHT- LIPID RELATIONSHIPS: LIPID PRODUCTION IN THE RIGHT LIGHT

The knowledge about the relationship between diversity and biomass/lipid production in primary producer communities for biofuel production is underestimated. However, basic ecological research studies on the growth of microalgal communities provide evidence of a

positive relationship between diversity and biomass production and show that the observed positive diversity-productivity-relationships are related to an increase in the efficiency of light use by diverse microalgal communities. I cultivated microalgae from all major freshwater algal groups in treatments that differed in their species richness and functional group richness. Polycultures with high functional group richness showed higher light use and algal lipid content with increasing species richness. Additionally, I could show a clear correlation between light use and lipid production in functionally diverse communities. Therefore, a powerful and cost effective way to improve biofuel production might be accomplished by incorporating diversity related resource-use-dynamics into algal biomass production.

DIVERSITY AND FOOD QUALITY: ADVANTAGES FOR AQUACULTURE FOOD WEBS

Determining the factors that control the energy transfer at the plant-animal interface is a key issue in ecology, because this transfer is highly variable and despite its global importance it is still not well understood. Food quality of primary producers seems to be a crucial factor influencing the transfer efficiency towards higher trophic levels. One major aspect of food quality is the biomass fatty acid composition in terms of essential ω3-polyunsaturated fatty acids (ω3-PUFAs) of primary producers, because all animals are incapable to synthesize them *de novo*. However, the influence of diversity on phytoplankton food quality in terms of lipid composition (e.g. ω3-PUFAs) remains unclear. I tested via a series of experiments controlled for diversity how the diversity of microalgal communities influences their fatty acid composition. My study shows the significant influence of diversity of primary producer communities on their fatty acid composition; especially on essential ω3-PUFA content.

MICROALGAL BIOMASS CONTROL VIA GRAZING: IMPACT OF MICROALGAL SIZE

The direction and strength of phytoplankton community responses to zooplankton grazing most probably depend on the size of phytoplankton species. To examine the influence of migrating (diel vertical migration, DVM) and non migrating zooplankton communities on different sized phytoplankton communities, I designed an experiment where I manipulated the size distribution of a natural phytoplankton community *a priori* in field mesocosms. Comparison of “migration” and “no migration” zooplankton treatments showed that nutrient availability and total phytoplankton biovolume were higher in “no migration” treatments with phytoplankton communities comprising mainly small algae and in “migration” treatments with phytoplankton communities of a broader size spectrum of algae. Additionally my results

showed experimentally that food size selection and migration behavior of *Daphnia hyalina* can cause a shift from small sized microalgae towards larger species.

NEW CULTIVATION TECHNIQUES FOR BIOMASS AND LIPID YIELD OPTIMIZATION IN MICROALGAE

For the installation of infrastructure for the large-scale production of biofuel from microalgae is essential to establish cultivation methods that maximize lipid production but which are also economically viable in terms of energy demand and resource supply. For this purpose, I compared different cultivation systems (semi-batch, continuous) to optimize simultaneously growth and biomass lipid content of *Botryococcus braunii*. To enhance both, biomass accumulation and lipid production at the same time I further investigated a two-stage cultivation method to replace one stage semi-batch cultivation systems. In the first step of this cultivation method a full growth medium allows an enhancement of biomass accumulation. In the next step, the culture was transferred into nitrogen limited growth medium, where a further accumulation of photosynthetic products in the form of lipids occurred. Two-stage cultivation cultures resulted in higher nutrient specific biomass production and lipid content of *B. braunii* compared to one stage cultivation. If a continuous cultivation of cultures with high biomass in stage one can be assured, an almost constant supply of huge amounts of algae with even high lipid content in the second step could be guaranteed.

My results clearly show that a better understanding of general ecological principles for biomass and lipid production of microalgae provides a cost effective and environmental friendly way to cultivate high yielding microalgal communities for commercial approaches. The enhancement of the yield efficiency of lipid production in diverse microalgal communities would be difficult to do only by technical means such as increasing resource supply. In addition, increasing the supply of resources is usually correlated with high energy requirements and therefore cost intensive. It is therefore important for biomass production systems to utilize all possible ecological options to increase the efficiency of the use of the supplied resources by integrating basic ecological principles into the cultivation systems.

CHAPTER 1

GENERAL INTRODUCTION

HISTORY OF ALGAL APPLICATIONS

The idea of making biofuels from biomass from primary producers is more than 100 years old and goes back to Rudolf Diesel, who designed his diesel engine to run with peanut oil. However, with the exploration of huge supplies of crude oil, petroleum became very cheap, leading to a reduction in the use of renewable energy, such as biofuels. Renewable biofuels degenerated to a minority “alternative” energy status.

However, today the predicted rises of over 30 % in greenhouse gas (CO_2) levels in the atmosphere (Chapin III et al. 2000), declining air and water quality, and human health concerns indicate that the use of fossil fuels is unsustainable. Renewable energy, such as wind and solar energy or biologically produced fuels have been identified as potential alternative energy sources (Smith et al. 2010).

The first generation of biofuels has been produced from organisms such as oil palm and coconut or soybean (Chisti 2007). However, the use of agricultural products for energy production instead of food resources has resulted in increased competition for fertile agricultural areas. The annual production needed for half of all U.S. transportation fuel would require an area equivalent to eight times the U.S. land area that is used for crop production (Chisti 2007). This creates a food versus energy dilemma (Tilman et al. 2009).

Using microalgae for biofuel feed stocks would result in a far smaller land footprint and a smaller ecological impact. To satisfy 50 % of the fuel demand of the U.S., microalgae would require a surface area of water equivalent to 1 - 3 % of US land used for agricultural crops, because of the higher growth rates and lipid content of many microalgae (see Tab. 1; Chisti 2007). Additionally, their cultivation is independent of soil fertility.

Another reason to consider microalgae as a potential bioenergy source is the fact that algae are the most important part of fossil oil deposits, which were built in the cretaceous period (Ratanasthien 1999). In shallow shelf seas, dead algae sank to the sea bottom and a high proportion of the organic compounds were preserved due to anoxic conditions in the sediments.

Over millions of years of high temperature, bacterial activity and high pressure resulted in the conversion of algal lipids to crude oil. Only a few microalgal species (e.g., *Botryococcus braunii*) have been identified as being responsible for these cretaceous oil deposits.

Table 1. Comparison of estimated biodiesel production efficiencies from vascular plants and microalgae[a] (modified after Smith et al. 2010).

Biodiesel feedstock	Area needed to meet global oil demand (10^6 hectares)	Area required as a percent of total global land	Area required as a percent of total arable global land
Cotton	15 000	101	757
Soybean	10 900	73	552
Mustard seed	8500	57	430
Sunflower	5100	34	258
Rapeseed/canola	4100	27	207
Jatropha	2600	17	130 (0)[b]
Oil palm	820	5.5	41
Microalgae (10 g/m^2/day, 30 % TAG)	410	2.7	21 (0)[c]
Microalgae (50 g/m^2/day, 50 % TAG)	49	0.3	2.5 (0)[c]

[a] Modified with rounding from Schenk et al. 2008.
[b] Jatropha is mainly grown on marginal land.
[c] Assuming that microalgal ponds and bioreactors are located on non-arable land.

The idea of using algae as an energy source was already being discussed over 50 years ago (Oswald and Golueke 1960). Later in the 70s, large screening programs in the US, known as the Aquatic Species Program (ASP) offered a concerted effort on developing algal energy production systems focusing on microalgae. However, due to low crude oil prices the program was stopped (Service 2011).

Recently, with increasing concerns about the potential of global climate change, declining air and water quality, and serious human health concerns, the development of biofuels, as a fossil fuel alternative, has re-emerged. Biofuels are currently mostly made from recycled vegetable oil and various feedstocks.

However, the advantages of microalgae, compared to terrestrial plants, seem to designate microalgae in the second run as the optimal resource for biofuel production.

Algal biomass and lipid content is currently also sought after for other applications than biofuel, e.g. health food, animal feeds and also fertilizers, as well as bioplastics and other applications for aquaculture (see Murphy 2006; Spolaore et al. 2006; Natrah et al. 2007; Plaza et al. 2008; Huerlimann et al. 2010).

ALGAE, PHOTOSYNTHESIS, LIPID PRODUCTION

The term “algae” is not a taxonomic classification, it is more to indicate a polyphyletic artificial assemblage of O_2-evolving, photosynthetic organisms. Algae occur in nearly all ecosystems (mostly in aquatic ecosystems) with very different forms, from microscopic cells (microalgae) to macroscopic multicellular (macroalgae) with complex leafy or blade forms, which contrast strongly with uniformity in vascular plants (Barsanti and Gualtieri 2006).

However, algae and vascular plants have one major ability in common: photosynthesis. Photosynthesis is the key process for the biological conversion of solar energy (1.35 kW m^{-2}) to chemical bond energy (Falkowski and Raven 2007), which is the usable energy for cell metabolism resulting in growth, production or storage compounds such as lipids. Microalgae are the most primitive form of autotrophic organism. While the mechanism of photosynthesis in microalgae is similar to that of vascular plants, they are generally more efficient converters of solar energy, because of their simple cellular structure. The transfer efficiency of solar energy into biomass can exceed 10 % for microalgae, whereas plants comprise only 0.5 % (Li et al. 2008).

Microalgae are very fast growing, they can double their biomass several times a day under good conditions (Reynolds 2006), whereas the life cycles of terrestrial plants are often weeks, months or even several decades. In addition, because the cells of microalgae grow usually in aqueous suspension, they have more efficient access to water and other nutrients.

Microalgae are categorized in a variety of classes. Generally the classification is based on the pigmentation of the microalgae, but also of their life cycle and basic cellular structure (van den Hoek 1984). Nearly all oxygenic phototrophs use chlorophyll-*a* as an important pigment. However, microalgae have a variety of secondary pigments (e.g., phycocyanin, fucoxanthin and phycoerythrin) and this results in the absorption spectra of microalgae

covering the photosynthetic active radiation (PAR) between 400 and 700 nm (see Fig. 1.1; Wilhelm and Jakob 2011). Therefore half of the solar spectrum is suitable for oxygenic photosynthesis.

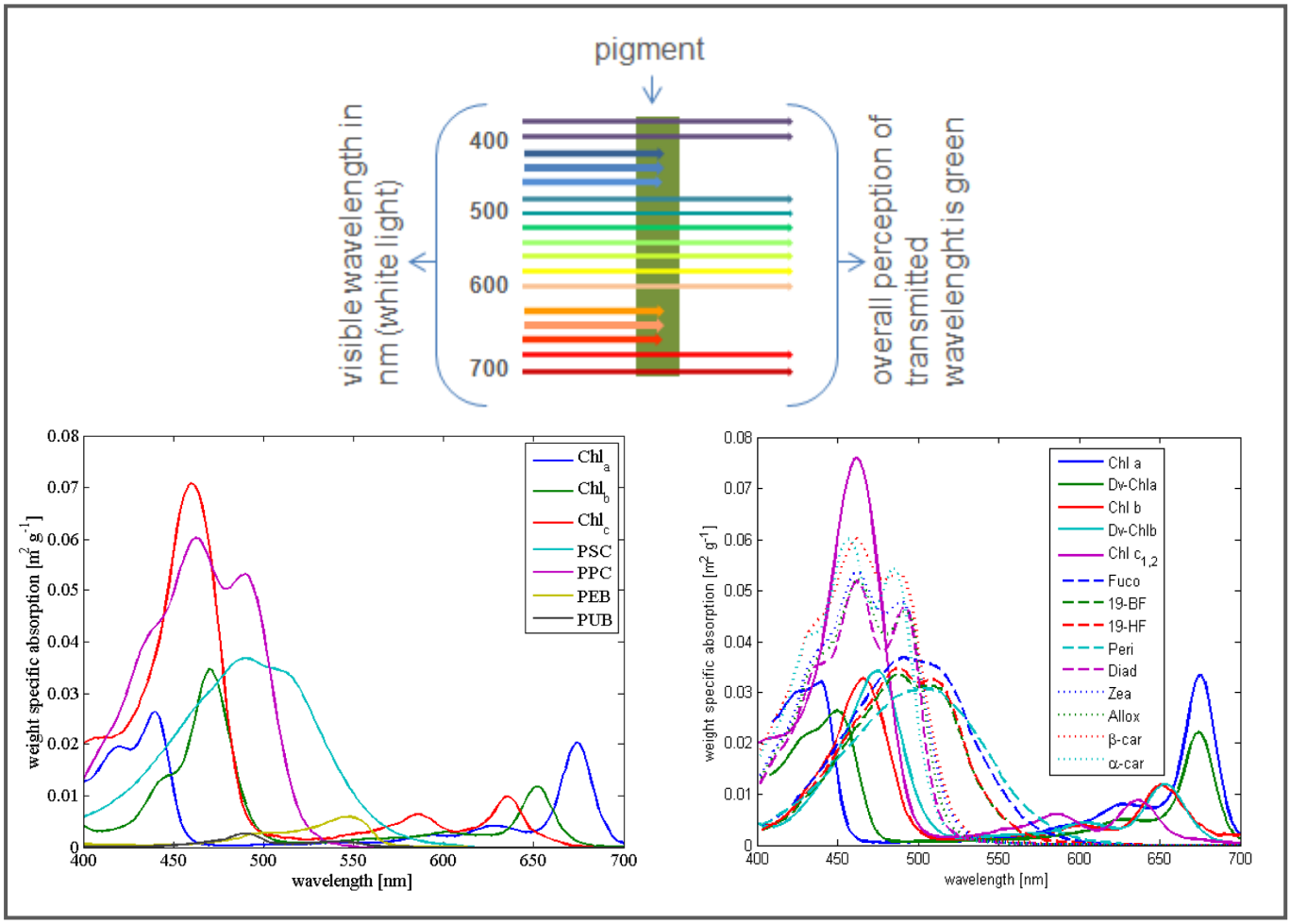

Fig. 1.1: Absorption and transmission of different wavelengths of light by a hypothetical pigment after Purves and Life (1997; above). Mass normalized absorption of phytoplankton pigment after Bricaud et al. (2004; below left) and Bidigare et al. (1990; below right).

The pigment variety of microalgae results in a high phylogenetic diversity. The four most important microalgal classes are:

- Diatoms (bacillariophyceae): These microalgae dominate the phytoplankton of the oceans, but are also found in fresh and brackish water. Diatoms contain polymerized silica in their cell walls. Diatoms store carbon in the form of natural oils or as a polymer of carbohydrates known as chrysolaminarin. Beside chlorophyll-*a* and -*c* they also contain diadinoxanthin and diatoxanthin (Fig. 1.1).
- Green algae (chlorophyceae): These are also quite abundant, especially in freshwater and occur as single cells or as colonies. As their name implies the green algae contain mainly chlorophyll-*a* and –*b* (Fig. 1.1). Starch is the main storage compound, though oils can be produced under certain conditions.
- Blue-green algae (cyanophyceae): Much closer to bacteria in structure and organization, these microalgae play an important role in fixing nitrogen from the atmosphere and are found in a variety of habitats. Besides chlorophyll-*a* of bacteria, further pigments are phycoerythrin and phycocyanin (Fig. 1.1).
- Golden algae (chrysophyceae): This group of microalgae is similar to the diatoms, but their pigment system is more complex. Chrysophyceae can appear yellow, brown or orange in color due to their fucoxanthin and xanthophylls (Fig. 1.1). Golden algae produce natural oils and carbohydrates as storage compounds.

The pigment composition is on average more similar in species from the same microalgal group (taxonomic group) than between species from different microalgal groups (Schlüter et al. 2006). Therefore, it is possible to create functional groups (functional meaning, in this case, different light harvesting capabilities of the different pigments) identical to taxonomic groups by the phylogenetic based pigment signatures, assuming that the microalgal pigments are a major criteria for the taxonomical classification of microalgae (van den Hoek 1984; Scheer 1999; Barsanti and Gualteri 2006).

Photosynthesis is the biochemical process of carbon fixation and produces hexoses or carbohydrates. About 90 % of carbon is bound as macromolecules, or in other words: proteins, lipids, carbohydrates (Wilhelm and Jakobs 2011). Lipids account for some 2 - 20 % of dry weight of phytoplankton. Some microalgae can reach a very high lipid content. For example, the already mentioned algal species, *Botryococcus braunii*, a main contributor to crude oil deposits, can show lipid concentrations of up to 80 % of its dry weight (Chisti 2007). Besides their capacity as a storage compound, lipids function as a buoyancy compartment. Most lipids are lighter than water, and inevitably, their presence counterbalances normal excess density to some limited extent (Reynolds 2006).

LIPID COMPOSITION OF MICROALGAE

Lipids generally consist of fatty acids and their derivates and also of substances, which are related functionally or biosynthetically to them. The lipid composition of microalgae is mainly divided into polar glycerolipids and nonpolar glycerolipids (Guschina and Harwood 2009). In general, the glycerolipid composition of microalgae is similar to higher plants. In most of the microalgal species the nonpolar triacylglycerols (TAG) are particularly accumulated as storage products and can be reutilized for polar lipid synthesis (Guschina and Harwood 2009).

Fatty acids are compounds synthesized in nature via condensation of malonyl coenzyme A units by a fatty acid synthase complex (Fig.1.2). They usually contain even numbers of carbon atoms in straight chains (commonly C_{14} to C_{24}). Fatty acids are class-divided into FAME (fatty acid methyl ester) or FFA (free fatty acids). FAMEs are grouped into SAFAs (saturated fatty acids), MUFAs (mono unsaturated fatty acids) and PUFAs (poly unsaturated fatty acids). PUFAs are an important part in all animal bodies and originate mostly from the diet.

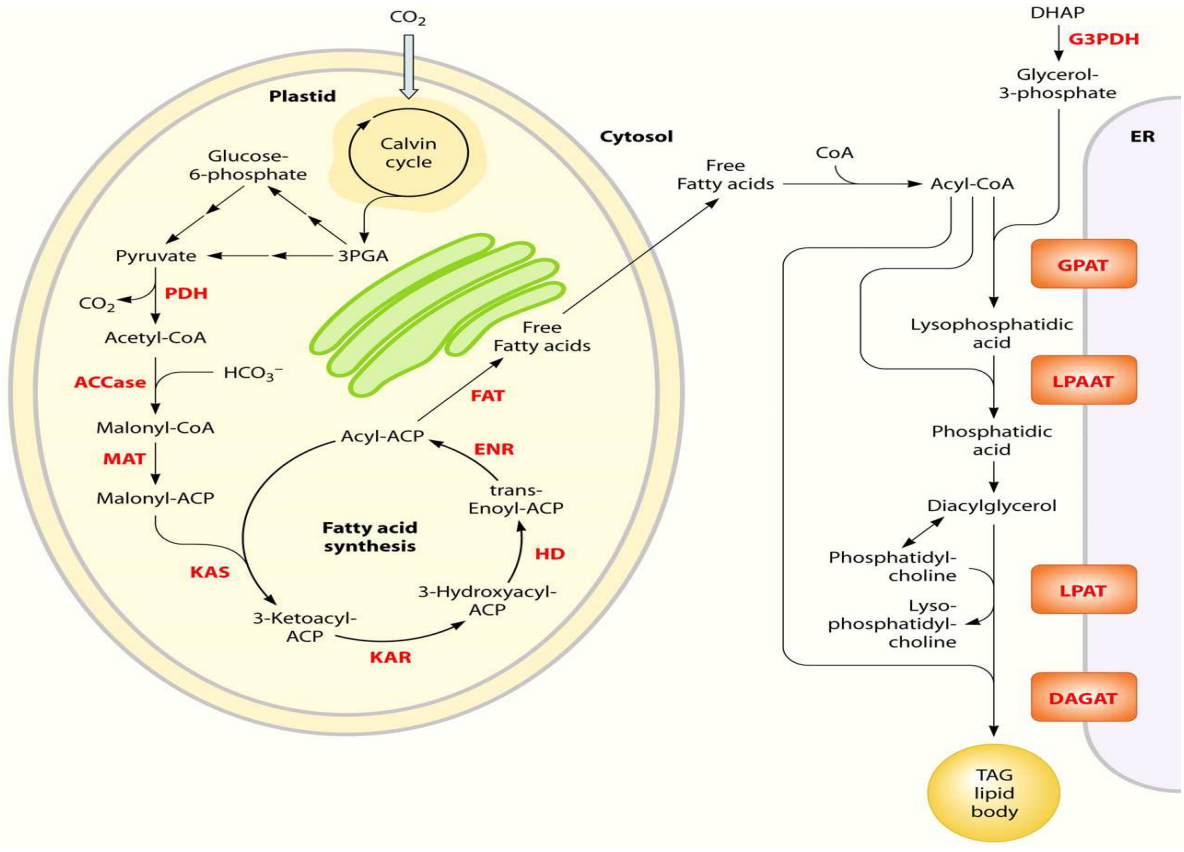

Fig.1.2: Simplified overview of the metabolites and representative pathways in microalgal lipid biosynthesis shown in black and enzymes shown in red (Radakovits et al. 2010).

The lipid content and quality of microalgae is highly variable within a species, as well as between different species. It can be affected by a number of environmental or culturing variables, such as growth phase, light intensity, temperature, salinity, carbon dioxide and nutrients.

Temperature generally has a high impact on the lipid content of microalgae. Several studies have shown that lower environmental temperatures generally cause variations in lipid content (Thompson 1996; McLarnon-Riches et al. 2003; Sushchik et al. 2003). The lipid composition is also influenced by shifts of temperature in the environment (Guschina and Harwood 2009).
Fatty acid synthesis is normally stimulated by light. Thompson (1996) showed that TAG is mostly synthesized in the light and then reutilized for polar lipid synthesis in the dark. Therefore, the overall lipid content of microalgae will reflect this change under different light conditions.
The nutrient availability in aquatic ecosystems has a significant influence on the lipid production of microalgae. Which particular nutrient is limiting is species dependent (e.g. silicon for species of the diatoms class). However, nitrogen is very important for the cell division process in all microalgal species. Generally, nitrogen limitation leads to higher lipid production in nearly all known microalgae (Smith et al. 2010). Microalgae switch carbon allocation from reproduction to oil production (Illman et al. 2000; Lv et al. 2010). The increase in lipids by nitrogen limitation is therefore inversely proportional, with growth resulting in a very low growth rate for nitrogen limited, lipid rich microalgae (Guschina and Harwood 2009). Many studies showed that the nitrogen starved cells can contain several times more lipids than non-starved cells (Shifrin and Chisholm 1980; Borowitzka 1988; Yamaberi et al. 1998; Griffiths and Harrison 2009).

New cultivation methods are needed to combine the advantages of biomass production and nitrogen starvation. Continuous cultures such as chemostats (or bioreactors) are comparable to steady state equilibrium, with the production and elimination of organisms, as well as consumption and supply of resources, being in balance (exponential phase; see Fig. 1.3; Lampert and Sommer 2007).

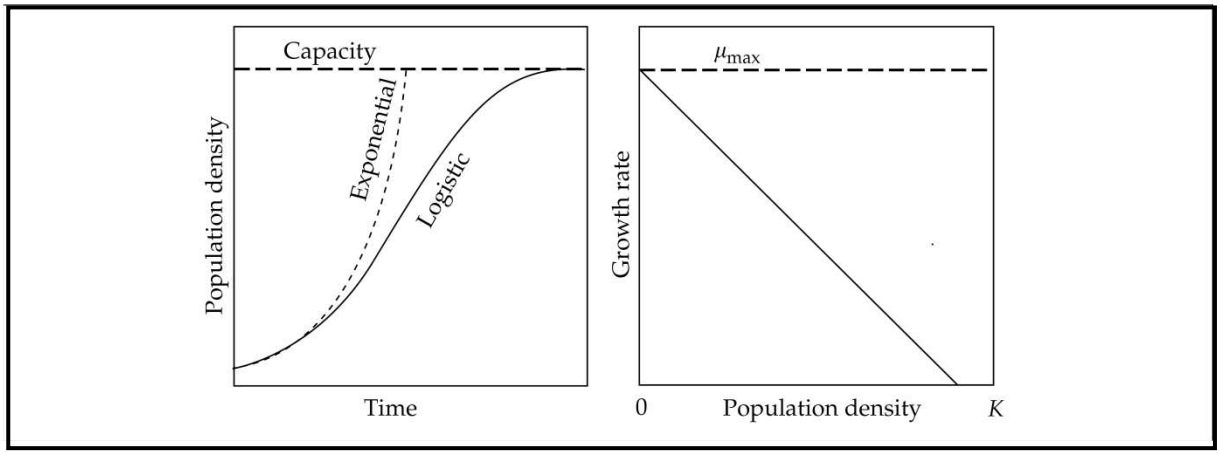

Fig.1.3: Population density development along time (left) and growth rate development along population density (right) in continuous culture (chemostat) after Lampert and Sommer (2007).

Whereas in static cultures, such as batch cultures (bioreactors where a small number of organisms is added to a known amount of medium with no further additions of medium or remove of culture liquid), the microalgal culture growth runs into nutrient limitation and the nutrients contained in each algal cell decreases (stationary phase; see Fig. 1.4; Lampert and Sommer 2007).

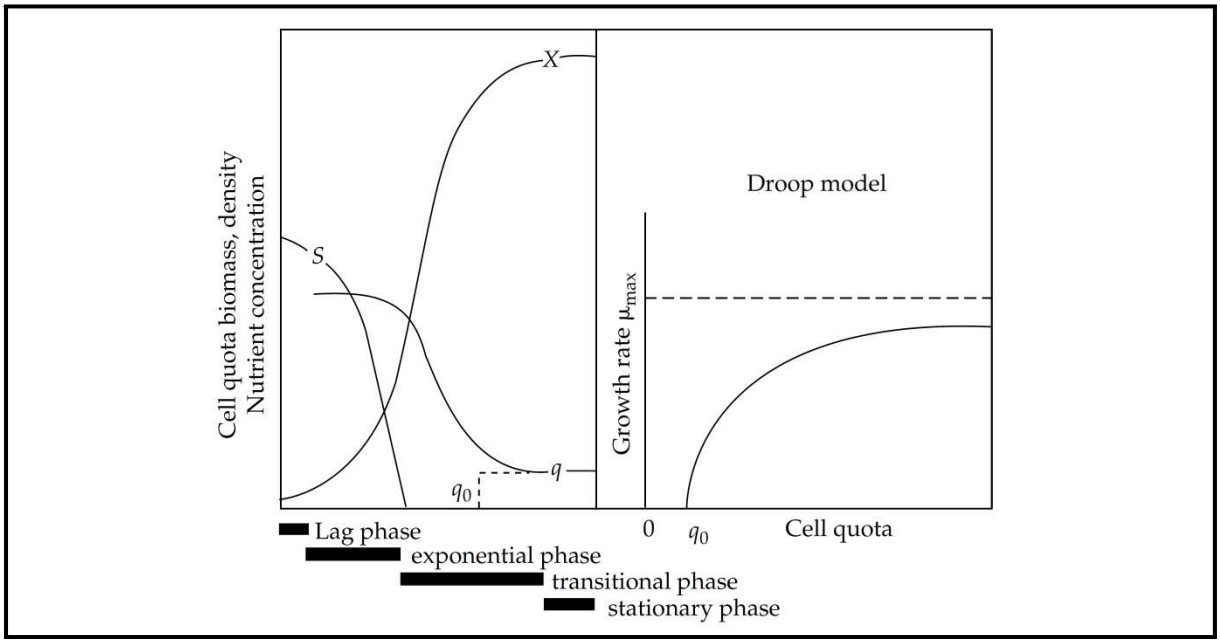

Fig. 1.4: Cell quota biomass (q), density (X) and nutrient concentration (S) in different growth phases of a static culture (batch culture) after Lampert and Sommer (2007).

Combining these two cultivation systems for optimized microalgal growth and reproduction in a first continuous stage, and high lipid production in a second stage, with batch conditions could be one option to optimize both total microalgal biomass production and its lipid content.

THE ROLE OF MICROALGAE IN AQUATIC FOOD WEBS

Making energy out of microalgae is now an important applied aspect of microalgal cultivation. There are two options for microalgal biomass production: closed systems and open ponds, both are considered as artificial ecosystems (Pulz 2001), characterized by controlled resource supply rates, regular harvest and predefined food web structures (Smith et al. 2010). However, these assembled systems follow the same important ecological principles as natural ones (Smith et al. 2010). In particular, open pond systems will not only contain monocultures of microalgae, but protozoa, rotifers and microcrusataceen will invade the system very quickly. The artificial pond system is then determined by both the bottom-up control of production by resource supply rates and the top-down control of biomass distribution via trophic cascades, including predation and the indirect effects of higher trophic levels on lower food web levels (Carpenter and Kitchell 1993).

Microalgae are therefore an important component of the aquaculture food chain, e.g. as live food for zooplankton and fish larval culture (Huerlimann et al. 2010). Zooplankton is highly dependent on the lipid composition of their food (Ravet et al. 2003; Arts et al. 2009; Martin-Creutzburg and von Elert 2009). PUFAs are especially important as they are involved in the regulation of physiological processes. PUFAs provide precursors for the biosynthesis of bioactive molecules such as prostaglandins, thromboxanes, leukotrienes and resolvins, which may affect egg-production, egg-laying, spawning and hatching, as well as other physiological functions in zooplankton (Brett und Müller-Navarra 1997; Mariash et al. 2011).

MICROALGAL DIVERSITY, LIGHT USE EFFICIENCY AND LIPID PRODUCTIVITY

Studies in lipid production by microalgae are often restricted to screening microalgae to find one very productive microalgal species in terms of biomass and lipid production (“a fat marathon runner”; Montero et al. 2011). Research in the last decades has focused mainly on the lipid content and fatty acid composition of distinct microalgal species (Sheehan et al. 1998; Alonso et al. 2000; Mansour et al. 2003). In natural systems, animals feed usually on a variety of foods. Additionally, the most common cultivation systems for microalgal biomass cultivation are open pond systems with a high degree of exposure to the environment. Due to perpetual biological input to these systems (wind, birds and rain, etc.), the desired monoculture does not persist very long and diverse microalgal communities will establish. However, diversity has previously been hardly considered as an important factor for microalgal production.

In basic ecology, scientists have devoted considerable effort to understanding the functional significances of diversity (Loreau et al. 2003; Hooper et al. 2005). This research has revealed the generally positive effects of diversity on the functioning of ecosystems and in particular the positive effects of plant species diversity (Balvanera et al. 2006; Cardinale et al. 2007) and microalgae diversity (Ptacnik et al. 2008; Striebel et al. 2009a, b) on primary productivity. In general, a combination of two liable mechanisms is discussed as being responsible for this positive diversity-productivity relationship: firstly, a "selection effect", whereby a highly productive species, which is more likely to be present in a highly diverse community, dominates the production of the observed system. Secondly, a "complementarity effect", which is interpreted as regulating niche differences and/or facilitating interactions among species (Loreau and Hector 2001; Fornara and Tilman 2008).

In understanding the functional significance of diversity, most experimental work has been conducted with synthetically assembled communities and has revealed generally positive effects of species diversity on primary production (Tilman et al. 2001; Balvanera et al. 2006; Cardinale et al. 2007; Striebel et al. 2009a). However, this is in contrast to natural community patterns that have been revealed by studies exploring productivity as a driver of species diversity (Jiang et al. 2009). The importance of resource use, complementarity and facilitation on diversity-productivity relationships in microalgal communities has already been recorded in natural ecosystems (Ptacnik et al. 2008; Striebel et al. 2009a, b; Vanelslander et al. 2009).

However, uncertainty remains as to whether cultures of selected, single microalgal species are actually more productive in terms of lipids than more diverse microalgal communities. Nevertheless, Smith et al. (2010) hypothesized in their review of the ecology of biodiesel production by microalgae, that naturally occurring multi species microalgal communities in open pond bioreactors might store more solar energy as lipids, compared to single species communities in closed photobioreactors.

Within an entire ecosystem, most of the studies referred to diversity as species richness, with other components of diversity being neglected (Diaz and Cabido 2001). However, species richness is only one way of viewing the diversity of a community and its organization (Hillebrand and Matthiesen 2009). Nowadays, it has become clear that the impact of diversity on ecosystem properties depends more on functional diversity, or functional composition, than on species richness (Diaz and Cabido 2001; Duru et al. 2012). The term "functional group" in this case represent a class of species, divided into groups based on their common biochemical and/or ecological functions (see Hood et la. 2006). Experiments on microalgal communities in which the diversity of functional groups was manipulated, showed that light

use efficiency increased when microalgal functional diversity was higher (Behl et al. 2011). It was postulated that complementary light use along the photosynthetically active radiation (PAR) spectrum (400 - 700 nm) could be the main mechanism behind microalgal diversity-productivity relationships (Striebel et al. 2009b; Behl et al. 2011). At the same time, it has also been reported that microalgal lipid metabolism is affected by light. Most of the studies deal with light intensities affecting the quality of lipids of distinct microalgal species; qualitative changes in lipids as a result of different light conditions are associated with alterations in chloroplast development (Harwood 1998). However, the understanding of the effects of the interactions between light and diversity on the lipid production of microalgae remains limited. To incorporate these findings into potential growth strategies for microalgal biomass production systems that generate lipids, a more mechanistic insight into these interactions is definitely required.

IS ALGAE BIOFUEL A COMMERCIAL REALITY?

As of now, there are about 150 algae companies pursuing efforts in various areas to make microalgae biofuels a commercial reality but no successful outcomes have yet been announced at a commercial level. One of the major challenges in making microalgae biofuels feasible is the harvesting of microalgae. The compromise between harvesting efficiency and cost is a critical problem in microalgal biofuel production and at the moment there are no low cost harvesting technologies available. The highly diluted biomass must be removed from a huge volume of water using harvesting processes such as centrifugation, micro-screening and flocculation. The process is estimated to contribute up to 20 - 30 % of the total cost, and thus, harvesting optimization has been emphasized as one of the key factors determining the feasibility of microalgal biofuel development in the future (Sheehan et al. 1998). The most cost effective method would be the natural sedimentation of microalgae due to gravity. Stokes' law describes the sinking velocity of a particle in a water column:

$$V_s = \frac{2}{9} g r^2 (q' - q) \mu^{-1} \Phi^{-1} \qquad \text{(I)}$$

Size (r) is the most influential parameter effecting sedimentation, a two-fold increase in size results in a four-fold faster sedimentation velocity. A selection towards larger microalgae in artificial open pond systems with diverse microalgal communities might be achieved by

introducing zooplankton, such as the cladoceran genus *Daphnia*. Sommer et al. (2001; 2003) showed that grazing has a greater effect on the composition of phytoplankton communities than on total microalgal biomass. In general, *Daphnia* graze on smaller microalgae (< 20 µm; Burns 1968), thereby reducing competition on large microalgae. Food size selection and grazing behavior of *Daphnia* might shift microalgal size structure from small to large species.

CENTRAL SCIENTIFIC ISSUES

The mass cultivation of microalgae still focuses many challenges. Although the cultivation of microalgae for special applications (e.g., dietary supplement) is well-established, the large scale cultivation of microalgae for biofuel production is not yet commercial reality. It requires long-term research to investigate the potential for ecological optimizations of microalgal growth. My research was motivated by a lack of knowledge how ecological concepts can be integrated into commercial aquatic biomass and lipid production. The following scientific issues are addressed and discussed in chapter 2- 6:

DIVERSITY-PRODUCTIVITY RELATIONSHIPS: THE ROLE OF DIVERSITY FOR MICROALGAL LIPID PRODUCTION

- Does a positive relationship between productivity and lipid production in microalgae exist?
- If a coupling between diversity and lipid production exists, is it based on a dominance of a single species or on resource partitioning among species?
- Is a with diversity increasing lipid content only resulting from increasing biomass production, or does diversity also positively influence the biomass specific lipid content of microalgae?

DIVERSITY- LIGHT- LIPID RELATIONSHIPS: LIPID PRODUCTION IN THE RIGHT LIGHT

- Is light exploited more efficiently in highly diverse microalgal communities?
- Is the effect of diversity on lipid production and light utilization stronger within communities consisting of more than one functional group? (species richness versus functional trait richness)
- Is there an underlying mechanism for increased lipid production and light utilization in more diverse communities and can it be declared by complementarity in resource (light) use?

DIVERSITY AND FOOD QUALITY: ADVANTAGES FOR AQUACULTURE FOOD WEBS

- To what extend is the ω3-polyunsaturated fatty acid (ω3-PUFA) content of microalgal communities influenced by its diversity?
- If diversity is affecting the ω3-PUFA content of algal communities, is it then dependent on species richness or on functional trait diversity?
- How does diversity influence a distinct ω3-PUFA of microalgae (α-linolenic) which is important for the life history of zooplankton (*Daphnia*)?

MICROALGAL BIOMASS CONTROL VIA GRAZING: IMPACT OF MICROALGAL SIZE

- How does algal size distribution interact with grazing by zooplankton of the common genus *Daphnia*)?
- Is this interaction further affected by behavioural responses of zooplankton to fish?
- Does an experimental manipulation of the size distribution of microalgal communities alter the interaction between zooplankton and microalgal community dynamics?

NEW CULTIVATION TECHNIQUES FOR BIOMASS AND LIPID YIELD OPTIMIZATION IN MICROALGAE

- Could a two-stage cultivation system provide an optimized cultivation method to ensure both, high biomass and high lipid production in microalgae?
- To what extend could such two-stage cultivation systems provide a starting point for large scale commercial production of lipid rich microalgal biomass?

All of my research questions were investigated via series of laboratory and large scale field experiments. Results are presented in chapter 2- 6. Chapter 2 and 5 are already published data, chapter 3 is in revision, chapter 4 and 6 are under review.

CHAPTER 2

THE EFFECT OF SPECIES DIVERSITY ON LIPID PRODUCTION BY MICRO-ALGAL COMMUNITIES

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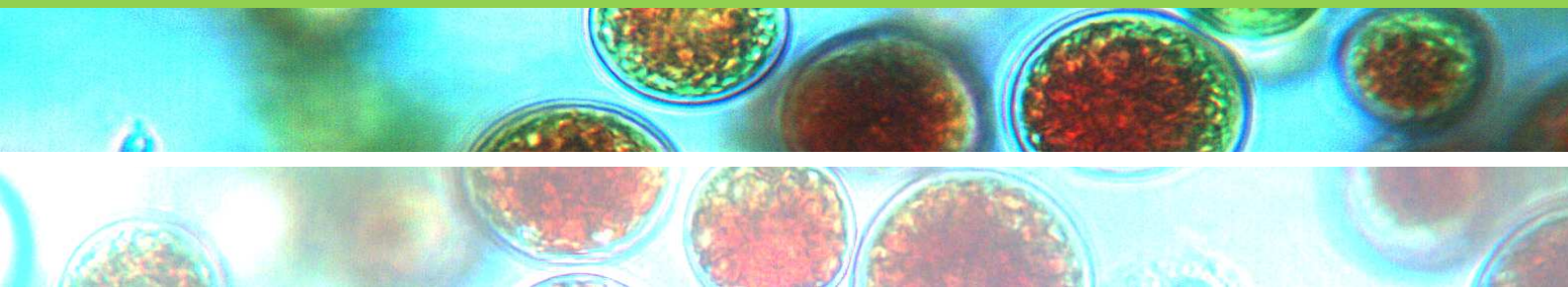

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The effect of species diversity on lipid production by micro-algal communities

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Abstract Current research investigating the importance of diversity for biofuel lipid production remains limited. In contrast, the relationship between diversity and productivity within terrestrial and algal primary producers has been well documented in ecology. Hence, we set out to investigate, experimentally, whether diversity may also affect lipid production in micro-algae. We investigated the growth and lipid production of micro-algae using species from all major algal groups. Algae were grown in a large number of treatments differing in their diversity level. Additionally, we compared the growth and lipid production of laboratory communities to natural lake and pond phytoplankton communities of different diversity. Our results show that lipid production increased with increasing diversity in both natural and laboratory micro-algal communities. The underlying reason for the observed 'diversity–productivity' relationship seems to be resource use complementarity. We observed higher lipid production of highly diverse algal communities under the same growth and resource supply conditions compared to monocultures. Hence, the incorporation of the ecological advantages of diversity-related resource-use dynamics into algal biomass production may provide a powerful and cost effective way to improve biofuel production.

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Introduction

The combination of rapid economic development, ever increasing population size and ever rising living standards will inevitably lead to fossil fuels being exhausted in the near future (Zhou et al. 2009). In parallel, predicted rises of over 30% in greenhouse gas (CO_2) levels in the atmosphere (Chapin et al. 2000) indicate that the use of fossil fuels is unsustainable (Schenk et al. 2008). Therefore, there is increasing effort to find alternative, renewable and therefore sustainable energy sources, such as solar and wind energy, or energy from plant biomass. One important energy source, which has received more focus during recent years, is the production of biofuels.

Today, the first generation of biofuels are being produced from foods, such as soybean, coconut, corn or palm (Chisti 2007). However, the use of agricultural products for energy production instead of food resources, results in increased competition for fertile agricultural areas. An alternative biomass source for biofuel production can be the growth of micro-algae. Micro-algae exhibit high growth rates, and may double their biomass several times a day under optimal growth conditions (Reynolds 2006). The lipid content of some micro-algal species, which is the most important aspect for biofuel production, is much higher (50% to 60% of dry weight) than that of terrestrial plants, such as rice or soybean (2% to 20% of dry weight; Sheehan et al. 1998; Spolaore et al. 2006). The installation of infrastructure for the large-scale production of biofuel from micro-algae is essential to establish cultivation methods that

maximise lipid production, but that are economically viable in terms of energy demand and resource supply. At the moment, the two most important methods for the mass cultivation of micro-algae are (1) monocultures growing in closed photobioreactors (PBRs), and (2) in open pond systems (Lehr and Posten 2009). The most noticeable difference, when comparing these two micro-algal production systems, is the degree of exposure of the respective systems to the environment. For example, PBRs are usually sophisticated systems that must be closed to maintain micro-algal monocultures, whereas open pond communities are completely exposed (Pulz 2001). This difference in production requirements means that PBRs require high maintenance, and hence are costly in comparison to simple open pond systems (Borowitzka 1999). While cultivating monocultures with single 'lipid-rich' species or strains in open ponds is possible, such monocultures would be unlikely to persist for long due to continual biological input from the environment (i.e. the addition of other micro-algae, protozoa, and zooplankton).

Ultimately, both options for micro-algal biomass production (closed systems versus ponds) are considered as artificial ecosystems (Pulz 2001). This is because the assembled ecosystems are characterised by controlled resource supply rates, regular harvests, and predefined food web structures (Smith et al. 2010). Artificial ecosystems follow the same important ecological principles as natural ones (Smith et al. 2010). Biomass accumulation in artificial pond ecosystems is determined by both the bottom-up control of production by resource supply rate and the top-down control of biomass distribution via trophic cascades, including predation and indirect effects of higher trophic levels on lower food web levels (Carpenter and Kitchell 1993). In fact, a recent review by Smith et al. (2010) showed how these processes may optimise the growth systems of algal biomass production for biofuels.

To operate such commercial growth systems, serious effort has been directed towards identifying the optimal algal strain(s) for the mass production for generating biofuels (Griffiths and Harrison 2009; Li et al. 2008; Miao and Wu 2004; Sheehan et al. 1998; Tran et al. 2009). However, uncertainty remains as to whether monocultures are actually more productive in terms of lipid accumulation than more diverse micro-algal communities.

Recent research is providing increasing evidence that the diversity of primary producer systems is often positively linked to biomass production (Downing and Leibold 2002; Power and Cardinale 2009; Ptacnik et al. 2008; Striebel et al. 2009a; Tilman et al. 2001). Generally, two mechanisms are considered responsible for observed positive diversity-productivity relationships. The first mechanism has been termed the 'selection effect'. Here, a highly productive species, which is more likely to be present in a highly diverse community, dominates the production of the observed system (Fox 2005). The second mechanism is termed the 'complementarity effect'. This mechanism is interpreted as deliberating niche differences and/or facilitative interactions among species (Fornara and Tilman 2008; Loreau and Hector 2001). Together, the complementarity and selection effect may be used to assess the total biodiversity effect (Fox 2005; Loreau and Hector 2001).

Recent research has provided evidence that such diversity–productivity relationships may also be observed in micro-algal communities. It has been found that highly diverse natural phytoplankton communities may be more productive than communities of low diversity (Ptacnik et al. 2008). The underlying reasons for such diversity–productivity relationships are most likely to be resource use complementarity and facilitation within micro-algal communities (Striebel et al. 2009a, b; Vanelslander et al. 2009). Experiments with phytoplankton communities have also provided clear evidence that the carbon to nutrient ratio of the biomass of micro-algal communities may increase with increasing diversity (Dickman et al. 2006; Striebel et al. 2009a), demonstrating improved resource use efficiency, and hence resource utilisation by highly diverse communities. Higher resource use efficiency of more diverse algal communities may therefore also increase the depletion rate of nutrients in growth systems. Previous studies have demonstrated that conditions causing nutrient stress, such as nitrogen starvation may induce higher lipid production in many micro-algal species (Dean et al. 2010; Li et al. 2008; Rodolfi et al. 2009). Indeed, a recent review about the ecology of biodiesel production by micro-algae hypothesize that naturally occurring multi species micro-algal communities in open pond bioreactors might store more solar energy as lipids compared to single species communities in closed photo-bioreactors (Smith et al. 2010).

Based on these arguments, we hypothesised that algal diversity may affect the lipid production of micro-algal communities. We applied the findings of recent studies, concerning the effects of diversity on productivity in phytoplankton, to investigate the following hypotheses: (1) is there a link between diversity and lipid production in micro-algal communities? (2) If such a link exists, is it based on the dominance of a single highly productive micro-algal strain, or on resource partitioning and facilitation among species? (3) Does diversity only positively influence biomass production, and thereby increase lipid yields of more diverse communities, or does diversity also influence the cell specific lipid content of micro-algae? These hypotheses were tested via a series of laboratory growth experiments under highly controlled environmental conditions. We assessed the growth and lipid production of micro-algae using 22 species from all major algal groups.

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Algae were grown in a large number of treatments with different levels of diversity. Additionally, the growth and lipid production of laboratory communities along a diversity gradient were compared to natural lake and pond phytoplankton communities of different diversities under the same environmental conditions. The results of this study are considered with respect to existing literature, providing suggestions on how biofuel production yields may be improved by using natural diverse communities as opposed to laboratory based monocultures.

Materials and methods

Laboratory experiments using artificial phytoplankton communities

We ran experiments using micro-algal communities with different levels of species richness that were created from a set of 22 micro-algal strains (SAG Culture Collection of Algae, Göttingen; UTCC, Toronto; Max-Planck-Institute (MPI) for Limnology, Plön) representing all major algal classes (Table 1). Each micro-algal strain was precultured in monoculture in Modified Woods Hole growth medium (WC-medium; Guillard and Lorenzen 1972) for several months before initiating the experiment.

We established a gradient for species diversity with four diversity levels, for all monocultures and polycultures, comprising two, three and four different algal species in each level.

Each diversity level had six different species compositions that were replicated three times, resulting in total of 120 communities. In all treatments, the initial total algal biovolume was set to be identical (2.4×10^6 fL mL^{-1}) at the beginning of the experiment. In other words, species contributed with equal initial biovolume to communities containing two, three or four species.

The experiment was run on translucent six-well (12 mL) cell culture plates (CELLSTAR, Greiner bio-one) for 7 days. Constant temperature (20°C) and light (90 μmol photons m^{-2} s^{-1}, PAR) in 12:12 h light:dark cycles were maintained during the experiment. The well plates were covered with PARAFILM® to avoid evaporation and contamination. The experiment was carried out in a modified fed-batch mode for seven days, with the daily addition of WC-medium that was 50% of the size of the current volume of each culture.

Experiments using natural phytoplankton communities

To compare the lipid production of laboratory communities to natural communities with shared evolutionary histories, we acquired phytoplankton samples from eight ponds and

Table 1 List of laboratory algal strains

Class	Algal strain (strain number; culture collection)
Chlorophytes	*Chlamydomonas reinhardtii* Dangeard (11-31; SAG, Göttingen)
	Monoraphidium minutum Nägeli (243-1; SAG, Göttingen)
	Scenedesmus obliquus Kützing (276-10; SAG, Göttingen)
	Selenastrum capricornutum Printz (37; UTCC, Toronto)
	Desmodesmus subspicatus Hegewald et Schmidt (86.81; SAG, Göttingen)
	Carteria sp. (8-5; SAG, Göttingen)
	Phacotus lentricularis Ehrenberg (61-1; SAG, Göttingen)
	Golenkinia brevispicula Hegewald et Schnepf (4.81; SAG, Göttingen)
	Staurastrum tetracerum (Meyen) Ralfs (7.94; SAG, Göttingen)
	Tetraedron minimum Hansgirg (44.81; SAG, Göttingen)
	Haematococcus pluvialis Flotow em. Wille (34-1 L; SAG, Göttingen)
	Choricystis minor (Skuja) Fott (17.98; SAG, Göttingen)
	Pediastrum simplex Meyen (21.85; SAG, Göttingen)
	Crucigenia tetrapedia W. et G.S. West (218-3; SAG, Göttingen)
	Botryococcus braunii Kützing (30.81; SAG, Göttingen)
Cyanobacteria	*Synechococcus* sp. (2156; SAG, Göttingen)
	Microcystis wesenbergii Kützing (own isolation, MPI for Limnology, Plön)
Cryptomonades	*Cryptomonas phasedus* Pascher (2013; SAG, Göttingen)
Crysophytes	*Poterioochromonas malhamensis* (Pringsheim) Péterfi (933-9; SAG, Göttingen)
Diatoms	*Fragilaria crotonensis* Kiton (28.96; SAG, Göttingen)
	Tabellaria fenestra Kützing (619; UTCC, Toronto)
	Navicula pelliculosa Hilse (1050-3; SAG, Göttingen)

lakes located in Southern Bavaria during June 2009. Three integrated water samples were taken from each pond and lake from the mixed surface layer. We filtered each sample through a 200-μm mesh to remove meso-zooplankton. Samples were stored in a common cooling box during transport. Subsequently, samples were stored in a climate chamber at 20°C for 1 day. The experiment was started with an initial total algal biovolume (2.4×10^6 fL mL^{-1}) from each lake/pond sample. Additionally, we determined micro-algal species richness and the total phosphorus (TP) content of each lake (Table 2). The collected plankton communities were cultured in the same way as described above for laboratory micro-algal communities. After inoculation of the collected phytoplankton samples in WC-medium, 50% of the culture volume was added daily for a total duration of 7 days.

Lipid analysis

The cell-specific lipid content of algae was estimated by staining neutral lipids with Nile Red, and measuring fluorescence in an imaging flow cytometer (FlowCAM® Fluid Imaging Technologies). For staining algae, 1 mg of fine-grained Nile Red (9-diethylamino-5 H-benzo[α]phenoxazine-5-one; HPLC grade, Sigma Aldrich) was dissolved in 4 mL acetone (HPLC grade; Lee et al. 1998). Subsequently, 20 μL Nile Red solution were added to 5 mL of algal solution and incubated for 30 min in a darkened container.

Fluorometric analyses were performed immediately, using FlowCAM® at an excitation peak wavelength of 532 nm and an emission peak wavelength of 645 nm (green laser). With the FlowCAM® it is possible to distinguish between the fluorescence of distinct species in communities. Thus, it was possible to quantify cell specific fluorescence, and thereby to estimate the cell-specific lipid content of each micro-algal species in diverse polycultures. A fluorescence–lipid content calibration curve was fitted along a linear gradient of cell density of *Saccharomyces cerevisiae* var. *carlsbergensis* suspended in WC-medium, with a cell-specific lipid content of 3.07×10^{-11} g and a cell specific fluorescence range of 2,680–2,800 units.

Table 2 Phytoplankton species richness and average total phosphorus content of water samples from the mixed layer of investigated ponds and lakes

Name of lake/pond	Species richness	Total phosphorus (μg L^{-1})
Lake Starnberger See	9	6
Lake Lustsee	10	3
Lake Stadler Weiher	16	22
Pond Biozentrum I	10	14
Pond Biozentrum II	7	10
Pond Max-Planck-Institute	19	302
Pond Martinsried	14	16
Pond Planegg	12	11

Microscopic counts and algal biovolume

Total algal biovolume and cell densities were estimated using a cell counter (CASY®-Cell-Counter, Schärfe-System) at the beginning and end of the experiment. At the end of the experiment, the species-specific composition of the different phytoplankton mixtures was determined by microscopic counting using the standard Utermöhl technique (Utermöhl 1958), in which the samples were fixed using Lugol's iodine. Species presence was recorded based on a minimum of 100 individuals by scanning a minimum of five perpendiculars transects or 20 randomly distributed distinct fields to keep the counting error at less than 10% (Lund et al. 1958). Biovolume of cells were determined by measuring two-dimensional live pictures using analySIS software (Pro 2.11.006, Soft-Imaging Software GmbH) and subsequent biovolume calculation by defining geometric shapes and mathematical equations according to Hillebrand et al. (1999) or own adjustments.

Calculations and statistical analyses

All analyses were performed 7 days after the initiation of the experiment. The net biodiversity effect (ΔY) was estimated by the tripartite partition method of Fox (2005):

$$\Delta Y = S \times \overline{M} \times \overline{\mathrm{RY}} + S \times \mathrm{Cov}\left(M_i, \frac{\mathrm{RY}_i}{\mathrm{RYT}} - \mathrm{RY}_{E,i}\right) + S \times \mathrm{Cov}\left(M_i, \mathrm{RY}_i - \frac{\mathrm{RY}_i}{\mathrm{RYT}}\right) \quad (1)$$

where S is the number of species in the community, $\overline{M}$ is the average monoculture biovolume or lipid content, $\overline{\mathrm{RY}} = \frac{1}{S} \times \sum_{i=1}^{S} \mathrm{RY}_i$ is the average relative yield of species in the mixture, with $\mathrm{RY}_i = \frac{O_i}{M_i}$ being the relative yield of species i (O_i is the biovolume of species i observed in the mixture, M_i its biovolume in monoculture), $\mathrm{RY}_{E,i} = \frac{1}{S}$ is the expected relative yield of species i in the community and $\mathrm{RYT} = \sum_{i=1}^{S} \mathrm{RY}_i$.

The diversity-related yield of algal biovolume, total algal lipid content and specific lipid content was calculated as the ratio of observed and expected values of these parameters at the end of the experiment. Expected values were based on a weighted average of the monoculture yields of the enclosed species in every community. For this purpose, we determined the proportion of each species within the experimental

communities by microscopy counting. The proportion of each species was multiplied by its species specific yield (biovolume) when growing in monoculture. These biomass values for each species in a mixture where added up to calculate the expected yield for each multi species treatment.

In addition, we calculated the contribution of complementarity and species identity (selection effect) to the observed yield according to Fox (2005).

Data are presented as the mean±1 standard error of the mean (mean±1 SE). Each mean is calculated from six different mixtures of the diversity levels two to four, and from 22 different strains of diversity level one. In addition, each community and all monocultures were replicated three times to estimate the degree of variation between identical algal community treatments.

Statistical analyses were completed using the mean values of the six different mixtures for each diversity level. Whenever possible, statistical analyses were performed using linear regression methods from the mean values of each diversity level. One-way analysis of variance (ANOVA) was used to compare differences in the mean values among treatment groups (communities and monocultures). If significant, we performed post hoc tests using all pair wise multiple comparison procedures (Holm Sidak method). Differences between the measured and expected mean values within a community were tested for statistical significance using an unpaired *t* test. To test whether the mean values of complementarity and selection effect where different from zero, we used one-sample *t* test.

Statistical analyses for treatments with natural phytoplankton communities were performed using the mean of the three replicates for each lake/pond.

Results

Total algal biovolume

On average, total algal biovolume ranged from 2.06×10^7 fL mL^{-1} in monocultures to 4.67×10^7 fL mL^{-1}in highest diverse communities. Species richness had a significant influence on the total measured algal biovolume (one-way ANOVA, $F_{3,39}=12.646$, $p<0.001$). A post hoc test (Holm Sidak) showed that the measured total algal biovolume in algal communities, with two ($p<0.001$), three ($p=0.002$) and four species ($p<0.001$) being significantly higher when compared to the total mean biovolume of the 22 monocultures. The expected values were generally lower than the measured values, producing a positive net biodiversity effect (Fig. 1).

The observed over yield in algal biovolume for all 18 algal communities appeared to be related to complementarity. Mean complementarity was positive and different from zero (one-sample *t* test, $p<0.001$, $t=8.835$, $df=17$). On average, the selection effect was negative, but not statistically different from zero (one-sample *t* test, $p=0.179$, $t=-1.402$, $df=17$).

Total algal lipid content

On average, total algal lipid content ranged from 3.26×10^6 pg mL^{-1} in monocultures to 1.08×10^7 pg mL^{-1} in communities with four species. The total lipid content had a tendency to increase with species richness ($y=2.36\times10^6x+2.49\times10^5$; $r^2=0.877$; $p=0.06$). Expected values were lower in communities with three and four species than that with measured values. The net biodiversity effect was therefore positive in communities with four species. The total algal lipid content in communities with four species was significantly higher than expected in communities with four species (unpaired *t* test, $p=0.021$, $t=2.439$, $df=34$; Fig. 2). The over yield in lipid production for all 18 communities were found to be related to complementarity. Mean complementarity was positive and different from zero (one-sample *t* test, $p=0.001$, $t=3.900$, $df=17$). On average, the selection effect was negative, but not statistically different from zero (one-sample *t* test, $p=0.171$, $t=-1.430$, $df=17$).

Specific algal lipid content

The specific algal lipid content is a quotient of the total algal lipid content with the total algal biovolume and was ranging from on average 1.58×10^{-1} pg fL^{-1} in monocultures to on average 4.70×10^{-1} pg fL^{-1} in communities with four species. Comparison of the expected values with the measured values of the specific algal lipid content,

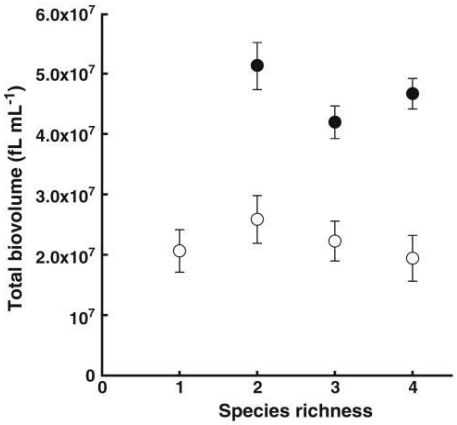

Fig. 1 Biomass, determined as total biovolume (fL mL^{-1}) as a function of species richness. Mean values of measured (*filled circles*) and expected (*open circles*) algal biomass are shown. *Error bars* represent ±1 SE

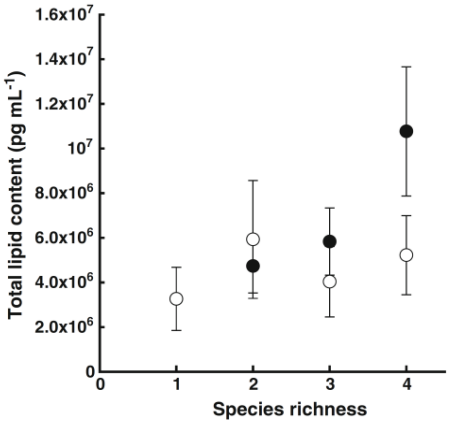

Fig. 2 Total lipid content of algal cultures (pg mL^{-1}) as a function of species richness. Mean values of measured (*filled circles*) and expected (*open circles*) lipid content are shown. *Error bars* represent ±1 SE

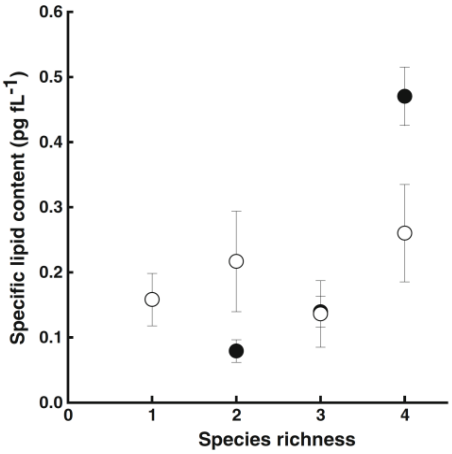

Fig. 3 Specific lipid content, determined as lipid content per biovolume unit (pg fL^{-1}) at the end of the experiment as a function of species richness. Mean values of measured specific lipid content (*filled circles*) and of expected mean values of specific lipid content (*open circles*). Error bars represent ±1 SE

showed that the measured specific algal lipid content for communities with four species were significantly higher than the calculated values from the respective monocultures (unpaired *t* test, $p=0.037$, $t=2.411$, $df=10$).

Species richness showed a significant influence on measured specific algal lipid content (one-way ANOVA, $F_{3,39}=8.361$, $p<0.001$). Post hoc tests (Holm Sidak) revealed a significantly higher specific algal lipid content in measured communities with four species compared to monocultures ($p<0.001$), as well as for communities with two ($p<0.001$) and three species ($p<0.001$; Fig. 3).

Total algal lipid content in natural phytoplankton communities

On average, the total algal lipid content ranged from 1.10×10^5 pg mL^{-1} (Lake Lustsee) to 5.17×10^6 pg mL^{-1} (Pond Max-Planck-Institute) in natural communities. There was a significant linear increase in total algal lipid content in correlation with species richness in natural communities ($y=3.42\times10^5x-2.72\times10^6$; $r^2=0.658$; $p=0.0146$). In addition, the average total algal lipid content of the eight natural communities (1.43×10^6 pg mL^{-1}) did not differ significantly from the average algal lipid content of the 22 laboratory monocultures (3.26×10^6 pg mL^{-1}; unpaired *t* test, $p=0.446$, $t=0.772$, $df=28$; Fig. 4).

Discussion

Similar to agricultural growth systems for biofuel production, there has been also extensive search effort to identify the most optimal cultured species or strains that would produce the highest yields of lipids in water based biomass production systems (Li et al. 2008; Miao and Wu 2004; Tran et al. 2009). This has resulted in a wide range of screening programmes that have investigated the growth potential and lipid production of a large variety of micro-algal species and strains (Li et al. 2008; Miao and Wu 2004; Tran et al. 2009). The optimisation of yield enhancement is mainly accomplished by technical engineering modifications of production systems, or by biological engineering of algal strains, such as the genetic modification of key processes of photosynthesis

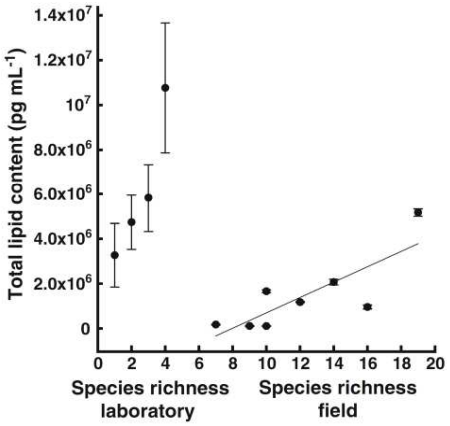

Fig. 4 Total lipid content of laboratory and natural algal community cultures (pg mL^{-1}) as a function of species richness. Mean values of total lipid content of laboratory algal communities (species richness one to four) and mean values of total lipid content of natural algal communities (species richness 7 to 19; $y=3.42\times10^5x-2.72\times10^6$; $r^2=0.658$; $p=0.0146$). *Error bars* represent ±1 SE of different algal communities with identical diversity levels (laboratory treatments) or within lake/pond sample replicates (natural phytoplankton treatments)

(e.g. Beckmann et al. 2009). However, in addition to technical and/or genetic engineering, the development of optimisations that are based on key ecological principles may provide cost effective yield enhancements, the potential of which has received limited attention in aquatic biomass production (Schenk et al. 2008; Smith et al. 2010).

The main objective of our study was to investigate how the lipid production of diverse multi species micro-algal communities compares to that of highly selected monoculture strains. Recent studies show a strong correlation between efficiency with respect to carbon accumulation in phytoplankton and algal diversity in lake ecosystems (Ptacnik et al. 2008). Experimental research has also provided additional evidence that the complementarity of light use along the spectral gradient of photosynthetic active radiation, may serve as an underlying mechanism of phytoplankton diversity–productivity relationships (Striebel et al. 2009a). However, at present, existing research on algal biomass production has not specifically considered the potential link between diversity and biomass/lipid production.

Diversity may influence lipid yield in micro-algae cultures through two possible mechanisms. First, an existing positive diversity–productivity relationship in micro-algae would result in increasing the biomass of algae with increasing diversity. Hence, the total amount of lipids would also increase, assuming that diversity does not influence the biomass specific lipid content of micro-algae. Second, diversity may also influence the specific lipid content of micro-algae, resulting in diversity–lipid production relationships. In this instance, higher resource use efficiency, due to increasing diversity in micro-algal communities, may lead to efficient nutrient limitation (e.g., phosphorus, nitrogen). The resulting accumulation of stress conditions, due to nutrient limitation, would increase the synthesis of lipids (Hu et al. 2008; Miao and Wu 2004). Our results show that both mechanisms seem to operate in high diverse communities. First, high diversity resulted in an increase of algal biomass and lipid production. Closer evaluation of treatments containing different mixtures of four algal species also indicated that specific algal lipid production was considerably higher than expected from the corresponding monocultures. These observations raised the question about whether the observed link between diversity and lipid production in micro-algal communities is based on the dominance of a single highly productive algal species, or on resource partitioning and facilitation among species. Our results clearly show that the dominance of a single highly productive species was not responsible for the observed positive effects of diversity on lipid production. Hence, the alternative major underlying mechanism behind the observed diversity–productivity relationships in our study may be accounted to complementarity. Complementarity effects in phytoplankton communities have been documented in light use, but may also arise in the use of mineral nutrients (Striebel et al. 2009b).

The comparison of eight natural lake phytoplankton communities against laboratory micro-algal communities in our study showed that the lipid production of selected laboratory monocultures was not significantly higher than that of field communities. This was an unexpected observation, as field phytoplankton communities were not habituated to the nutrient-rich growth medium and the environmental conditions of the laboratory, unlike the laboratory cultures. Additionally, as species richness increased in the natural communities, lipid production also increased, which supported the diversity–lipid production relationship found in laboratory communities, as well as the diversity–productivity relationships found in natural phytoplankton communities in Scandinavian and Bavarian lakes (Ptacnik et al. 2008; Striebel et al. 2009a).

Experiments investigating lipid production in micro-algal communities in which a high number of replicates are present must use fast, cost effective but reliable methods for quantification. On completion of our experiment, we determined the total lipid content of micro-algae communities by using the neutral lipid-staining fluorescent dye Nile Red. This is a simple and well-established method for the rapid determination of algal lipids (Eltgroth et al. 2005; Elsey et al. 2007; McGinnis et al. 1997). Lee et al. (1998) found a significant relationship between fluorescence and the lipid content of algae, and suggested that the method of staining algal cells with Nile Red to be equally viable as the gravimetric method, which is commonly used for lipid determination. Another advantage of Nile Red is that it selectively stains neutral lipids in algal cells, which are the most important lipid class for biofuels production (Cooksey et al. 1987). According to Sheehan et al. (1998), a major problem of Nile Red is that species vary in their uptake of this dye. However, in our analyses, we compared multi species mixtures with their respective monocultures; so this effect should not have biased our results on lipid production as a function of diversity. Additionally, our analyses of the micro-algae lipid content with Nile Red fluorescence and using a imaging flow cytometer (FlowCAM®) has the unique advantage to estimate the lipid content of each algal cell in diverse communities without requiring the (practically impossible) physical separation of algal cells.

Algae are organisms that have a high potential for dispersal. Water, wind, birds and insects serve as vectors for the fast distribution of algae and their spores (Cellamare et al. 2010; Chrisostomou et al. 2009; Messikommer 1943). Therefore, the development of systems that supply large amounts of light and nutrients to facilitate the optimal growth of micro-algae are potentially prone to invasion by other micro-algal species. Such invasions may increase the diversity of existing communities, beyond selected single

species or strain cultivations, potentially reducing high growth and production rates. However, our results demonstrate that in fact highly diverse communities produce higher yields of biomass in terms of both carbon and lipids when compared to monocultures. For example, in communities with mixtures of four different species, we measured almost double the lipid content that would be expected from monocultures. Furthermore, this enhancement of high yield efficiency of lipid production recorded in the diverse algal communities would be difficult to replicate by technical means for monocultures. In addition, increasing the supply of technical growth enhancements is often not viable, as they are difficult to obtain and are usually correlated with high energy requirements and thus high costs. In contrast, an increase of biological efficiency is self-financing. It is therefore important for biomass production systems to utilise all possible options to increase the efficiency of supplied resources by using the ecological parameters of the biota.

An additional positive aspect of high algal diversity may be that the invasion of communities becomes increasing difficulty as diversity increases (McGrady-Steed et al. 1997). Furthermore, highly diverse communities are also considered to produce more temporally stable ecosystem services, due to complementary effects among species that perform similar ecosystem functions (Ptacnik et al. 2008; Tilman 1996). This so called diversity–stability hypothesis states that species diversity mediates the functional stability of a community by compensating interactions to environmental fluctuations among the systems of concurrent performing species (McNaughton 1977). In accordance to the diversity–lipid production relationship, observed in our experiments, diversity parameters should be given more consideration with respect to biomass production, as a number of additional positive aspects of diversity on biomass production may be identified.

Outlook

Our results strongly support ideas that naturally occurring multi species micro-algae communities in open pond bioreactors may store more solar energy as lipids compared to single species communities in closed photo-bioreactors (Smith et al. 2010). Additionally, a recent review on micro-algal lipid production revealed that the lipid production of 20 micro-algal species was higher when grown in outdoor ponds than in the laboratory (Griffiths and Harrison 2009). The use of outdoor pond bioreactors, which will typically contain multi species communities, must therefore not be inferior for lipid production compared to technically optimised single species photo-bioreactors. Future studies should investigate which level of diversity would be ideal for the optimisation of lipid production, and how diversity interacts with environmental parameters of algal cultivation (i.e. nutrient and light supply, temperature). The diversity effect on algal lipid production might saturate at high diversity levels. A recent study shows that the positive slope of the relationship between diversity and resource use efficiency in phytoplankton communities decrease with increasing diversity (Ptacnik et al. 2008). Additionally, it has to be determined how long a gradient of diversity would persist in multi species micro-algal cultures. Several mechanisms are important for maintaining diversity including disturbance, spatial and temporal heterogeneity. The number of species at equilibrium will be determined by the number of limiting resources (Sommer et al. 1993). However, the persistence of diversity is also regulated by the frequency of disturbances and the temporal and spatial heterogeneity of the environment (Connell 1978; Gaedeke and Sommer 1986). This may differ considerably between photo-bioreactors and open pond systems. Fluctuations of environmental conditions (nutrients, light, turbulences, etc.) but also internal population dynamics can lead to higher diversity than expected from the number of limiting resources (Huisman and Weissing 1999; Hutchinson 1961). Species sorting, that results from the operation of local environmental filters can also influence diversity and result in very small species richness; this process is more important in very small, closed systems or in natural systems exposed to exceptionally high nutrient loading (Smith et al. 2005). Experiments at different spatial and temporal scales are definitely needed to investigate the importance of these processes for micro-algal growth systems. However, the results of the current study have already provided strong evidence that further research on the diversity of micro-algal communities is essential to improve micro-algal biomass production for the long-term sustainable generation of biofuels.

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CHAPTER 3

FUNCTIONAL GROUP RICHNESS: IMPLICATIONS OF BIODIVERSITY ON LIGHT USE AND LIPID YIELD IN MICROALGAE

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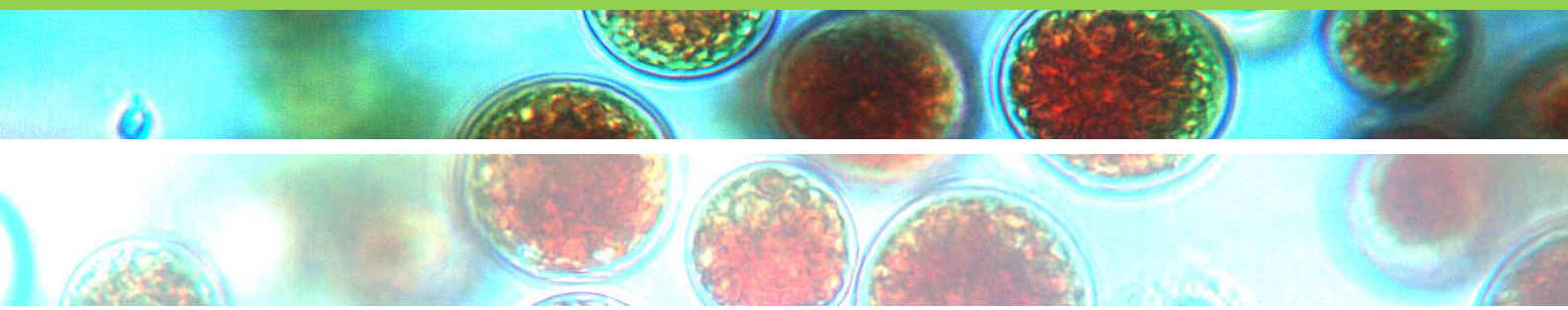

Abstract

Currently, very few studies address the relationship between diversity and biomass/lipid production in primary producer communities for biofuel production. However, basic research studies on the growth of microalgal communities provide evidence of a positive relationship between diversity and biomass production. Recent studies have also shown that the observed positive diversity-productivity-relationships are related to an increase in the efficiency of light use by diverse microalgal communities. Here, we hypothesize that there is a relationship between diversity, light use, and microalgal lipid production in phytoplankton communities. Microalgae from all major freshwater algal groups were cultivated in treatments that differed in their species richness and functional group richness. Polycultures with high functional group richness showed higher light use and algal lipid content with increasing species richness. There was a clear correlation between light use and lipid production in functionally diverse communities. Hence, a powerful and cost effective way to improve biofuel production might be accomplished by incorporating diversity related resource-use-dynamics into algal biomass production.

Keywords: Algal neutral lipids, Biofuel, Diversity, Functional groups, Light use, Nile Red, PAR absorbance, Phytoplankton, Resource-use-efficiency, Species richness

Introduction

Algae are important components of aquatic ecosystems, accounting for more than half the total global primary production, with their lipids serving as major dietary sources for primary consumers (Guschina and Harwood 2009). Lipids are vital for maintaining somatic and population growth, survival, and reproductive success (Brett and Müller-Navarra 1997). However, the lipids of microalgae have gained increasing attention in recent years, as they may provide a new source for biofuel production in times when fossil fuels are running out. Hence, at present, great effort is being invested towards finding the best microalgal strain or species that could provide the highest growth and lipid yields for biofuel production (Sheehan et al. 1998, Miao and Wu 2004, Li et al. 2008, Griffiths and Harrison 2009, Tran et al. 2009).

The most common growth systems for the mass production of microalgae are closed photobioreactors and open ponds. Closed systems are not easily contaminated, but are expensive to build and operate; alternatively, open ponds are relatively inexpensive but are usually open to the environment, and monocultures of selected strains do not persist for very long (Sheehan et al. 1998). Recent studies have shown that monocultures of selected microalgal strains may not be superior, in terms of lipid production, compared to diverse microalgal communities: In controlled growth experiments, diverse microalgal communities showed higher lipid production and biomass specific lipid content compared to corresponding monocultures (Stockenreiter et al. 2011). However, to incorporate these findings into potential cultivation systems for microalgal biomass production that generate lipids, a more mechanistic insight into the biodiversity - lipid productivity relationship is required.

In general, two mechanisms, operating in combination, are believed to be responsible for diversity - productivity relationships. The first mechanism is termed the “selection effect,” whereby a highly productive species, which is more likely to be present in a highly diverse community, dominates the production of the observed system (Fox 2005). The second mechanism is termed the “complementarity effect,” and is interpreted as regulating niche differences and/or facilitative interactions among species (Loreau and Hector 2001, Fornara and Tilman 2008). The importance of resource use complementarity and facilitation on diversity - productivity relationships in microalgal communities has already been documented in natural ecosystems (Ptacnik et al. 2008, Striebel et al. 2009a, b, Vanelslander et al. 2009). Although phytoplankton compete for both light and nutrient resources, recent studies indicate that complementarity in light use along the PAR spectrum (400–700 nm) is the key mechanism behind microalgal diversity - productivity relationships (Striebel et al. 2009b, Behl et al. 2011). Considering that the spatial and temporal availability of light can be quite heterogeneous, even in homogeneous open systems (Kirk 2011), a rich variety of photosynthetic pigments obtained from diverse microalgal communities might exploit the

existing light supply more efficiently, due to their utilizing different wavelengths in the photosynthetically active radiation (PAR) spectrum (Falkowski et al. 2004, Stomp et al. 2004).

Diversity is often referred to as species richness, with other components of diversity being neglected (Diaz and Cabido 2001). However, species richness is only one way of viewing the diversity of a community and its organization (Hillebrand and Matthiesen 2009). Diaz and Cabido (2001) showed that most of the recorded positive effects of species richness are the effect of functional richness and/or functional composition, in which the term “functional group” represents a set of species that is collected into groups based on their shared biochemical and/or ecological functions (Hood et al. 2006). While the influence of species richness on the functioning of communities within functional groups has been well studied (Cardinale et al. 2006, Duffy et al. 2007, Bruno and Cardinale 2008), our understanding about the influence of species richness across functional groups remains limited (Scrosati et al. 2011). Experiments with microalgae, in which the diversity of microalgal functional groups was increased, showed that increased light-use was complementarity with a strong increase in biomass-specific absorbance yield when algal functional diversity was higher (Behl et al. 2011).

Based on these arguments, and our results from a previous study suggesting that species richness is positively linked to algal lipid-production (Stockenreiter et al. 2011), we investigated the following hypotheses: (1) microalgal diversity leads to higher lipid production; (2) light is exploited more efficiently in highly diverse communities; (3) the effect of diversity on lipid production and light utilization is stronger within communities consisting of more than one functional group; and (4) the underlying mechanism for increased lipid production and light utilization in more diverse communities is related to complementarity. These hypotheses were tested by using laboratory growth-experiments under highly controlled environmental conditions.

We used 23 species from the most abundant microalgal freshwater groups to assess resource use efficiency, in terms of light usage along the PAR spectrum and the lipid production of microalgae communities. We created a gradient of functional group diversity using the four major freshwater microalgal classes (chlorophyta, bacillariophyta, cyanophyta, and chrysophyta). In addition to the ubiquitous chlorophyll-*a*, algal species contain several other pigments. These pigments are often taxon specific, thereby linking microalgal phylogenetic diversity to functional diversity in terms of light use. Communities assembled from species of several microalgal classes normally show larger differences in pigmentation compared to communities assembled from species of a single microalgal class (Schlüter et al. 2006; Behl et al. 2011).

We compared lipid production and light use from species from single functional groups to that of species from two, three, or four functional groups. Based on these results, we compare here the advantages and disadvantages of using single and multiple functional groups in algal biofuel production, and we suggest future studies to further our knowledge on this subject.

Methods

Experimental design

We performed experiments using laboratory microalgal communities that had different levels of species richness, both within and among functional groups (representing the major taxonomic groups of microalgae: chlorophytes, diatoms, cyanophyta, and chrysophyta). We used a set of 23 microalgal strains (SAG Culture Collection of Algae, Göttingen; UTCC, Toronto; Max Planck Institute (MPI) for Limnology, Plön) (Table 1). Each microalgal strain was pre-cultured in monoculture in modified Woods Hole growth medium (WC-medium; Guillard and Lorenzen 1972) for several months before initiating the experiment. In each functional group (except chrysophytes, due to lack of different species in our culture collection), we established a diversity gradient of all monocultures and polycultures of two and four species. In addition, we established a species richness gradient among the functional groups containing sets of four, six, and eight different species. To accomplish this, we combined algal species from two, three, and four functional groups. Each diversity level was replicated four times, with different random species compositions, which resulted in 83 experimental microalgal communities (Table 2).

At the beginning of the experiment, the initial total microalgal biovolume was set to be identical ($2.4 \cdot 10^6$ fL mL^{-1}) in all treatments. The experiment was arranged in a fed-batch-mode of cultivation (50 % of the existing culture volume was added daily with WC-growth medium) in 650 mL cell culture flasks (CELLSTAR, Greiner bio-one). Cultures were kept at ambient room temperature (26 ºC) for nine days. Constant light (90 µmol photons $m^{-2}s^{-1}$, PAR) in 12:12 h light:dark cycles was maintained throughout the experiment.

Measurements

At the beginning of the experiment, total microalgal biovolume and cell densities were estimated using a cell counter (CASY®- Cell-Counter, Schärfe-System). All other measurements were made at the end of the experiment.

The species specific composition of the different microalgal polycultures was determined by microscopic counting, using the standard Utermöhl technique (Utermöhl 1958), in which the samples were fixed using Lugol's iodine in an inverted microscope. Species presence was recorded based on a minimum of 100 individuals, by scanning a minimum of five perpendiculars transects or 20 randomly distributed distinct fields, to maintain the counting error at less than 10% (Lund et al. 1958). The biovolume of cells were determined by measuring 2-dimensional live pictures using analySIS software (Pro 2.11.006, Soft-Imaging Software GmbH), which was followed by a biovolume calculation, with geometric shapes and mathematical equations being defined according to Hillebrand et al. (1999) or our own adjustments.

In addition, microscopic counting was required to calculate the expected values for polycultures, to compare expected with measured values. Expected values were based on the weighted average of the monoculture yields of the enclosed species in every polyculture. Therefore, we determined the proportion of each species within the experimental polycultures by microscopy counting. The biovolume proportion of each species was multiplied by its species specific yield (biovolume) when growing in monoculture. These biovolume values for each species in a given polyculture were summed to calculate the expected yield for each multi species treatment.

The spectral *in vivo* absorption was measured from 350 to 800 nm at 1 nm intervals with a dual-beam spectrophotometer (Perkin Elmer Lambda 650, Massachusetts, USA). The samples were measured in a 1 cm quartz cuvette, which was placed in front of a 150 mm integrating sphere. The PAR absorption coefficient (a (λ) m^{-1}) was calculated from the optical density of a sample ($OD(\lambda)$), and the length of the cuvette (L = 0.01 m):

$$a(\lambda) = 2.303 \cdot OD(\lambda) / L \qquad \text{(I)}$$

Spectral absorption was averaged for the PAR range (400–700 nm), and normalized to a 10 mMol phytoplankton carbon biomass. This parameter was termed PAR_{abs}.

To estimate particular organic carbon (POC) content, samples from each treatment were filtered onto pre-combusted and acid-washed GF/C filters (Whatman ®, UK). Measurements were performed with an Elemental Analyzer (CE Instruments, Milan, Italy), using standard methods (Wetzel and Likens 1991).

Microalgal lipid content was estimated by staining neutral lipids with Nile Red. To stain algae, 1 mg of fine-grained Nile Red (9-diethylamino-5 H-benzo[α]phenoxazine- 5-one; HPLC grade, Sigma Aldrich) was dissolved in 4 mL acetone (HPLC grade; Lee et al. 1998). Subsequently, 12 µL Nile Red solution was added to 3 mL microalgal solution (pre-frozen at -20 ℃), and incubated for 30 min in a darkened cont ainer. Fluorescence was measured with spectrofluorometer (Cary Varian Eclipse, Agilent Technologies, Inc., Colorado, USA) at an excitation peak wavelength of 530 nm and an emission peak wavelength of 580 nm, following pre-scans of excitation and emission of neutral lipid standards (Chen et al. 2009). Fluorescence readings were corrected for background fluorescence of Nile Red in culture media and for microalgal fluorescence.
To meet statistical assumptions (Sokal and Rohlf 1981), the fluorescence values were ln-transformed and were termed algal neutral lipids in the remainder of the manuscript.

A major problem of using Nile Red staining to estimate algal lipid content is the variability in the penetration of dye through the cell walls of different microalgae. However, in our analyses we compared multi-species polycultures with their respective monocultures; hence, this effect should not have biased our results on lipid production as a function of diversity.

The diversity related yield (Δ*Y*) of total microalgal lipid content and spectral absorbance were calculated as the ratio of observed and expected values of these parameters at the end of the experiment. A complementarity as well as a selection effect was estimated by the tripartite partition method of Fox (2005):

$$\Delta Y = S * \overline{M} * \overline{RY} + S * Cov\left(M_i, \frac{RY_i}{RYT} - RY_{E,i} \right) + S * Cov\left(M_i, RY_i - \frac{RY_i}{RYT} \right) \quad \text{(II)}$$

where S is the number of species in the polyculture, $\overline{M}$ is the average monoculture biovolume or lipid content, $\overline{RY} = \frac{1}{S} * \sum_{i=1}^{S} RY_i$ is the average relative yield of species in the polyculture, with $RY_i = \frac{O_i}{M_i}$ being the relative yield of species *i* (O_i is the biovolume of species *i* observed in the polyculture, M_i is the biovolume of species *i* in monoculture), $RY_{E,i} = \frac{1}{S}$ is the expected relative yield of species *i* in the polyculture and $RYT = \sum_{i=1}^{S} RY_i$.

Statistical analyses

Statistical analyses were completed using the values of each monoculture and polyculture for each diversity level. Whenever possible, statistical analyses were performed using linear regression methods from the values of each diversity level. Differences between measured and expected values within a polyculture were tested by comparing the slopes of the regression lines for statistical significance using the Student's t-test.

Results

Microalgal neutral lipid content

A) Effects of species richness on Microalgal neutral lipid production within single functional groups

Within the treatments comprising just species from single functional groups, there was no significant relationship between increasing species richness and microalgal neutral lipids. In polycultures consisting of only chlorophyta and only cyanophyta, the measured microalgal neutral lipid content tended to increase with species richness; however, linear regressions were not significant (Fig. 1a, linear regression: $r^2 = 0.08$; $P = 0.3$; Fig. 1b, linear regression: $r^2 = 0.15$; $P = 0.2$). In addition, the slopes of the linear regression lines for species richness versus expected and measured values of microalgal neutral lipids did not differ significantly (Fig. 1a, b; Table 3). Microalgal neutral lipid content tended to decrease with species richness in communities consisting of only diatoms; however, the linear regression was not statistically significant (Fig. 1c, linear regression: $r^2 = 0.04$; $P = 0.5$). Comparisons of the slopes for diatom species richness versus expected and measured values of microalgal neutral lipid content also showed no statistical difference (Fig. 1c; Table 3).

B) Effects of species richness on microalgal neutral lipid production in polycultures consisting of different functional groups

Among polycultures consisting of more than one functional group, the increase of microalgal neutral lipids with species richness was statistically significant in communities consisting of two (Fig. 1d, linear regression: $r^2 = 0.20$; $P = 0.03$), three (Fig. 1e, linear regression $r^2 = 0.33$; $P = 0.0009$), and four (Fig. 1f, linear regression: $r^2 = 0.25$; $P = 0.0049$) functional groups. Comparison of the slopes for species richness versus measured and expected values of microalgal lipid content in polycultures with two different functional groups showed no significant difference (Fig. 1d; Table 3). Polycultures consisting of three different functional groups showed significantly higher measured microalgal neutral lipid content than expected

from the corresponding monocultures. Comparison of the slopes for species richness versus measured and expected values of microalgal lipid content differed significantly (Fig. 1e; Table 3). Comparison of the slopes for species richness versus measured and expected values of microalgal neutral lipid content in polycultures consisting of four different functional groups showed no statistical significance (Fig 1f; Table 3).

Carbon-specific average PAR absorbance coefficient in vivo (PAR_{abs})

A) Effects of species richness on PAR abs within single functional groups

We found a statistically significant linear increase in PAR_{abs} with increasing species richness within one single functional group, which was the polycultures of chlorophyta (Fig. 2a, linear regression: $r^2 = 0.23$; $P = 0.05$). There was no significant difference between the measured and expected slopes of the linear regression values in polycultures of chlorophyta (Fig. 2a; Table 3). PAR_{abs} of cyanophyta tended to increase with species richness (Fig. 2b, linear regression: $r^2 = 0.16$; $P = 0.14$). There was no significant difference in the measured and expected slopes of the linear regression values in polycultures of cyanophyta and diatoms (Figure 2b, c; Table 3).

B) Effects of species richness in polycultures consisting of different functional groups on PAR_{abs}

Among polycultures consisting of more than one functional microalgal group, we found a statistically significant linear increase in PAR_{abs} with increasing species richness in polycultures consisting of two (Fig. 2d, linear regression: $r^2 = 0.60$; $P < 0.0001$), three (Fig. 1e, linear regression: $r^2 = 0.45$; $P < 0.0001$), and four (Fig. 2f, linear regression: $r^2 = 0.21$; $P = 0.007$) different functional groups. Comparison of the slopes for species richness versus measured and expected PAR_{abs} values in polycultures with two, three, and four different functional groups all showed a significantly higher slope of measured PAR_{abs} values (Fig. 2d; Table 3).

Correlation of PAR_{abs} and microalgal neutral lipid content

A) Treatments with species from a single functional group

In polycultures comprising just chlorophyta, there was no statistical significant linear relationship between PAR_{abs} and microalgal neutral lipid content (Fig. 3a, linear regression: $r^2 = 0.08$; $P = 0.3$). A similar result was obtained in polycultures comprising only cyanophyta

(Fig. 3b, linear regression: r^2 = 0.1; P = 0.29). A negative relationship between PAR_{abs} and microalgal neutral lipid content was evident in polycultures consisting of diatoms; however, it was not statistically significant (Fig. 3c, linear regression: r^2 = 0.05; P = 0.42).

B) Treatments with species from different functional groups

In polycultures consisting of species from two different functional groups, there was no significant positive relationship between PAR_{abs} and microalgal neutral lipids (Fig. 3d, linear regression r^2 = 0.08; P = 0.17). However, in polycultures consisting of three different functional groups, there was a significant positive relationship between PAR_{abs} and microalgal neutral lipids (Fig. 3e, linear regression r^2 = 0.18; P = 0.018). There was also a significant positive relationship between PAR_{abs} and microalgal neutral lipids in polycultures consisting of species from four different functional groups (Fig. 3f, linear regression r^2 = 0.13; P = 0.047).

Complementarity and selection effect

On average, the observed over-yields in PAR_{abs} were related to complementarity in all polycultures. Mean complementarity was positive (0.5219 ± 0.025 SE) and different from zero (one-sample t-test, P < 0.001, t = 20.95, df = 49). On average, the selection effect (0.012 ± 8.48 x 10^{-3} SE) was not statistically different from zero (one-sample t-test, P = 0.17, t = 1.39, df = 49). On average, the observed over-yields in microalgal neutral lipid content were also related to complementarity in all polycultures. Mean complementarity was positive (1.69 ± 0.08 SE) and different from zero (one-sample t-test, P < 0.001, t = 20.80, df = 49). On average, the selection effect (-0.07 ± 0.05 SE) was not statistically different from zero (one-sample t-test, P = 0.15, t = -1.47, df = 49).

Discussion

Identifying the most efficient method of converting light energy into chemical energy and biomass for biofuels is one of the major problems in the development of microalgal biomass production systems. In a recent review, Smith et al. (2010) suggested that naturally occurring multi species microalgal communities in open pond bioreactors might store more solar energy as lipids compared to single species communities in closed photo-bioreactors. Furthermore, recent studies are providing increasing evidence that the diversity of primary producer systems is often positively linked to biomass production. However, it is argued that this evidence is based on the more efficient utilization of the photosynthetically active radiation light spectrum by niche differentiation (Striebel et al. 2009b, Behl et al. 2011). Based on these findings, and recent results showing that microalgal diversity is positively linked with neutral lipid production (Stockenreiter et al. 2011), we attempted to identify whether a higher light use of diverse communities is indeed linked to lipid production.

The diversity of primary producers in biofuel production units might be important for two reasons. First, it might be of interest to include diversity into biofuel production concepts. This may prove a useful strategy to increase the efficiency of biofuel production by ecological means. However, for such a concept to work, it would be necessary to analyze whether over-yields in lipid production are either transgressive or non-transgressive in diverse mixtures of selected microalgae. Transgressive over-yielding means that diverse communities are more productive than any monoculture of the component species. Non-transgressive over-yielding means that the yield of mixtures is higher than the average yield of monocultures, but with the yield of one of the component monocultures being higher than that of the mixture (e.g. Hector et al. 2002). Hence, in the case of non-transgressive over-yielding, it might be more efficient to use the highly productive monoculture rather than the diverse mixture.

Second, in many microalgal mass cultivation systems, e.g., open ponds, diversity is unavoidable, due to constant species input from the environment. For such production systems, it might be important to identify the existing potential of well-suited microalgal group combinations for biomass or lipid production at a certain location. Then, it would be possible to support the growth of desired microalgal groups by favorably manipulating the conditions required for optimal environmental growth (i.e., through resource supply, resource ratios, etc.), as proposed in a recent review by Smith et al. (2010). However, regardless of the system used, it is necessary to identify the mechanisms behind diversity – productivity relationships.

The effect of functional group richness on microalgal lipid production

There is growing evidence that functional group diversity is most probably the component of biodiversity that is highly important for ecosystem functioning (Diaz and Cabido 2001, Hooper et al. 2005, Wright et al. 2006). Indeed, our results show that there are differences between polycultures consisting of only one or several functional groups, with respect to microalgal neutral lipid content. In our study, there was no increase in microalgal neutral lipid content with species richness in polycultures consisting of members originating from a single functional group. In fact, in the case of diatoms, increasing species richness actually showed a slight decrease in measured microalgal lipid content. However, a clear increase in lipid production was observed in all communities consisting of different functional groups. Not all polycultures consisting of different functional groups showed transgressive over-yielding; however, measured values of microalgal neutral lipid content were mostly higher than the expected values, especially in polycultures consisting of three or four functional groups.

Light use in polycultures and the role of functional groups

In general, the ecological diversity-productivity theory proposes that multi species ecosystems should produce higher (microalgal) biomass yields, and thus resources should be used more efficiently (Ptacnik et al. 2008, Striebel et al. 2009a, b). While there was no significant increase in microalgal neutral lipid content in polycultures consisting of species from one single functional group, in communities consisting only of chlorophyta, there was, on average, measurably higher PAR_{abs} with increasing species richness. However, in all polycultures that consisted of species from two, three, or four functional groups, light usage significantly increased with species richness. These results support previous laboratory experiments, showing that functional diversity is more important for carbon fixation in microalgal polycultures than species richness (Behl et al. 2011). Even if not all polycultures consisting of different functional groups were transgressive over-yielding, the measured values of light usage were, in most cases, higher than expected.

Diversity-light-lipid relationship

One key question is whether higher light usage with increasing functional group diversity leads to higher lipid production. For polycultures consisting of one functional group, we could not find a significant relationship between light usage and microalgal neutral lipid content. Significant microalgal neutral lipid content dependency on light usage was observed in polycultures consisting of three and four functional groups. This finding supports our results on microalgal neutral lipid content and light usage as a function of species richness.

One central question is what level of microalgal diversity is required to optimize biomass production. Our results show that higher species richness might not necessarily lead to higher lipid yields. We found that species richness *per se* was not a good predictor for the strength of diversity effects. In fact, there is growing evidence that diversity, in terms of functional group diversity, might often exceed the impact of species richness (Cardinale et al. 2011). The integration of diversity into concepts of biomass production should be based on the functional niche properties of the individual species within the production communities. Our results support the hypothesis (the ecological diversity-productivity theory, Smith et al. 2010) that communities of greater diversity produce higher microalgal yields (Ptacnik et al. 2008). Our results indicate that diversity needs to be characterized with respect to both species richness and functional group richness, when examining diversity as a factor that potentially increases biomass production in cultivation systems. Furthermore, possible complementarities in resource use between species should to be identified. Screening programs for the selection of suitable microalgal species for lipid production could provide such information, by including analyses of resource uptake characteristics.

Complementarity and selection effects

We hypothesized that the underlying mechanism for diversity - light use and the diversity - microalgal neutral lipid relationship is related to complementarity. In general, both effects, selection and complementarity, act together (Loreau and Hector 2001), but in most cases just one is emphasized. In our study, the mean over-yield of the 50 polycultures in PAR_{abs} and microalgal neutral lipids was found to be related to complementarity. However, the importance of complementarity did not increase with increasing functional group richness, and instead remain relatively stable for one, two, three, or four different functional groups. This result contrasts with the findings of Behl et al. (2011); however, in addition to other differences, the experimental duration of our study was just half that of Behl et al. (2011), which might have affected the results. For example, Cardinale et al. (2007) showed that complementarity effects tend to become stronger with time. Hence, the observed importance of the complementarity effect may have been caused by the short duration of our experiments, with longer durations possibly leading to a shift towards increasing complementarity with increasing functional groups.

The importance of the diversity - productivity relationship of primary producers for biofuel production has been invoked or observed in only a few studies (Adler et al. 2009, Smith et al. 2010, Stockenreiter et al. 2011). However, diversity could also provide other advantages for biomass production, which could lead to further increases in production efficiencies. For example, such advantages might include the production of more stable biomass (Tilman

1996) or reduced vulnerability to invasion from undesired species (Elton 1958). Microalgal diversity is usually highly dynamic. Hence, future studies targeted towards integrating species diversity into biofuel production, should examine how diversity and desired species combinations might be maintained for longer periods in production systems to ensure a constant quantity (lipid content) and quality (fatty acid composition) of microalgae. In addition, studies should be developed to learn how to produce storage lipids with multi species communities. In conclusion, our study confirmed theoretical predictions that microalgal polycultures comprising different functional groups show a clear increase in lipid production, which has potential application towards improving the productivity of current biofuel production.

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Table 1. List of laboratory microalgal strains

Class	*Microalgal strain (strain number; culture collection)*
Chlorophyta	***Scenedesmus obliquus* Kützing (276-10; SAG, Göttingen)**
	***Selenastrum capricornutum* Printz (37; UTCC, Toronto)**
	***Desmodesmus subspicatus* Hegewald et Schmidt (86.81; SAG, Göttingen)**
	***Golenkinia brevispicula* Hegewald et Schnepf (4.81; SAG, Göttingen)**
	***Staurastrum tetracerum* (Meyen) Ralfs (7.94; SAG, Göttingen)**
	***Tetraedron minimum* Hansgirg (44.81; SAG, Göttingen)**
	***Haematococcus pluvialis* Flotow em. Wille (34-1l; SAG, Göttingen)**
	***Pediastrum simplex* Meyen (21.85; SAG, Göttingen)**
	***Crucigenia tetrapedia* W. et G.S. West (218-3; SAG, Göttingen)**
Cyanobacteria	***Synechococcus sp.* (2156; SAG, Göttingen)**
	***Microcystis wesenbergii* Kützing (own isolation, MPI for Limnology, Plön)**
	***Anabaena cylindrica* Lemmermann (1403-2; SAG, Göttingen)**
	***Chroococcus minutus* Kützing (4179; SAG, Göttingen)**
	***Pseudoanabaena galeata* Böcher (13.83; SAG, Göttingen)**
	***Merismopedia glauca* Ehrenberg (48.79; SAG, Göttingen)**
	***Planktothrix rubescence* Anagnostidis et Komárek (5.89; SAG, Göttingen)**
Crysophyta	***Synura petersenii* Korshikov (24.86; SAG, Göttingen)**
Bacillariophyta	***Fragilaria crotonensis* Kiton (28.96; SAG, Göttingen)**
	***Tabellaria fenestra* Kützing (619; UTCC, Toronto)**
	***Navicula pelliculosa* Hilse (1050-3; SAG, Göttingen)**
	***Cyclotella meneghiniana* Kützing (2136; SAG, Göttingen)**
	***Asterionella formosa* Hassall (8.95; SAG, Göttingen)**
	***Nitzschia palea* Kützing (1052-3a; SAG, Göttingen)**

Table 2. Experimental design of microalgal polycultures consisting of one to eight species from one or several functional groups, to produce species richness and functional group diversity gradients.

No. of polycultures	*No.of algal species in polyculture*	*No. of functional groups in polyculture*
23	1	1
12	2	1
12	4	1
4	4	2
4	4	3
4	4	4
4	6	2
4	6	3
4	6	4
4	8	2
4	8	3
4	8	4

Table 3. Statistical analyses (t-test) comparing the linear regression slopes for species richness versus measured and expected values of PAR absorbance (PAR_{abs}), and species richness versus microalgal neutral lipids, within polycultures comprising one to four different functional groups.

No. functional groups	PAR_{abs}	Microalgal neutral lipids
1 (chlorophyta)	*P*=0.42;*t*=0.81;*df*=32	*P*=0.09;*t*=1.70;*df*=30
1 (cyanophyta)	*P*=0.33;*t*=0.99;*df*=28	*P*=0.16;*t*=1.42;*df*=24
1 (diatoms)	*P*=0.43;*t*=-0.78;*df*=26	*P*=0.82;*t*=-0.22;*df*=26
2	***P*=0.15**;*t*=-1.44;*df*=66	*P*=0.17;*t*=1.39;*df*=48
3	***P*=0.01**;*t*=-2.59;*df*=64	***P*=0.01**;*t*=2.66;*df*=58
4	***P*<0.001**;*t*=-3.56;*df*=52	*P*=0.10;*t*=1.66;*df*=56

Figure legends

Figure 1. Microalgal neutral lipids as a function of species richness for expected values (open circles) and measured values (filled circles). Lines for measured values (solid line) and expected values (dashed line) represent linear regressions.

Within a single functional group: (a) chlorophyta; linear regressions: measured: y = 0.1x – 0.25; r^2 = 0.08; *P* = 0.3; expected: y = -0.1x – 0.35; r^2 = 0.11; *P* = 0.2; (b) cyanophyta; linear regressions: measured: y = 0.32x – (-0.14); r^2 = 0.15; *P* **=** 0.2; expected: y = -0.07x – 0.13; r^2 = 0.004; *P* **=** 0.85; (c) bacillariophyta; linear regressions: measured: y = -0.17x – 1.78; r^2 = 0.04; *P* **=** 0.5; expected: y = -0.1x – 1.53; r^2 = 0.02; *P* = 0.65.

Within different functional groups: (d) two functional groups; linear regressions: measured: y = 0.1x – (-0.05); r^2 = 0.19; *P* **= 0.003**; expected: y = 0.01x – 0.15; r^2 = 0.003; *P* **=** 0.78;, (e) three different functional groups; linear regressions: measured: y =0.14x – (-0.04); r^2 = 0.32; *P* **= 0.0009;** expected: y = -0.003x – 0.11; r^2 = 0.0002; *P* **=** 0.95; (f) four different functional groups; linear regressions: measured: y =0.13x – (-0.06); r^2 = 0.25; *P* **= 0.005;** expected: y = 0.013x – 0.11; r^2 = 0.003; *P* = 0.79.

Significant linear regressions are marked by asterisks.

Figure 2. Carbon specific average PAR absorbance (PAR_{abs}) as a function of species richness for expected values (open circles) and measured values (filled circles). Lines for measured values (solid line) and expected values (dashed line) represent linear regression.

Within a single functional group: (a) chlorophyta; linear regressions: measured: y = 0.04x – 0.41; r^2 = 0.23; *P* **= 0.05**; expected: y = 0.02x – 0.41; r^2 = 0.10; *P* = 0.2; (b) cyanophyta; linear regressions: measured: y = 0.04x – 0.44; r^2 = 0.16; *P* = 0.14; expected: y = 0.001x – 0.46; r^2 = 0.0002; *P* **=** 0.96; (c) bacillariophyta; linear regressions: measured: y = -0.007x – 0.46; r^2 = 0.02; *P* **=** 0.6; expected: y = 0.006x – 0.45; r^2 = 0.03; *P* = 0.57.

Within different functional groups: (d) two functional groups; linear regressions: measured: y = 0.03x – 0.41; r^2 = 0.60; *P* **< 0.0001**; expected: y = 0.005x – 0.43; r^2 = 0.04; *P* **=** 0.33; (e) three different functional groups; linear regressions: measured: y = 0.04x – 0.37; r^2 = 0.45; *P* **< 0.0001;** expected: y = 0.01x – 0.4; r^2 = 0.07; *P* **=** 0.15; (f) four different functional groups; linear regressions: measured: y = 0.02x – 0.39; r^2 = 0.20; *P* **= 0.007;** expected: y = 0.007x – 0.41; r^2 = 0.028; *P* **=** 0.35.

Significant linear regressions are marked by asterisks.

Figure 3. Microalgal neutral lipids as a function of carbon specific average PAR absorbance (PAR_{abs}) in communities comprising a single functional group (a) chlorophyta; linear regression, y = 1.15x – (- 0.11); r^2 = 0.08; *P* = 0.30, (b) cyanophyta; linear regression, y = 3.9x – (- 1.66); r^2 = 0.1; *P* = 0.29, (c) bacillariophyta; linear regression, y = -4.54x – 3.42 r^2 = 0.05; *P* = 0.42; and within communities comprising different functional groups (d) two different functional groups: linear regression, y = 1.49x – (- 0.49; r^2 = 0.08; *P* = 0.17; (e) three different functional groups: linear regression, y = 2.43x – (- 0.86; r^2 = 0.18; *P* = **0.018**; (f) four different functional groups: linear regression, y = 1.74x – (-0.6); r^2 = 0.13; *P* = **0.047**.

Significant linear regressions are marked by asterisks.

Figure 1

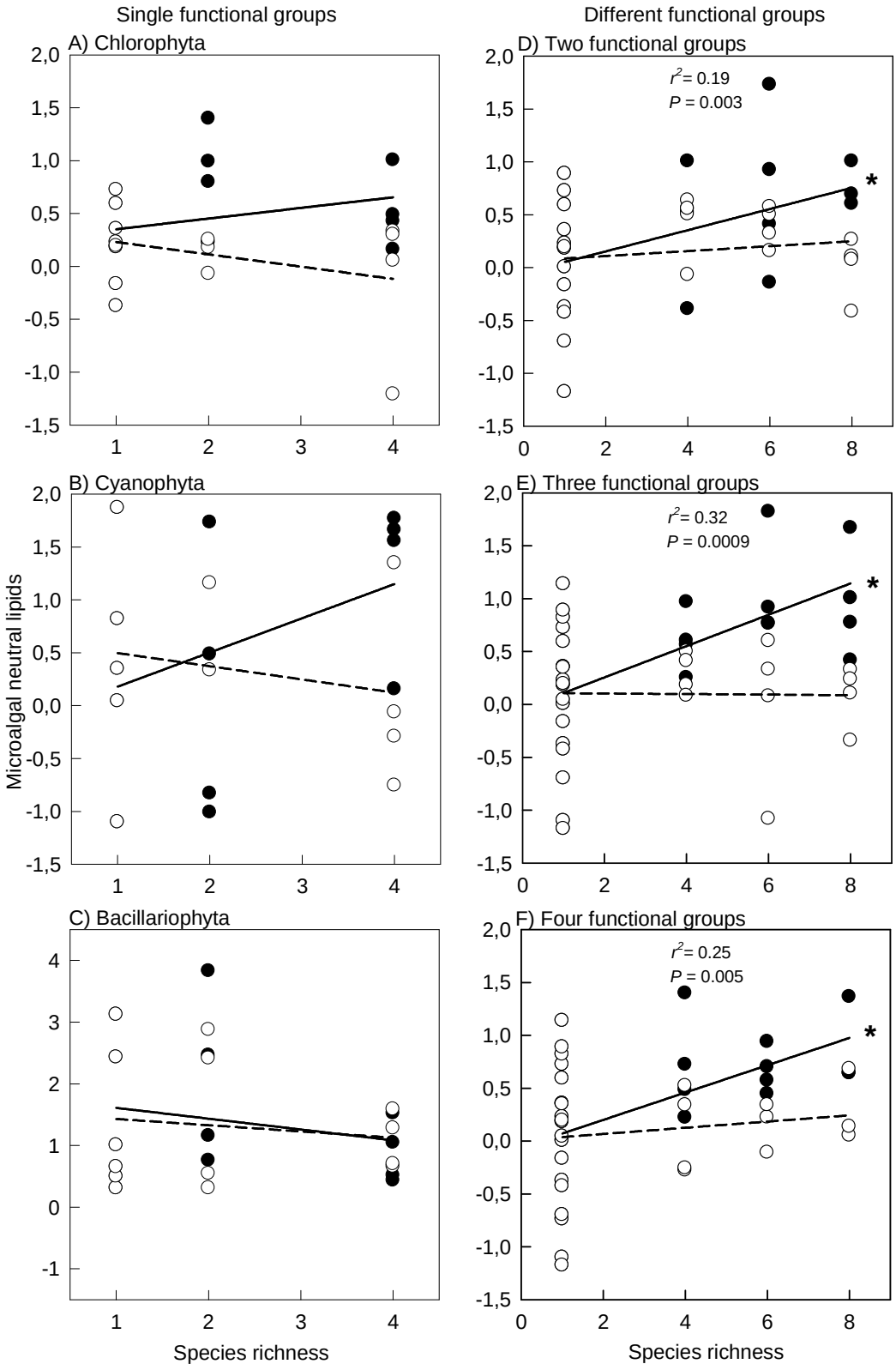

Single functional groups
Different functional groups
A) Chlorophyta
B) Cyanophyta
C) Bacillariophyta
D) Two functional groups
E) Three functional groups
F) Four functional groups
r2= 0.19
P = 0.003
r2= 0.32
P = 0.0009
r2= 0.25
P = 0.005
*
Microalgal neutral lipids
Species richness
Species richness

Figure 2

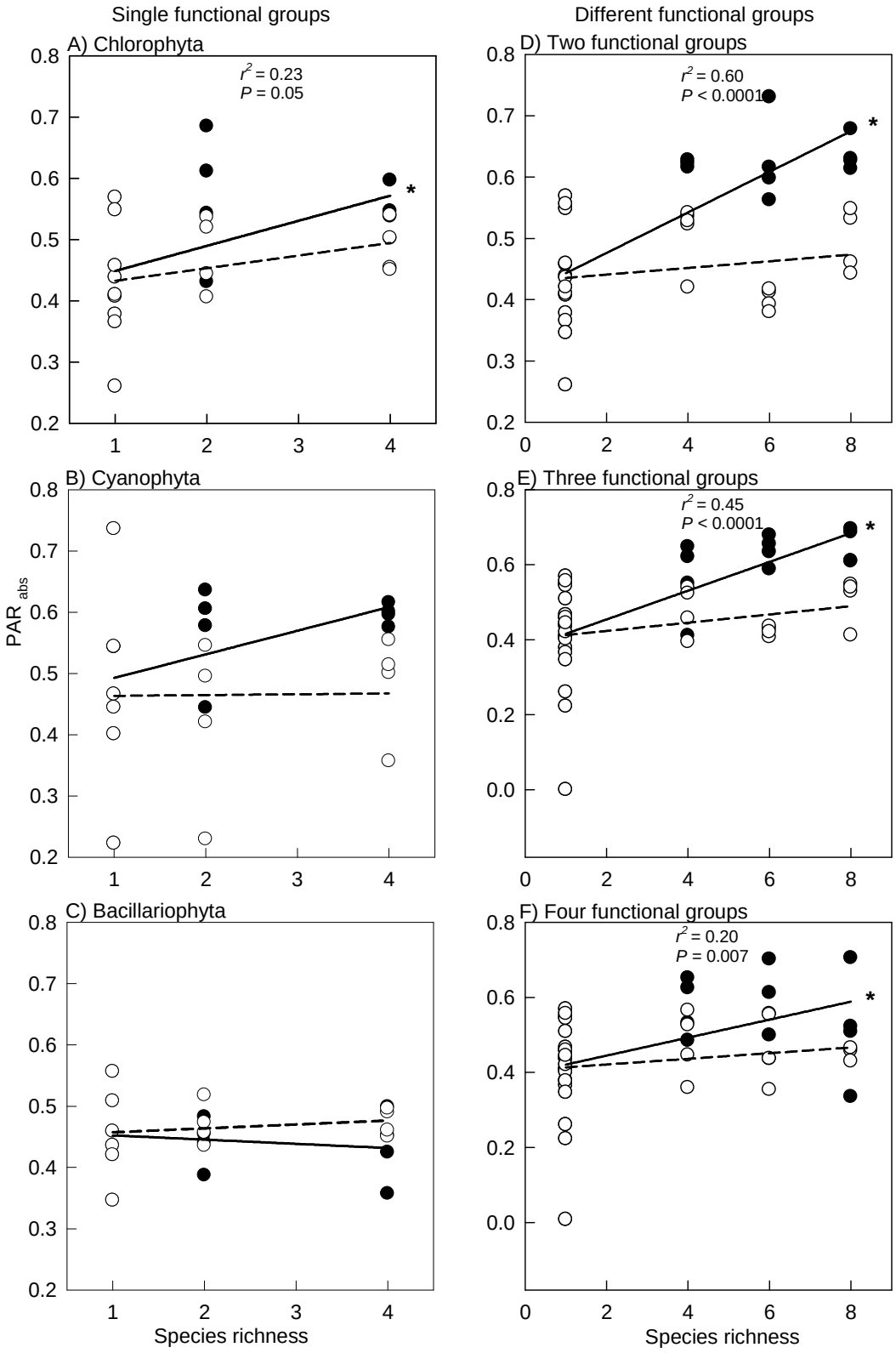
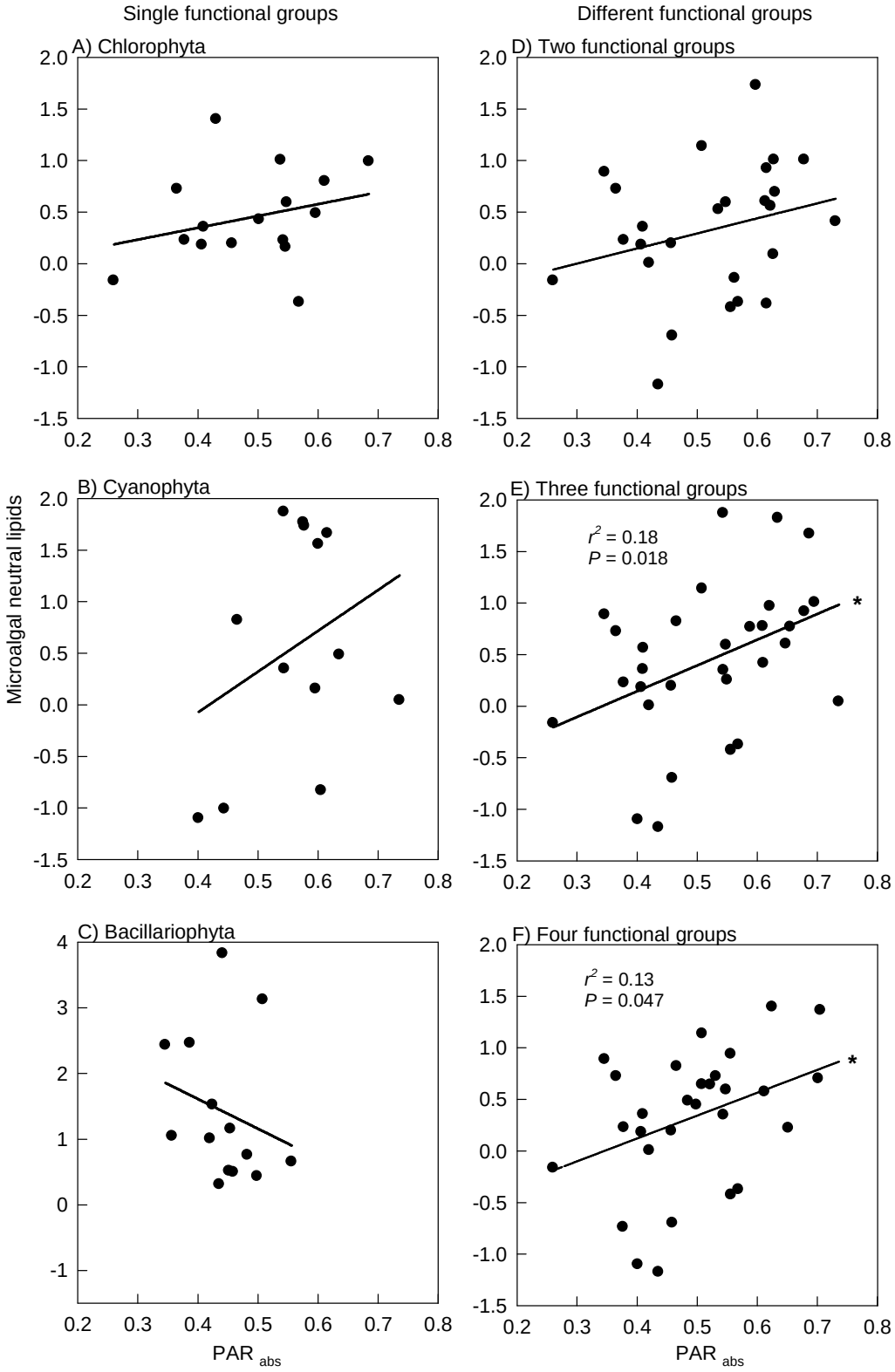

Single functional groups
Different functional groups
A) Chlorophyta
r^2 = 0.23
P = 0.05
D) Two functional groups
r^2 = 0.60
P < 0.0001
B) Cyanophyta
E) Three functional groups
r^2 = 0.45
P < 0.0001
C) Bacillariophyta
F) Four functional groups
r^2 = 0.20
P = 0.007
PAR abs
Species richness
Species richness

Figure 3

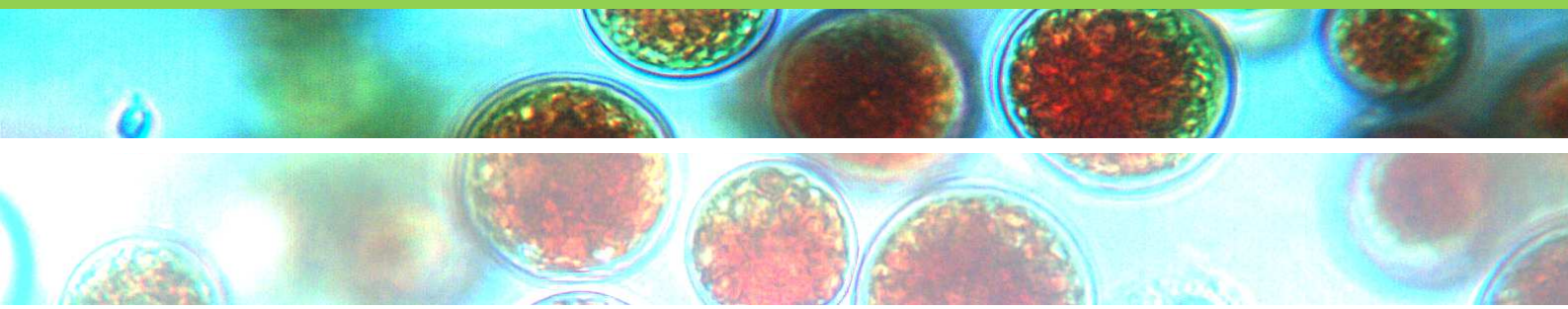

Single functional groups
Different functional groups
A) Chlorophyta
D) Two functional groups
B) Cyanophyta
E) Three functional groups
C) Bacillariophyta
F) Four functional groups
Microalgal neutral lipids
PAR abs
r² = 0.18
P = 0.018
r² = 0.13
P = 0.047
*
*

CHAPTER 4

PHYTOPLANKTON DIVERSITY INFLUENCES ALGAL FATTY ACID COMPOSITION- IMPLICATIONS FOR THE AQUATIC FOOD WEB

M. STOCKENREITER, F. HAUPT, K. SPILLING, J. SEPPÄLÄ, D. ENSS, H. RISCHER , H. STIBOR AND T. TAMMINEN

FUNCTIONAL ECOLOGY, UNDER REVIEW



Summary

1. Determining the factors that control energy transfer at the plant–animal interface is a key issue in ecology, because this transfer is highly variable and despite its global importance it is still not well understood. The food quality of primary producers seems to be a crucial factor influencing the efficiency of transfer towards higher trophic levels. One major aspect of food quality is the fatty acid composition in terms of essential ω3-polyunsaturated fatty acids (ω3-PUFAs) in primary producers, because all animals are incapable of synthesizing them *de novo*.
2. In aquatic ecosystems primary consumers feed on complex assemblages of phytoplankton species, which vary in taxonomic and functional diversity. The nutrient status of aquatic ecosystems also influences the diversity of their primary producers, which in turn will affect their resource use efficiency and productivity. However, the influence of diversity on phytoplankton food quality in terms of lipid composition (e.g. ω3-PUFAs) remains unclear.
3. Here we show that the diversity of phytoplankton communities affects their fatty acid composition. We quantified the fatty acid composition of 23 species originating from four major phytoplankton groups in monoculture and 48 communities assembled from these monocultures and controlled for species- and functional group richness.
4. We found that the proportion of ω3-PUFAs in total lipids was higher in more diverse communities and increased with increasing functional group richness. A main driver of this pattern was the higher content of the essential ω3-PUFA α-linolenic acid (ALA) in polycultures assembled from different functional groups. The ω3-PUFA ALA content is an important predictor for primary consumer growth and ALA serves as a precursor for other essential ω3-PUFAs.
5. Our data provide the starting point for further studies regarding the impacts of diversity on ecosystem functioning beyond primary producer productivity. Diversity-dependent primary producer ω3-PUFA content could be an important mechanism behind biodiversity–ecosystem functioning relationships. Furthermore, our findings support arguments for the preservation of biodiversity in order to maintain important ecosystem services.

Keywords: food quality, functional groups, lipids, microalgae, PUFA, species richness.

Introduction

Phytoplankton forms the basis of nearly all aquatic food webs and is responsible for more than 50% of global primary production (Falkowski & Raven 2007). Therefore its biomass composition strongly influences major energy and nutrient fluxes in aquatic ecosystems. The efficiency of biomass and energy transfer at the phytoplankton–animal interface of aquatic ecosystems is highly variable (Brett & Goldman 1997). Despite the global importance of this interface we lack a detailed understanding of the factors influencing this variability. However, there is general agreement that phytoplankton food quality is a crucial factor (Müller-Navarra *et al.* 2000; Wacker & von Elert 2001). As primary consumers, herbivorous zooplankton use phytoplankton as their main food source and the growth and reproduction of zooplankton is heavily dependent on the quality of their phytoplankton diet.

The food quality of phytoplankton is determined by its cellular composition in terms of chemical elements, carbohydrates, lipids, proteins etc. In general, low carbon-to-nutrient (especially phosphorus and nitrogen) biomass ratios seem to result in high quality food for herbivorous zooplankton (Urabe & Sterner 1996; Hill, Rinchard & Czesny 2011). However, beside variations in the carbon-to-nutrient ratio, the availability of nutrients can also affect the fatty acid content of phytoplankton (Müller-Navarra *et al.* 2004; Hill *et al.* 2011). The quality of phytoplankton species as food for zooplankton is – irrespective of its nutrient content – often strongly linked to certain fatty acids in the phytoplankton (Ravet, Brett & Müller-Navarra 2003; Martin-Creuzburg & von Elert 2009). Polyunsaturated fatty acids (PUFAs) are especially important for zooplankton growth and reproduction (Mariash *et al.* 2011).

Some of these PUFAs are of general importance for trophic interactions: ω3-PUFAs containing 18–22 carbon atoms (C18–C22) are essential for all animals (Wacker & von Elert 2001) because, unlike autotrophs, animals are unable to synthesize ω3-PUFAs *de novo* (Cook & McMaster 2002). Studies suggest that ω3-PUFAs are important constituents of the zooplankton diet, particularly single ω3-PUFAs such as eicosapentaenoic acid (EPA) (Müller-Navarra *et al*. 2000) and α-linolenic acid (ALA) (Wacker & von Elert 2001).

Most studies dealing with the influence of ω3-PUFAs and fatty acid composition focused on distinct algal species in monocultures and their fatty acid profiles; only a few studies have considered the influence of a mixture of two algal species and their positive impact on zooplankton performance (see Boersma & Vijverberg 1995). However, in natural aquatic

ecosystems herbivores feed on a diverse assemblage of microalgae consisting of more than two algal species originating from different functional groups.

There is broad consensus that a diversity of primary producers increases their productivity (Tilman *et al.* 2001; Downing & Leibold 2002; Power & Cardinale 2009) whereas there is no clear understanding of how diversity influences the quality of primary producers. Striebel, Behl & Stibor (2009) observed an increasing C:P ratio with increasing taxon richness, which could indicate lower food quality for zooplankton with increasing richness. However, diversity will not only influence the stoichiometry of phytoplankton communities. Algal species are characterized by individual fatty acid profiles and therefore different algal communities differ substantially in their overall profile. For this reason, food quality in terms of phytoplankton fatty acid composition is probably as important as the biomass C:P ratio. Müller-Navarra *et al*. (2000) have already shown that fatty acids are among the most important nutritional factors affecting the fitness of zooplankton, by supplying essential compounds for life history parameters. In theory, increasing diversity could therefore also lead to higher fatty acid richness with higher probabilities for the presence of ω3-PUFAs.

Diversity is often considered to equate to species richness, although species richness is only one way of describing the diversity of a community and its organization (Hillebrand & Matthiesen 2009). In fact, there is growing evidence that diversity, in terms of functional group diversity, might often exceed the impact of species richness (Cardinale 2011). Functional groups refer to classes of species that are divided into groups based on their common biochemical and/or ecological functions (Hood *et al*. 2006), for example the phytoplankton functional groups chlorophyta, cyanophyta, bacillariophyta and chrysophyta. However, our understanding of the impact of diversity across functional groups on phytoplankton ω3-PUFA quantity and quality remains limited.

We therefore tested the following hypotheses: (1) Algae from different functional groups differ in their ω3-PUFA composition. (2) The ω3-PUFA content is positively influenced by diversity and is therefore higher in mixed communities than in monocultures. (3) Communities consisting of algal species from different functional groups show higher ω3-PUFA ratios than those comprising algal species originating from a single functional group. (4) Functional diversity also positively influences the content of single ω3-PUFAs.

Using a series of growth experiments under highly controlled environmental conditions, we tested the above-described hypotheses about the effect of diversity on the fatty acid profile of microalgal communities. We quantified the fatty acid profile of 23 species in monoculture and 48 communities assembled from the 23 monocultures, the latter differing in functional group diversity and species richness.

Methods

EXPERIMENTAL SETUP

We used a set of 23 microalgal strains (SAG Culture Collection of Algae, Göttingen, Germany; UTCC, Toronto, Canada; Max Planck Institute (MPI) for Limnology, Plön, Germany) from four different functional groups (representing the major taxonomic groups of phytoplankton: chlorophyta, bacillariophyta, cyanophyta and chrysophyta). Each microalgal strain was pre-cultured as a monoculture in modified Woods Hole growth medium (WC-medium; Guillard & Lorenzen 1972) for several months before initiating the experiment. In each functional group (with the exception of chrysophyta, due to the lack of sufficient species in our culture collection), we established a diversity gradient of all monocultures and polycultures of four species (one functional group). In addition, we established a species richness gradient among the functional groups containing four, six or eight different species; to accomplish this we combined algal species from two, three or four functional groups. Each diversity level was replicated four times, with different random species compositions, resulting in 71 experimental algal communities.

The initial total algal biovolume was identical ($2.4 \cdot 10^6$ fL mL^{-1}) in all treatments at the beginning of the experiment. The experiment was arranged in a fed-batch-mode of cultivation (50% of the existing culture volume was added daily with WC-growth medium) in 650 mL cell culture flasks (Cellstar, Greiner bio-one, Kremsmünster, Austria). Cultures were kept at ambient room temperature (26 ºC) for 9 days. Constant light (90 µmol photons $m^{-2}s^{-1}$, PAR) was maintained throughout the experiment in a 12:12 h light:dark cycle.

MEASUREMENTS

At the beginning of the experiment, total algal biovolume and cell densities were estimated using a cell counter (FlowCAM®, Fluid Imaging Inc., Yarmouth, USA). All other measurements were made at the end of the experiment. The species-specific composition of the different phytoplankton mixtures was determined by microscopic counting, using the standard Utermöhl technique (Utermöhl 1958), in which the samples were fixed using Lugol's iodine in an inverted microscope. Species presence was recorded based on a minimum of 100 individuals, by scanning a minimum of five perpendicular transects or 20 randomly distributed fields, to ensure a counting error of less than 10% (Lund, Kipling & le Cren 1958).

FATTY ACID ANALYSIS

Fatty acids were separated and evaluated quantitatively using a capillary gas chromatograph (7890A with sampler CTC Analytics GC-PAL System, Agilent Technologies, Santa Clara, USA) and a standard protocol (Dagmar Enss, unpublished data).

We identified and quantified a total of 31 fatty acids or fatty acid methyl esters. We identified six ω3-PUFA fatty acids: C18:3 ω3-α-linolenic acid (ALA), C18:4 ω3-stearidonic acid (SDA), C20:3 ω3-eicosatrienoic acid (ETE), C20:5 ω3-eicosapentaenoic acid (EPA), C22:5 ω3-docosapentaenoic acid (DPA) and C22:6 ω3-docosahexaenoic acid (DHA).

STATISTICAL ANALYSIS

To estimate general differences between monocultures and communities we used the independent sample t-test. To determine the impact of functional group diversity, we plotted the proportion of total fatty acids against the ω3-PUFA and ALA content of communities against functional group richness using linear regression. To test the impact of species richness on the proportion of total fatty acids and the ω3-PUFA and ALA content we plotted the residuals from the linear regression of ω3-PUFA and ALA content and total fatty acid proportions against functional group richness. We plotted the residuals against species richness to determine whether the deviations between the measured and calculated (regression-based) ω3-PUFA and ALA proportion and content to total fatty acids were a function of species richness. To ensure normality (Shapiro-Wilk test), the data were either arcsine square root transformed (proportions) or ln (x+1)-transformed (total content).

Results

We calculated the proportion of ω3-PUFA to total fatty acids in all monocultures and mixed communities. For all monocultures combined, the proportion of ω3-PUFA to total fatty acids was on average 12.2 (± 2.6); for all mixed communities combined, the proportion was on average 17.8 (± 1.4) (mean ± 1SE). These differences in proportions between monocultures and mixed communities were significant according to the t-test: $t_{(69)} = 2.69$; $p = 0.009$.

In mixed communities the mean proportion of ω3-PUFA to total fatty acids was 12.1 ± 2.4 (one functional group), 18.3 ± 2.5 (two functional groups), 19.7.6 ± 2.3 (three functional groups) and 21.5 ± 3.1 (four functional groups) (mean ± 1 SE). The proportion of ω3-PUFA to total fatty acids increased as functional group richness increased (Fig. 1a; $R^2 = 0.13$; $p = 0.013$). The residuals of the linear regression between functional group richness and ω3-PUFA proportion were not influenced by the species richness of the mixed communities (Fig. 1b; $R^2 = 0.00$; $p = 0.66$).

The mean ω3-PUFA content in mixed communities was 7.2 ± 2.3 (one functional group), 11.4 ± 3.3 (two functional groups), 11.6 ± 2.0 (three functional groups) and 14.5 ± 3.5 (four functional groups) µg L^{-1} (mean ± 1 SE). The ω3-PUFA content increased as functional group richness increased (Fig. 1c; $R^2 = 0.09$; $p = 0.037$). The residuals of the linear regression between functional group richness and ω3-PUFA content were not influenced by the species richness of the mixed communities (Fig. 1d; $R^2 = 0.01$; $p = 0.61$).

We then calculated the proportion of C18:3 ω3-α-linolenic acid (ALA) to total fatty acid content in all monocultures and mixed communities. In all monocultures combined the proportion of ALA to total fatty acids was on average 9.9 (± 2.7); in all mixed communities combined the proportion was on average 14.8 (± 1.5) (mean ± 1SE). These differences in proportions of ALA to total fatty acids between monocultures and mixed communities were significant according to the t-test: $t_{(69)} = 2.24$; $p = 0.028$.

In mixed communities the mean proportion of ALA to total fatty acids was 7.6 ± 2.8 (one functional group), 18.3 ± 2.5 (two functional groups), 19.7.6 ± 2.3 (three functional groups) and 21.5 ± 3.1 (four functional groups) (mean ± 1 SE). The proportion of ALA to total fatty acids also increased with higher functional group richness (Fig. 2a; $R^2 = 0.19$; $p = 0.003$). The residuals of the linear regression between functional group richness and ω3-PUFA

proportion were not influenced by the species richness of the phytoplankton communities (Fig. 2b; $R^2 = 0.01$; $p = 0.50$).

The mean ALA content in mixed communities was 3.2 ± 2.2 (one functional group), 10.0 ± 3.1 (two functional groups), 9.7 ± 2.1 (three functional groups) and 13.0 ± 3.5 (four functional groups) µg L^{-1} (mean ± 1 SE). The ALA content of mixed communities increased significantly as functional group richness increased (Fig. 2c; $R^2 = 0.20$; $p = 0.002$). The residuals of the linear regression between functional group richness and ALA content were not influenced by the species richness of the mixed communities (Fig. 2d; $R^2 = 0.01$; $p = 0.48$).

Discussion

Lipids are important for the growth and reproduction of aquatic organisms and have a strong impact on fitness and life history parameters (Brett & Goldman 1997; Müller-Navarra *et al.* 2000; Ahlgren *et al.* 2005). ω3-PUFAs are of significant nutritional importance for aquatic organisms, because they affect metabolic activity, individual and population growth rates and reproduction. In aquatic ecosystems these ω3-PUFAs are almost exclusively produced by phytoplankton. Given their inability to synthesize these fatty acids *de novo*, higher trophic levels are highly dependent on these ω3-PUFAs (Cook & McMaster 2002).

The fatty acid composition and proportion of ω3-PUFAs in algae are highly species-specific (Müller-Navarra *et al*. 2000) and are strongly influenced by culture conditions (Sterner, Hagemeier & Smith 1993; van Donk & Hessen 1993). However, chlorophyta are known to have high amounts of ω3-PUFAs (e.g. 40% in *Scenedesmus acutus*, see DeMott & Müller-Navarra 1997). Indeed, in our study, chlorophyta, together with bacillariophyta, contained higher levels of ω3-PUFA when grown in monoculture in comparison to cyanophyta and chrysophyta.

However, on average, the mixed communities contained even higher proportions of ω3-PUFAs than the monocultures. In addition, the proportion of ω3-PUFAs increased with increasing functional group richness in the mixed communities. In natural ecosystems the persistence of diversity is regulated by the frequency of disturbance and the temporal and spatial heterogeneity of the environment (Gaedeke & Sommer 1986) as well as the trophic

status of the ecosystem (Dodson, Arnott & Cottingham 2000); however, in our study diversity was the only parameter that varied, while nutrients and cultivation conditions were kept constant. This implies that combining dissimilar species (e.g. in terms of functional traits) has a greater effect on ecosystem functioning than combining similar species (Carey & Wahl 2011), which means in our study that the increase in the proportion of ω3-PUFAs in the algae was dependent on functional diversity. However, phytoplankton species from one functional group are known to exhibit a similar fatty acid composition (Burns, Brett & Schallenberg 2011), akin to the similar pigment composition of species within one functional group.

Aquatic herbivores show a strong response to the fatty acid composition of their food: experiments revealed that higher ω3-PUFA levels in food led to higher somatic growth and higher egg production in herbivorous zooplankton (*Daphnia*) (Müller-Navarra *et al*. 2000; Wacker & von Elert 2001). The transfer efficiencies of these ω3-PUFAs are usually high and they are thereby accumulated in the biomass of upper trophic levels (Burns *et al*. 2011; Gladyshev *et al*. 2011).

Some of these ω3-PUFAs i.e. ALA, EPA and DHA (Müller-Navarra *et al*. 2000; Wacker & von Elert 2001) are critical for food web processes. ALA is central as the precursor for the subsequent bioconversion products EPA and DHA (Burns *et al*. 2011). The phytoplankton ALA content seems to be a good predictor for herbivorous zooplankton population growth. Wacker & von Elert (2001) showed a strong increase in *Daphnia galeata* growth rate with increasing ALA content in their food: the growth rate at high ALA levels was almost three times higher than at low ALA levels. The mean ALA content of the phytoplankton in our study was within the range reported by Wacker & von Elert (2001): it increased in mixed communities from 3.2 µg L^{-1} (one functional group) to 13.0 µg L^{-1} (four functional groups). Our observed, functional diversity-based increase in phytoplankton ALA content correlated with the findings of Wacker & von Elert (2001), and would therefore account for an increase in *Daphnia* somatic growth rate of about 20% (from 0.45 d^{-1} to 0.55 d^{-1}).

An explanation for the observed positive influence of functional diversity on ω3-PUFAs and ALA might be found in the positive correlation between diversity and light use. Recent studies suggest that light harvest in functionally diverse algal assemblages is higher than that expected in their respective monocultures, and the reason for this is probably the higher variability in light-harvesting pigments (Behl, Donval & Stibor 2011; Maria Stockenreiter,

unpublished data). Additionally, ω3-PUFAs are primarily found in photosynthetically active membranes (Hill *et al*. 2011). Hence, there might be a link between light harvest and ω3-PUFA production in these mixed communities that remains to be investigated.

Our data provide the starting point for further studies regarding not only the production-based impacts of diversity on ecosystem functioning, but also its effects on qualitative traits. The ω3-PUFA content in phytoplankton seems to influence the overall matter and energy transfer efficiency in aquatic ecosystems because ω3-PUFAs constitute a considerable part of the bulk carbon biomass in organisms at different trophic levels (Gladyshev *et al*. 2011). Müller-Navarra *et al*. (2000; 2004) hypothesised that a low concentration of polyunsaturated acids in phytoplankton results in a bottleneck for carbon transfer from producers to consumers. The phytoplankton ω3-PUFA content therefore seems to be key to our understanding of ecological processes. Aquatic ecosystems play a unique role in the biosphere as the principal source of ω3-PUFA for all animals including the inhabitants of terrestrial ecosystems. Our finding that the proportion of ω3-PUFA within the total fatty acids of phytoplankton is affected by diversity supports arguments to preserve biodiversity in order to maintain important ecosystem services.

Acknowledgements

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Figure legends

Fig. 1a, Linear regression between functional group richness and arcsine ω3-PUFA proportion to total fatty acids of mixed communities: arcsine ω3-PUFA proportion = 17.6 + 2.6 x functional group richness. $R^2 = 0.13$; $p = 0.013$.

b, Linear regression between species richness and residuals of the linear regression between functional group richness and arcsine ω3-PUFA proportion to total fatty acids of mixed communities: residuals arcsine ω3-PUFA proportion = -1.6 + 0.30 x species richness. $R^2 = 0.00$; $p = 0.66$.

c, Linear regression between functional group richness and ln (ω3-PUFA content µg L^{-1} + 1) of mixed communities: ln (ω3-PUFA content µg L^{-1} + 1) = 1.60 + 0.23 x functional group richness. $R^2 = 0.09$; $p = 0.037$.

d, Linear regression between species richness and residuals of the linear regression between functional group richness and ln (ω3-PUFA content µg L^{-1} + 1) of mixed communities: residuals ln (ω3-PUFA content µg L^{-1} + 1) = -0.21 + 0.04 x species richness. $R^2 = 0.01$; $p = 0.61$.

Fig. 2a, Linear regression between functional group richness and arcsine ALA proportion to total fatty acids of mixed communities: arcsine ALA proportion = 9.9 + 4.19 x functional group richness. $R^2 = 0.19$; $p = 0.003$.

b, Linear regression between species richness and residuals of the linear regression between functional group richness and arcsine ALA proportion to total fatty acids of mixed communities: residuals arcsine ALA proportion = -3.28 + 0.60 x species richness. $R^2 = 0.01$; $p = 0.50$.

c, Linear regression between functional group richness and ln (ALA content µg L^{-1} + 1) of mixed communities: ln (ALA content µg L^{-1} + 1) = 0.73 + 0.43 x functional group richness. $R^2 = 0.20$; $p = 0.002$.

d, Linear regression between species richness and residuals of the linear regression between functional group richness and ln (ALA content µg L^{-1} + 1) of mixed communities: residuals ln (ALA content µg L^{-1} + 1) = -0.34 + 0.06 x species richness. $R^2 = 0.01$; $p = 0.48$.

Figures

Fig. 1

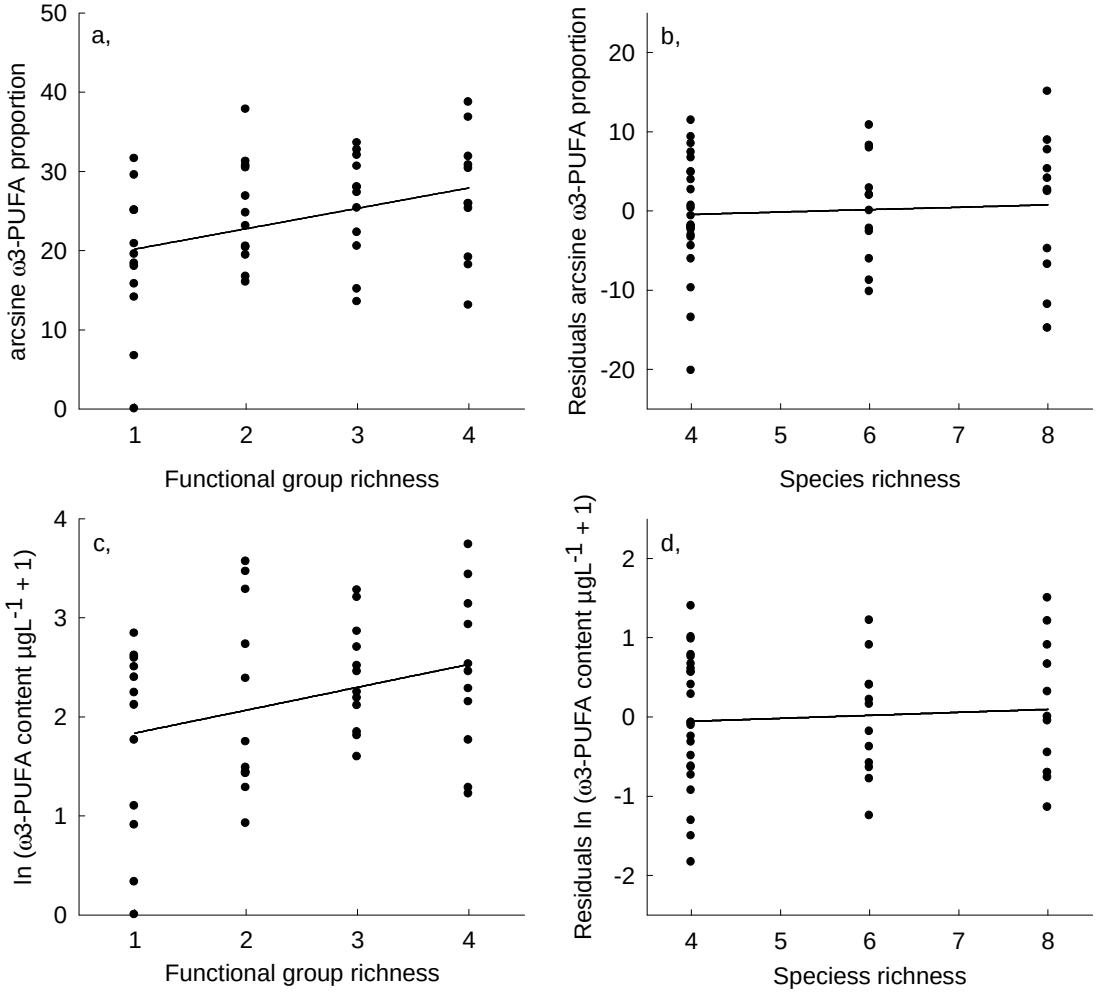
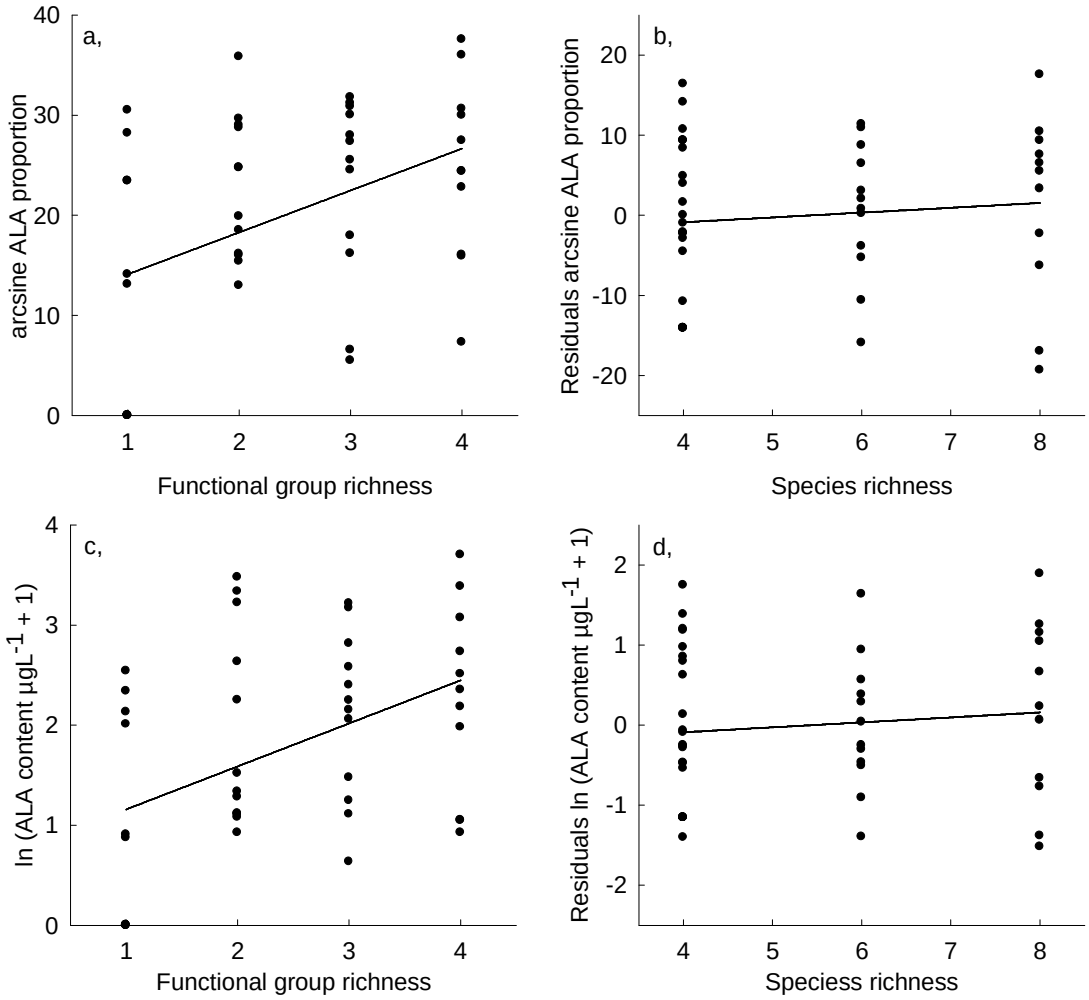


Fig. 2


a,
arcsine ALA proportion
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ln (ALA content µgL-1 + 1)
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Residuals ln (ALA content µgL-1 + 1)
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CHAPTER 5

INITIAL SIZE STRUCTURE OF NATURAL PHYTOPLANKTON COMMUNITIES DETERMINES THE RESPONSE TO *DAPHNIA* DIEL VERTICAL MIGRATION

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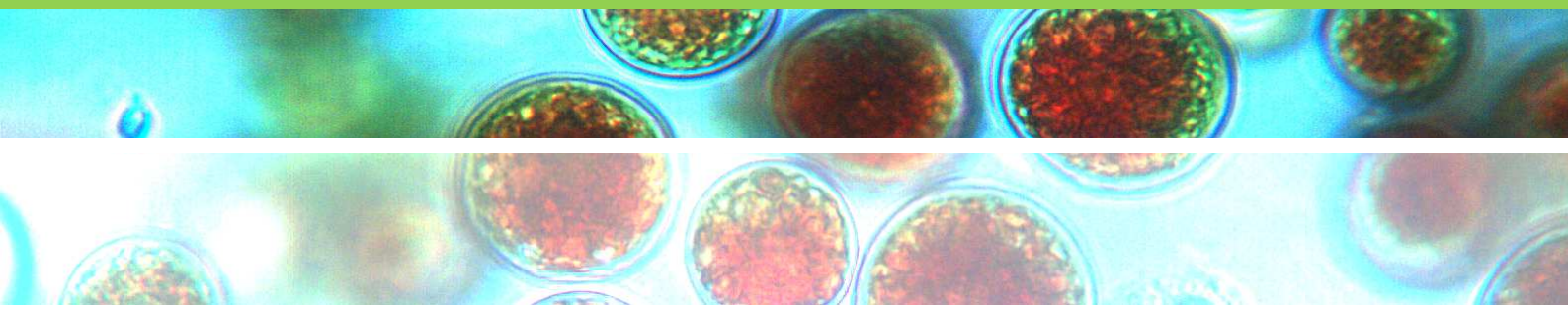

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Initial size structure of natural phytoplankton communities determines the response to *Daphnia* diel vertical migration

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ABSTRACT

Diel vertical migration (DVM) is a common behavior of many pelagic herbivorous zooplankton species in response to predation pressure. It is characterized by a twice daily habitat shift of the zooplankton species: staying in the epilimnion only during night time and migrating down in the crack of dawn in deeper water layers, staying there during the day time. This causes a discontinuous grazing regime and previous studies have shown that the direction and strength of phytoplankton community responses to zooplankton DVM most probably depends on the size of phytoplankton species. To examine the influence of zooplankton DVM on different sized phytoplankton communities, we designed an experiment where we manipulated the size distribution of a natural phytoplankton community a priori in field mesocosms. We investigated the influence of DVM of the cladoceran Daphnia hyalina *on two different phytoplankton communities, by the use of deep (10 m) field enclosures. Epilimnetic lake water, containing a summer phytoplankton community, was filtered with two different mesh sizes (11 µm and 64 µm). The 11 µm phytoplankton community ("small") contained mainly small algal species, while the 64 µm community ("large") had a wider range of phytoplankton sizes. To simulate zooplankton DVM,* D. hyalina *were placed in mesh cages that were lowered or raised ("migration") as dictated by the study design; a "no migration" (representing absence of DVM) treatment was also tested. Phytoplankton abundance was measured using chlorophyll-*a *and biovolume; size distribution of the algae and nutrient availability was also determined in each treatment. The results indicated that DVM had contrasting effects on the two evaluated phytoplankton communities. Comparison of "migration" and "no migration" zooplankton treatments showed that nutrient availability and total phytoplankton biovolume was higher in (1) "no migration" treatments with phytoplankton communities comprising mainly small algae and (2) "migration" treatments with phytoplankton communities of a broader size spectrum of algae. Hence our study showed two different mechanisms of how zooplankton DVM may influence the phytoplankton community dynamics. Nutrient cycling was an important factor in phytoplankton communities of mainly small algae, whereas the refuge effect was the main driver of phytoplankton dynamics in phytoplankton communities of a large size spectrum of algae*

Key words: behavior, algae, trait mediated interactions, nutrients, refuge effect.

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INTRODUCTION

Natural primary producer communities typically comprise many species of various taxonomic levels with vastly different body sizes (Gaston, and Lawton 1988). Body size is an important feature in many food web models because of its importance in numerous ecological interactions, including inter-species competition and prey selection by predators (Cohen et al. 1993; Williams, and Martinez 2000). The impact of size structure on ecological interactions in pelagic ecosystems may be substantial. For example, predatorprey relationships are almost exclusively based on larger organism eating smaller organisms, with non-lethal herbivory being practically absent in these systems.

The PEG model (Sommer et al. 1986) of annual plankton succession in lakes demonstrates the significant relationship between size structure and pelagic ecosystem dynamics. Interactions are linked to the size structure and composition of the plankton community, which are subject to substantial seasonal fluctuations. Seasonal changes are further influenced by, and linked to, other abiotic and biotic factors, such as temperature gradients, nutrient availability, intra- and interspecific competition, and predation. The greatest annual community shift in temperate pelagic freshwater ecosystems, described by the PEG model, generally occurs at the end of the "clear water" phase in late spring/early summer. At this time, the community of small algae transforms into the summer community of large, grazing-resistant algae, thus influencing food availability for zooplankton.

The transition between spring and summer phytoplankton communities is often characterized by a high abundance of juvenile fish, which start to populate the pelagic zone and prey on the zooplankton. Strong predation pressure

triggers avoidance behavior in many zooplankton species (such as *Daphnia*), resulting in "diel vertical migration" (DVM) behavior. DVM is one of the most important escape responses exhibited by aquatic herbivores (Hays 2003) and it has been shown, that DVM can be a feature in a lake nearly throughout the whole year (Huber et al. 2011). Zooplankton species performing DVM spend the night primarily in upper water layers, migrating down the water column at dawn to spend the day in deeper, darker and, colder waters (Lampert 1989). Because fish feed visually, behavioral studies have clearly established predator avoidance as the primary ultimate driver for DVM (Zaret, and Suffern 1976). The immediate triggers initiating vertical migration behavior by zooplankton are the changes in light intensity around dawn and dusk (Ringelberg 1991), while the presence of a chemical substance (kairomone) that is released by predatory fish affects the motivation of zooplankton to respond to these triggers (Loose et al. 1993).

While many studies have investigated the effects of DVM on the population dynamics of the zooplankters (Lampert et al. 1988; Loose, and Dawidowicz 1994; Reichwaldt et al. 2005), little research has been directed toward the potential consequences of DVM for phyto-plankton dynamics, possibly because of the difficulties in inducing and regulating migration behaviour in controlled experiments. Conceivably are different mechanisms through which DVM of zooplankton can influence epilimnetic phytoplankton communities. Perhaps the most substantial impact is reduced grazing pressure, due to lower zooplankton densities. For example, migrating zooplankton populations encounter lower temperatures in the hypolimnion than in the epilimnion. These lower temperatures lead to slower somatic growth, which may ultimately lead to lower population growth (Loose, and Dawidowicz 1994). The lower density of migrating compared to non-migrating zooplankton populations is certainly only the case if one leaves out predation as a potential mortality factor, because if predation was present, a non-migrating population would also have lower growth due to this predation (Stich, and Lampert 1984).

The second possible mechanism affecting epilimnetic phytoplankton communities is that zooplankton migration leads to the daytime period being generally free of grazing, which results in intermittent grazing pressure on the phytoplankton community in the epilimnion. Because both of these mechanisms may lead to reduced grazing pressure on phytoplankton, it is assumed that both may significantly enhance phytoplankton biomass (Lampert et al. 1986; Reichwaldt et al. 2004). Results of recent studies also suggest a third mechanism, whereby the migration of zooplankton may have a significant effect on epilimnetic nutrient supplies due to a change in nutrient recycling. Specifically, Lampert, and Grey (2003) showed that DVM by *Daphnia* may result in the upward transport of nitrogen, while Haupt et al. (2010) showed an enrichment of upper water layers with phosphorus by *Daphnia* DVM.

Theoretical models have been developed using available data to estimate the impacts of zooplankton DVM on phytoplankton communities, in which discontinuous zooplankton grazing under DVM is indicated to enhance phytoplankton biomass by fostering small and fast growing algal species (Lampert 1987). A model developed by Petzoldt et al. (2009) showed that reduced zooplankton grazing and changed nutrient recycling under DVM are important mechanisms influencing plankton dynamics. The few experiments that have been conducted to investigate the effects of zooplankton DVM on pelagic ecosystems show that it may affect phytoplankton abundance, species composition, and diversity (Reichwaldt, and Stibor 2005; Haupt et al. 2009).

One emerging hypothesis from the post hoc analyses of experimental studies (Reichwaldt, and Stibor 2005; Reichwaldt et al. 2004; Haupt et al. 2009), is that the effects of zooplankton DVM on phytoplankton communities are species-specific, probably depending on the size of particular phytoplankton taxa and the acceptable food-size range of zooplankton. Because small phytoplankton species normally have higher growth rates than larger species (Reynolds, and Irish 1997), they may use spatial and temporal refuges created by zooplankton DVM more efficiently. Hence, small fast growing edible algae may benefit more from DVM than larger slow-growing taxa within the community. Conversely, communities that consist mainly of large inedible algae may benefit from relatively constant uninterrupted grazing by non-migrating zooplankton on the few edible taxa. Additionally, zooplankton release nutrients through sloppy feeding and excretion, which further increases the abundance of inedible algae within the community.

Hence, we hypothesize that differences in the size structure of phytoplankton communities will affect the response of phytoplankton communities to zooplankton DVM. If correct, experimental manipulation of the size distribution of a phytoplankton community should alter its response to zooplankton DVM. To investigate this hypothesis, we manipulated the size distribution of a natural phytoplankton community in large (7000 L) field mesocosms, representing two differently size structured phytoplankton communities (either with mainly small species or with a larger size spectrum of species). The resulting communities were exposed to migrating and non-migrating populations of *Daphnia*. We consider our results against theories of phytoplankton community responses to *Daphnia* DVM, and potential trophic web impacts.

MATERIALS AND METHODS

The study was conducted in an experimental enclosure system deployed in oligotrophic Lake Brunnensee, south-

ern Germany (47°59'N, 12°26'E), in the summer (June-July) of 2007. This small (5.8 ha), deep (18.6 m), hard-water lake is strongly phosphorus-limited (total P: 12 ng L^{-1}), with a high nitrate concentration (NO_3: 5 mg L^{-1}) during the summer. To investigate the effects of vertically migrating zooplankton on two differently size structured phytoplankton communities, we moved *Daphnia* populations up and down the water column using cages. To create the two different phytoplankton communities, epilimnetic lake water containing a summer phytoplankton community was filtered using meshes (Sefar Petex, Sefar AG, Switzerland) with either a 11 μm or 64 μm mesh size.

The submersible cages used in this study had already been successfully applied in earlier experiments (Reichwaldt, and Stibor 2005; Haupt et al. 2009). Although predation is considered to be one of the most important causes of zooplankton DVM (Zaret, and Suffern 1976), attempts to establish a predatory dynamic by fish stocking have proven very difficult, primarily due to potential indirect effects on phytoplankton caused by nutrients excreted by enclosed fish (Schindler 1992; Vanni, and Layne 1997; Attayde, and Hansson 1999). In practice, it is also not possible to induce zooplankton DVM behavior using kairomones because too little is known about the structure and dose-effect relationship of these chemical signals.

Experimental design

Twenty four cylindrical enclosures (transparent Trikoron bags, Rheinische Kunststoffwerke Worms, Germany) were suspended vertically from a raft to a depth of 10 m. Each 0.9 m diameter enclosure was heat-sealed at the bottom and open to the atmosphere. In the enclosures, we mimicked an unmixed, 6 m deep hypolimnion and a well-mixed, 4 m deep epilimnion. The latter was produced by intermittently bubbling compressed air (3 min on, 40 min off) through PVC-tubes at a depth of 4 m. To prevent a vertical temperature gradient in the enclosures, all were surrounded by a 15 m deep, transparent silage film (0.2 mm), which acted as a homogenous, tempered water bath. Uniform mixing in the water bath was achieved by the intermittent injection of compressed air (5 min on, 20 min off) at a depth of 12 m.

Homogenous temperature along the vertical gradient was necessary to achieve similar growth in migrating and non-migrating *Daphnia* populations. Reichwaldt, and Stibor (2005) showed a fluctuating temperature regime had a significantly negative impact on the population growth and hence abundance of migrating *Daphnia*. In this study, we aimed to investigate the refuge effect of *Daphnia* DVM on phytoplankton communities of different size structures, and the consequences of DVM on nutrient dynamics in such communities. Therefore we used a modification of the experimental setup of Reichwaldt, and Stibor (2005) to separate refuge effects from temperature effects. This method, constructs a well-mixed water bath around all enclosures, allowing the refuge effect of zooplankton DVM to be examined under field conditions without significant temperature differences between upper and deeper water layers.

Twelve enclosures were filled with 64 μm filtered epilimnetic water, and another 12 were filled with 11 μm filtered epilimnetic water. The 11 μm filtered communities consisted mainly of small phytoplankton species, whereas the 64 μm filtered communities consisted of a variety from small to large phytoplankton species (Fig. 1). From this point onwards, we therefore refer to the 11 μm filtered communities as "small" communities and the 64 μm-filtered communities as "large" communities. For a detailed description of the natural summer phytoplankton community in Lake Brunnensee see Haupt et al. (2009). Filtration and the filling of the enclosures began on 19 June 2007, which took approximately 48 h. The enclosures were filled haphazardly with either "small" or "large" phytoplankton. After filling the enclosures, the "small" community enclosures were enriched with 10 μg KH_2PO_4 L^{-1} to attain similar particulate phosphorus concentrations in all treatments, due to the particulate material having been removed from these enclosures.

Daphnia were placed in a cylindrical mesh cage (224 μm mesh aperture, diameter 0.7 m, length 3.5 m; Sefar Petex, Sefar AG, Switzerland) inside each enclosure. This mesh aperture ensured that all *Daphnia* were retained within the cages, while allowing the free exchange of algal cells. Each cage had a mesh cap that could be re-sealed to allow sampling. The volume of the cages was approximately 50% of the epilimnion. To simulate DVM, cages were moved up and down the water column within the enclosures in a diurnal rhythm.

For the "migration" treatment group, cages containing *Daphnia* were kept in the epilimnion (top of cage: 0.25 m depth) at night (20:00-08:00 h), and then lowered into the hypolimnion (top of cage: 5.5 m depth) during the day (08:00 h to 20:00 h). Cages were manually moved as slowly as possible (maximum speed: 0.05 m s 1). For the "no migration" treatment group, the cages containing *Daphnia* were kept permanently in the epilimnion. Although previous studies detected no plankton or nutrient dynamic effects from the movement of the cages (Reichwaldt, and Stibor 2005; Haupt et al. 2009), we again evaluated this possibility by installing enclosures with migrating empty (no *Daphnia*) cages, and enclosures with non-migrating empty cages. Therefore, the twelve "small" enclosures and the twelve "large" enclosures included three *Daphnia* "migration" treatments, three *Daphnia* "no migration" treatments, three migrating empty cages, and three non-migrating empty cages.

We used a clone of *Daphnia hyalina* originating from

Lake Brunnensee, which is known to perform DVM in this lake (H. Stibor, unpublished data), to stock the cages. Prior to the experiment, *Daphnia* were reared in 30 L buckets, with an artificial culture medium in an environmental chamber at a constant temperature of 20°C. They were fed *Scenedesmus obliquus* (>1 mg C L^{-1}) every other day, and 50% of their medium was renewed every 5 d. Two days before the beginning of the experiment, all *Daphnia* were transferred to 30 μm filtered, epilimnetic lake water. At the beginning of the experiment, *Daphnia* were released into the *Daphnia* "migration" and *Daphnia* "no migration" treatment cages at a starting density of five individuals L^{-1} within the epilimnion, which is a density that is typical for this species in Lake Brunnensee (H. Stibor, unpublished data).

The experiment began with the stocking of *Daphnia* on 25 June 2007 (day 0), 5 d after filling the enclosures, to compensate phytoplankton growth from the losses caused by the 11 μm filtration in the "small" community treatments. The experiment lasted for four weeks until 24 July 2007 (day 29). This has proven to be an ecologically rational time span for enclosure experiments, because it is long enough to show strong effects on the monitored parameters, but short enough to prevent the occurrence of artificial effects in the enclosures, such as extensive wall growth (Reichwaldt, and Stibor 2005; Haupt et al. 2009).

Sampling program

Water temperature was measured weekly at 1 m vertical intervals using a WTW model Lf 191 meter with LT1/T probe (Wissenschaftlich-Technische Werkstätten, Germany). Vertical profiles of photosynthetically-active radiation (PAR) were measured once in all enclosures on day 14, using a LI-139SA spherical quantum sensor (LiCor, USA). In the "no migration" treatment groups, where cages remained in the epilimnion throughout the day, light intensity was measured with the cages in place, to account for possible shading effects. In both "migration" and "no migration" conditions, PAR was measured stepwise at 1 m intervals from the surface to a depth of 7 m, and was used to calculate the depthaveraged light attenuation coefficient (Diehl et al. 2002) for each enclosure. A *t*-test revealed no significant differences in PAR between the "migration" and "no migration" treatments in the "small" and "large" enclosures ("small" community: $t_{(10)}$=0.02; p=0.98; "large" community: $t_{(10)}$=0.39; p=0.70). This data validated that "migration" and "no migration" treatments were not impacted by different shading regimes in either phytoplankton community.

At the start (day 1) and end (day 29) of the experiment, water samples were collected from outside the cages in each enclosure at a depth of 0.5 m (epilimnion) and 7 m (hypolimnion) using a hand pump. All samples were collected before the "migration" treatment cages were lowered to the hypolimnion. The samples were filtered through a 250 μm mesh screen, and immediately analyzed for biological and chemical parameters. Concentrations of soluble reactive phosphorus (SRP) and silicate (SiO_2) were measured following standard methods (Wetzel, and Likens 1991). Nitrate concentration was measured by ion chromatography (Model 300, Dionex Corporation, USA). Chlorophyll-*a* concentrations were determined fluorometrically (TD 700, Turner Design, USA).

To analyze the total biovolume and size spectrum of the two phytoplankton communities, we immediately preserved subsamples of the collected water samples with acid Lugol's iodine. These samples were measured with a particle counter (Casy 1, Schärfe Systems, Germany). Plankton particles were sorted according to equivalent spherical diameter (ESD). The ESD was then used to determine 22 size classes. For each size class, we pooled the biovolume of all particles around±0.5 μm of each respective ESD size class. Hence the smallest size class was 4 μm ESD, including the biovolume of all particles between 3.5 μm and 4.5 μm ESD, while the largest size class was 25 μm ESD, including the biovolume of all particles between 24.5 μm and 25.5 μm ESD.

At the end of the experiment (day 29), zooplankton samples from all cages were collected to test the potential effects of the migrating cage on *Daphnia* growth. To accomplish this, in the morning before the migrating cages were lowered, all cages were opened at the top and mixed with a Secchi disc (the Secchi disc was lowered and brought up two times in each cage) to uniformly distribute the zooplankton. A vertical net haul from the bottom to the top inside the cage (net diameter: 0.25 m; mesh size: 150 μm) was then taken. This sampling method allowed direct comparisons between enclosures, although it probably under-sampled actual *Daphnia* densities inside the cages, because *Daphnia* that remain near to the cage bottom are not effectively caught (Haupt et al. 2009). The samples were preserved in 4% sucrose-formaldehyde solution (Haney, and HalL^{-1}973), and all zooplankton individuals were counted under a dissecting microscope.

Data processing

In this study we were interested in the mechanisms of how discontinuous grazing caused by zooplankton DVM may influence phytoplankton communities. Therefore, we used the data from the end of the experiment (day 29), in which we expected to observe the largest effects on the monitored parameters, for the analysis of the algal communities. Because all available theoretical models investigating zooplankton DVM are focused on the effects of DVM on epilimnetic algal communities, we primarily report data from this layer.

The total biovolume and biovolume of each size class of the phytoplankton communities were used to calculate

the percentage biovolume of each size class at the start (day 1) and the end of the experiment (day 29). We used this data to predict the development of phytoplankton biomass $r_{(i)}$ of each size class during the experiment from the logarithms of the biovolume percentage. We used for the calculations a modified version of the population growth rate (PGR) formula:

$$r_{(i)}=(\ln BVP(i)_{end} \ln BVP(i)_{start}) \qquad (1$$

where $BVP(i)_{end}$ is the biovolume percentage in size class i at the end of the experiment, and $BVP(i)_{start}$ is the biovolume percentage in size class i at the start of the experiment. We analyzed the biomass development r of the phytoplankton size classes in "migration" and "no migration" treatments by using standard regression models. Lack of fit tests were used to determine the validity of linear models, and ANCOVA methods were used to compare the slope and intercepts of linear regressions.

Cage effects were analyzed using *t*-tests to compare migrating and non-migrating empty cage data. Two-way analysis of variance (ANOVA) (with phytoplankton community type and *Daphnia* migration treatment as fixed factors) was used to compare soluble reactive phosphorus concentrations, chlorophyll-*a* and total phytoplankton biovolume between *Daphnia* "migration" and *Daphnia* "no migration" treatments. If a significant interaction between fixed factors was indicated, we performed post hoc tests using all pair wise multiple comparison procedures (Holm-Sidak method). Data are mainly presented as mean±one standard error of the mean. Where appropriate to meet statistical assumptions (Sokal, and Rohlf 1981), data were lntransformed.

RESULTS

Success of the experimental design

Filtration and initial conditions

Total phytoplankton biovolume at the start of the experiment (day 1) was $2.8\times10^9\pm1.6\times10^8$ μm³ L^{-1} in the "small" and $2.7\times10^9\pm3.8\times10^8$ μm³ L^{-1} in the "large" communities. *T*-tests revealed no significant differences in total biovolume between both phytoplankton communities in all 12 *Daphnia* treatments: $t_{(22)}=0.67$, $p=0.51$.

Linear regressions were calculated to test for significant differences between size class biovolume percentages in the "small" and "large" phytoplankton communities at the start of the experiment of all *Daphnia* treatments. Biovolume percentages after filtration may be described as a linear function of size classes, with the linear regressions being significant for both communities: "small" community: y=0.57 x+12.08, $R^2=0.56$, $F_{1,87}=111.91$, p <0.001; "large" community: y=0.29 x+7.85, $R^2=0.31$, $F_{1,85}=37.61$, p <0.001 (Fig. 1). The

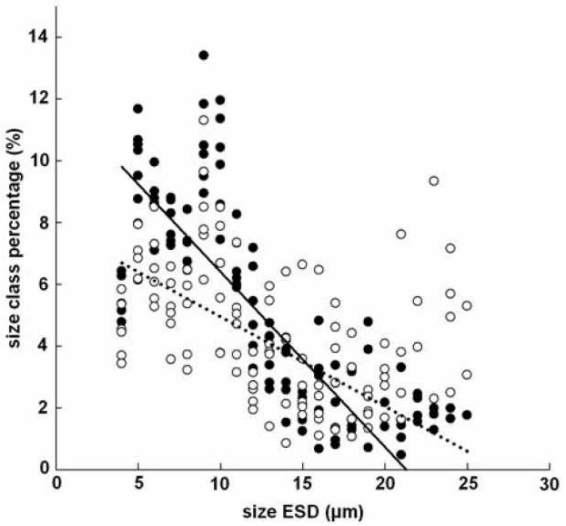

Fig. 1. Biovolume percentages of size classes in "small" (filled circles) and "large" (open circles) phytoplankton communities at the beginning of the experiment. Lines represent linear regressions: "small" community, y=0.57x+12.08, $R^2=0.56$, p<0.001 (solid line); "large" community, y=0.29x+7.85, $R^2=0.31$, p<0.001 (dotted line).

analysis of covariance revealed statistical differences in the biovolume percentage of size classes in both communities: slopes: $F_{1,172}=14.88$, p<0.001; intercepts: $F_{1,173}=15.02$, p<0.001. Therefore, filtration was successful, with the "small" phytoplankton community containing more small algae size classes (size <15 μm ESD), while the "large" community contained larger algae size classes (individual size >15 μm ESD).

Initial soluble reactive phosphorus (SRP) concentrations showed no significant differences between "small" (3.3±0.1 μg P L^{-1}) and "large" (3.1±0.1 μg P L^{-1}) phytoplankton communities in all 12 *Daphnia* treatments on day 1: $t_{(10)}=1.65$, $p=0.13$.

General conditions of the experiment

Dissolved nitrate (2.8 mg L^{-1}±0.1) and silicate (3.1 mg L^{-1}±0.2) were measurable in high concentrations at the end of the experiment in all enclosures, and therefore not limiting during the experimental duration. Water temperature was constant in all enclosures, averaging 17.4°C±0.03 at all depths. There was virtually no vertical temperature gradient, with the difference between temperature at the surface and maximum depth (10 m) being just 1.5°C±0.07.

Daphnia densities inside the cages averaged 4.7±0.6 ind L^{-1} based on total epilimnion volume. We found no significant differences in *Daphnia* densities between *Daphnia* "migration" and *Daphnia* "no migration" treat-

ment groups for both phytoplankton communities: "small": $t_{(4)}$=0.65, p=0.55; "large": $t_{(4)}$=1.07, p=0.35. Although control treatments were not initially stocked with *Daphnia*, some animals were present in the water, and a *Daphnia* population did develop. However, *Daphnia* densities in the control treatments were always less than 0.1 ind L^{-1}. Additional mesozooplanktonic organisms were, for the most part, excluded by the initial filtration, although some animals, mainly copepods, were found at densities of less than 0.1 ind L^{-1}.

Control treatments (empty cages)

Analysis using *t*-tests revealed no significant differences between migrating and non-migrating control treatments for any of the measured parameters: "small" communities: soluble reactive phosphorus (SRP) concentration: $t_{(4)}$=0.85, p=0.44; chlorophyll-*a* concentration: $t_{(4)}$=0.02, p=0.98; total phytoplankton bio-volume: $t_{(4)}$=0.07, p=0.95. "Large" communities: SRP concentration: $t_{(4)}$=1.84, p=0.14; chlorophyll-*a* concentration: $t_{(4)}$=1.81, p=0.14; total phytoplankton bio-volume: $t_{(4)}$=1.65, p=0.17. To evaluate the possible effects of the cages on large diatoms, we compared the silicate (SiO_2) concentrations between migrating and non-migrating empty cages, with no significant differences being found: "small" communities: $t_{(4)}$=1.74, p=0.16; "large" communities: $t_{(4)}$=0.91, p=0.41.

Experimental results

Nutrients

Two-way ANOVA indicated a significant interaction effect of phytoplankton community type and migration behavior on soluble reactive phosphorus (SRP) ($F_{(1,8)}$=5.67, p=0.044) (Fig. 2a). In the "small" communities post hoc analyses revealed no significant effects of migration treatments on SRP concentrations: 2.0±0.03 µg P L^{-1} in the "migration" treatments and 2.3±0.3 µg P L^{-1} in the "no migration" treatments. Post hoc analyses showed that the SRP concentrations in "large" communities were significantly higher in the "migration" (2.1±0.01 µg P L^{-1}) treatments than in the "no migration" treatments (1.5±0.02 µg P L^{-1}): p=0.036. When considering only the "no migration" treatments, SRP concentrations in the "small" communities were significantly higher than in the "large" communities (p=0.018).

Phytoplankton abundance

There was a significant interaction effect of phytoplankton community type and migration behavior on chlorophyll-*a* concentrations ($F_{(1,8)}$=7.01, p=0.029) (Fig. 2b). Again, in the "small" communities post hoc analyses showed no significant effect of migration treatments on

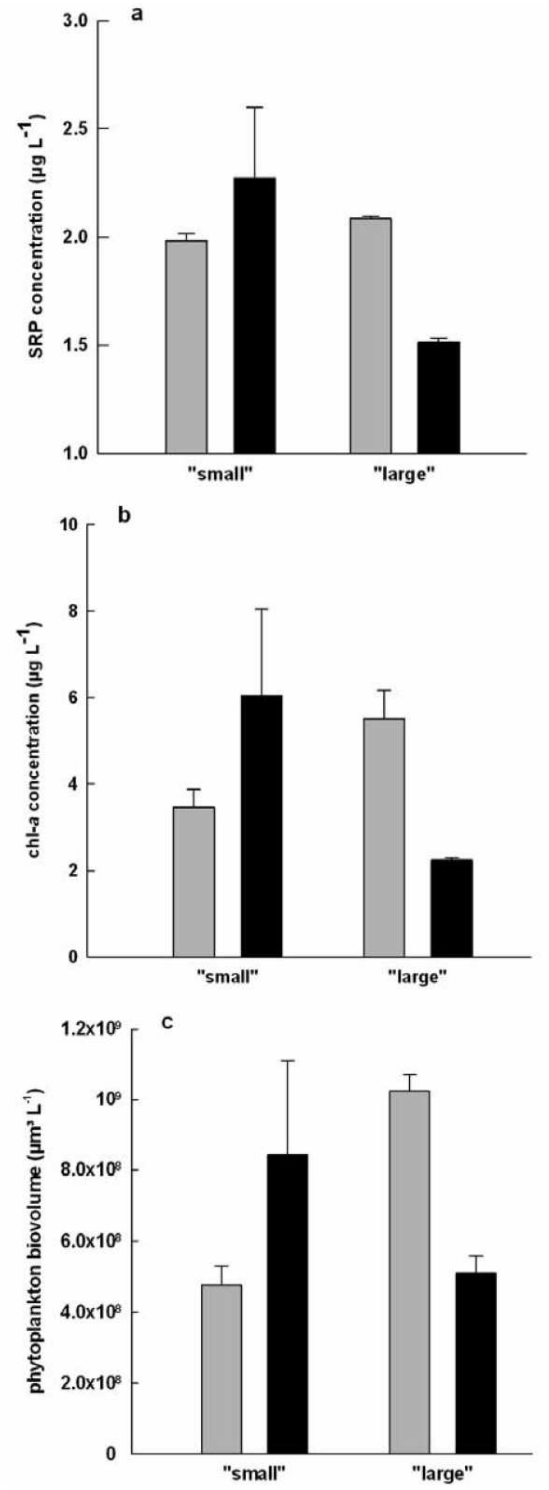

Fig. 2. Mean (±1 SE) SRP (soluble reactive phosphorus) concentrations (a), chlorophyll-*a* concentrations (b) and total phytoplankton biovolume (c) in *Daphnia* "migration" (light grey) and *Daphnia* "no migration" (dark grey) treatments of the "small" and "large" communities.

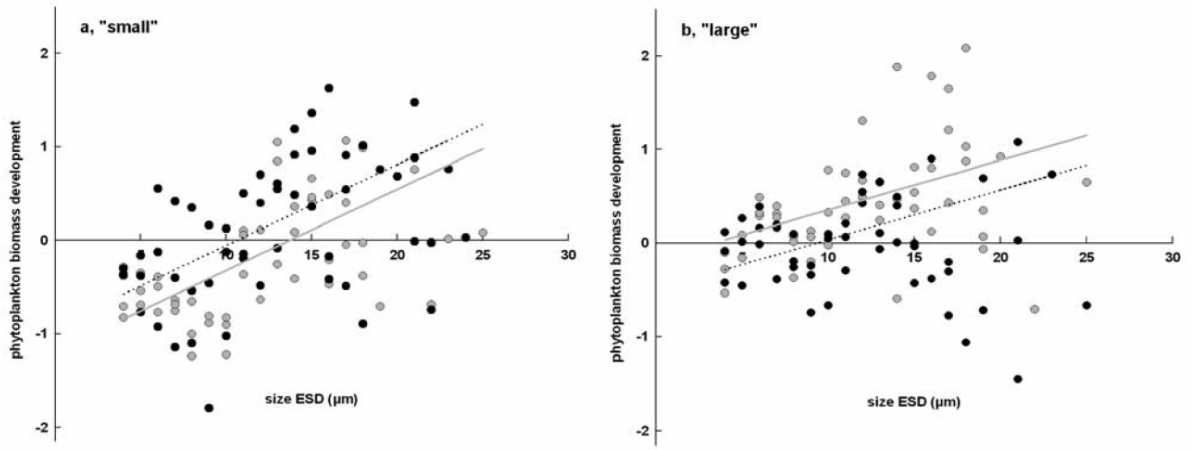

Fig. 3. Phytoplankton biomass size class development in "migration" (gray circles) and "no migration" (black circles) treatments of "small" (a) and "large" (b) communities. Lines represent combined linear regressions. "Small" communities: "migration" treatments, y=0.09 x-1.19; R^2=0.41 (gray line); "no migration" treatments, y=0.09 x-0.92; R^2=0.22 (dotted line). "Large" communities: "migration" treatments, y=0.05 x-0.18; R^2=0.27 (gray line); "no migration" treatments, y=0.05 x-0.50; R^2=0.07 (dotted line).

mean chlorophyll-*a* concentrations: 3.5±0.4 µg chl a L^{-1} in the "migration" treatments and 6.0±2.0 µg chl a L^{-1} in the "no migration" treatments. However, post hoc analyses indicated that in the "large" communities chlorophyll-*a* concentrations of the "migration" treatments (5.5±0.7 µg chl a L^{-1}) were significantly higher than in the "no migration" treatments (2.2±0.1 µg chl a L^{-1}): p=0.029. Also, when considering only the "migration" treatments, chlorophyll-*a* concentrations of the "large" communities were significantly higher (p=0.038) than in the "small" communities.

As with chlorophyll-*a*, there was a significant interaction effect of phytoplankton community type and migration behavior on total phytoplankton biovolume ($F_{(1,8)}$=7.55, p=0.025) (Fig. 2c). Post hoc analyses revealed no significant differences in mean total phytoplankton biovolume between migration treatments in the "small" communities: "migration" treatments ($4.6\times10^8\pm5.4\times10^7$ µm³ L^{-1}) and "no migration" treatments ($8.4\times10^8\pm2.7\times10^8$ µm³ L^{-1}).

Post hoc analyses showed, that in the "large" communities, biovolume in migration treatments ($1.0\times10^9\pm4.6\times10^7$ µm3 L^{-1}) was significantly higher than in the "no migration" treatments ($5.1\times10^8\pm4.8\times10^7$ µm³ L^{-1}): p=0.044. Also, considering only the "migration" treatments, phytoplankton biovolume of the "large" communities was significantly higher (p=0.030) than the "small" communities.

Phytoplankton community size dependent growth rates

To identify size dependent responses of both phytoplankton communities to the "migration" and "no migration" treatments, we analyzed the biomass development of phytoplankton *(r)* as a function of size.

Linear regressions of phytoplankton biomass development as a function of size in the "small" communities were significant for both migration treatments: "migration": y=0.09x 1.24, R^2=0.41, $F_{1,43}$=29.63, p <0.001; "no migration": y=0.08x 0.87, R^2=0.22, $F_{1,42}$=12.16, p=0.001. Analysis of covariance revealed no statistical differences between slopes ($F_{1,85}$=0.12, p=0.73), but there were statistical differences between the intercepts of the regression ($F_{1,85}$=15.02, p<0.001). These results allow a new calculation of linear regressions with a combined mean slope: "migration" treatments, y=0.09x-1.19, R^2=0.41, p <0.001; "no migration" treatments, y=0.09x-0.92, R^2=0.22, p=0.001 (Fig. 3a). The results indicate higher biomass development in the "no migration" treatments compared to "migration" treatments of the "small" communities. Additionally, growth rates were positive for phytoplankton species larger than 11 µm ESD for "no migration" treatments, whereas this was only the case for size classes larger than 14 µm ESD in the migration treatments.

Linear regressions of phytoplankton biomass development as a function of size in the "large" communities were only significant for "migration" treatments: y=0.07x-0.33, R^2=0.27, $F_{1,46}$=17.32, p<0.001. "No migration" treatments showed no significant relation-ship between biomass development and size: y=0.03x 0.23, R^2=0.07, $F_{1,36}$=2.53, p=0.12. Analysis of covariance revealed no statistical differences in the slopes ($F_{1,82}$=2.70, p=0.10), but there were statistical differences in the intercepts ($F_{1,83}$=9.88, p=0.002) between regressions. These results allow a new calculation of linear regressions with a

combined mean slope: "migration" treatments, y=0.05x-0.18, R^2=0.27; "no migration" treatments, y=0.05x-0.50, R^2=0.07 (Fig. 3b). The results indicate higher biomass development in the "migration" treatments compared to "no migration" treatments of the "large" communities. Additionally, biomass development was positive for all phytoplankton size classes in "migration" treatments. "No migration" treatments had no clear effect on size dependent bio-mass development in the "large" communities.

DISCUSSION

We experimentally manipulated the size distribution of a natural summer phytoplankton community in a small oligotrophic lake. We exposed the resulting communities to migrating and non-migrating zooplankton populations. In general, both phytoplankton communities responded with the higher growth of larger algae when exposed to grazing by *Daphnia*, which was indicated by the positive relationship between biomass development and algal size. This general response was similar between "migration" and "no migration" treatments, as shown by the similar slopes of the size-bio-mass development relationships. Nevertheless, zoo-plankton DVM had a different effect on phytoplankton growth, which was dependent on phytoplankton size structure.

We could observe a loss in total phytoplankton biovolume during our experiments caused by grazing; however, the grazing losses were to some extent counterbalanced by DVM related effects. Those effects were different between "small" and "large" communities, which can be seen on the different total phytoplankton biovolume patterns. Our hypothesis that different phytoplankton size distributions could affect the direction and strength of the community response to zooplankton DVM is therefore supported by the results. We were able to show experimentally, that the effects of zooplankton DVM on phytoplankton may be modified by phytoplankton size structure manipulations. However, our general expectations were mainly met by the results from treatments with the "large" communities.

The "large" communities followed the general predictions (stated in the introduction) that zooplankton DVM would cause higher phytoplankton abundance by promoting algae that are able to use the temporal refuge from grazing for growth. However, it seems that a full phytoplankton community size spectrum was necessary for zooplankton DVM to induce a refuge effect for algae. Phytoplankton only profited from zooplankton DVM in treatments containing large algae. However, contrary to the expectations stated in the introduction that mainly small algae should profit, larger algae also profited from "migration" treatments in the "large" communities. The results obtained from the "small" communities, which were mainly absent of large algae, suggest impacts to the contrary. For example, continuous grazing instead of discontinuous grazing resulted in higher phytoplankton biomass.

The results of the "small" treatments fit well to an earlier mesocosm study in the same lake, in which a non-manipulated phytoplankton community was exposed to zooplankton DVM (Haupt et al. 2009). In the above mentioned study, permanent grazing resulted in higher phytoplankton abundance by fostering small phytoplankton species with gelatinous sheaths. Theoretical concepts and empirical studies suggest that under oligotrophic conditions, the benefits of grazing mediated by nutrient recycling may balance or even overyield mortality related grazing losses (Sterner 1990; Elser, and Urabe 1999; Nugraha et al. 2010). Other possible explanations could be based on the interactions between microzooplankton, such as ciliates, and *Daphnia* (Juergens 1994). Phytoplankton communities consisting of mainly small algae suffering from serious predation by ciliates could benefit from the continuous presence of *Daphnia*, which are known to be able to drastically reduce microzooplankton biomass (Zoellner et al. 2003). Hence, more detailed studies are necessary to disentangle the different possibilities of how small, ingestible algae in natural lake communities are able to still profit from permanent grazing.

Since all other variables were controlled in the experiment, the observed differences in phytoplankton response to zooplankton DVM were directly associated with the manipulation of phytoplankton size structure. The phosphorus data also suggest that nutrient recycling by *Daphnia* appeared to be crucial for phytoplankton development in "small" treatments containing high proportions of small, algae. In the "small" treatments with continuous grazing, sustained removal of edible algae resulted in noticeably more dynamic nutrient recycling with higher phosphorus availability. Boersma, and Wiltshire (2006) showed that *Daphnia* excrete up to about 80% phosphorus as soluble reactive phosphorus (SRP), which means that higher nutrient recycling by grazing should be coupled with a higher release of SRP. This hypothesis is supported in our study, whereby significantly higher SRP concentrations in the "small" community "no migration" treatments compared to the "migration" treatments with discontinuous zooplankton grazing.

Obviously, the response of phytoplankton communities to zooplankton DVM was dependent on the presence or absence of large algae. The phytoplankton data, together with the nutrient measurements, indicate that the refuge effects of zooplankton DVM were stronger in "large" communities compared to the effects of nutrient recycling. Large algae have the potential to store nutrients more effectively, and remove larger parts of the dissolved phosphorus pool (Wen et al. 1997). Furthermore, their lower edibility would also lead to lower recycling of phos-

phorus in communities with a higher proportion of large algae. In direct contrast, small algae with lower storage abilities for phosphorus and higher edibility would foster higher nutrient turnover and recycling. Therefore, in communities mainly consisting of small algae the effect of nutrient recycling (which would be even higher in "no migration" treatments with constant grazing) may be more important than the refuge effects of zooplankton DVM. The observed size dependent interactions of zooplankton DVM with phytoplankton community structure support that both the refuge effects and size structure depend on nutrient recycling as the main drivers of how zooplankton DVM affects phytoplankton abundance.

The question arises in which kind of environment small or large algae are favored and influenced by zooplankton DVM. One important parameter determining the size of phytoplankton is nutrient availability (Sommer 2000): low nutrient levels promote small size classes of algae, whereas high nutrient levels result in the opposite. DVM in oligotrophic systems may therefore result in phytoplankton responses similar to our "small" treatments, while eutrophic systems are more likely to show responses towards DVM as our "large" treatments.

CONCLUSIONS

Diel vertical migration of zooplankton is a classic example of a so called trait mediated effect. Trait mediated effects describe trophic cascades that are not mediated by direct mortality but by the behavioral responses of herbivores through predators (Schmitz et al. 2004). Our experimental results suggest that the direction and strength of trait mediated effects may depend on the distribution of functional traits within a community. If functional traits, such as body size, determine the flow of energy and matter within trophic cascades, the distribution of these functional traits should also influence the strength and the direction of cascade flows. In our experimental system, algal cell size not only influenced direct mortality by grazers, but also the supply of dissolved nutrients available for total phytoplankton growth. Substantial dominance by small algae resulted in trait mediated trophic cascades that were different in strength and direction from that observed for the community in which size classes were more evenly distributed, and where large species were more common. Whether the indirect trophic cascade mediated by zooplankton DVM resulted in a positive or negative effect on the trophic level of primary producers, it was clearly a function of the size distribution of the phytoplankton.

Since trait mediated trophic cascades appear to depend on functional trait distributions within primary producer communities, significant alterations in environmental factors could severely affect conditions within lake ecosystems. Global warming may be one such factor. For example, increasing spring air temperatures could result in earlier stratification and spring algae blooms (Winder et al. 2004; Berger et al. 2010). In contrast, zooplankton dynamics are primarily governed by water temperature (Bottrell 1975; Reichwaldt et al. 2004) and only to a lesser extent by earlier stratification. Accelerated stratification processes caused by global warming may lead to an earlier "clear water" phase, which would lead to earlier phytoplankton community succession (Berger et al. 2010) from smaller fast growing spring species to larger and slower growing summer phytoplankton species. Therefore a significant grazing on bloom forming algae occurs only after a lag phase caused by zooplankton population development. That could result in a mismatch between phytoplankton and zooplankton cycles. If zooplankton misses the opportunity to capitalize on a highly edible spring bloom of small algal species, they may be forced to rely on less edible post-bloom phytoplankton communities. This negative impact on zooplankton growth could cascade to young fish, which consume zooplankton as a significant part of their diet. Therefore, the complex interaction between phytoplankton size structure, fish predation, and zooplankton DVM may adjust in response to increasing warming.

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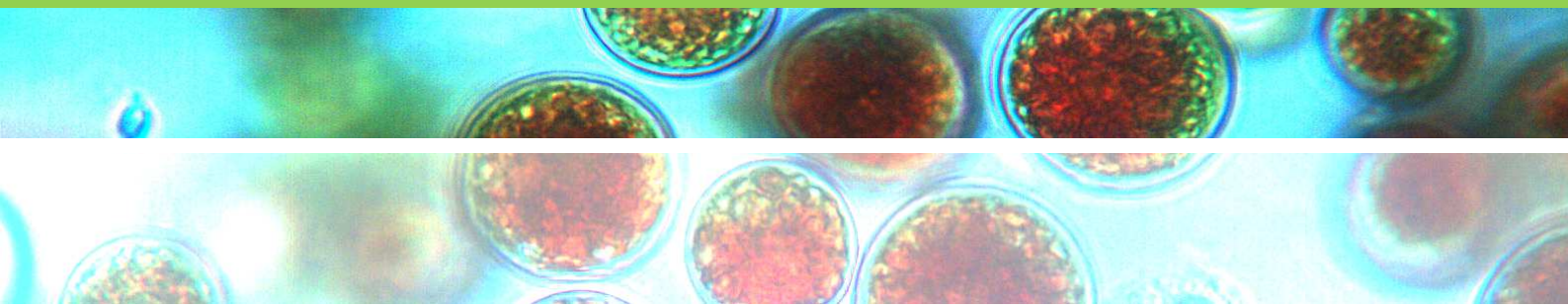
CHAPTER 6

ALGAL CULTIVATION TECHNIQUES FOR BIOMASS AND LIPID YIELD OPTIMIZATION IN *BOTRYOCOCCUS BRAUNII*

M. STOCKENREITER, F. HAUPT AND H. STIBOR

BIORESOURCE TECHNOLOGY, UNDER REVIEW



Abstract

Microalgae are currently in the focus of research for biofuel production systems. Finding the most productive microalgal strain and also the best way of cultivation is a topic that has bothered scientists for over two decades. However, the production of biofuel from algae is still not a commercial reality. *Botryococcus braunii* is currently one of the most promising microalgal species for biofuel production due to its very high lipid content. However, its growth rate is reported to be very low. In this study different cultivation systems (semi-batch, continuous) and growth conditions were compared in order to optimize simultaneously the growth and biomass lipid content of *B. braunii.* The same parameters were also investigated under conditions of nitrogen starvation. Population parameters, such as algal biomass production dynamics under different nutrient supplies, the biomass yield per unit of limiting nutrients and the lipid contents of biomass, were estimated. Finally, a cultivation method (two-stage cultivation system) was developed which allows high biomass and lipid production simultaneously in *B. braunii* cultures.

Key words: Two-stage, Nitrogen, Microalgae, Biomass, Biofuel.

1. Introduction

Algae have recently received a lot of attention as a new biomass source for the production of renewable energy. Several studies report that the most feasible biodiesel source for the US is microalgae (Chisti 2007; Scott et al. 2010). Algae grow rapidly and can double their biomass several times a day during exponential growth (Reynolds 2006). Additionally, algae are efficient producers of natural oils, can sequester carbon dioxide thereby reducing greenhouse gases, and do not compromise a food stock or deplete soil nutrients. Finding high lipid producing strains (Griffiths and Harrison 2009; Rodolfi et al. 2009) and selecting the appropriate culturing and processing conditions (Rodolfi et al. 2009; Brennan and Owende 2010; Huang et al. 2010) are critical for realising the potential and large-scale adoption of advanced algal biofuels. However, most of the results of the large-scale programs in the recent past were deemed to be uneconomical, because of the high culturing costs (Robertsen et al. 2011).

To achieve yield enhancements via cultivation mechanisms, attempts were made to develop new production culture techniques. Some of these trials were directed towards developing a continuous culture system for the production of aquatic organisms (Su et al. 2011; Wjiffles and Barbosa 2010; Csavina et al. 2011). However, current industrial microalgal mass cultivation systems mainly comprise batch cultures (static cultures), as this method has proven to be less complex. In static cultures, like batch cultures (bioreactors where a small number of organisms is added to a known amount of medium with no further additions of medium or remove of culture liquid), the algal culture growth results in a nutrient limitation over time and the biomass specific nutrient content in the algae decreases (Lampert and Sommer 2007).

Nutrient availability has a significant impact on the lipid- and fatty acid-composition of algae (Guschina and Harwood 2009). When placed in stressful environments (such as nutrient starvation), algae are able to switch their carbon allocation from reproduction to lipid production (Illman et al. 2000). Many studies have already shown that nitrogen-starved cells can contain significantly more lipids than non-starved cells (up to four times the amount) (Sheehan et al. 1998; Griffiths and Harrison 2009; Borowitzka 1988; Yamaberi et al. 1998; Alonso et al. 2000). The slower growth of algae due to starvation conditions leads to deposits of photosynthetic products into storage lipids instead of the synthesis of new membrane compounds (Thompsons 1996). However, the lipid content tends to be indirectly proportional to the growth rate (Metting 1996; Smith et al. 2010). Therefore, the net oil productivity can actually decrease in a stressful environment, even with the increased biomass-specific oil content due to reduced growth (Sheehan et al. 1998). Therefore, a new cultivation method is

needed to combine the advantages of biomass production with nitrogen starvation (see also Csavina et al. 2011).

In continuous cultures (like chemostats) fresh medium is continuously added while the culture suspension is continuously removed, which keeps the culture volume constant. Hence, the elimination rate corresponds to the flow rate, which simulates the mortality of the organisms (Lampert and Sommer 2007). The algal growth in continuous cultures can be kept close to the optimum, due to the possibility of maintaining conditions for exponential growth (Lampert and Sommer 2007). Chemostats were developed primarily to investigate microorganism growth, especially in bacteria and algae. However, chemostats are capable of producing high amounts of biomass if supplied with a constantly high nutrient supply. At the same time, these conditions will not allow a simultaneous increase of the carbon to nutrient biomass ratios, which indicates an increase in nutrient limitation, as typically seen in batch cultures that results in increased lipid production. It is obvious that there is a need for a better understanding of these metabolic processes (biomass and lipid accumulation) in order to design new cultivation methods which achieve a significant increase in algal lipid productivity via specifically controlled nutrient depletion (Sheehan et al. 1998).
Semi-static cultures, such as batch cultures (bioreactors), where a small number of organisms is added to a known amount of medium with the daily removal and addition of a distinct amount of medium, can be used to each a stationary growth phase and run algae into nutrient depletion at high densities. This distinguishes them from chemostats (with a constant influent and effluent amount), which are designed to maintain permanently high growth rates (log phase of culture growth). Nevertheless, semi-batch cultures are probably the most frequent type of reactor used on a commercial scale due the ease of handling, however the maintenance of a nutrient-limited (stationary growth phase) culture in a semi-batch culture can be challenging.

A combination of the two described cultivation systems (continuous and semi-batch) could provide a solution to overcome the usual mismatch between biomass accumulation and lipid yield. A two-stage cultivation system is suggested here in which algal growth and the subsequent algal biomass can be optimised in the first stage, while lipid productivity is maximised in the second stage. The first stage represents a continuous culture with optimal growing conditions for high biomass yields. The second stage represents a semi-batch culture, which could harvest a distinct amount of algal cells from the first stage once a day, leading them into nutrient starvation and therefore to higher lipid productivity levels. The described two-stage cultivation model was tested on a laboratory scale in terms of biomass

and lipid productivity, and was compared to the common single semi-batch culture conditions.

2. Methods

2.1. Microalgal cultures and culture medium:

The algal strain *Botryococcus braunii* Kützing (30.81; SAG, Göttingen; further described as *B.braunii*) used for the experiment is favoured in the literature as a highly suitable algal species for the lipid production of biofuel. The strain was pre-cultured over a period of several months prior to the experiment in a common growth medium, WC growth medium (Woods Hole Growth Medium; Guillard and Lorenzen 1972), which was used also in the experiment.

For optimal growth conditions during the experiment the unmodified WC growth medium was used, and for nutrient limitation in the experiment a nitrogen-limited growth medium was used, which was a modified WC growth medium.

2.2. Experimental set up:

2.2.1 Semi-batch cultivation:

B. braunii was cultivated in semi-batch cultures for two weeks with WC growth medium and a full nutrient supply. Afterwards, the culture was divided into three replicates and further cultivated with WC growth medium. The three replicates were then cultivated with a modified growth medium with reduced nitrogen ($1.6 \mu g L^{-1}$ NO_3^{2-}) supply. 15% of the cultures were replaced by the corresponding fresh medium. In all treatments, the initial total algal biovolume was set to be identical (1.0×10^6 fL mL^{-1}) at the beginning of the experiment. Each treatment was replicated three times, resulting in six cultures (300mL).

2.2.2 Two-stage cultivation:

In the first stage (chemostat; 300mL), an unmodified WC- growth medium was used to ensure high population growth. In the second stage (semi-batch; 300mL), which was inoculated daily with 15% (45mL) of algal culture grown in the first stage, the culture was supplied with a modified WC-growth medium with reduced nitrogen content ($1.6 \mu g L^{-1}$ NO_3^{2-}). The remaining amount of the effluent of algal culture of stage one was rejected. Simultaneously, the same amount (45mL) was removed on a daily basis from the second stage. In chemostat treatments, the initial total algal biovolume was set to be identical (1.0×10^6 fL mL^{-1}) at the start of the experiment. Each treatment was replicated three times resulting in a total of six cultures.

The temperature and light intensity for both set-ups was maintained at 20℃ and 100µMol photons m^{-2} s^{-1} with a 12 h:12 h light:dark cycle.

2.3. Measurements:

2.3.1 Biovolume:

Total algal biovolume and cell densities were estimated using a cell counter (CASY®- Cell-Counter, Schärfe-System) at the beginning and end of the experiment.

2.3.2 Lipid content:

The cell specific lipid content of algae was estimated by staining neutral lipids with Nile Red, and measuring fluorescence in an imaging flow cytometer (FlowCAM® Fluid Imaging Technologies). For staining algae, 1mg of fine-grained Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one; HPLC grade, Sigma Aldrich) was dissolved in 4mL of acetone (HPLC grade; Lee et al. 1998). Subsequently, 20µL of Nile Red solution was added to 5mL of algal solution and incubated for 30 min in a darkened container. Fluorometric analyses were performed immediately, using the FlowCAM® at an excitation peak wavelength of 532nm and an emission peak wavelength of 645nm (green laser). A fluorescence–lipid content calibration curve was fitted along a linear gradient of cell density of *Saccharomyces cerevisiae var. carlsbergensis*, which was suspended in WC-medium, with a cell-specific lipid content of $3.07x10^{-11}$g and a cell-specific fluorescence range of 2680-2800 units according to Stockenreiter et al. (2012).

2.3.3 Nutrient estimation:

The nitrate (NO_3^{2-}) concentration of the modified nitrogen-limited WC growth medium was measured by ion chromatography (Model 300, Dionex Corporation) and accounted for 1.6µg L^{-1} NO_3^{2-} at the highest value. Therefore, low nitrogen supply was guaranteed for the whole experiment in nitrogen-limited cultures. The amount for high nitrogen supply was throughout the whole experiment 14mg L^{-1} NO_3^{2-} .

2.4. Statistical analysis:

Data are presented as the mean ±1 standard error of the mean (mean ±1 SE). Each mean is calculated from three replicates of each culture.

Statistical analyses were completed using each value of the three replicates for each treatment. One-way analysis of variance (ANOVA) was used to compare differences in the mean values among treatment groups (between the two algal species and nitrogen supply stages). To meet statistical assumptions (Sokal and Rohlf 1995) data were transformed (log) where appropriate.

3. Results and Discussion

3.1 Biomass accumulation in semi-batch and two-stage cultures with different nitrogen supplies:

Total algal biovolume (fL mL^{-1}) of *B. braunii* decreased in semi-batch cultures from $1.35x10^{7}$fL mL^{-1}± $1.04x10^{7}$ in high nitrogen supply to $1.15x10^{5}$fL mL^{-1} ± $8.24x10^{3}$ in low nitrogen supply. However, despite the large mean difference, this decrease was not significant ($F_{1,5}$ = 5.31; p = 0.08; Figure 1) due to the high biovolume variances in treatments with high nitrogen supply. Nevertheless, the results show that *B. braunii* was strongly influenced by nutrient supply as less than 1% of the biomass established at high nitrogen supply was accumulated at low nitrogen supply.

However, in the two-stage cultivation system outlined here, where a continuous culture (first stage) was connected to a semi-batch culture (second stage), no biomass reduction was identified in the second stage (where nitrogen was reduced) with $2.54x10^{7}$fL mL^{-1}± $1.10x10^{7}$, compared to the first stage $2.28x10^{7}$fL mL^{-1}± $8.88x10^{6}$: $F_{1,5}$ < 0.001; p = 0.99 (Figure 2). Microalgae were harvested from the first stage where exponential growth conditions where prevailing, and the very low nitrogen concentration in the second stage (1.6µg L^{-1}) was then sufficient to maintain the high biomass accumulated in the first stage.

Additionally, in continuous cultures, the nutrient demand of microalgae was more controllable than in semi-batch cultures (Hirsbrunner 1988). Therefore an optimal microalgal biomass supply could be guaranteed with a continuous culture as a first stage, providing a continuous seed culture for a final lipid accumulation stage. The biomass production per unit of nitrogen was also different between the semi-batch cultures and the two-stage cultivation method. The accumulation of biomass of *B. braunii* per unit of nitrogen in low nitrogen treatments was significantly higher in the two-stage cultivation method ($1.59x10^{13}$fL $mg^{-1}(NO3^{2-})$ ± $6.85x10^{12}$)

compared to that in the semi-batch cultures ($7.21x10^{10}$fL $mg^{-1}(NO3^{2-})$ ± $5.15x10^{9}$: $F_{1,5}$ = 29.03; p = 0.006; Fig. 3).

A major factor to consider during the mass cultivation of microalgae is a high and stable biomass provision. *B. braunii*, which is one of the most favored algal strains due to its high lipid yield (Metzger and Largeau 2005; Zhila et al. 2005), is usually a very slow-growing algae. However, the results of this study show that the biomass and lipid production of this important species can be further optimised by combining two different algal growing methods.

3.2 Lipid accumulation due to nitrogen starvation:

A recent study showed that, in a similar set-up to that described here, a two-stage cultivation system can result in a higher algal biomass accumulation (Csavina et al. 2011). However, the study did not report results for the algal lipid production in the two-stage systems. Higher lipid concentrations per microalgal cell were observed in nitrogen-reduced treatments of the semi-batch and two-stage cultivation systems. The total lipid content of the cultures was then compared with low nitrogen supply in the semi-batch and second stage of two-stage cultivation, and a higher total lipid content ($4.22x10^{6}$pg mL^{-1} ± $8.80x10^{5}$) was found at a low nitrogen supply in the two-stage cultivation system compared to semi-batch cultures ($1.78x10^{4}$pg mL^{-1} ± $3.20x10^{3}$: $F_{1,5}$ = 18.65; p <0.001 (Figure 4)).

Hence, the question of whether the lipid yield increases only due to the biomass increase in the two-stage cultivation system or whether the cultivation system itself influences the specific lipid production of the algal cells arises. Mansour et al. (2003) showed that the lipid content of a specific algal species can increase two-fold from the logarithmic to the stationary growth phase. They stated that the maximum lipid content was obtained during stationary growth, which indicates that the second stage of a two-stage system should be maintained at conditions for stationary growth. However, these issues need further investigations.

3.3 Specific lipid content of B. braunii in semi-batch and two-stage cultivation systems:

The cell specific lipid content in the cultures of *B. braunii* was calculated in semi-batch cultivation systems and two-stage cultivation systems. Although the specific lipid content in two-stage cultures (0.38pg fL^{-1} ± 0.18) at low nitrogen supply was on average two-fold higher than in the semi-batch culture (0.158pg fL^{-1}± 0.02) with low nitrogen supply this difference was not significant ($F_{1,5}$ = 0,98; p = 0.38, Figure 5). Therefore, it was reasoned that the higher total algal lipid content resulted most likely from the higher biomass in the two-stage

cultivation system and was not influenced by the specific algal lipid content in different cultivation systems.

3.4 Two-stage cultivation systems as starting point for large scale hybrid systems:

The two-stage culture system presented here could be the cornerstone of a hybrid system consisting of two cultivation methods: a first stage would involve a PBR (photo-bio-reactor) functioning as a chemostat, and a connected open pond, with the second stage then being continuously inoculated by the effluent of the PBR. Nutrients for the first nutrient-rich PBR stage can be provided by a waste water plant (see Wu et al. 2012). For the second stage, where nutrient limitation of algal growth is preferable to induce lipid accumulation, purified nutrient-poor water of the treatment plant could be used. Besides the microalgal biomass and lipid production, such a waste water-based system would additionally provide an environmental service. Another advantage of the hybrid system would be the continued provision of a dense algae culture from the first stage as an inoculum, which is important for optimal growth and oil accumulation (Knoshaug and Darzin 2011).

The second stage of a hybrid algal growth system could be a cost-effective open pond, which is, however, prone to invasions from other species due to high exposure to the environment. A single very productive species might be then out-competed by other microalgal species and/or a diverse community could be established. However, this might not be a disadvantage for microalgal production systems. Stockenreiter et al. (2012) showed positive effects of diversity on the lipid production of microalgae. In their study, highly diverse communities produced significantly higher total lipid contents than monocultures, and the specific lipid content in these microalgal communities were over two-fold higher than those of monocultures. Furthermore, highly diverse communities are also thought to produce more temporally stable ecosystem services, due to complementary effects among species that perform similar ecosystem functions (Ptacnik et al. 2008; Tilman 1996) and are, therefore, more likely to provide a permanent stable supply of microalgal biomass in large scale production systems.

4. Conclusion

Over the last few decades great advantages have been made to develop large-scale cultivation systems for algae (Borowitzka 1999), resulting in different commercial production technologies. With the two-stage cultivation system suggested here a basic method for evolving large scale hybrid systems could be provided where high biomass and lipid production of microalgae can be guaranteed. Additionally, implications of diversity for microalgal biomass production (Stockenreiter et al. 2012) raise the question of whether the two-stage cultivation suggested here is feasible with diverse algal communities, an aspect which needs further investigation.

Acknowledgements

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Figure captions

Fig. 1:

Total algal biovolume (fL mL^{-1}) in semi-batch cultures of *B.braunii.* The black bar represents the mean total algal biovolume (±1 SE) in semi-batch cultures of *B.braunii* at high nitrogen supply; the grey bar represents the mean total algal biovolume (±1SE) of *B. braunii* in semi-batch cultures at low nitrogen supply at the end of the experiment. (ANOVA, $F_{1,5} = 5.31$; $p = 0.083$)

Fig. 2:

Total algal biovolume (fL mL^{-1}) in two-stage cultures of *B.braunii.*. The black bar represents the mean total algal biovolume (±1SE) in first stage (continuous) cultures of *B.braunii* at high nitrogen supply; the grey bar represents the mean total algal biovolume (±1SE) of *B. braunii* in second stage (semi-batch) cultures at low nitrogen supply at the end of the experiment. (ANOVA, $F_{1,5} < 0.001$; $p = 0.99$)

Fig. 3:

Total algal biovolume per nitrogen (f L $mg^{-1}(NO_3^{2-})$ supply of cultures of *B.braunii* in semi-batch cultures and two-stage cultures, both with low nitrogen supply. The black bar represents the mean total algal biovolume per nitrogen (±1SE) of *B. braunii* in semi-batch culture at low nitrogen supply; the grey bar represents the mean total algal biovolume per nitrogen (±1SE) of *B. braunii* in two-stage culture at low nitrogen supply. (ANOVA, $F_{1,5} = 29.03$; $p = 0.006$)

Fig. 4:

Total neutral lipid content (pg mL^{-1}) of *B.braunii* in semi-batch and two-stage cultures, both at low nitrogen supply. The black bar represents the mean total neutral lipid content (±1SE) of *B. braunii* in semi-batch culture at low nitrogen supply; the grey bar represents the mean total neutral lipid content (±1SE) of *B. braunii* in two-stage culture at low nitrogen supply. (ANOVA, $F_{1,5} = 182.65$; $p < 0.001$).

Fig.5:

Specific algal lipid content (pg mL^{-1}) of *B.braunii* in semi-batch and two-stage cultures, both at low nitrogen supply. The black bar represents the mean specific algal lipid content (±1SE) of *B. braunii* in semi-batch culture at low nitrogen supply; the grey bar represents the mean specific algal lipid content (±1SE) of *B. braunii* in two-stage culture at low nitrogen supply. (ANOVA, $F_{1,5} = 0.98$; $p = 0.38$).

Figure 1

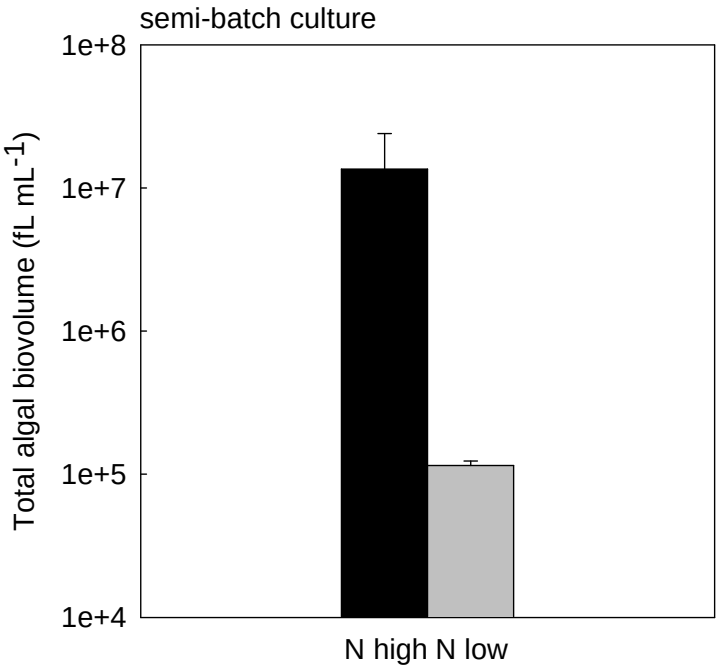


Figure 2

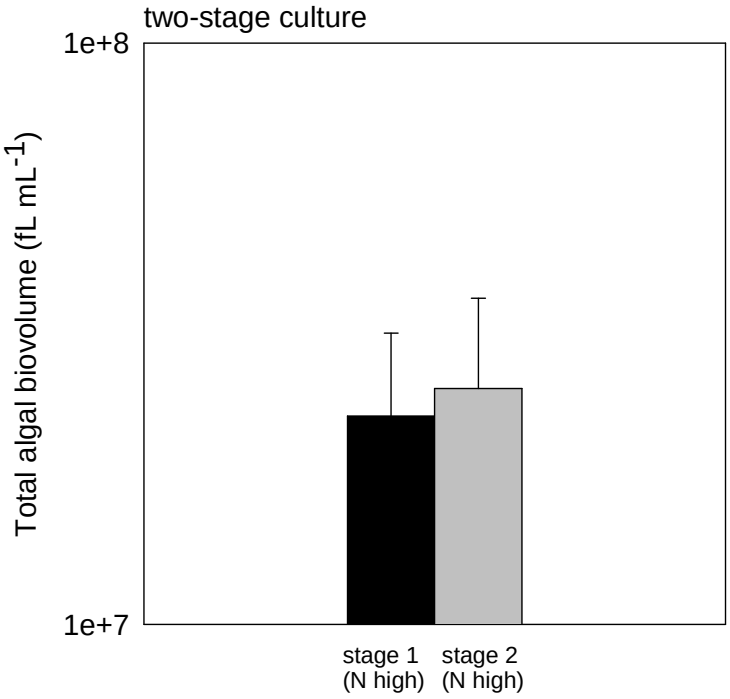


Figure 3

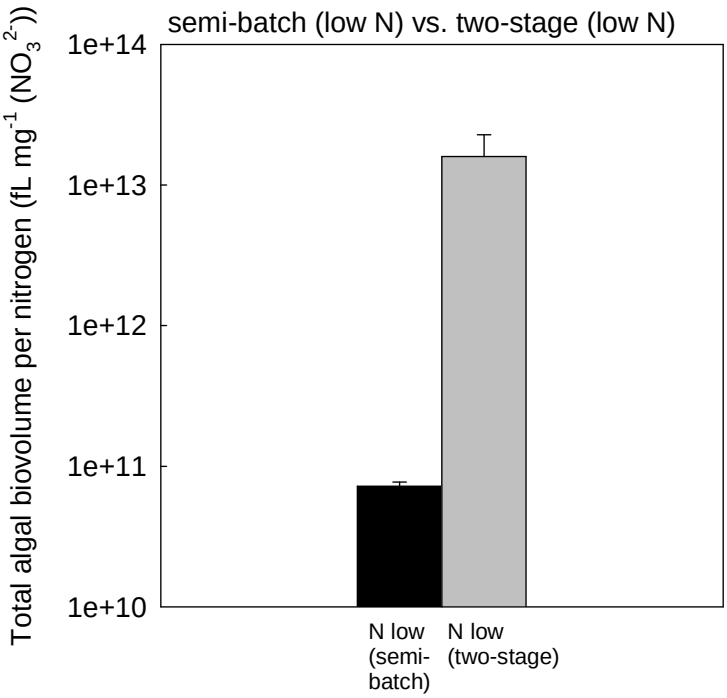


Figure 4

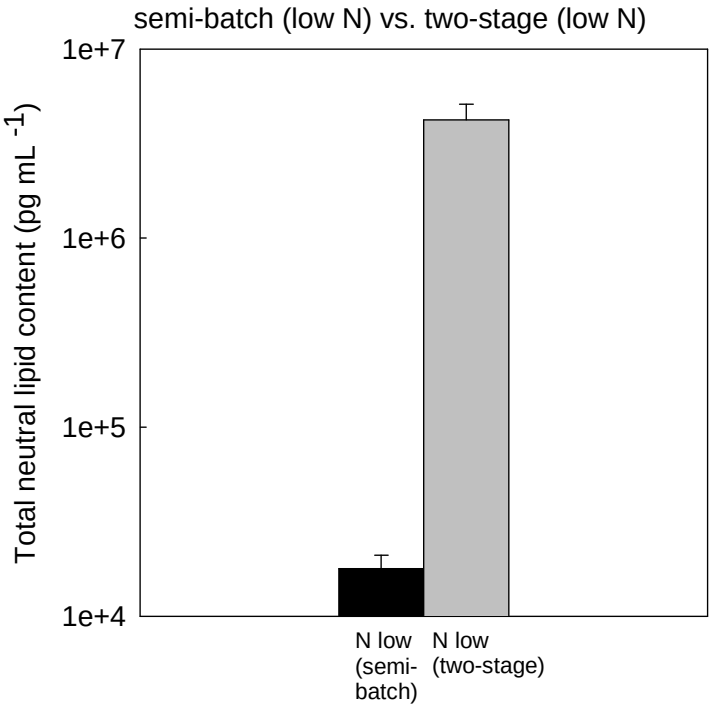
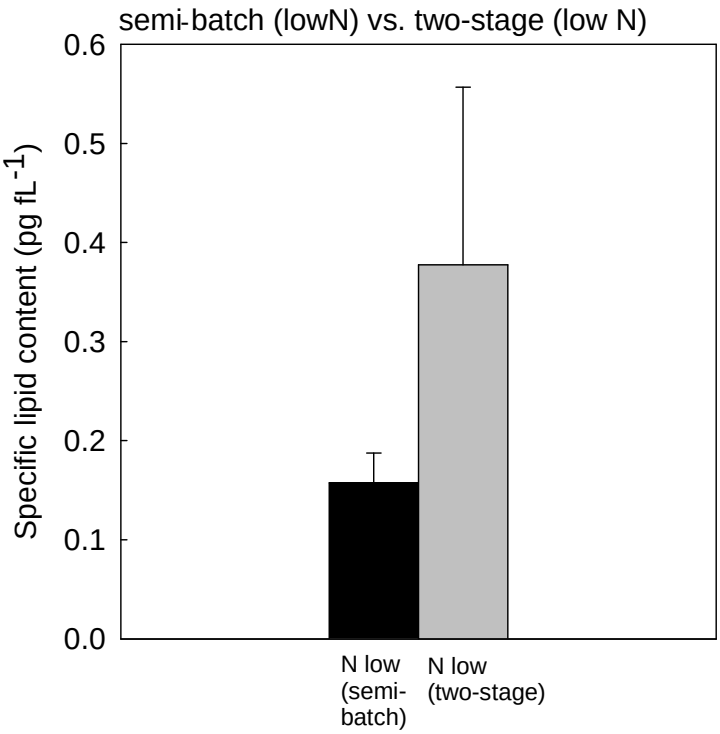


Figure 5


semi-batch (lowN) vs. two-stage (low N)
Specific lipid content (pg fL-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
N low (semi-batch)
N low (two-stage)

CHAPTER 7

GENERAL DISCUSSION

In my thesis, I have shown how ecological key principles and new analytical methods can be used to establish efficient microalgal systems for biomass and lipid production.

The chapters 2-6 showed that there are many “ecological” possibilities available to optimize the biomass and lipid production of microalgae without the need of (bio)technical engineering, e.g. genetic transformation. Preceding the general discussion of my results I will briefly discuss some of the methods, which I used in my study. Given that my experimental studies on the effect of diversity on the lipid production of microalgae are the first to be done, some methods to analyze important aspects of diversity-lipid production relationships had to be developed by myself.

LIPID CONTENT ESTIMATION – NILE RED AND THE FLOWCAM®

Bligh and Dyer (1959) developed a very traditional analysis of lipid content in living cells, which requires solvent extraction and gravimetric determination. Neutral lipid quantification requires separation of the crude extractions and quantification of the lipid fractions by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) or gas chromatography (GC) (Eltgroth et al. 2005). A major disadvantage to these conventional techniques is that they are expensive, time consuming and require intensive laboratory work (Elsey et al. 2007; Chen et al. 2009).

Since the identification of high-yield species for commercial production and an ongoing observation of the lipid content of these microalgae is important, it is timely to revisit a high throughput screening method for microalgal lipids (Elsey at al. 2007; Bertozzini et al. 2011). Rapid measurements are required for their integration into on-site quality control measurements. Therefore, increasing attention has focused on the *in situ* measurement of microalgal lipid content using the lipid-soluble fluorescent vital dye Nile Red (Fig. 7.1, 7.2; Cooksey et al. 1987; McGinnis et al. 1997; Lee et al. 1998; Elsey et al. 2007; Chen et al. 2009).

This method is simple and well established for the rapid determination of microalgal lipids (McGinnis et al. 1997; Eltgroth et al. 2005; Elsey et al. 2007). Lee et al. (1998) found a significant relationship between Nile Red fluorescence and the lipid content of microalgae (Fig. 7.1), and suggested that the method of staining microalgal cells with Nile Red is equally viable as the gravimetric method, which is commonly used for lipid determination (chapters 2, 3 and 6).

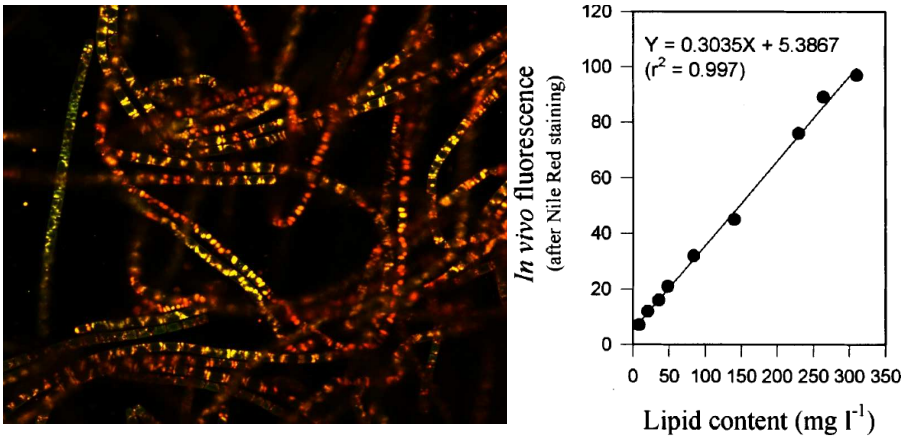


Fig. 7.1: Nile Red stained filamentous green algae. Yellow areas show Nile Red stained lipids (*The Plant Lab© HollySmith-Baedorf*) (left). Significant relationship between *in vivo* fluorescence after Nile red staining and lipid content of the green algae *Botryococcus braunii* (Lee et al. 1998) (right).

However, there are many modified methods for the use of Nile Red, especially for individual microalgal species and many different detailed instructions are described (McGinnis et al. 1997; Wawrik and Harriman 2010; Bertozzini et al. 2011). The inconsistency of the Nile Red method in determining the neutral lipid content in some green algae, due to the composition and structure of cell walls preventing the binding of Nile Red, in particular has led to intensive studies on the modification of the methods (Chen et al. 2009). In my analysis, I also faced this problem and used the simple physical process of freezing the samples before staining with Nile Red in order to make the cell walls porous. Pre-analysis (unpublished data) showed a higher Nile Red uptake in frozen samples.

A major problem lies in estimating the lipid content of single algal strains when grown in multi species communities. To overcome this problem I combined the above described modified method of Nile Red measurements with the ability of the FlowCAM® to work as an imaging flow cytometer, with the unique advantage of estimating the lipid content by image analysis of each algal cell. In this, I was able to estimate the lipid content (neutral lipids or triglyceride,

Fig. 7.2) of each microalgal cell in diverse communities without requiring the practically impossible physical separation of algal cells (chapters 2, 6).

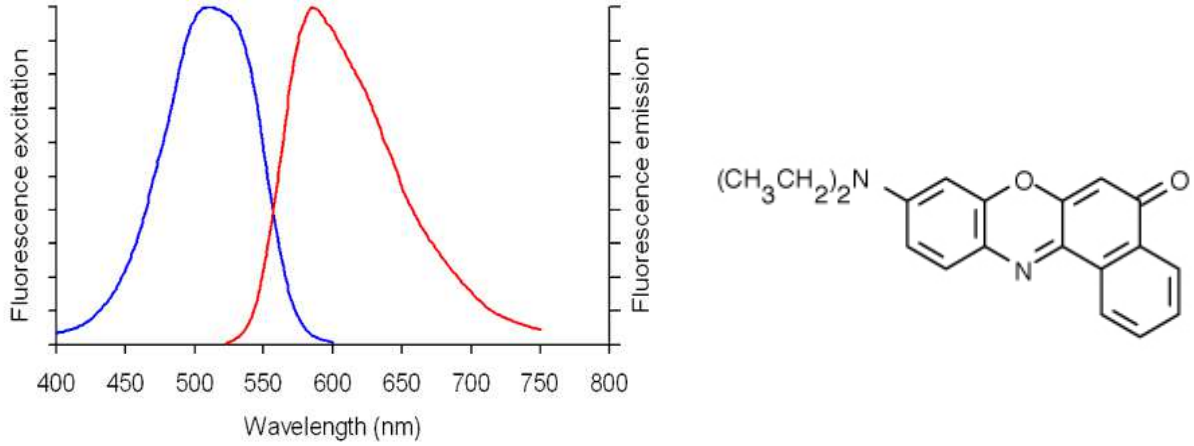


Fig.7.2: Nile Red fluorescence excitation and emission for triglycerides (left); chemical structure of Nile Red (9-diethylamino-5-benzo[α]phenoxazinone) (right) (© *Invitrogen*™).

However, to estimate the exact fatty acid composition of microalgal lipids, which is an important aspect for biofuel production for industrial aquaculture and for phytoplankton-zooplankton interactions, further analyses of samples by gas chromatography (GC; chapter 4) are essential.

EXPERIMENTAL SETUP – MICROCOSM COMMUNITIES

Currently, microalgal mass cultivation is conducted mainly in photobioreactors (PBRs) and open ponds. An intensive discussion is currently going on about which of these methods would be the best for cultivating microalgae for commercial biomass production. A combination of open ponds and PBRs is probably the most logical choice for cost-effective cultivation of single high yielding strains or diverse microalgal communities for biofuels. The inoculation of a specific stock culture of microalgae has always been a part of microalgal aquaculture. Open ponds are inoculated with desired microalgal strains that were invariably cultivated in a bioreactor, which could be a simple plastic bag or a high tech fiber optic bioreactor. The realization of industrial scale production of microalgal biomass faces many hurdles and its success may require progress and development in many scientific disciplines (Packer et al. 2011). All experiments described in the chapters 2-4 and 6 were conducted at the laboratory scale representing PBR conditions. However, it was not the aim of this work to investigate PBRs, though PBRs were the method of choice to investigate patterns behind diversity-lipid productivity relationships under controlled laboratory conditions.

DIVERSITY-PRODUCTIVITY RELATIONSHIP: THE ROLE OF DIVERSITY FOR MICROALGAL LIPID PRODUCTION

Smith et al. (2010) stated in their review on microalgal biomass production that the development of optimizations based on key ecological principles in addition to technical and/or genetic engineering may provide cost effective biomass yield enhancements. This potential has received only limited attention in aquatic biomass production. Currently, the optimization of yield enhancement is mainly accomplished by engineering modifications of production systems, or by biological engineering of single microalgal strains, e.g. the genetic modification of key processes of photosynthesis (e.g. Beckmann et al. 2009). Only 15 different species out of 40,000 identified species of microalgae are currently used for mass cultivation in industrial approaches (Wilkie et al. 2011). To what extend the diversity of a microalgal community influences the lipid production of these aquaculture systems has been completely underestimated until now.

Smith et al. (2010), however, suggested that there could exist a potential positive link between diversity and biomass/lipid production in diverse microalgal assemblages. First, this could be based on principles of ecological diversity-productivity theory, which proposes that highly diverse communities can produce more biomass than low diversity systems. I showed (chapter 2) that indeed communities with higher diversity showed higher primary production and therefore also accumulated higher biomass than monocultures (see also Ptacnik et al. 2008, Striebel et al. 2009b). The higher biomass production resulted also in higher lipid amounts (chapters 2 and 3). However diversity might not necessarily influence the biomass specific lipid content of microalgae. However, after a closer evaluation of my results, a significant increase in the biomass specific lipid content with increasing diversity was also measurable (chapter 2). To my knowledge, this is the first experimental evidence for a diversity-specific lipid productivity relationship in microalgae.

In the light of positive diversity-productivity relationships found in natural phytoplankton communities in Scandinavian and Bavarian lakes (Ptacnik et al. 2008; Striebel et al. 2009), I also assumed there might be a diversity-lipid productivity relationship in natural phytoplankton systems, which was supported by results from experiments described in chapters 2 and 3. Natural communities with shared evolutionary histories showed similar lipid production as laboratory communities assembled from highly selected microalgal strains. However, my assumption was that increasing diversity may lead to increasing lipid productivity also in natural communities. Therefore, it was necessary to cultivate these natural samples in the same way as the laboratory samples, with identical nutrient loading to exclude influences of different trophic levels (chapter 2).

DIVERSITY-LIGHT-LIPID RELATIONSHIP: LIPID PRODUCTION IN THE RIGHT LIGHT

The positive relationship of diversity and ecosystem processes is neither simple nor universal, meaning that diversity is not only simply species richness, moreover there are also functional richness and functional compositions describing the diversity of an ecosystem (Diaz and Cabido 2001; Hooper et al. 2005; Wright et al. 2006). Diaz and Cabido (2001) presented in their review, which tested 35 ecosystems of synthetic assemblages and manipulation of natural communities, and addressed all three components of diversity, that functional group diversity is probably the most important component of diversity for ecosystem functioning. The results of my study presented in chapter 3, provided again the positive link between diversity and lipid production in microalgae, however, mostly apparent at highest diversity levels. My results showed distinct differences between polycultures consisting of only one or several functional groups with respect to their lipid content. The results point towards the importance of functional diversity and not only species richness for maintaining diversity-productivity relationships.

The ecological diversity-productivity theory proposes not only that multi species ecosystems should produce higher biomass yields, but also that resources should therefore be used more efficiently (see also Ptacnik et al. 2008; Striebel et al. 2009b).

Light and nutrients are summarized under the concept of essential resources. Light as a key resource for photosynthesis is therefore irreplaceable for the mass cultivation of microalgae. Diversity in pigments and coincidental light use strategies is much higher in microalgae compared to terrestrial plants (Gantt and Cunningham 2001). This reduces an overlap in the used wavelength spectrum and promotes coexistence in microalgal communities (Stomp et al. 2004; 2007). Therefore increasing the diversity of microalgal species can result in an increasing diversity of pigments.

Ptacnik et al. (2010) expected higher trait variance in light acquisition than in mineral resource acquisition traits; hence higher microalgal diversity should maximize light use and thus carbon fixation. An assumption, which also covers the idea of Smith et al. (2010), that solar energy fixation, could be higher in diverse communities. With this background and my findings in the first two experiments (chapter 2 and 3), it seems obvious that one can suggest the increased absorbance of light by highly diverse communities as the basic mechanism behind increasing lipid production with increasing diversity. There was a significant correlation between absorbance and lipid production mediated by increasing functional diversity, though species richness *per se* was not a good predictor for the strength of the

diversity-light effect on microalgal lipid production, which supports arguments that functional group diversity often exceeds the impact of simple species richness (Cardinale et al. 2011).

Studies regarding the effects of light on lipid synthesis in microalgae have, until now, been restricted to different light conditions, showing that various light intensities are associated with alterations in cellular structures relevant for lipid synthesis. Several studies demonstrated that high light conditions can alter the lipid storage strategies of several algal species (Fabregas et al. 2004; Khotimchenko and Yakovleva 2004), but also low light conditions had an effect on algal lipids (Napolitano 1994). Nevertheless, in these studies, the availability of light was considered to be homogeneous and was only manipulated regarding intensity.

Light can also be seen as a heterogeneous resource, respective to its wavelength spectrum. A community including high functional diversity in absorbing light at different wavelength can use the full light spectrum more efficiently. Additionally, a recent review on microalgal lipid production revealed that the lipid production of 20 microalgal species was higher when grown in outdoor ponds than in the laboratory (Griffiths and Harrison 2009). This yield enhancement, due to the optimal exploitation of solar energy, might therefore demonstrate a very important ecological benefit for the use of outdoor ponds, even in areas with lower incidence of solar radiation (Fig. 7.3). Thereby, the ability of microalgae to survive across a wide range of environmental conditions is reflected by the tremendous diversity of cellular lipids as well as by the ability to modify lipid metabolism efficiently in response to changes in environmental conditions, e.g. light (Guschina and Harwood 2006).

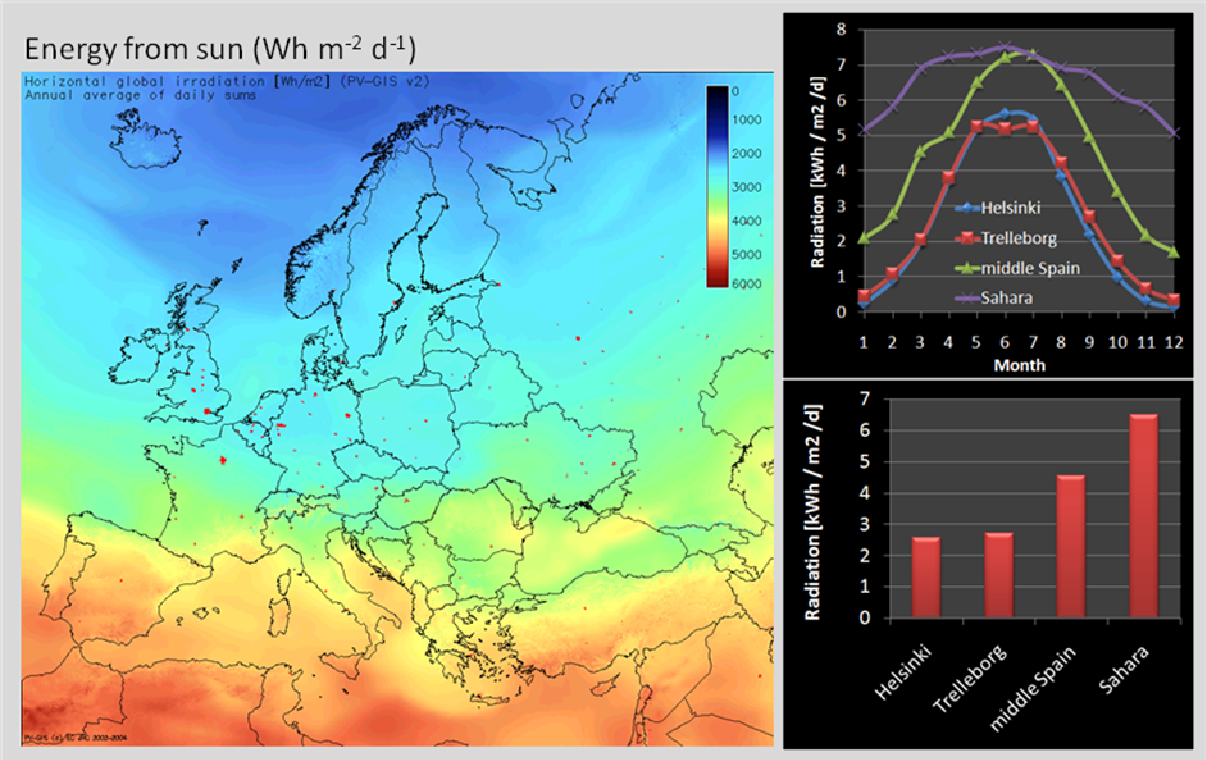


Fig. 7.3: Is there enough light? Available energy from sun in Europe (see presentation Jukka Seppälä at SubMariner project meeting: "Present and potential uses of algae - a cooperation event"; Trelleborg, Sweden.

CULTIVATING MICROALGAE: LIGHT AND NUTRIENTS

Microalgae as alternative energy resource should provide a net energy gain, and should produce large quantities of biomass without reducing food supplies (Hill et al. 2006; Smith et al. 2010). It is therefore essential to use sunlight as a freely available energy source for commercial algae production. Artificial light on the other hand allows continuous production, but needs an immensely higher energy input into the system. Additionally, for cultivating monocultures it is advantageous to provide only special wavelengths of the PAR spectrum for optimal production: e.g. diatoms generally have photosynthetic pigments including chlorophyll-*a* and -*c*, whereas chlorophyll-*a* and -*b* is mainly provided by green algae (Brennan et al. 2010). However, my experiments clearly show that the integration of diversity into concepts of biomass production can increase light use efficiency and can be based on functional niche properties (light can be considered as a cluster of resources for microalgae) of the individual species within a producer community (Fig. 7.4).

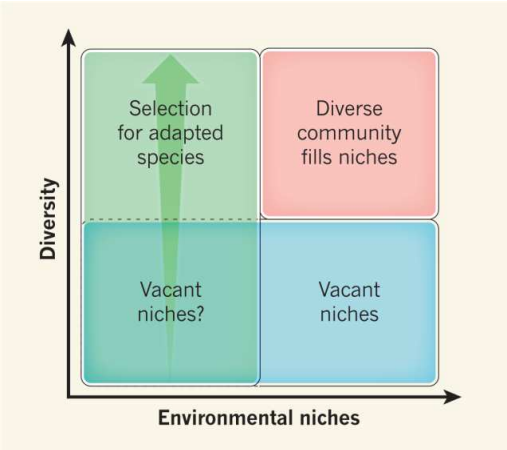


Fig. 7.4: Complex environments require a diverse community of complementary species (see Hector 2011).

Nevertheless, niche partitioning in functionally diverse communities, which are able to gather the photosynthetic relevant light spectrum more efficiently, raises the question of an underlying mechanism responsible for this positive diversity-light-lipid relationship. According to my results, diversity dependent productivity of microalgal communities is a result of the complementary use of resources (such as light), which is mainly responsible for biomass and lipid yield enhancement. The complementary use of light and the concurrent complementarity in lipid production in microalgae (chapters 2 and 3) indicates a clear link between diversity, light and lipid production in highly diverse microalgal communities.

Mineral nutrients are the second important class of resources for microalgal production. Usually a reduction of nutrients, especially nitrogen, seems to be an efficient mechanism to increase microalgal lipid concentrations (Shifrin and Chisholm 1980, Sheehan et al. 1998, Alonso et al. 2000). A major problem of this cultivation method is that cell division is stopped after reducing nitrogen supply and further biomass accumulation is not possible (chapter 6). In this way, a continuous and high availability of algal biomass for biofuel production is not guaranteed.

Genome sequence and transformation methods are currently utilized to facilitate investigations of the lipid biosynthesis of certain microalgal strains which permits genetic engineering strategies to further improve highly productive species (Radakovits et al. 2012). However, the use of genetic engineered microalgae may include a major problem for the natural environment: microalgae can easily escape culture environments due to their small size and their enormous dispersal abilities. Until know it has not been investigated whether genetically modified microalgae could affect dynamics of natural aquatic ecosystems

(Knoshaug and Darzins 2011). As shown in chapter 6, it is also possible to improve biomass and lipid production by modifying culture methods.

My experiments covered only monocultures, however, results will be similarly valid for polycultures as nitrogen starvation is frequently reported to increase lipid levels in a variety of algal species (Shifrin and Chisholm 1980, Sheehan et al. 1998, Alonso et al. 2000). A two stage cultivation allowed the simultaneous increase of both biomass and lipid production (chapter 6). The first stage enabled high biomass production, whereas the growth conditions within the second stage resulted in high lipid content. If a continuous cultivation of cultures with high biomass in the first stage can be assured, an almost constant supply of huge amounts of microalgae with concomitant high lipid content in the second step could be guaranteed (chapter 6).

DIVERSITY AND FOOD QUALITY: ADVANTAGES FOR AQUACULTURE FOOD WEBS

Microalgae are currently touted as a hope for renewable energies, however, microalgae have been used until now mainly in other commercial areas. The second largest market, after the dietary supplements and cosmetics market of microalgae, with 30 % of the produced algae biomass, is the feed market, with algae being mainly used as an additive to fish feed in aquaculture (DECHEMA Report 2012). The food quality of microalgae is therefore very important for the successful aquaculture farming of fish and/or zooplankton. Modern aquaculture normally provides special microalgal strains, which have several quantitative and qualitative properties (e.g., *Scenedesmus, Chlorella* or *Spirulina*). However, in natural ecosystems, zooplankton and fish are exposed to a variety of food organisms, which are normally living in communities assembled from a mixture of different species.

The food quality of microalgae is determined by its cellular composition of chemical elements, carbohydrates, lipids and proteins, etc.. In general, low carbon to nutrient (especially phosphorus and nitrogen) biomass ratios seem to result in high quality food for herbivorous zooplankton (Urabe and Sterner 1996; Hill *et al.* 2011). However, the food quality of microalgae is not only determined by its cellular composition of chemical elements, such as proteins, carbohydrates and lipids which are also highly important. Determining the factors that control the energy transfer at the plant-animal interface is a key issue in ecology, as this transfer is highly variable and, despite its global importance, is still not well understood. One major aspect of food quality is the biomass fatty acid composition in terms of the ω3-polyunsaturated fatty acids (ω3-PUFAs) of primary producers, as all animals are incapable of synthesizing them *de novo*.

In aquatic ecosystems it has been shown, that the system nutrient (phosphorus) amount correlates to the phytoplankton ω3-PUFA content (Müller-Navarra et al. 2004). The nutrient status of aquatic ecosystems also influences the diversity of their primary producers, which in turn will affect their resource use efficiency and productivity. However, the influence of diversity on phytoplankton food quality in terms of lipid composition (e.g. ω3-PUFAs) remains unclear. In chapter 4, I provided for the first time, experimental evidence that there is a significant influence of diversity of primary producer communities on their fatty acid composition, especially on essential ω3-PUFA content. Therefore the observed correlations between nutrients and phytoplankton fatty acid composition could be mediated by diversity. My results represent an initial point for studies regarding impacts of diversity on ecosystem functioning in artificial systems, like open ponds, beyond primary producer productivity in terms of carbon.

MICROALGAL BIOMASS CONTROL VIA GRAZING: IMPACT OF MICROALGAL SIZE

Open pond systems for microalgal mass production will, in particular, not contain a community of microalgae alone; herbivorous zooplankton will invade creating a simple, two trophic level food web, which can end up having strong day-to-day variance in microalgal biomass due to the grazing activities of the zooplankton (Smith et al 2010). A strong decrease of algal biomass could result in an unpredictable reduction in the mass cultivation of microalgae for biofuel production (Smith et al. 2010). Undesirable oscillations can be dampened by introducing zooplankton consuming fish species, following the ecological principle of the top-down control (Fig. 7.5).

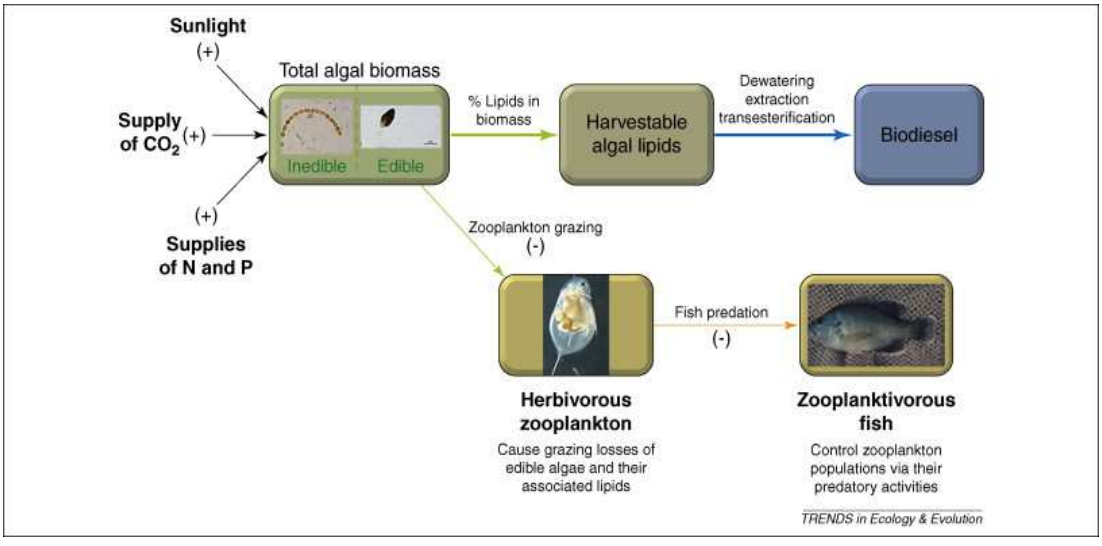


Fig.7.5: A simple artificial pond system is determined by bottom-up control of production by resource supply rate and the top-down control of biomass distribution via trophic cascades. Undesirable grazing losses of edible microalgae can be reduced by adding zooplanktivorous fish (see Smith et al. 2010)

However, Fig. 7.5 presents a simplified description of a pond food web, not taking into account size selective effects of grazing and nutrient recycling by zooplankton, which additionally may also show flexible migration behavior. In chapter 5, I provide a more detailed insight into a typical two trophic level food web including *Daphnia hyalina* and microalgae, which might extend the above described view of Smith et al. (2010). My results showed experimentally that food size selection and migration behavior of *Daphnia hyalina* can cause a shift from small sized microalgae towards larger species. Similarly, Shapiro and Wright (1984) demonstrated that *Daphnia* abundance and size can be promoted via biomanipulation (elimination of planktivorous fish), which resulted in a strong shift towards large, inedible species (Fig. 7.6).

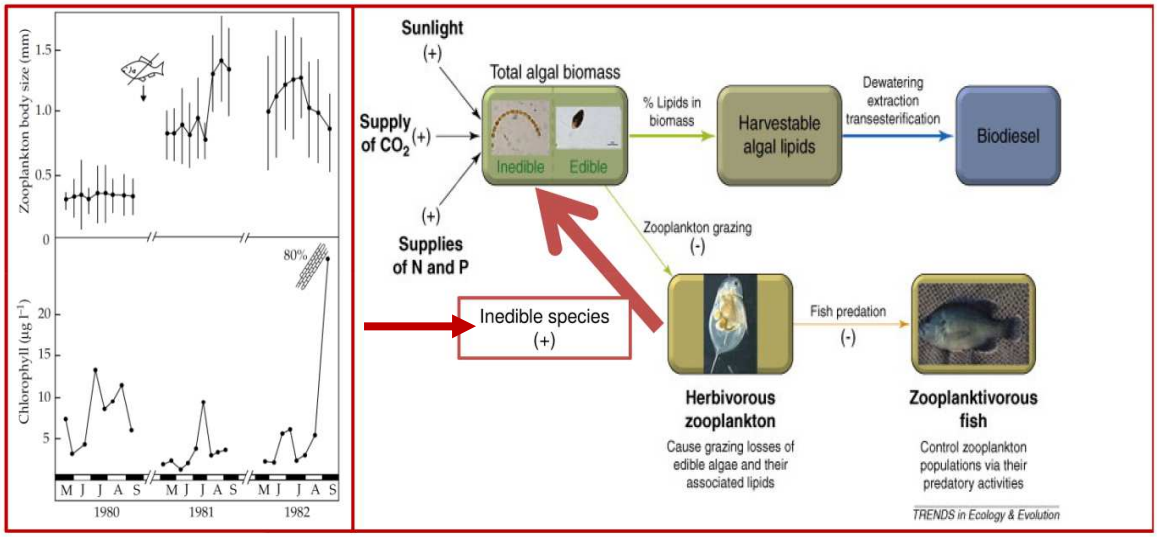


Fig. 7.6: Modified food web structure; Implications of zooplankton (*Daphnia*) on size structure of microalgae, as there could be losses of edible microalgae and therefore their lipids. Grazing of large bodied zooplankton might lead to larger, inedible microalgal species, which are fast sinking species suitable for uncomplicated harvesting (left side Lampert and Sommer (2007) after Shapiro and Wright (1984); modified food web structure after Smith et al. (2010)).

The intensity of the shift towards larger microalgal species in natural communities (a natural community is represented by “large” community in chapter 5) depends additionally on the migration behavior of *Daphnia hyalina*.

Daphnia often migrate between the surface of a lake where most of the population stays during the night, and deeper and darker waters layers, which are used during the day as a refuge from optical orientated fish predators (Zaret and Suffern 1976). Usually, ponds for microalgal mass cultivation are of shallow depth to allow light supply to most of the water column, additionally they seldom will be inhabited by fish species. Therefore *Daphnia* will probably not show a typical duel vertical migration behavior (as described in Lampert 1989) in these ponds. However, most of the data regarding the effects by *Daphnia* on

phytoplankton come from studies carried out in lakes where *Daphnia* normally migrate. Therefore, a transfer of this knowledge to open ponds should only be done with caution. Nevertheless, my experiments (which were the first studies investigating the effects of *Daphnia* on phytoplankton size structure simultaneously for migrating and non-migrating *Daphnia hyalina* populations), show that even non-migrating *Daphnia* are beneficial in promoting growth of large, fast sinking algae. As all major microalgal groups include large, fast sinking species, quality of lipid profiles can still be influenced by selecting growth conditions (for example by resource supply ratios) for desired microalgal groups, depending on commercial needs.

In conclusion, lipid and biomass productivity are benchmarks for microalgal aquaculture. My findings presented in chapter 2-6 may provide a first step to overcome ecological hurdles before the large scale production of microalgae for biofuels and other applications can become reality in a commercial context.

CHAPTER 8

OUTLOOK

So far in my work I have discussed some general ecological principles for biomass and lipid production of microalgae, in the following I will point out some areas which need to be addressed for economically viable and sustainable commercial scale of microalgal cultivation for biomass and lipid production.

TRAIT-BASED APPROACHES FOR MICROALGAL LIPID PRODUCTION

The observation that diversity leads to higher lipid yields presents an ecological method to increase production, but which has the potential to be optimized. Distinct species combinations might represent higher yields than others. Therefore, it is important to identify relevant traits and trade-offs in microalgae, which in general helps to explain the mechanism of species coexistence and diversity (Litchman et al. 2010). Different environmental conditions, such as different light intensities, light colors and nutrients have distinct impacts on microalgal composition. Therefore, it is advantageous to identify, which species occur at which environmental conditions to provide a modular system to allow assembly of microalgal communities suitable for different growth environments. Communities assembled by *a priori* analysis of how microalgal traits match growth conditions might provide a predictable growth system for a guaranteed supply of biomass and lipids. Trait-based approaches are being already used in terrestrial plant ecology (Lavorel and Garnier 2002; Westoby and Wright 2006), hence such approaches must also be included in microalgal biomass and lipid production.

PHYSIOLOGICAL CONTROLS OF MICROALGAE - USING THE RIGHT MICROALGAL MIXTURE

I could show that diversity promotes lipid quantity and -quality in microalgae by using light more efficiently, however, microalgal lipid production is also very sensitive to nutrient limitation patterns. Optimal nitrogen (N): phosphorus (P) ratio for optimal growth is very species specific and can vary between 20:1 and 50:1 (molar ratio) (Guilford and Hecky 2000;

Geider and LaRoche 2002). Microalgal biofuel production systems including combinations of species varying in their stoichiometric (carbon) C:N:P ratios could be used to maximize lipid production per unit of limiting nutrient. Therefore, further experiments with different nutrient supply ratios, given either continuously or in pulses, are definitely needed to evaluate the possibility of controlling microalgal diversity and thereby lipid production by resource supply management.

As described in my thesis, light can be a very heterogeneous resource in terms of quality. However, the quantity of light depends on daily, and also seasonal, changes influencing the productivity of microalgae in open ponds. Future studies should test experimentally how microalgal traits associated with light use can be included in microalgal biomass production. Similar to the above described variations in biomass stoichiometry, variations in light adaption (for example high light adapted microalgae growing on snow, low light adapted microalgae growing in deep waters), could be used to optimize light use efficiently in environment specific trait-based assembled microalgal communities.

A modular system based on both light and nutrient uptake specific traits of microalgae might provide a promising method to optimize microalgal cultivation for commercial uses and for particular environmental conditions.

ALTERNATIVE SOURCES OF RESOURCE SUPPLY

The supply of nutrients and freshwater to the place of production of microalgal mass cultivation definitely influences its economical feasibility. The combination of wastewater treatment and large scale microalgae cultivation may be a solution for large scale bioenergy and/or biofuel production in the future. Wastewater streams can originate from households, industry and poultry. Wastewater streams are usually very rich in macronutrients, such as phosphorus and nitrogen, as well as in essential trace metals necessary for photosynthesis. Those nutrients in wastewater streams are derived without direct costs and have to be removed because they are responsible for the eutrophication of fresh and seawater ecosystems. As a last, but also very important point, the nutrients are already suspended in the water which is the most important resource for the growth of microalgae. Therefore, the use of wastewater streams for the cultivation of microalgae has a high economical potential and provides an ecological service at the same time, therefore definitely warranting further investigation.

MAINTAINING DIVERSITY - USING THE EFFECTS OF DISTURBANCE

To include diversity into commercial microalgal growth systems, it has to be determined for how long a gradient of diversity would persist in microalgal cultures and which parameters could promote high diversity over longer time periods. Several mechanisms are important for maintaining diversity, including spatial and temporal heterogeneity. In general, disturbance in intermediate frequency and intensity positively influences diversity. Disturbances can include fluctuations in resource supply (light and nutrients) or physical parameters (e.g. turbulence). Diversity promoting factors therefore differ between photobioreactors with constant environmental conditions and open pond systems with higher disturbance regimes. Future studies should therefore test the importance of environmental disturbances in maintaining microalgal biodiversity and its effect on both biomass quantity and quality in microalgal biomass and lipid cultivation systems.

AQUATIC FOOD WEB CONFIGURATION – MULTI-TROPHIC EFFECTS ON MICROALGAE

Open pond systems, which are the only cost effective way of microalgal mass production until now, are exposed to a perpetual biological input because ponds cannot be closed to the environment. Therefore, microalgae in open ponds are sooner or later affected by invasions of herbivorous zooplankton resulting in multi-trophic food webs. To my knowledge, most of the studies regarding mass cultivation of microalgae lack estimation of the potentially positive effect of introducing intentional food webs, as these commercial systems will mostly follow the same ecological principles (bottom-up control via resources and top-down control via grazers/predators) as natural systems. However, it is important to realize that artificial systems will exhibit more strongly compressed aquatic food webs than most natural lakes, resulting in communities being more sensitive to dynamic instabilities.

Therefore, the complex ecological interactions between microalgal communities and herbivorous zooplankton have to be studied in artificially assembled commercial food webs in more detail, in order to be able to integrate these interactions into commercial production systems to optimize biomass production. Additionally, future studies should provide a more detailed insight into how many trophic levels are needed (e.g. adding fish or not), to produce high yields of biomass and therefore lipids.

UPSCALING - FINALLY, THERE IS NO WAY AROUND

Currently, microalgal biofuel is not economically sustainable. Compared to fuels from crude oil it is too much expensive. Several aspects of the production chain such as harvesting and nutrient supply are still to cost-intensive. Microalgal biofuel can only be successful if the price decreases. In my work I have investigated possible ecological optimizations of microalgae production, which might supersede optimizations based on technical engineering, such as the construction of sophisticated, expensive photobioreactors, genetic engineering or complex artificial light supply. Due to the initial stage of this research field, most studies are still performed in small laboratory scale systems. However, to make the whole process economically feasible, upscaling is inevitable. Before transferring the knowledge of ecological optimizations of microalgal communities obtained in small scale studies towards large scale production processes, it is necessary to test experimentally whether the mechanisms observed in the laboratory have the same impact in large scale treatments.

> *"Combining ecological principles with insights from the disciplines of algal physiological ecology, limnology, and ecological stoichiometry can provide important new guidance for the design and successful operation of microalgae-based biofuel production systems."*
>
> *Smith et al. 2010*

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PERSONAL NOTES

Name: Maria Stockenreiter

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EDUCATION

Ludwig-Maximilians-University, Aquatic Ecology Group, München, Germany:

Ph.D. student in Biology. **Since 04/2008**

Dissertation: “Ecological optimization of biomass and lipid production by microalgae”. Supervisor: Prof. Dr. Herwig Stibor.

Ludwig-Maximilians-University, München, Germany:

Diploma in Biology (very good) **09/2007**

Diploma thesis: “The influence of diel vertical migration (DVM) of *Daphnia* on the phytoplankton community – main focus: hypolimnion.” (in German)

Main subject: Ecology; Minor subjects: Zoology and Neuroscience.

Ludwig-Maximilians-University, München, Germany:

Basic study period in Chemistry **10/2000- 10/2002**

Karolinen- Gymnasium, Rosenheim, Germany:

Matriculation standard (Allgemeine Hochschulreife) **06/2000**

RESEARCH EXPERIENCE

Ludwig-Maximilians-University, Aquatic Ecology Group, München, Germany:

Research assistant in the group of Prof. Dr. Herwig Stibor. **11/2011 - Present**

SYKE Finnish Environment Institute, Marine Research Laboratory, Helsinki, Finland:

Invited and salaried associate scientist in the group of Dr. Timo Tamminen.

08/2011 – 11/2011

Planned, performed and analyzed experiments with microalgae in waste water streams for biomass and biofuel production in the projects ALGISEL (Algae for biodiesel production, Finland) and SubMariner (Sustainable use of Baltic marine resources, international).

Ludwig-Maximilians-University, Aquatic Ecology Group, München, Germany:

Research assistant in the group of Prof. Dr. Diehl and Dr. Herwig Stibor.

08/2008 – 07/2011

Full responsibility for the project **„LIPIDO – Optimizing lipid production by planktonic algae".** International project in the N-INNER (Northern European Innovative Energy Research) program with partners from public (SYKE Finish Environment Institute, Finland, University of Oslo, Norway, University of Science and Technology, Norway, Ludwig-Maximillians-University München, Germany) and private research organization (VTT Technical research centre of Finland, Finland) and industry (Blue Lagoon Research Group, Iceland). Planned, performed and analyzed all experiments and managed all administrative issues including a one month stay in Finland for joint experiments and SYKE.

DFG, german research fundation project – Auswirkung der tagesperiodischen Vertikalwanderung von Zooplanktern auf die Lebensgemeinschaft des Pelagials:

Associate scientist **04/2007 – 03/2008**

Conducted and analyzed experiments with large field mesocosm experiments and laboratory experiments. Project was funded by DFG, German research foundation. Expert in counting freshwater phytoplankton organisms.

HydraLab III project, Hopervagen, Norway:

Associate scientist **08/2009**

Operated marine enclosure mesocosm infrastructure, planned and analyzed experiments with marine plankton communities in this international project (ICBM University of Oldenburg Germany; WasserCluster Lunz, Austria; University of Salzburg, Austria; Ludwig-Maximilians-University München, Germany; IUEM, Technopôle, Brest-Iroise, France).

IUEM, Technopôle, Brest-Iroise, France:

Visiting scientist **08/2009 – 09/2011**

Several research visits at IUEM, Brest for conducting several experiments, data analyzing and conferences.

Max-Planck-Institute for Limnology, Plön, Germany:

Visiting Scientist **09/2006**

Conducted experiments in the “Plankton Towers” with labeled (33P) algae and migrating zooplankton communities.

TEACHING EXPERIENCE

Ludwig-Maximilians-University, Aquatic Ecology Group, München, Germany:

Teaching assistant for ecology and limnology internships **09/2007 - Present**

Prepared course material for lectures and practical work in the field and laboratory. Instructed students during experiments and analyzes.

Supervision of Diploma, Master and Bachelor students **04/2008 - Present**

Planned and performed experiments with students, supplied help during analyzing data and reviewed their thesis.

Ludwig-Maximilians-University, Limnological Research Station, Seeon, Germany:

Project day “ Limnology” for schools, lecture and practical course **04/2008 - Present**

Created and taught a curriculum in theoretical and practical limnology with comprehensive course materials.

Instruction for bog-guides, lecture and practical course. **05/2011**

Created and taught a curriculum of Limnology for bog-guides for the Austrian nature conservation organization NATOPIA.

PUBLICATIONS

Peer reviewed

Haupt, F., **Stockenreiter, M.**, Boersma, M. and Stibor, H. (2012) The effect of phytoplankton size structure on its response to *Daphnia* diel vertical migration. *Journal of Limnology*, **71**, 125-134.

Stockenreiter, M., Graber, A.-K., Haupt, F. and Stibor, H. (2012) The effect of species diversity on lipid production by micro-algal communities. *Journal of Applied Phycology*, **24**, 45-54.

Haupt, F., **Stockenreiter, M.**, Reichwaldt, E. S., Baumgartner, M., Lampert, W., Boersma, M. and Stibor, H. (2010) Upward phosphorus transport by *Daphnia* diel vertical migration. *Limnology and Oceanography*, **55**, 529-534.

Haupt, F., **Stockenreiter, M.**, Baumgartner, M., Boersma, M. and Stibor, H. (2009) *Daphnia* diel vertical migration: Implications beyond zooplankton. *Journal of Plankton Research*, **31**, 515-524.

Manuscripts for peer reviewed journals

Stockenreiter, M., Haupt, F., Graber, A.-K., Seppälä, J., Spilling, K., Tamminen, T. and Stibor, H. (2012) Functional group richness: Implications of diversity on light use and lipid content in micro-algae for biofuels. *Journal of Phycology,* in revision.

Stockenreiter, M., Haupt, F., Enss, D., Seppälä, J., Spilling, K., Tamminen, T. and Stibor, H. (2012) Phytoplankton diversity influences algal fatty acid composition – Implications for the aquatic food web. *Functional Ecology,* under review.

Stockenreiter, M., Haupt,F. and Stibor, H. (2012) Algal cultivation techniques for biomass and lipid yield optimization in *Botryococcus braunii*. *Bioresource Technology,* under review.

Non peer reviewed

Stockenreiter, M., Haupt, F. and Stibor, H. (2011) Optimising microalgal lipid production: enhanced cultivation methods and effects of species diversity. In: Algae: the sustainable biomass for the future. *Perspectives from the Submariner project algae cooperation event.Trelleborg, Sweden – September 28-29, 2011*.

Stockenreiter, M., Haupt, F. and Stibor, H. (2011) Optimizing lipid production in planktonic algae. In: Optimizing lipid production in planktonic algae: LIPIDO. Final report. Edited by Kristian Spilling.

Striebel, M., Ptacnik, R., Stibor, H., Behl, S., Berninger, U., Haupt, F., Hingsamer, P., Mangold, C., Ptacnikova, R., Steinböck, M., **Stockenreiter, M.**, Wickham, S. and Wollrab, S. (2010) Water column stratification, phytoplankton diversity and consequences for resource use and productivity. *Proccedings of the HYDRALAB III joint user meeting, Hannover, Febuary 2010*.

PRESENTATIONS

Talks

Diel, S., Striebel, M, Behl, S., **Stockenreiter, M.** And Stibor, H. (2011) Spectral niche complementarity and the diversity-productivity relationship in phytoplankton. 96th ESA annual meeting, Austin, Texas. **INVITED TALK**.

Haupt, F., **Stockenreiter, M.**, Boersma, M., and Stibor, H. (2011) Does diel vertical migration of *Daphnia* influence the phytoplankton community structure? ASLO aquatic science meeting, San Juan, Puerto Rico.

Stockenreiter, M., Graber, A.-K., Haupt, F. and Stibor, H. (2011) The effect of species diversity on lipid production by micro-algal communities. ASLO aquatic science meeting, San Juan, Puerto Rico.

Stibor, H., **Stockenreiter, M.** and Haupt, F. (2011) Integrating ecological concepts into industrial biomass growing systems. ENSIETA , Brest, France.

Stockenreiter, M., Haupt, F. and Stibor, H. (2011) Enhancing the lipid production of micro-algal communities – the effect of species diversity. Submariner meeting, Trelleborg, Sweden.

Spilling, K., Seppälä, J., **Stockenreiter, M.**, Haupt, F., Rischer, H., Enss, D. and Tamminen, T. (2011) Growing algae in Northern Europe: possibilities and limitations. ALGIESEL meeting, Göteburg, Schweden.

Stockenreiter, M., Steinböck, M., Haupt, F., Peterson, K. and Stibor, H. (2010) FlowCAM Technology- Optimizing lipid production by planktonic algae- Microalgae as energy source. NAA meeting, Houston, USA. **INVITED TALK**.

Stockenreiter, M., Haupt, F., Graber, A.-K. and Stibor, H. (2010) Optimizing lipid production by planktonic algae - Does diversity enhance micro-algal lipid production? SYKE, Helsinki, Finland.

Stockenreiter, M., Haupt, F., Graber, A.-K. and Stibor, H. (2010) Optimizing lipid production by planktonic algae - Does diversity enhance micro-algal lipid production? Annual Lipido- meeting, Helsinki, Finland.

Stockenreiter, M., Haupt, F. and Behl, S. (2010) Die Grünalge *Mougeotia* sp. Algenstammtisch Bund Naturschutz, Seeon, Germany.

Stockenreiter, M., Haupt, F., Graber, A.-K. and Stibor, H. (2010) The effect of species diversity on lipid production by micro-algal communities. Final Lipido- meeting, Brest, France.

Haupt, F., **Stockenreiter, M.**, Baumgartner, M., Reichwaldt, E. S., Boersma, M. and Stibor, H. (2009) Upward nutrient transport by *Daphnia* diel vertical migration. ASLO aquatic science meeting, Nice, France.

Stockenreiter, M., Steinböck, M., Haupt, F. and Stibor, H. (2009) Optimizing lipid production by planktonic algae. ASLO aquatic science meeting, Nice, France.

Haupt, F., **Stockenreiter, M.**, Baumgartner, M., Reichwaldt, E. S., Boersma, M. and Stibor, H. (2008) Aufwärtsgerichteter Nährstofftransport durch vertikalwandernde Daphnien. DGL Jahrestagung, Konstanz, Germany.

Haupt, F., **Stockenreiter, M.**, Baumgartner, M., Boersma, M. and Stibor, H. (2007) Does diel vertical migration of *Daphnia* influence the phytoplankton community structure? SIL international meeting, Montreal, Canada.

Posters

Stockenreiter, M., Haupt, F., Peterson, K., Kurtz, V. (2012) Enhancing the lipid production of micro-algal communities – the effect of species diversity. 5. Bundesalgenstammtisch (DECHEMA), Linde AG, Pullach, Germany.

Stockenreiter, M., Haupt, F., Peterson, K., Kurtz, V., Tamminen, T. and Stibor, H. (2011) Enhancing the lipid production of micro-algal communities – the effect of species diversity. International algae congress, Berlin, Germany.

Stockenreiter, M., Haupt, F., Kurtz, V., Peterson, K., Tamminen, T. and Stibor, H. (2011) Enhancing the lipid production of micro-algal communities – the effect of species diversity. ABO meeting, Mineapolis, USA.

Haupt, F., **Stockenreiter, M.**, Baumgartner, M., Reichwaldt, E. S., Boersma, M. and Stibor, H. (2009) Aufwärtsgerichteter Nährstofftransport durch vertikalwandernde Daphnien. Munich Interact, München, Germany.

Stockenreiter, M., Steinböck, M. and Stibor, H. (2009) Optimizing lipid production by planktonic algae. Munich Interact, München, Germany.

Stockenreiter, M., Steinböck, M., **Haupt, F.** and Stibor, H. (2008) Lipidproduktion in planktischen Algen: Screening von Algen bezüglich Lipidgehalt mittels batch cultures und Durchflussfluoreszenzmikroskopie (FlowCAM®). DGL Konstanz, Germany.

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DECLARATION

Diese Promotion wurde im Sinne §12 der Promotionsordnung von Prof. Dr. Herwig Stibor betreut. Ich erkläre hiermit, dass die Dissertation keiner anderen Prüfungskommission vorgelegt worden ist und dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

EIDESSTATTLICHE VERSICHERUNG

Ich versichere an Eides Statt, dass die vorgelegte Dissertation von mir selbständig, ohne unerlaubte Hilfe angefertigt wurde.

München, den 29.05.2012	Maria Stockenreiter
ORT, DATUM	UNTERSCHRIFT

BEITRÄGE DER KOAUTOREN UND EIGENER BEITRAG

CHAPTER 2

Anne-Kathrin Graber und Florian Haupt waren an der Durchführung und an der Auswertung des Experiments beteiligt. Herwig Stibor unterstützte mich bei der Konzipierung des Versuchs, war an der Diskussion der Ergebnisse beteiligt und half beim Anfertigen des Manuskripts.

CHAPTER 3

Anne-Kathrin Graber, im Rahmen ihrer Masterarbeit, und Florian Haupt waren an der Durchführung und Auswertung des Experiments beteiligt. Florian Haupt, Jukka Seppälä, Kristian Spilling und Timo Tamminen gaben hilfreiche Kommentare zur Fertigstellung des Manuskripts. Herwig Stibor unterstützte mich bei der Konzipierung des Versuchs, war an der Diskussion der Ergebnisse beteiligt und half beim Anfertigen des Manuskripts.

CHAPTER 4

Florian Haupt war an der Durchführung der Versuche sowie beim Schreiben des Manuskripts beteiligt. Jukka Seppälä, Kristian Spilling und Timo Tamminen waren an der Durchführung beteiligt und gaben hilfreiche Kommentare zur Fertigstellung des Manuskripts. Heiko Rischer und Dagmar Enss waren an der Durchführung beteiligt. Herwig Stibor unterstützte mich bei der Konzipierung des Versuchs, war an der Diskussion der Ergebnisse beteiligt und half beim Anfertigen des Manuskripts.

CHAPTER 5

Ich war an der Konzeption, Durchführung und Auswertung des Experiments (Auszählen der Phytoplanktonproben) sowie am Verfassen des Manuskripts beteiligt. Desweiteren war ich der Bearbeitung der Kommentare der Reviewer beteiligt. Florian Haupt war an der Konzeption, Durchführung und Auswertung der Experiments, sowie beim Verfassen des Manuskripts und Bearbeitung der Kommentare der Reviewer beteiligt. Maarten Boersma unterstützte bei der Konzipierung der Versuche und half beim Anfertigen des Manuskripts. Herwig Stibor unterstützte bei der Konzipierung des Versuchs und war an der Diskussion und Anfertigen des Manuskripts beteiligt.

CHAPTER 6

Florian Haupt war an der Konzipierung und der Durchführung der Versuche, sowie beim Verfassen des Manuskripts beteiligt. Herwig Stibor unterstützte mich bei der Konzipierung des Versuchs, war an der Diskussion der Ergebnisse beteiligt und half beim Anfertigen des Manuskripts.

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