Effects of natural phytoplankton trait diversity on *Daphnia* spp.

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“Had to have high, high hopes for a living
Didn't know how but I always had a feeling
I was gonna be that one in a million
Always had high, high hopes”

- Brendon Urie (Panic! at the Disco)

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Zusammenfassung

Durch den anthropogenen Einfluss verursachte Umweltveränderungen zählen zu den größten Antrieben des globalen Biodiversitätverlusts in verschiedenen Arten von Ökosystemen. Während sich unzählige Studien mit dem Einfluss des Biodiversitätverlusts auf die Ökosystemfunktionen befasst haben, ist wenig über die zugrundelegenden Mechanismen hinter der Beziehung zwischen der Biodiversität und den Ökosystemfunktionen bekannt. In den letzten Jahren folgten Studien einer „Trait"-basierten Herangehensweise, bei der die Rolle der Art- und Taxon-spezifischen funktionellen Traits (i.e. Eigenschaften) untersucht wird, wie z.B. die Aufnahme von Ressourcen oder die biochemische Zusammensetzung. Der Verlust solcher Traits verändert die Schlüsselprozesse der Ökosysteme wie Produktivität und Nachhaltigkeit. In aquatischen Ökosystemen wird vermutet, dass der Biodiversitätverlust auf der Ebene der Produzenten eine kaskadierende Auswirkung auf multiple trophische Ebenen haben könnte. Die Phytoplankton-Zooplankton-Verbindung ist insbesondere vom Interesse, da der Phytoplanktongehalt an mehrfachungesättigten Fettsäuren (PUFAs) entscheidend für die Fitness des herbivoren Grazers *Daphnia* ist, welche nicht in der Lage ist, solche PUFAs *de novo* zu synthetisieren. Daher wird vermutet, dass essentielle PUFAs ein funktionelles Phytoplanktontrait sind, welches die Effizienz des trophischen Energietransfers und die Dynamiken zwischen Primärproduzent und Primärkonsument beeinflusst. Allerdings unterscheidet sich die Fettsäurenzusammensetzung des Phytoplanktons zwischen den einzelnen taxonomischen Gruppen. Daher stellte ich die Hypothese auf, dass eine veränderte Phytoplanktongemeinschaft verändernde Effekte auf die Diversität der Fettsäuren haben kann. Dies würde wiederrum den innerartlichen Konkurrenzkampf in *Daphnia* Populationen beeinflussen.

Um diese Hypothesen zu adressieren, wurden zunächst zwei häufig verwendete Methoden für die indirekte Bestimmung der Zusammensetzung der Phytoplanktongemeinschaft verglichen. Aus den erlangten Ergebnissen konnte man schlussfolgern, dass die Pigment-basierte Methode für eine *in vitro* Bestimmung der Phytoplanktondiversität eine höhere Auflösung der Diversität bietet verglichen zu der spektrofluorometrischen *in vivo* Methode. Zweitens, konzentrationsabhängige Wachstumsversuche wurden mit zwei verschiedenen *Daphnia* Arten durchgeführt um die relative Bedeutung zweier C_{20}-PUFAs aus verschiedenen ω-Familien für die Fitness von Daphnien zu vergleichen. Dabei wurden Sättigungskonzentrationen der beiden PUFAs für das Wachstums und die Reproduktion bestimmt und zwischen den beiden *Daphnia* Arten verglichen. Anders als erwartet, weisen die Daten auf eine gleich starke Bedeutung von ω3- und ω6-PUFAs für die Fitness von Daphnien hin. Drittens, die Variabilität innerhalb einer natürlichen Population von koexistierenden *Daphnia longispina* Genotypen wurde in Hinsicht auf ihre Anfälligkeit für Fitness-Limitierung durch die Verfügbarkeit der essentiellen PUFAs bestimmt. Dabei wurden zunächst die *D. longispina* Individuen aus einem oligo-mesotrophen See isoliert und die verschiedenen Genotypen mittels der Mikrosatellitenanalyse identifiziert. Daraufhin wurden Wachstumsexperimente durchgeführt um die juvenile Wachstumsrate der verschiedenen *D. longispina* Genotypen in An- und Abwesenheit von drei verschiedenen essentiellen PUFAs zu bestimmen. Aus den erlangten Ergebnissen wurde die Anfälligkeit jedes Genotyps für das Vorhandensein der supplementierten PUFAs bestimmt. Dabei wurden innerartliche Unterschiede zwischen den verschiedenen *D. longispina* Genotypen gefunden.

Letztlich wurde ein Common garden Experiment mit einer manipulierten natürlichen Phytoplanktongemeinschaft und natürlich koexistierenden *D. longispina* Genotypen mit ausgeprägten Unterschieden in Ihrer Anfälligkeit für die Fitness-Limitierung durch die Abwesenheit von PUFAs durchgeführt. Die Daten demonstrieren, dass die Phytoplanktondiversität mit der Diversität von PUFAs korreliert, und dass einzelne PUFAs direkt den innerartlichen Konkurrenzkampf zwischen den getesteten *D. longispina* Genotypen beeinflussen. Somit deutet diese Studie auf eine potentielle Verbindung zwischen der Diversität der Phytoplankton-Traits (im Sinne von PUFA Verfügbarkeit) und den Dynamiken innerhalb der *Daphnia* Population hin.

Abstract

Anthropogenically induced environmental changes are ranked among the major drivers of global biodiversity loss in different types of ecosystems. While there have been numerous studies that examined the impact of biodiversity loss on ecosystem functioning, it is still not much known about the underlying mechanisms behind the biodiversity-ecosystem functioning relationship. In recent years, studies started to follow a trait-based approach, i.e. examining the role of species and taxon specific functional traits, such as resource uptake or biochemical composition. Loss of such traits has been shown to alter key processes of ecosystems, such as productivity and sustainability. In aquatic ecosystems, biodiversity loss on producer level is believed to have cascading effects on multiple trophic levels. In particular the phytoplankton-zooplankton interface is of high interest, as the phytoplankton content of dietary polyunsaturated fatty acids (PUFAs) was found to be crucial for the fitness of the herbivorous grazer *Daphnia,* which is not capable of *de novo* synthesis of such PUFAs. Essential PUFAs are thus assumed to be a functional phytoplankton trait that affects the trophic transfer efficiency and dynamics between the primary producers and consumers. However, fatty acid composition of phytoplankton was shown to be taxon-specific. Thus, I hypothesized that altered phytoplankton community composition will result in an altered composition of dietary fatty acids. This in turn will affect the intraspecific competition in *Daphnia*. To address these hypotheses, I first compared two commonly used methods for the indirect assessment of the phytoplankton community composition. From the obtained results, I concluded that the pigment-based method for the *in vitro* estimation of phytoplankton diversity provides a higher resolution compared to the *in vivo* method based on fluorescence spectra.

Second, I performed dose-response growth experiments with two different *Daphnia* species in order to compare the relevance of two C_{20}-PUFAs from different ω-families for *Daphnia*'s fitness. I estimated the growth and reproduction saturation thresholds of both PUFAs and compared these between the species. Other than expected, the results of my experiments provide evidence for equal relevance of ω3- and ω6-PUFAs for *Daphnia*.

Third, I assessed the intrapopulation variability in susceptibility of naturally coexisting *Daphnia longispina* genotypes to limitations by the availability of essential dietary PUFAs. I first isolated *D. longispina* individuals from an oligo-mesotrophic lake and identified different genotypes via microsatellite analyses. Subsequently, I performed standardized growth assays and determined the juvenile somatic growth rate of different *D. longispina* genotypes in presence and absence of three different essential PUFAs. From the obtained data, I estimated the susceptibility of each genotype to the availability of supplemented PUFAs and found intraspecific differences between the tested *D. longispina* genotypes. Finally, I performed a common garden experiment with diversity-manipulated natural phytoplankton community and naturally coexisting *D. longispina* genotypes with pronounced differences in their susceptibility to limitations by essential dietary fatty acids. The obtained data demonstrate that the phytoplankton diversity is correlated with the composition of polyunsaturated fatty acids (PUFAs), and that single PUFAs directly affect competitive interactions between the tested *D. longispina* genotypes. Therefore, the present study provides evidence for a potential link between phytoplankton trait diversity (in terms of PUFA availability) and *Daphnia*'s population dynamics.

General Introduction and aim of the study

Anthropogenically induced environmental changes, such as global warming and eutrophication, are ranked among the major drivers of global biodiversity loss in different types of ecosystems (Cardinale et al., 2006; Cardinale, 2012; Loreau, 2001, 2010; Naeem et al., 2009; Hooper et al., 2012). While there have been numerous studies that examined the impact of species loss on ecosystem functioning and community dynamics, it is still not much known about the underlying mechanisms behind the biodiversity-ecosystem functioning relationship. Therefore, novel approaches were developed to provide new insights into processes coupled with the response of ecosystem properties to species loss and allow for further predictions on the consequences of the ongoing global diversity decline.

Trait-based approaches are more and more frequently applied to study mechanisms that link ecosystem functioning, community structure and eco-evolutionary dynamics within various ecosystems (Fussmann et al., 2007; Litchman and Klausmeier, 2008; Litchman et al., 2013). Such studies address different levels of organization and complexity, ranging from individuals to whole populations and ecosystems (Ackerly and Cornwell, 2007; Litchman et al., 2007, 2010). In this context, traits are defined as measurable morphological, physiological or phenological characteristics of an organism, while functional traits are considered to affect the organismal growth, reproduction and survival, thus affect its fitness and performance (McGill et al., 2006; Violle et al., 2007; Cadotte et al., 2015). Loss of such traits has been shown to alter key processes of ecosystems, such as productivity and sustainability (Striebel et al., 2009b, 2009a).

Furthermore, functional traits are crucial for the response of an individual to the surrounding environment and may play an important role in intra- and interspecific interactions (Jung et al., 2010; Bolnick et al., 2011; Violle et al., 2012). Variability in a functional trait within and among species is the basis for competition and selection and thus a driver of evolution in natural populations.

In previous studies, it has been shown that, although often underestimated or neglected, intraspecific variability occurs within populations in both terrestrial (Eichenberg et al., 2015; Bu et al., 2017) and aquatic ecosystems (De Bruin et al., 2004; White et al., 2011; Werner et al., 2018). For example, White et al. (2011) documented an intraspecific variation in vulnerability of co-occuring genotypes of the toxic and colony-forming cyanobacterium *Microcystis aeruginosa* to herbivory by the filter-feeding zebra mussel *Dreissena polymorpha*. Furthermore, with a combination of single-trait and multi-trait analyses of five functional traits measured for 13 plant species, Albert et al. (2010) found a pronounced intraspecific variation, accounting for approximately 30% of the overall trait variability. Within a single species, a high variation in a functional trait (given either as genetical diversity or phenotypic plasticity) can promote coexistence of different genotypes by decreasing their niche overlap (Violle and Jiang, 2009; Bolnick et al., 2011). On the other hand, given that the trait-variation arises from genetic variation, such standing genetic diversity may promote local adaptation and increase the resilience to changing environmental conditions and other biotic and abiotic factors such as predation risk, toxicity and resource availability (Barrett and Schluter, 2008; Jung et al., 2010), thus buffering biodiversity losses (Reusch, 2005; Albert et al., 2010).

The water flea *Daphnia*, a herbivorous crustacean zooplankton, is a well established model organism in ecology, in particular regarding trophic interactions and effects of trait-variation on eco-evolutionary dynamics (Hairston et al., 1999; Ebert, 2011; Stoks et al., 2016). *Daphnia* is a key species in freshwater food webs, as it feeds directly on phytoplankton and serves as a food source for planktivorous fish (Gaedke and Straile, 1998). Under optimal environmental conditions, *Daphnia* spp. produce clonal females through parthenogenesis (Stross and Hill, 1965). As *Daphnia* are non-selective filter feeders (DeMott, 1986), their fitness depends strongly on the diet present.

Two major factors that determine the nutritional value and thus the food quality of *Daphnia*'s diet are the elemental and biochemical composition of the phytoplankton (Ahlgren et al., 1990a; Müller-Navarra, 1995a; Park et al., 2002; Becker and Boersma, 2003). Besides dietary carbon to nutrient ratios (Sterner et al., 1993; Urabe et al., 1997; Ravet and Brett, 2006), *Daphnia*'s performance has often been associated with the availability of polyunsaturated fatty acids (PUFAs) in their diet (Müller-Navarra, 1995b; Wacker and von Elert, 2001; von Elert, 2002), i.e. fatty acids with two or more double bonds in their carbon chain. Essential PUFAs are thus assumed to be a functional phytoplankton trait that affects the trophic transfer efficiency and dynamics between the primary producers and consumers. However, fatty acid composition of the phytoplankton was shown to be taxon-specific (Ahlgren et al., 1990a; Lang et al., 2011). Therefore, biodiversity loss on producer level might result in a decreased trait diversity, which can have cascading effects on multiple trophic levels. Furthermore, the outcome of such cascading effects on the level of consumers might depend of consumer's specific functional traits coupled to their fitness and susceptibility to limitations by poor food quality.

In **Chapter 1**, I compare two methods commonly used to indirectly determine the phytoplankton community composition. This is of particular interest, as the estimation of producer's diversity builds the basis of trait-based approaches addressing cascading effects of biodiversity loss on other trophic levels. Although other methods are available, such as microscopic counting or DNA metabarcoding, they have several disadvantages which make them unsuitable for fast assessment of the phytoplankton community complexity. Therefore, I discuss the advantages and disadvantages of a pigment-based method, which includes extraction and separation of phytoplankton pigments and subsequent complex algorithmic calculations via the matrix-factorization program CHEMTAX. On the other hand, I present the data obtained from a spectrofluorometric method and compare both methods in terms of reliability and resolution regarding the estimated phytoplankton diversity.

In **Chapter 2**, via dose-response growth experiments, I compare two C_{20}-PUFAs from different ω-families, namely eicosapentaenoic acid (EPA, 20:5 ω3) and arachidonic acid (ARA, 20:4 ω6), regarding their relevance for the fitness of two *Daphnia* species. Although EPA has been shown previously to strongly affect the fitness of different *Daphnia* species (von Elert, 2002; Becker and Boersma, 2003; Ravet et al., 2012), the role of ARA is not yet clear, which is apparent from inconsistent findings in the present literature.

In **Chapter 3**, I assess the intrapopulation variability in susceptibility of naturally coexisting *Daphnia longispina* genotypes to availability of α-linolenic acid (ALA, 18:3 ω3), EPA and ARA. Here, susceptibility is suggested to be a consumer's functional trait that might affect intraspecific competition and community dynamics within natural *Daphnia* populations.

Finally, in **Chapter 4**, I perform a common garden experiment to study effects of biodiversity on phytoplankton functional trait diversity. Such biodiversity loss is suggested to alter the food quality of the phytoplankton and result in a decreased PUFA availability and diversity, which in turn might affect the population dynamics of the consumer. On the other hand, the susceptibility of different *D. longispina* genotypes, assessed in **Chapter 3**, might affect the outcome of competitive interactions within the *Daphnia* community. I discuss the role of sestonic PUFA content (i.e. phytoplankton functional trait) and *Daphnia*'s susceptibility to limitations by PUFA availability (i.e. consumer functional trait) as potential links between biodiversity and ecosystem functioning.

In summary, the present study follows a trait-based approach to study possible links and mechanisms behind the biodiversity-ecosystem functioning relationship in aquatic ecosystems. Furthermore, this study allows for suggestions on improvement of phytoplankton diversity assessment and provides evidence for an equal relevance of a ω3- and ω6-PUFAs for the fitness of *Daphnia* spp.. Finally, this thesis addresses the role of intraspecific variation in a fitness-related functional trait for competitive interactions and provides evidence for a potential link between phytoplankton trait diversity (in terms of PUFA availability) and *Daphnia*'s population dynamics.

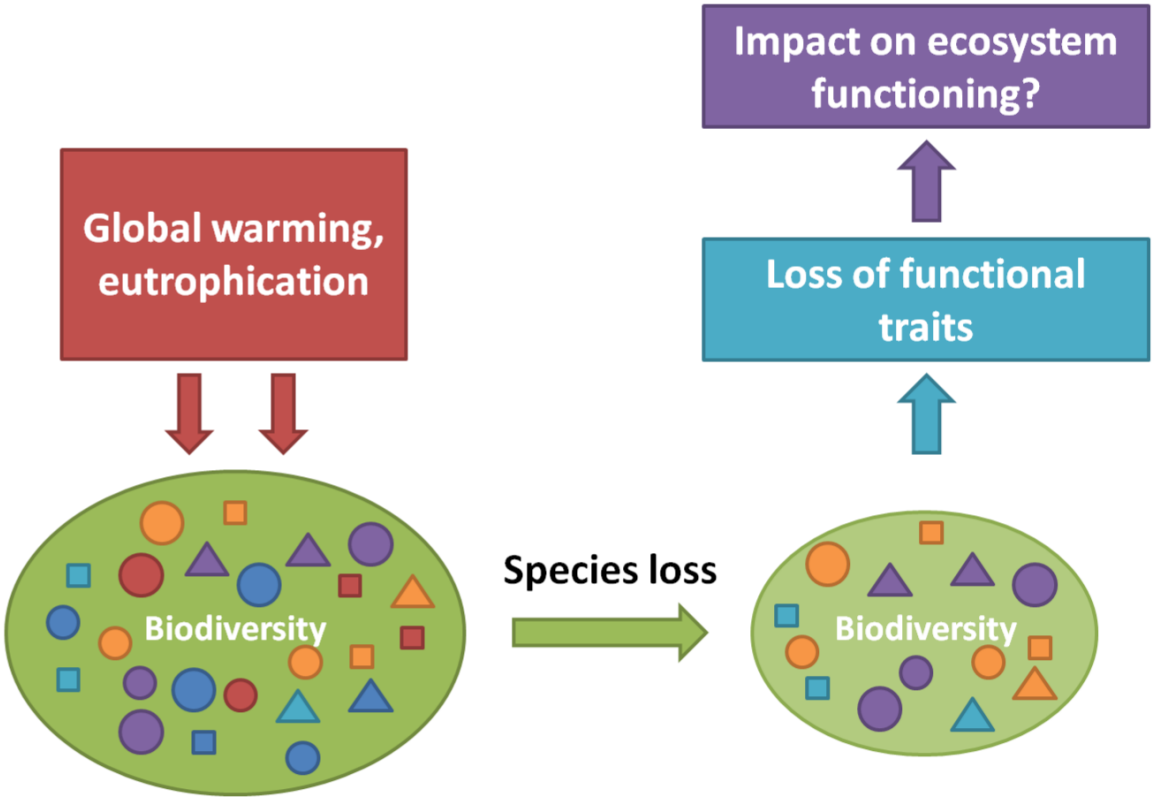

Fig. I: The general concept illustrating the potential role of functional traits as a link between biodiversity loss and ecosystem functioning.

Chapter 1

Determination of phytoplankton biodiversity in lakes of different trophic state through pigment and fluorescence proxies

1.1 Introduction

Phytoplankton community composition has often been used as an indicator of the ecosystem productivity and trophic status of lakes (Reynolds et al., 2002) and of food quality for e.g. herbivorous zooplankton (Behl and Stibor, 2015). In the past few decades, the global biodiversity has experienced a strong decline (Cardinale, 2012; Hooper et al., 2012). One of the major drivers of global biodiversity loss is the anthropogenic eutrophication, which further causes the formation of cyanobacterial blooms (Hooper et al., 2012; O'Neil et al., 2012). Such cyanobacterial blooms can be toxic, as some cyanobacteria are producing toxins like microcystins, anatoxins, cylindrospermopsin and nodularins, with microcystins being the most commonly detected cyanobacteria toxin (Pilotto et al., 2004; Koreivienė et al., 2014; Zamyadi et al., 2016). Microcystins have been shown to be carcinogenic and cause liver damage ((Falconer et al., 1994; Falconer, 2005; Koreivienė et al., 2014). Thus, the formation of toxic, in particular microcystin-rich cyanobacterial blooms, may have negative impacts on ecosystem properties and services such as the provision of drinking water (Jurczak et al., 2005). To ensure the sustainable use of aquatic ecosystems and to manage the water treatment operations, the European Community legislation has introduced the EU Water Framework Directive (WFD, Directive 2000/60/EC). The WFD defines the composition of the phytoplankton community as one of the most important biological parameters that determine the quality and ecological status of surface water bodies (Sarmento and Descy, 2008; Catherine et al., 2012; Escoffier et al., 2015). Because of the rapid respond of the phytoplankton community to environmental changes (Richardson et al., 2010) and a usually high number of samples that have to be processed, it is necessary to use time saving methods for the assessment of phytoplankton community composition and

in particular the monitoring of cyanobacteria abundances. This is of high importance, as the time delay between sampling and data processing has to be as short as possible, to ensure a fast management of necessary water treatment actions. Traditionally, the phytoplankton community composition is estimated via microscopic counting. However, this method is extremely time consuming and highly depended on the taxonomic knowledge of the respective person who identifies and counts the phytoplankton taxa. Also, phytoplankton of a small size (picoplankton, < 5 µm) cannot be differentiated accurately(Groendahl et al., 2017). Another approach, which is new and not yet completely implemented, is DNA metabarcoding, e.g. using 18S rDNA. Although this method could help to distinguish between small phytoplankton species, it is still very costly and limited by available databases that still lack many reference sequences (Simmons et al., 2016; Groendahl et al., 2017). Thus, chemotaxonomic alternatives have been proposed, such as pigment-based spectrofluorometry and high pressure liquid chromatography (HPLC) of pigments. Both approaches are based on the differences in pigment composition of the main phytoplankton groups. For example, Dinoflagellates contain the pigment peridinin, which is a Dinoflagellate-specific pigment, while alloxanthin and lutein are pigments that are group-specific for Cryptophytes and Chlorophytes, respectively (Gieskes and Kraay, 1983). Thus, the pigment composition of the phytoplankton is commonly used for the assessment of phytoplankton community composition. The advantage of both methods is that they are less time consuming then microscopy or DNA metabarcoding. However, they only allow for a relatively low taxonomic resolution (class level, compared to microscopy and DNA metabarcoding which both allow for genus or even species level).

Traditionally, fluorescence-based chlorophyll *a* quantification methods were applied for both *in vitro* and *in vivo* (Yentsch and Menzel, 1963; Yentsch and Phinney, 1985) measurements of chlorophyll *a*. Subsequently, spectrofluorometric methods that use multiple excitation and/or emission wavelengths were developed and became the standard application for phytoplankton monitoring (Beutler et al., 2002; Richardson et al., 2010). The chlorophyll *a* fluorescence is mostly determined by the peripheral and core antennae of photosystem II (Beutler et al., 2002). While the evolutionarily conserved core antenna contains the chlorophyll *a* molecules in all phytoplankton taxa, the peripheral antenna includes species-dependent light-absorbing accessory pigments, which are responsible for selective excitation spectra and thus represent the fundament of the spectrofluorometric differentiation of phytoplankton groups. Based on these observations, Beutler et al. (2002) introduced a novel spectrofluorometer, the Algae Lab Analyser (bbe Moldaenke, Kiel, Germany) as a bench-top device which is commonly used by water authorities and routine laboratories. The Algae Lab Analyser contains five different light-emitting diodes (LEDs) with λ 450 nm, 525 nm, 570 nm, 590 nm and 610 nm, respectively. Based on the group-specific excitation spectra, also called norm spectra, the Algae Lab Analyser allows for differentiation of four spectral groups: the green group (Chlorophytes), the blue-green group (Cyanobacteria), the brown group (Chromophytes, which includes Chrysophytes, Diatoms and Dinoflagellates) and mixed-group (Cryptophytes and phycoerythrin-containing algae, Beutler et al., 2002). Calculation of the contribution of each phytoplankton group to the total chlorophyll *a* is based on linear unmixing (solving linear equations). For details and algal species used to determine norm spectra and calculation algorithms for all four spectral algal groups, see Beutler et al. (2002). The *in vivo* measurement is very fast (few minutes) and the data is transferred online to a PC or lap top. Using the actual five-point excitation spectrum of a

water sample, the allocation of the measured chlorophyll *a* concentration to the four phytoplankton groups is rapidly obtained. Thus, Algae Lab Analyser seems to be very suitable for fast monitoring and assessment of the phytoplankton community composition.

Another commonly used method for the assessment of the phytoplankton community composition is the HPLC analysis of photopigments, combined with the matrix factorization programme CHEMTAX. HPLC is not only a fast and cost-effective alternative to microscopic counts, but also allows for identification of species that are usually not detectable by microscopic counting (e.g. picoplankton), as the detection limits of diagnostic pigments are usually low (Schlüter et al., 2016). The HPLC approach to analyze the pigment composition of phytoplankton communities has been widely used and is nowadays often combined with the matrix factorization programme CHEMTAX, which was developed in 1996 by Mackey et al. for marine systems, but has been used and updated since then for both marine and freshwater ecosystems (Descy et al., 2000, 2009; Buchaca, 2004; Lewitus et al., 2005; Sarmento and Descy, 2008; Lauridsen et al., 2011; Armbrecht et al., 2015; Schlüter et al., 2016). The CHEMTAX approach is based on group-specific pigments (e.g. peridinin, lutein etc. as mentioned above) and uses input ratio matrices containing ratios of such pigments to chlorophyll *a*, which can be found in the literature. For example, Schlüter et al. (2006) provide an excellent summary on pigment : chlorophyll *a* ratios of several freshwater phytoplankton groups, estimated in laboratory cultures, which have been cultured under different light intensities, to account for different environmental conditions that can cause changes in pigment : chlorophyll *a* ratios of the phytoplankton species. Recently, such ratio matrices have been developed using data from 46 German lakes along a phosphorus gradient (oligotrophic, mesotrophic and eutrophic lakes) by

Schlüter et al. (2016). Depending on the pigment ratio matrix and observed concentrations of the pigments, CHEMTAX provides the best fit of contributions of the predefined phytoplankton groups to the total chlorophyll *a* concentration and is able to differentiate between more than 4 groups (to which the Algae Lab Analyser is limited). The number of discernible groups by CHEMTAX depends on the number of analyzed diagnostic pigments and the previous knowledge about the phytoplankton community composition of the water body of interest (Mackey et al., 1996).

Both the Algae Lab Analyser and the HPLC/CHEMTAX approach are limited in the taxonomic resolution, as they only allow a differentiation on the group level. This may not be a major constraint, as multiple studies have shown functional phytoplankton diversity to be a better predictor of ecosystem functioning than species richness (Striebel et al., 2009b; Behl et al., 2011; Stockenreiter et al., 2013). This means that both approaches might be good alternative tools to assess the phytoplankton community composition and investigate research questions. In particular for cyanobacteria monitoring, many different methods have been used, e.g. microscopic counting, quantitative polymerase chain reaction (qPCR), pigments extraction and fluorometric probes. An excellent review of such methods as well as further recommendations and proposals on cyanobacterial monitoring were recently summarized by Zamyadi et al. (2016). Furthermore, some studies compared the suitability of spectrofluorometry and HPLC/CHEMTAX for the assessment of the phytoplankton community composition, e.g. usage of the bbe Moldaenke FluoroProbe (Catherine et al. 2012) and the bbe Moldaenke Algae Online Analyser (Richardson et al. 2010) compared to CHEMTAX-derived community composition data. However, both these studies did not compare lakes across a gradient of trophic status, which is done here for the first time.

We here focused on the following specific hypotheses: (i) both methods give a good representation of the phytoplankton community composition, (ii) CHEMTAX allows for a higher resolution of the phytoplankton biodiversity as it can distinguish between more than four taxonomic groups depending on the specific marker pigment calibrated and (iii) the Algae LabAnalyser allows a more accurate determination of cyanobacteria, as the lipophilic solvent extraction of pigments used for the HPLC method does not capture the water-soluble cyanobacterial pigments phycocyanin and phycoerythrin.

1.2 Materials and Methods

1.2.1 Field experiment and sampling

During the summer in 2014, we simultaneously conducted large-scale mesocosm field experiments in three lakes of different trophic state situated in Upper Bavaria (Germany): Brunnsee (47° 59' 01" N, 12° 26' 12" E, area: 5.8 ha, maximal depth: 20 m), Klostersee (47° 58' 26" N, 12° 27' 10" E, area: 47.0 ha, maximal depth: 16 m) and Thalersee (47° 54' 16" N, 12° 20' 17" E, area: 3.8 ha, maximal depth: 7 m, Fig. S1). Depending on the average epilimnetic total phosphorus concentration (TP, ± standard deviation) determined in summer 2014 Brunnsee can be classified as an oligotrophic (TP = 5.62 ± 1.09 µg L^{-1}), Klostersee as an oligo-mesotrophic (TP = 9.88 ± 2.47 µg L^{-1}) and Thalersee as a mesotrophic lake (16.80 ± 5.18 µg L^{-1}, Nürnberg, 1996).

The mesocosms were made of white plastic foil, forming cylindrical enclosures closed at the bottom and open at the top to allow for gas exchange with the atmosphere. They had a diameter of 0.95 m and a length of 5 m (Thalersee) and 6 m (Brunnsee and Klostersee), resulting in a total volume of approx. 3.5 – 4.2 m^3, respectively. Per lake, 20 mesocosms were installed and filled with surrounding water from the respective lake, which was pre-filtered over a 250 µm gaze, to exclude zooplankton and thus prevent grazing. We used disturbances of the water column via compressed air to manipulate the natural phytoplankton community in the mesocosms, in order to establish a diversity gradient (Flöder and Sommer, 1999; Hammerstein et al., 2017). We regularly (usually once a week) took water samples from the mesocosms and from the lake itself, using a 2 L integrated water sampler (KC Denmark), from the depth of 0.5 m to 2.5 m.

The water samples were transported to the laboratory and were kept cool and dark until further analyses (within a few hours). In total, we analyzed 562 samples (186 from Brunnsee, 187 from Klostersee and 189 from Thalersee).

1.2.2 In vivo fluorometric analysis (Algae Lab Analyser)

The fluorometric measurement of the chlorophyll *a* concentration was done using the Algae Lab Analyser (bbe Moldaenke). For this, the water samples were carefully shaken and 25 ml were transferred into a cuvette and measured *in vivo*, using the manufacturer's default settings. The bbe++ Windows software was used to calculate the best sum of the four specific excitation norm spectra (corresponding to the four phytoplankton groups: Chlorophytes, Chromophytes, Cryptophytes and Cyanobacteria) from the measured fluorescence signal. With this, we obtained the contribution of each of the four phytoplankton groups to the total chlorophyll *a* concentration, given in micrograms per liter. For details on calibration of the fluorometer, default settings and mathematical equations used for the calculation of the contribution of the four phytoplankton groups to the total chlorophyll *a*, see Beutler et al. (2002).

1.2.3 In vitro chromatographic analysis (HPLC)

For the HPLC analyses, up to 1000 ml of the water samples from the lakes were filtered onto precombusted glass fiber filters (VWR GF/F, Ø 25 mm). The filters were wrapped in aluminium foil and stored at -20 °C until analysis. Seston samples were extracted with each 3.5 ml 100% acetone (Alfa Aesar, HPLC grade), sonicated for 2 min and then placed on ice for 1 min. This was repeated 5 times, resulting in a total of 10 min sonication and extraction time. Subsequently, the filters were kept at 4°C over night to allow for further extraction. On the following day, the filters were removed from the tubes and the

extracts were centrifuged for 15 min at 4500 x g (Eppendorf Centrifuge 5804) to remove cell and filter debris. 1 ml of the extracts were transferred to new tubes, evaporated to dryness under a gentle stream of nitrogen gas, re-dissolved in 100 µl acetone and transferred to HPLC vials. To correct for sample loss during the evaporation, we used trans-β-apo-8'-carotenal (Sigma Aldrich) as an internal standard (ISTD). We added 100 ng of the ISTD to 1 ml of extract prior to evaporation. 25 - 50 µl per sample were injected into the HPLC system. All samples were measured within 72 hours after extraction.

A Prominence HPLC System from Shimadzu equipped with a binary pump (LC-20AB), an autosampler SIL-A20C, a column oven CTO-10AC set at 40°C and a diode array detector (PDA) SPD-M20A was used for the analysis of phytoplankton pigments. We used a reverse phase Spherisorb ODS2 column (stationary octadecyl-phase (C_{18}), dimensions: 25 cm x 4.6 mm, particle size: 5 µm). The pigments were separated with a method modified after Garridol and Zapata (1993): The solvents used were methanol : 1 M ammonium acetate : acetonitrile (50:20:30, v/v, Solvent A) and acetonitrile : ethyl acetate (50:50, v/v, Solvent B). The gradient system used was as follows: 0 min: A: 90%, B: 10%; 2 min: A: 90%, B: 10%; 26 min: A: 40%, B: 60%; 28 min: A: 10%, B: 90%; 30 min: A: 10%, B: 90%. The composition of the solvents was returned to initial conditions over a 1 min gradient, followed by 2 min of system re-equilibration before the next sample was injected. The flow rate was 1 ml min^{-1}. Absorbance was recorded in the PDA from 350 to 700 nm. Pigments were identified by the retention times and the absorption spectra, which were obtained from previous measurements of the pure pigment standards. Peak areas were integrated at 436 nm and corrected for internal standard. For the quantification of the pigments, calibration curves were estimated by measuring at least 5 different amounts of each pigment standard in triplicates and fitting a linear regression between the amount of the pigment and the observed peak area at 436 nm.

We determined the limit of detection and the limit of quantification as described in Hooker et al. (2005).

Based on our previous knowledge on phytoplankton groups that are usually present in the examined lakes (data from long-term monitoring), we chose 10 pigment standards, of which 9 were obtained from DHI Water (Hoersholm, Denmark): alloxanthin (marker pigment for Cryptophytes), β-carotene, chlorophyll *a*, chlorophyll *b* (marker pigment for Chlorophytes), diatoxanthin, echinenone (marker pigment for Cyanobacteria), fucoxanthin (marker pigment for Chrysophytes and Diatoms), lutein (another marker pigment for Chlorophytes) and zeaxanthin (usually used as the only marker pigment for Cyanobacteria (Havskum et al., 2004; Llewellyn, 2004; Lewitus et al., 2005), but also shared with other groups like Chlorophytes). Peridinin (marker pigment for Dinoflagellates, extracted from *Symbiodinium* spp. following the protocol from Rogers and Marcovich, 2007) was kindly provided by D. Langenbach from the group of M. Melkonian at the University of Cologne. With the solvent gradient described above, we were able to separate all pigment peaks to the baseline except for lutein and zeaxanthin (Fig. 1.1). Although well separated, diatoxanthin was excluded from the subsequent CHEMTAX analysis as it was detected in very low amounts and in only few samples. Also, we excluded β-carotene as it did not have any effect on the output data (previous CHEMTAX runs, data not shown).

1.2.4 CHEMTAX and data analysis

The recently published (Schlüter et al. 2016) pigment : chlorophyll *a* ratio matrices had been established for oligo- and for meso- and eutropic lakes, including all three lakes from our study. These ratio matrices should thus be highly suitable for our study and were used in the present study to calculate the contribution of six phytoplankton

groups, namely Chlorophytes, Cryptophytes, Cyanobacteria, Chrysophytes, Diatoms and Dinoflagellates, to the total chlorophyll *a* via CHEMTAX (Mackey et al., 1996; version 1.95 provided by S. Wright).

For the CHEMTAX calculations, 60 different ratio matrices were generated from the initial ratio matrices (separately for the oligotrophic lake and for the oligo-meso/mesotrophic lakes). 10% (n=6) of the matrices with the lowest residual root mean square (RMS) were averaged and used as new input ratio matrices. The runs were repeated using final ratio matrices from every previous run as input ratio matrix for the next run. This was repeated until the ratios became stable. For details on this procedure, see Latasa (2007) and Higgins et al. (2011). The parameters used within CHEMTAX were set as recommended by Mackey et al. (1996) and S. Wright (pers. comm.): ratio limits: 500 (this allowed initial pigment ratios to vary from $r/6$ to $6r$, in total a 36-fold change), weighting: bounded relative (error by pigment, see Latasa, 2007), iteration limit: 100, epsilon limit: 0.0001, initial step size: 10, step ratio: 1.3, cutoff step: 100, elements varied: 5, subiterations: 1, weight bound: 30. For explanations, see Mackey et al. (1996).

Subsequently, we used the HPLC derived pigment concentrations and the CHEMTAX derived biomasses of the phytoplankton groups (in units of chlorophyll *a*) to calculate Shannon-Diversity Indices as estimates of pigment and phytoplankton diversity (Shannon and Weaver, 1949). For this, we used the equation (Eq. 1):

$$H' = -\sum p_{\mathrm{i}} \times \ln(p_{\mathrm{i}})$$

where p_{i} is the proportion of the pigment or phytoplankton class relative to the total amount of the pigments or the total biomass, respectively.

When the Shannon-Diversity Indices were calculated from the CHEMTAX data, we followed two approaches: first, we summed up the contribution of Chrysophytes, Diatoms and Dinoflagellates to the total chlorophyll *a* to one single data point per sample, which was comparable to the biomass estimates of Chromophytes via Algae Lab Analyser. With this, we were able to calculate and compare the Shannon-Indices based only on the biomass estimates of the four groups (Chlorophytes, Chromophytes, Cryptophytes and Cyanobacteria) from both methods (spectrofluorometrically and chromatographically estimated biomass). The second approach was to calculate the Shannon-Indices using the biomass estimates for all 6 phytoplankton groups, as CHEMTAX was able to discriminate between the subgroups of the Chromophytes (see above).

To compare the biomass (given as total chlorophyll *a*, in the following abbreviated as TChl *a*) and biodiversity estimates from the Algae Lab Analyser with those from HPLC and CHEMTAX, we estimated the Spearman correlation coefficient, r_s, as the data was not normally distributed. Additionally, we calculated the ratio between the estimates from the Algae Lab Analyser and CHEMTAX, $R_{LAB/CHEM}$. The ratios and the biodiversity estimates were tested for normality with the Shapiro-Wilk's test, while the homogeneity of variances was tested with Levene's test. We performed one-way ANOVAs for all four phytoplankton groups, with the ratio $R_{LAB/CHEM}$ as the dependent variable and trophic status of the lakes as the independent variable, followed by the Tukey's HSD *post hoc* test ($\alpha = 0.05$). Alternatively, when the data was not normally distributed and variances were heterogeneous, we applied the nonparametric Kruskal-Wallis test on both the ratios $R_{LAB/CHEM}$ and the biodiversity estimates, followed by Dunn's *post hoc* test. For all calculations, statistics and figures, we used R (version 3.3.2, R Core Team, 2016) and RStudio (version 1.1.383, RStudio Team, 2016).

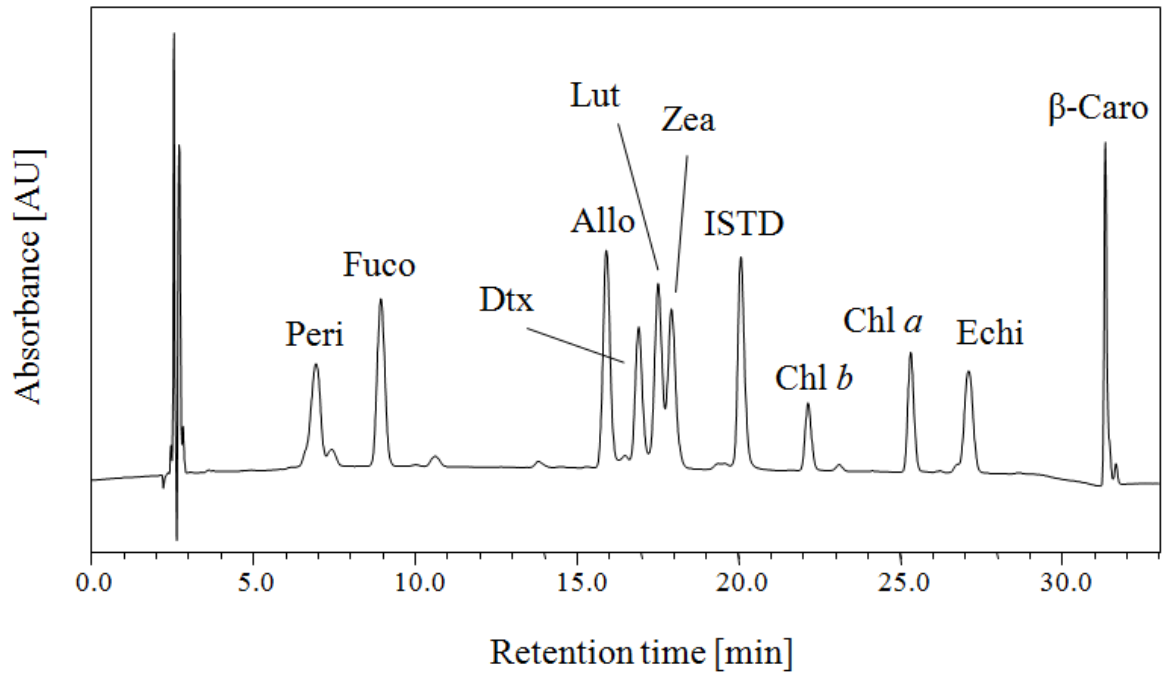

Fig. 1.1: HPLC chromatogram recorded at 436 nm. Shown are peaks of the pigments peridinin (Peri), fucoxanthin (Fuco), alloxanthin (Allo), diatoxanthin (Dtx), lutein (Lut), zeaxanthin (Zea), chlorophyll *b* (Chl *b*), chlorophyll *a* (Chl *a*), echinenone (Echi) and β-carotene (β-Caro). The pigment standard trans-β-apo-8'-carotenal was used as an internal standard (ISTD) and was well separated from the other pigments.

1.3 Results

1.3.1 Pigment composition

The most abundant pigment in the oligotrophic lake was zeaxanthin (37%), followed by fucoxanthin (36%), while the relative abundance of alloxanthin (marker pigment for the Cryptophytes) was even below 0.5%. In both the oligo-mesotrophic and the mesotrophic lake, the most abundant pigment was fucoxanthin (34% and 30%, respectively). Also, in both lakes, zeaxanthin and chlorophyll *b* (maker pigment for the Chlorophytes) were found in high relative abundances (Fig. 1.2). While peridinin (marker pigment for the Dinoflagellates) was moderately abundant in both the oligotrophic (14%) and the mesotrophic (16%) lake, we found only 1% of peridinin in the oligo-mesotrophic lake. The pigment diversity per sample ranged from 0.57 to 1.39 and was on average 1.11.

1.3.2 CHEMTAX final output ratio matrices

The final output ratio matrices from CHEMTAX calculations for all three lakes can be found in the Tab. 1.1. Both the final output peridinin : chlorophyll *a* and echinenone : chlorophyll *a* ratios were lower in all three lakes compared to the input ratios from Schlüter et al. (2016).

The zeaxanthin : chlorophyll *a* ratios for the Cyanobacteria were found to be higher in the output ratio matrices from the oligotrophic and the oligo-mesotrophic lake, while the final output zeaxanthin : chlorophyll *a* and chlorophyll *b* : chlorophyll *a* ratios for the Chlorophytes were lower compared to the input ratios. In the mesotrophic lake, the opposite was the case.

While the fucoxanthin : chlorophyll *a* ratios between the Diatoms and the Chrysophytes were similar in both input ratio matrices, the ratios changed during the CHEMTAX calculations: in the oligotrophic lake, the final output fucoxanthin : chlorophyll *a* ratio for Chrysophytes was higher than the fucoxanthin : chlorophyll *a* ratio for Diatoms (0.463 and 0.104, respectively). Interestingly, in both the oligo-mesotrophic and the mesotrophic lake, the final output fucoxanthin : chlorophyll *a* ratio for Chrysophytes was found to be much lower than the fucoxanthin : chlorophyll *a* ratio for Diatoms (0.032 and 0.685 in the oligo-mesotrophic lake and 0.044 and 0.399 in the mesotrophic lake, respectively).

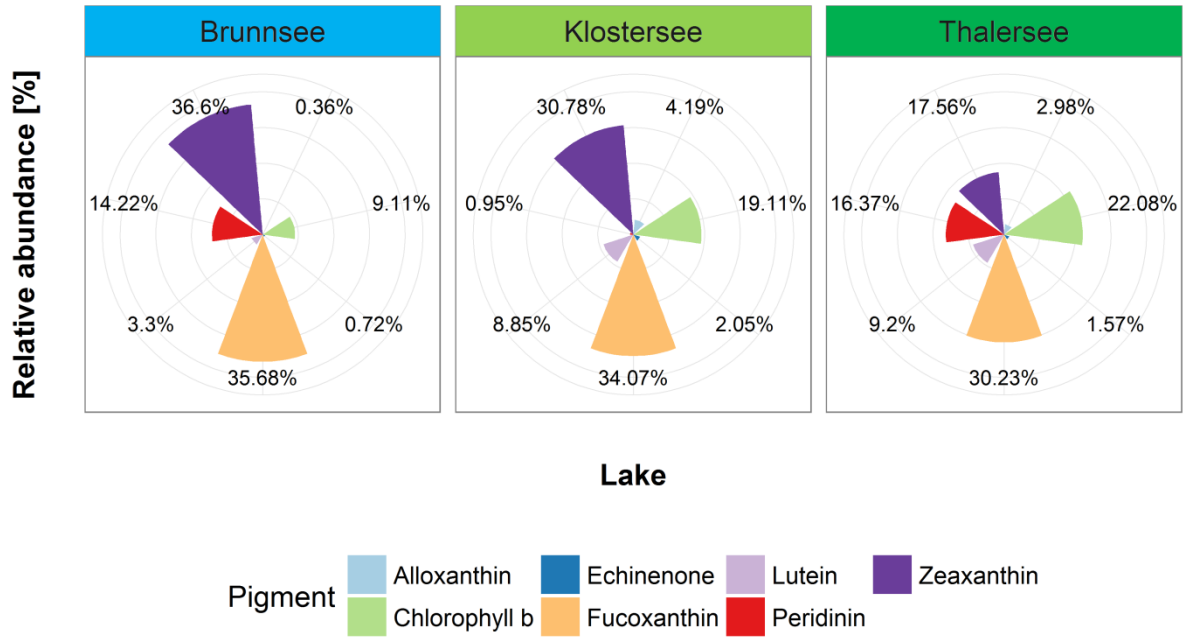

Fig. 1.2: Pigment composition of the natural phytoplankton communities of the lakes Brunnsee (oligotrophic), Klostersee (oligo-mesotrophic) and Thalersee (mesotrophic). Shown are relative abundances of pigments alloxanthin, chlorophyll *b*, echinenone, fucoxanthin, lutein, peridinin and zeaxanthin, given as an average over the whole duration of the experiment, including both mesocosms and the lake itself.

Tab. 1.1: Final pigment : chlorophyll *a* ratio matrices after CHEMTAX calculations for each of the lakes: oligotrophic (Brunnsee), oligo-mesotrophic (Klostersee) and mesotrophic lake (Thalersee). Allo: alloxanthin, Chl *b*: chlorophyll *b*, Echi: echinenone, Fuco: fucoxanthin, Lut: lutein, Peri: peridinin, Zea: zeaxanthin.

	Allo	Chl *b*	Echi	Fuco	Lut	Peri	Zea
Oligotrophic lake							
Chlorophytes	0	0.276	0	0	0.131	0	0.002
Cryptophytes	0.228	0	0	0	0	0	0
Cyanobacteria	0	0	0.012	0	0	0	0.554
Chrysophytes	0	0	0	0.463	0	0	0.014
Diatoms	0	0	0	0.104	0	0	0.019
Dinoflagellates	0	0	0	0	0	0.340	0
Oligo-mesotrophic lake							
Chlorophytes	0	0.264	0	0	0.139	0	<0.001
Cryptophytes	0.162	0	0	0	0	0	0
Cyanobacteria	0	0	0.024	0	0	0	0.538
Chrysophytes	0	0	0	0.032	0	0	<0.001
Diatoms	0	0	0	0.685	0	0	0.002
Dinoflagellates	0	0	0	0	0	0.367	0
Mesotrophic lake							
Chlorophytes	0	0.363	0	0	0.165	0	<0.001
Cryptophytes	0.147	0	0	0	0	0	0
Cyanobacteria	0	0	0.030	0	0	0	0.400
Chrysophytes	0	0	0	0.044	0	0	<0.001
Diatoms	0	0	0	0.399	0	0	<0.001
Dinoflagellates	0	0	0	0	0	0.401	0

1.3.3 Total biomass

The biomasses per sample, given as TChl *a*, ranged between 0.01 and 11.51 µg TChl *a* L^{-1}, as determined with the Algae Lab Analyser, and between 0.22 and 12.92 µg TChl *a* L^{-1}, as determined via HPLC (Fig. 1.3a). The average TChl *a* per lake was higher when determined with Algae Lab Analyser (0.86 in the oligotrophic, 1.27 in the oligo-mesotrophic and 3.19 µg L^{-1} in the mesotrophic lake) compared to the values determined via HPLC (0.53, 1.26 and 2.01 µg L^{-1}, respectively). Despite those differences, we found a high positive correlation for the estimated TChl *a* between the two methods (r_S = 0.82, Tab. 1.2) across all three lakes. The ratio $R_{LAB/CHEM}$ for TChl *a* was 1.47 and differed significantly from the 1:1 relationship (Tab. 1.3). The best match between the two methods was found in the oligo-mesotrophic lake (Kruskal-Wallis test, $X^2_{2,559}$ = 144.57, $p < 0.001$, Tab. 1.4), where the ratio $R_{LAB/CHEM}$ was not significantly different from 1 (value of 1 included in the 95% confidence interval, Tab. 1.3).

1.3.4 Phytoplankton community composition and biodiversity

The phytoplankton communities of all three lakes were strongly dominated by Chromophytes, as determined by Algae Lab Analyser (Fig. 1.4). Their relative abundance ranged from 55% in the oligo-mesotrophic lake to 76% as found in the oligotrophic lake. Second most abundant group in the oligo-mesotrophic and the mesotrophic lake were Cryptophytes (32% and 23%, respectively), while the Chlorophytes were the second most abundant group in the oligotrophic lake (16%). Cyanobacteria were found only in very low abundances being even below 2% (Fig. 1.4).

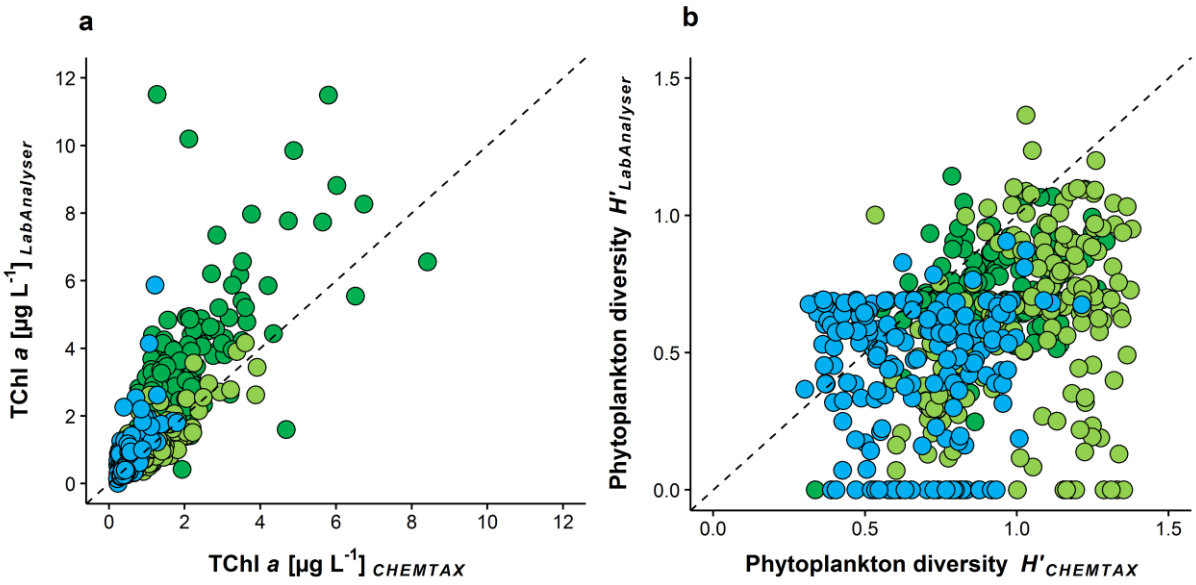

Fig. 1.3: (a) Total chlorophyll *a* concentration (µg L^{-1}) and (b) phytoplankton diversity *H'* (Shannon-Index) determined spectrofluorometrically *in vivo* with Algae Lab Analyser (*y*-axis) and chromatographically *in vitro* via HPLC and CHEMTAX (*x*-axis). The dashed lines represent the 1:1 relationship. Color of the circles represents the trophic state of the lakes, blue: oligotrophic (*n*=186); light green: oligo-mesotrophic (*n*=187); dark green: mesotrophic (*n*=189); n in parentheses indicates the number of water samples per lake used in the study.

With CHEMTAX, we were able to differentiate between the subgroups of Chromophytes (Chrysophytes, Diatoms and Dinoflagellates) and thus received a higher taxonomical resolution of the phytoplankton community composition compared to Algae Lab Analyser. According to the CHEMTAX calculations, in the oligotrophic lake, the Diatoms were the most abundant phytoplankton group (46.44%), followed by Cyanobacteria (15.22%), Dinoflagellates (14.12%) and Chrysophytes (14.06%), while the relative abundance of the Cryptophytes was below 1% (Fig. 1.5), as indicated by very low amount of alloxanthin in the oligotrophic lake (Fig. 1.2). In both the oligo-mesotrophic and the mesotrophic lake, we found high relative abundances of Chrysophytes (43.05% and 36.24%, respectively). As indicated by high amounts of zeaxanthin, chlorophyll *b* and lutein in the oligo-mesotrophic lake (Fig. 1.2), the Chlorophytes (21.98%) were the

second most abundant phytoplankton group in this lake, followed by Cyanobacteria (15.77%, Fig. 1.5). In the mesotrophic lake, which was dominated by Chrysophytes and Diatoms (in total 55.23%), as indicated by high amounts of fucoxanthin, the other 4 phytoplankton groups were all present in relatively similar abundances, ranging from 8.05% (Cryptophytes) to 13.02% (Chlorophytes, Fig. 1.5). Interestingly, in more than 63% of the samples, we found only 1 or 2 functional groups when using Algae Lab Analyser (47 and 311 samples, respectively) while 3 or 4 groups were found in 184 and 20 samples, respectively. With CHEMTAX, we found all four phytoplankton groups in 484 out of 562 samples, while 78 samples had a functional richness of 3 (Fig. 1.6).

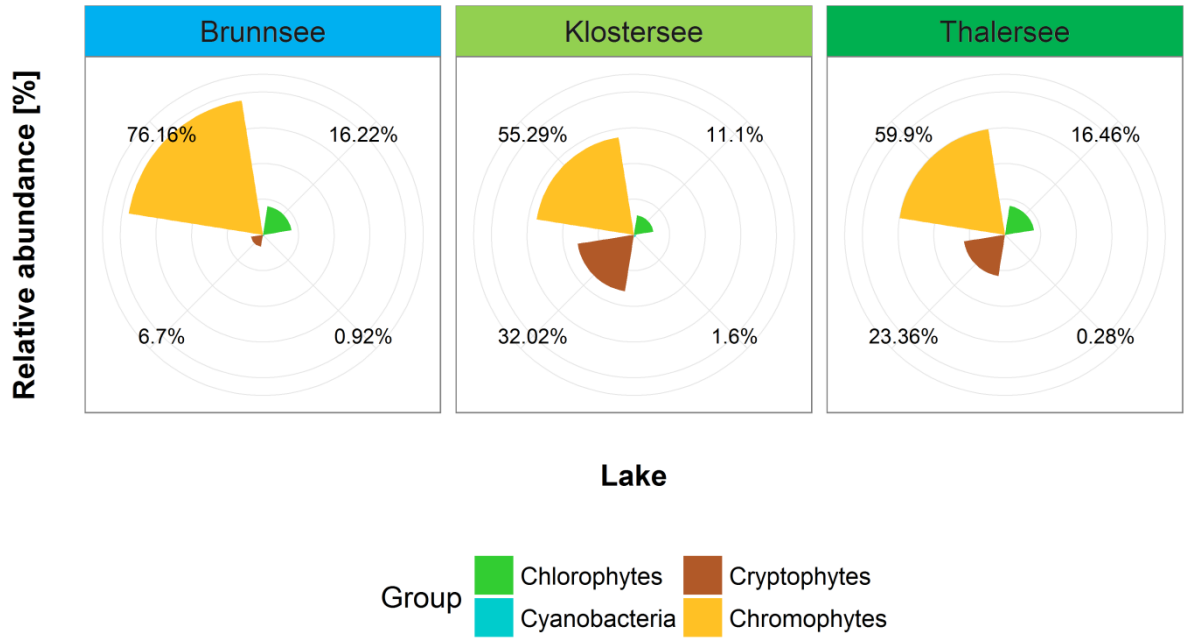

Fig. 1.4: Composition of the natural phytoplankton communities of the lakes Brunnsee (oligotrophic), Klostersee (oligo-mesotrophic) and Thalersee (mesotrophic), as determined via Algae Lab Analyser. Shown are relative abundances of phytoplankton groups Chlorophytes, Chromophytes, Cryptophytes and Cyanobacteria, given as an average over the whole duration of the experiment, including both mesocosms and the lake itself.

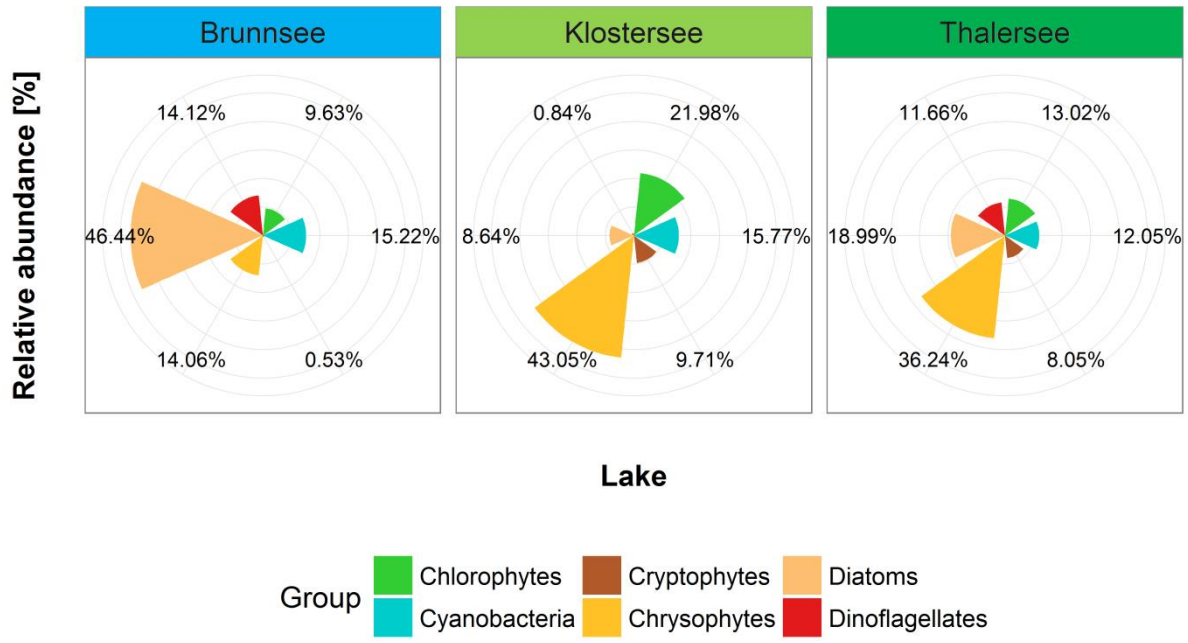

Fig. 1.5: Composition of the natural phytoplankton communities of the lakes Brunnsee (oligotrophic), Klostersee (oligo-mesotrophic) and Thalersee (mesotrophic), as determined via HPLC and CHEMTAX. Shown are relative abundances of phytoplankton groups Chlorophytes, Cryptophytes, Cyanobacteria, Chrysophytes, Diatoms and Dinoflagellates, given as an average over the whole duration of the experiment, including both mesocosms and the lake itself.

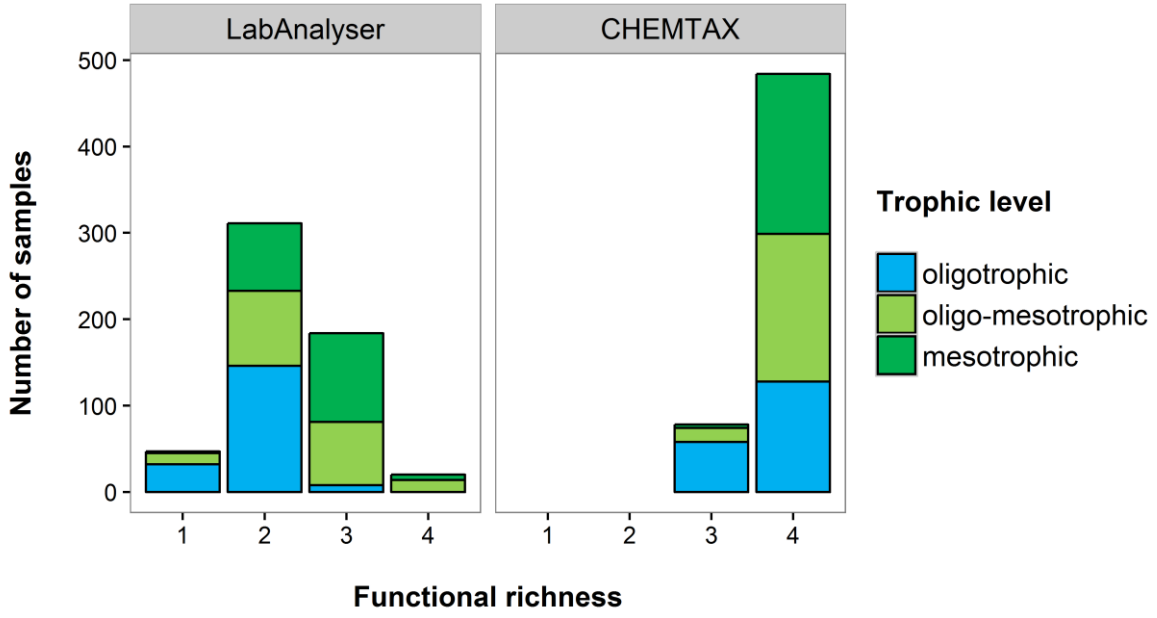

Fig. 1.6: Functional richness of the natural phytoplankton communities in each sample from the lakes Brunnsee (oligotrophic, *n*=186), Klostersee (oligo-mesotrophic, *n*=187) and Thalersee (mesotrophic lake, *n*=189), as determined via Algae Lab Analyser (left panel) and CHEMTAX (right panel). Only the four phytoplankton groups Chlorophytes, Chromophytes, Cryptophytes and Cyanobacteria are included.

The phytoplankton diversity (given as Shannon-Diversity Index) based on the biomass estimates of four phytoplankton groups (Chlorophytes, Chromophytes, Cryptophytes and Cyanobacteria) from the Algae Lab Analyser ranged from 0 (only one group present) to 1.37 and was on average 0.59 across all three lakes. The average phytoplankton diversity based on CHEMTAX biomass estimates was higher (0.88) and ranged between 0.30 and 1.38 (Fig. 1.3b), resulting in a ratio significantly below 1 ($R_{LAB/CHEM}$ = 0.7±0.03 (95% confidence interval), Tab. 1.3). The highest average diversity of the phytoplankton community was found in the oligo-mesotrophic lake (*H'* = 1.03±0.21 (mean ± standard deviation); Kruskal-Wallis test, $X^2_{2,559}$ = 199.34, p < 0.001) based on CHEMTAX biomass estimates, while the mesotrophic lake was the most diverse lake based on biomass estimates from Algae Lab Analyser (*H'* = 0.72±0.15; Kruskal-Wallis test, $X^2_{2,559}$ = 131.52, p < 0.001). We found the lowest average phytoplankton diversity in the oligotrophic lake as indicated by both the Algae Lab Analyser and CHEMTAX biomass estimates (0.43 and 0.66, respectively).

1.3.5 Comparison of the biomass estimates from both methods

We found a very low, but nevertheless significant correlation between the two methods for the biomass estimates of the Chlorophytes (r_s= 0.19, p < 0.001, Tab. 1.2). As determined via the Algae Lab Analyser, the contribution of the Chlorophytes to the TChl *a* was on average 0.36 µg L^{-1} and ranged from 0 to 8.05 µg L^{-1}. With CHEMTAX, we found lower average contribution of the Chlorophytes to the TChl *a* (0.20 µg L^{-1}), with a maximum value of only 3.21 µg L^{-1} in the mesotrophic lake (Fig. 1.7a), which resulted in an average ratio $R_{LAB/CHEM}$ of 2.97 (Tab. 1.3). The best correlation for the Chlorophytes was found in the oligo-mesotrophic lake (r_s = 0.41, p < 0.001, Tab. 1.2 and Tab. 1.4),

which was also the only lake where the average contribution of the Chlorophytes to the TChl *a* was higher when determined via CHEMTAX than Algae Lab Analyser ($R_{LAB/CHEM}$ = 0.58, Tab. 1.3 and Fig. 1.8a).

The highest correlation between the biomass estimates from both methods was found for the Chromophytes (r_s = 0.77, p < 0.001, Tab. 1.2). The average contribution of the Chromophytes to the TChl *a* was 1.07 (Algae Lab Analyser) and 0.82 µg L^{-1} (CHEMTAX, Fig. 1.7b). Again, we found the best fit between the two methods in the oligo-mesotrophic lake (r_s = 0.72, p < 0.001, Tab. 1.2 and Tab. 1.4), with an average ratio $R_{LAB/CHEM}$ of 1.17 (Tab. 1.3). Compared to the biomass estimates of the other phytoplankton groups, the ratios $R_{LAB/CHEM}$ for Chromophytes were the closest to 1 in all three lakes (Fig. 1.8b).

The contribution of the Cryptophytes to the TChl *a* ranged from 0 to 6.75 µg L^{-1} and from 0 to 1.25 µg L^{-1} as determined via Algae Lab Analyser and CHEMTAX, respectively (Fig. 1.7c). On average, we found only 0.09 µg L^{-1} of the Cryptophytes in our samples according to the CHEMTAX calculations, while with the Algae Lab Analyser, the average biomass of the Cryptophytes was 4 times higher (0.36 µg L^{-1}). Still, we found a highly significant positive correlation between the biomass estimates from the two methods across all lakes (r_s = 0.62, p < 0.001, Tab. 1.2), while the best fit was found in the mesotrophic lake (r_s = 0.5, p < 0.001, Tab. 1.2). The worst fit for the Cryptophytes was found in the oligotrophic lake, but was still significant (r_s = 0.15, p < 0.05, Tab. 1.2). The ratios $R_{LAB/CHEM}$ found for the biomass estimates of the Cryptophytes were in many cases very high and ranged up to about 2 x 10^6 as found in the oligo-mesotrophic lake (Fig. 1.8c), which was due to very low concentrations of alloxanthin in the samples and thus, a very low contribution of the Cryptophytes to the TChl *a* as determined via CHEMTAX.

The lowest correlation between the two methods was found for the Cyanobacteria (r_S = 0.07, p > 0.05, Tab. 1.2 and Fig. 1.7d). Although the biomass estimates for the Cyanobacteria from Algae Lab Analyser and CHEMTAX were in a very similar range (0 to 0.86 µg L^{-1} and 0.01 to 0.88 µg L^{-1}, respectively), the overall ratio $R_{LAB/CHEM}$ was only 0.1 (Tab. 1.3 and Fig. 1.8d). Interestingly, we found Cyanobacteria in all samples as determined via CHEMTAX, but the same was the case in only 69 samples when using Algae Lab Analyser. In 493 samples (88% of all samples), Cyanobacteria were not found at all according to the Algae Lab Analyser. In the oligotrophic and the mesotrophic lake, this was even the case in 96% and 93% of the samples, respectively. The only positive correlation between the two methods was found in the oligotrophic lake, but was very low (r_S = 0.17, p < 0.05, Tab. 1.2). For both the Cryptophytes and the Cyanobacteria, no significant differences between the ratios $R_{LAB/CHEM}$ were found between the lakes (Tab. 1.4).

Tab. 1.2: Spearman rank correlation coefficients (r_s) between the chlorophyll *a* concentrations (µg L^{-1}) determined fluorometrically with Algae Lab Analyser and chromatographically via HPLC and CHEMTAX estimated across all lakes and for each lake separately; significance levels are indicated with asterisks: * p < 0.05, *** p < 0.001.

Chlorophyll *a* (µg L^{-1})	All lakes	oligotrophic	oligo-mesotrophic	mesotrophic
	r_s (n = 562)	r_s (n = 186)	r_s (n = 187)	r_s (n = 189)
Total	0.82***	0.56***	0.59***	0.71***
Chlorophytes	0.19***	-0.06	0.41***	0.26***
Chromophytes	0.77***	0.66***	0.72***	0.62***
Cryptophytes	0.62***	0.15*	0.33***	0.5***
Cyanobacteria	0.07	0.17*	0	-0.12

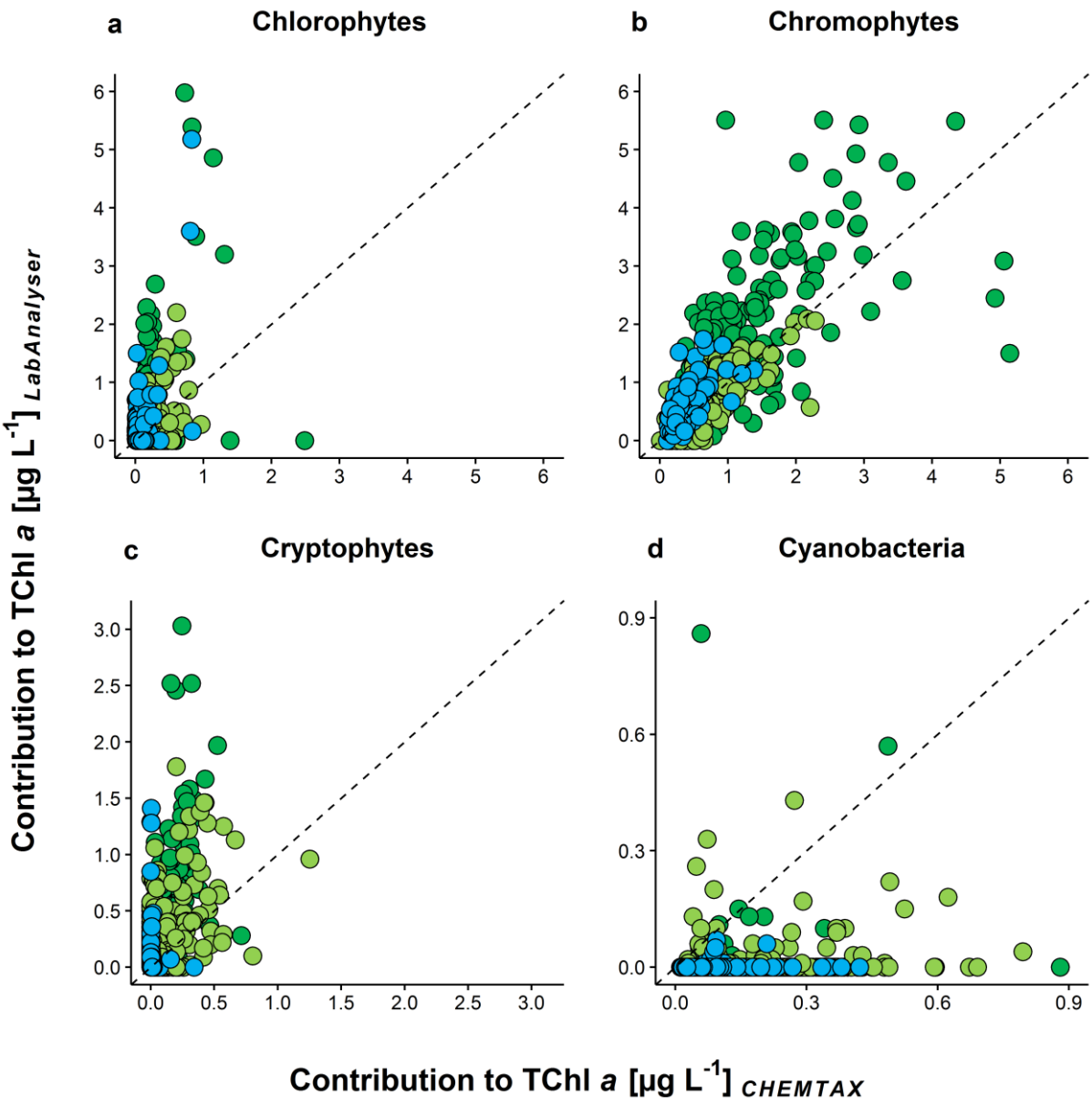

Fig. 1.7: Contribution of (a) Chlorophytes, (b) Chromophytes (Chrysophytes, Diatoms and Dinoflagellates), (c) Cryptophytes and (d) Cyanobacteria to the total chlorophyll *a* concentration (µg L^{-1}) determined spectrofluorometrically *in vivo* with Algae Lab Analyser (*y*-axis) and chromatographically *in vitro* via HPLC and CHEMTAX (*x*-axis). The dashed lines represent the 1:1 relationship. Color of the circles represents the trophic state of the lakes, blue: oligotrophic (*n*=186); light green: oligo-mesotrophic (*n*=187); dark green: mesotrophic (*n*=189); n in parentheses indicates the number of water samples per lake used in the study.

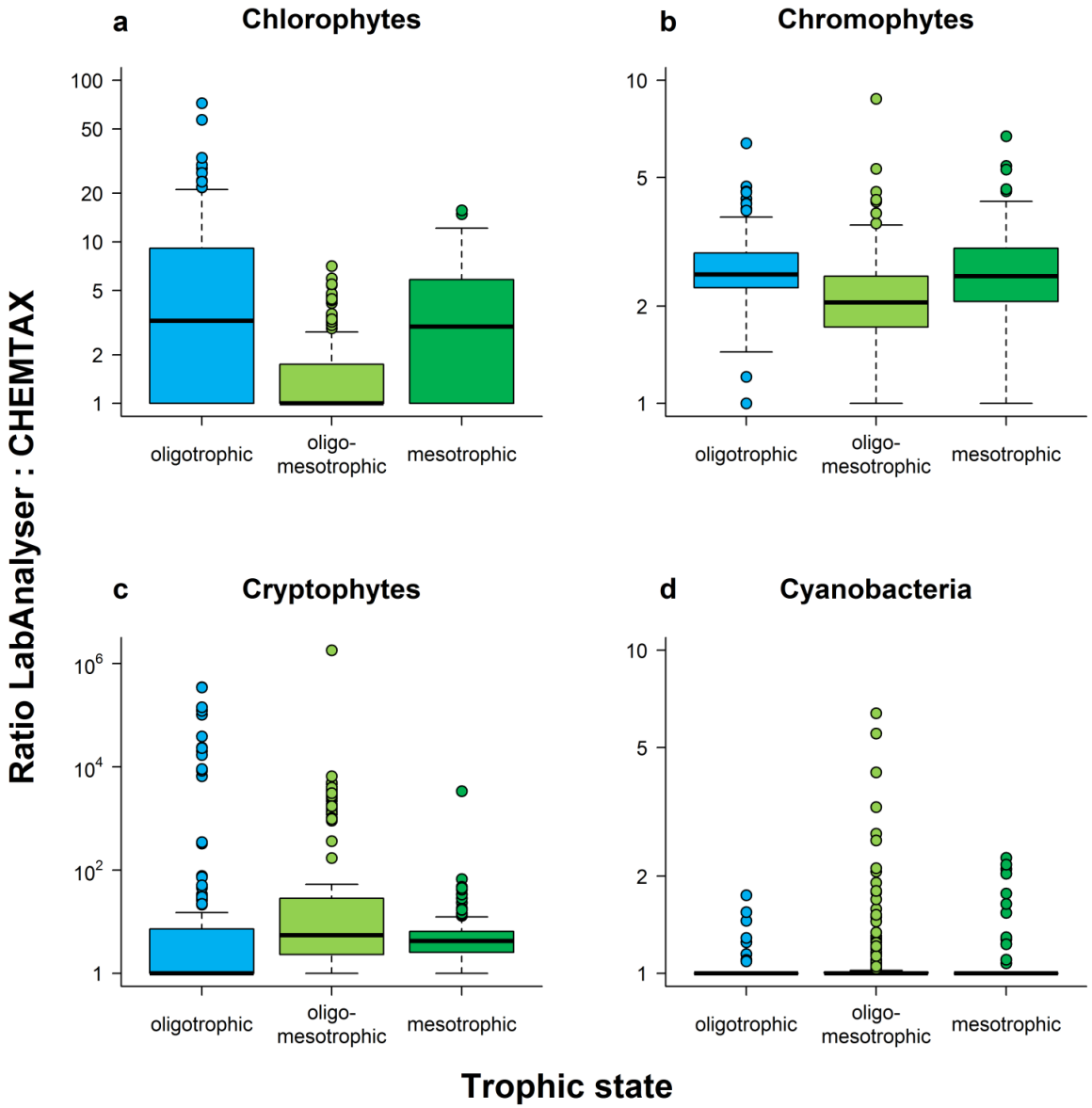

Fig. 1.8: The distribution of the ratios ($R_{LAB/CHEM}$ + 1, to be able to use a logarithmic scale for the *y*-axis) between the contribution of four phytoplankton groups (a) Chlorophytes, (b) Chromophytes (Chrysophytes, Diatoms and Dinoflagellates), (c) Cryptophytes and (d) Cyanobacteria to the total chlorophyll *a* determined spectrofluorometrically *in vivo* with Algae Lab Analyser and chromatographically *in vitro* via HPLC and CHEMTAX for all three lakes of different trophic status.

1.3.6 CHEMTAX derived phytoplankton diversity including all 6 phytoplankton groups

The average ratio between the Shannon-Diversity Indices from two the methods was even lower when all 6 phytoplankton groups (Chlorophytes, Cryptophytes, Cyanobacteria, Chrysophytes, Diatoms and Dinoflagellates) were included into CHEMTAX derived phytoplankton diversity (Fig. 1.9a). Here, the correlation coefficient r_S between Algae Lab Analyser and CHEMTAX derived diversity was only 0.3, while the average ratio $R_{LAB/CHEM}$ across all lakes was 0.44 (Tab. 1.3).

When we compared the HPLC derived pigment diversity (including chlorophyll *a*) and phytoplankton diversity determined via CHEMTAX (all 6 groups included, Fig. 1.9b), we found a highly significant positive correlation ($r_S = 0.67$, $p < 0.001$), which was found to be the highest in the mesotrophic lake ($r_S = 0.82$, $p < 0.001$). The average ratio $R_{phytoplankton/pigments}$ was 1.23 across all lakes and ranged between 0.67 and 1.50. The ratio most closely to 1 was found in the oligo-mesotrophic lake ($R_{phytoplankton/pigments} = 1.13$; Kruskal-Wallis test, $X^2_{2,559} = 290.9$, $p < 0.001$).

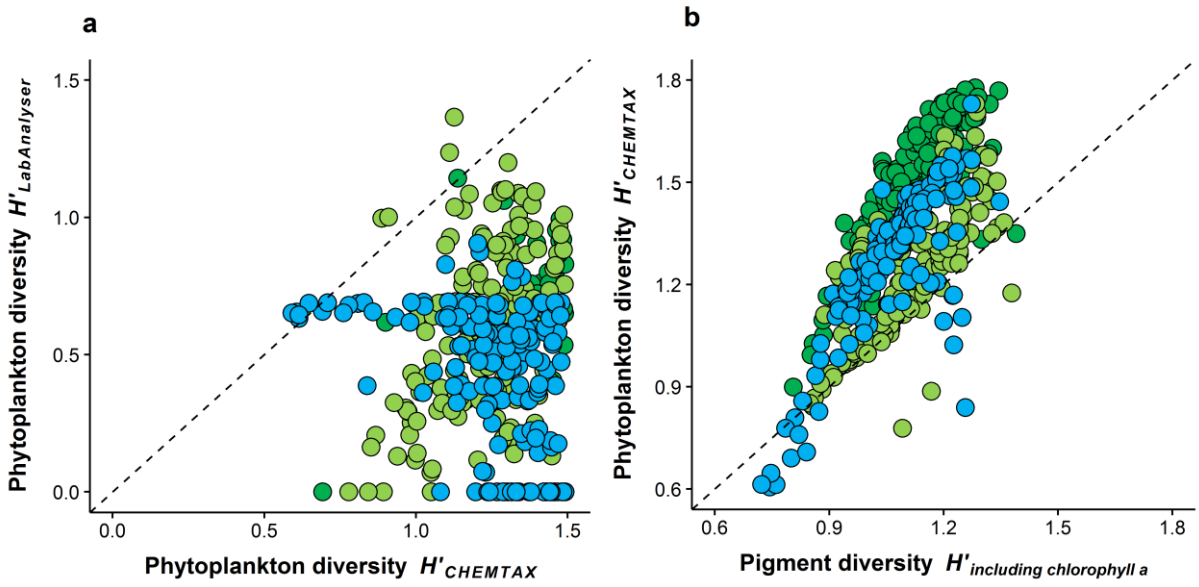

Fig. 1.9: (a) Correlation between the phytoplankton diversity *H'* (Shannon-Index) determined spectrofluorometrically *in vivo* with Algae Lab Analyser (*y*-axis) and chromatographically *in vitro* via HPLC and CHEMTAX (*x*-axis, including all 6 taxonomic groups: Chlorophytes, Cryptophytes, Cyanobacteria, Chrysophytes, Diatoms and Dinoflagellates). (b) Correlation between the phytoplankton diversity estimated with HPLC and CHEMTAX (including all 6 taxonomic groups) and pigment diversity (including chlorophyll *a*). The dashed line represents the 1:1 relationship. For legend, see Fig. 1.3.

Tab. 1.3: Average ratios $R_{LAB/CHEM}$ between the estimates from Algae Lab Analyser and CHEMTAX for the total biomass (TChl *a*, in µg L^{-1}), contribution of the four phytoplankton groups to the chlorophyll *a* (µg L^{-1}) and the phytoplankton diversity (4: including only four groups, Chlorophytes, Chromophytes, Cryptophytes and Cyanobacteria; 6: including all 6 taxonomic groups determined via CHEMTAX, Chlorophytes, Cryptophytes, Cyanobacteria, Chrysophytes, Diatoms and Dinoflagellates). Given are average ratios ± 95% confidence intervals calculated across all lakes and for each lake separately.

Average ratio $R_{LAB/CHEM}$	All lakes	oligotrophic	oligo-mesotrophic	eutrophic
	n = 562	*n* = 186	*n* = 187	*n* = 189
TChl *a*	1.47 ± 0.06	1.65 ± 0.11	1.04 ± 0.06	1.72 ± 0.12
Chlorophytes	2.97 ± 0.49	5.57 ± 1.30	0.58 ± 0.16	2.79 ± 0.44
Chromophytes	1.47 ± 0.07	1.63 ± 0.10	1.17 ± 0.12	1.59 ± 0.12
Cryptophytes	6661.32 ±7618.32	10434.63 ± 8315.19	11017.03 ± 20647.46	23.69 ± 35.83
Cyanobacteria	0.100 ± 0.06	0.014 ± 0.01	0.168 ± 0.09	0.118 ± 0.15
Phytoplankton diversity 4	0.70 ± 0.03	0.73 ± 0.07	0.59 ± 0.04	0.79 ± 0.03
Phytoplankton diversity 6	0.44 ± 0.02	0.36 ± 0.04	0.47 ± 0.03	0.48 ± 0.02

Tab. 1.4: Effects of the trophic status of the lakes on the ratios $R_{LAB/CHEM}$ for (a) total chlorophyll *a* (TChl *a*), biomass estimates for (b) Chlorophytes, (c) Chromophytes, (d) Cryptophytes and (e) Cyanobacteria and for phytoplankton diversity, including either (f) 4 or (g) 6 groups from CHEMTAX. One-way ANOVA was performed for homoscedastic data, while Kruskal-Wallis test (KW) was applied to heteroscedastic data. The different letters in the column "Group" are indicating significant differences between the trophic states (after Tukey's HSD and Dunn's *post hoc* tests following ANOVA and Kruskal-Wallis analyses, respectively). The lakes are ordered depending on the mean values of the given variable (in descending order).

$R_{LAB/CHEM}$	Group	Test	X^2 or F	p
(a) TChl *a*				
mesotrophic	a			
oligotrophic	a	KW	$X^2_{2,559} = 144.57$	< 0.001
oligo-mesotrophic	b			
(b) Chlorophytes				
oligotrophic	a			
mesotrophic	a	KW	$X^2_{2,559} = 64.579$	< 0.001
oligo-mesotrophic	b			
(c) Chromophytes				
oligotrophic	a			
mesotrophic	a	KW	$X^2_{2,559} = 58.576$	< 0.001
oligo-mesotrophic	b			
(d) Cryptophytes				
oligo-mesotrophic	a			
oligotrophic	a	ANOVA	$F_{2,481} = 0.904$	0.406
mesotrophic	a			
(e) Cyanobacteria				
oligo-mesotrophic	a			
mesotrophic	a	ANOVA	$F_{2,559} = 2.196$	0.112
oligotrophic	a			
(f) Phytoplankton diversity 4				
mesotrophic	a			
oligotrophic	b	KW	$X^2_{2,559} = 46.627$	< 0.001
oligo-mesotrophic	c			
(g) Phytoplankton diversity 6				
mesotrophic	a			
oligo-mesotrophic	a	KW	$X^2_{2,559} = 42.477$	< 0.001
oligotrophic	b			

1.4 Discussion

1.4.1 General

Both the Algae Lab Analyser and CHEMTAX allowed for a good general overview of the composition of natural phytoplankton communities. Despite the somewhat limited taxonomic resolution of both methods, the overall patterns of phytoplankton group abundances matched well with both the *in vivo* and the *in vitro* assay. Further, phytoplankton biomass estimates (determined as total chlorophyll *a*) were very similar with both methods. This demonstrates the general utility of both approaches, in contrast to other methods and devices such as FluoroProbe and Algae Online Analyser (both from bbe Moldaenke) which frequently underestimate the total chlorophyll *a* (Gregor and Maršálek, 2004; Catherine et al., 2012; Harrison et al., 2016). As both our methods require relatively little time in comparison to e.g. microscopic counts or DNA-metabarcoding approaches, this makes them highly suited for monitoring and routine phytoplankton analyses.

Despite their general comparability, both methods differed markedly in some important aspects. This applies in particular, but not exclusively, to the determination of cyanobacterial abundances, which are a major focus of phytoplankton community assessment in the context of water quality management. In our study, the Algae Lab Analyser was frequently unable to detect any Cyanobacteria in the lakes' phytoplankton, even though the detection of echinenone in the HPLC gave clear indications of cyanobacterial presence which is supported by microscopic observations of a subset of the samples that showed a presence of Cyanobacteria in the majority of analysed samples.

It has to be noted that manufacturer suggests to calibrate the Algae Lab Analyser with phytoplankton species isolated from the water bodies of interest to get a more accurate assessment of the phytoplankton community composition. However, this seems not realistic in practice, in particular for routine laboratories and water authorities that monitor numerous different lakes and other aquatic systems.

An important aspect that might explain the observed differences between the two methods is the possibility to adjust the sensitivity of the HPLC/CHEMTAX method via the filtered volume of samples. While only 25 ml of the water samples are measured in the Algae Lab Analyser, we filtered 500 – 1000 ml of water for each sample for the pigment-analyses via HPLC. Thus, the concentration of the pigments extracted from the filters and detected via HPLC was higher compared to the pigment concentrations in the water sample measured *in vivo* with Algae Lab Analyser. This probably allowed for the higher sensitivity of the CHEMTAX method and its accuracy in the estimation of low Cyanobacteria abundances.

1.4.2 Comparative assessment of methods

Three specific aspects in the comparative evaluation of the HPLC-based and the *in vivo* method merit particular attention: The first applies to the distinction between Cryptophytes and Cyanobacteria, which is of particular relevance for water quality assessment and monitoring (Gregor et al., 2005; Catherine et al., 2012): The detection of Cryptophytes by the Algae Lab Analyser depends not only on the main Cryptophyte marker pigment alloxanthin, but further on the specific absorption of phycoerythrin (Beutler et al., 2002, 2004), which is also an important pigment for many “red” and “blue” Cyanobacteria (Bryant, 1982; Gregor et al., 2005; Haverkamp et al., 2009).

As the lipophilic extraction commonly applied prior to the HPLC separation of pigments does not capture the hydrophilic pigment groups of phycoerythrins and phycocyanins, these pigments cannot be evaluated by the CHEMTAX approach. This led us to the assumption that CHEMTAX may underestimate the abundance of Cyanobacteria in the lake phytoplankton, as this method does not consider these two main groups of pigments typical for Cyanobacteria.

Interestingly, our data indicated quite the opposite, i.e. a much higher relative abundance of Cyanobacteria in the phytoplankton community assessment via CHEMTAX as compared to the Algae Lab Analyser. Catherine et al. (2012) also reported a "potentially strong misattribution towards Cryptophytes of "red" Cyanobacteria" when they compared the biomass estimates of Cryptophytes and Cyanobacteria from FluoroProbe to the microscopic counts. When examining cyanobacterial blooms in reservoirs, in some samples dominated by Cyanobacteria, Gregor et al. (2005) detected certain amounts of Cryptophytes (approx. 1-20% of TChl *a*) via FluoroProbe, although microscopic counts revealed no Cryptophyte abundances. This may be explained by the inclusion of phycoerythrins into the detection of Cryptophytes by the Algae Lab Analyser (and FluoroProbe). Admittedly, there have been attempts to account for this potential problem by the manufacturers of the Algae Lab Analyser (Beutler et al., 2003, 2004). Nevertheless, our data indicate that under certain conditions, the CHEMTAX approach may be more sensitive for the detection of low cyanobacterial abundances in comparison to the *in vivo* approach of the Algae Lab Analyser.

Beyond the distinction between Cryptophytes and Cyanobacteria, it may also be challenging to distinguish Chlorophytes from Cyanobacteria under certain conditions. Most published HPLC gradients have difficulties in separating the peaks of lutein and zeaxanthin (Latasa et al., 1996; Van Heukelem and Thomas, 2001; Stoń-Egiert and

Kosakowska, 2005). This was also the case for our HPLC gradient. As a consequence, lutein may be frequently underestimated, which would lead to an underestimation of Chlorophytes relative to Cyanobacteria. In our HPLC data, no lutein peak could be identified in some samples, although microscopic counts indicated the presence of Chlorophytes. Such an underestimation of Chlorophyte abundances due to an insufficient separation of lutein and zeaxanthin may explain the lower Chlorophytes : Cyanobacteria ratio detected by CHEMTAX in comparison to the Algae Lab Analyser.

CHEMTAX estimates the relative abundance of Chlorophytes mainly based on the occurrence of lutein and chlorophyll *b*. If chlorophyll *b*, but no lutein is detected, this is probably a consequence of the above mentioned weak separation of the lutein and zeaxanthin peaks in the HPLC. An alternative explanation could be the occurrence of euglenophytes that are characterized by the possession of chlorophyll *b* without a concomitant abundance in lutein (Fietz and Nicklisch, 2004; Schlüter et al., 2006; Sarmento and Descy, 2008). However, microscopic observations of our samples give no indications of common occurrences of euglenophytes in our study lakes.

The third important difference of the two methods is related to the distinction of Diatoms and Chrysophytes. As both share the characteristic pigment fucoxanthin, the Algae Lab Analyser does not allow for a distinction between these algal groups. This is somewhat unfortunate, as these two algal groups often dominate in oligo- and mesotrophic lakes (Buchaca, 2004; Ptacnik et al., 2008; Järvinen et al., 2013; Poxleitner et al., 2016; Schlüter et al., 2016). CHEMTAX provides the distinct advantage of separating Chrysophytes from Diatoms based on their specific fucoxanthin : chlorophyll *a* ratios (Tab. 1.1). As mentioned before, the final output ratio of fucoxanthin : chlorophyll *a* differed between the oligotrophic lake and the oligo-mesotrophic and the

mesotrophic lake, resulting in a switched dominance of either the Diatoms or the Chrysophytes comparing the lakes (oligotrophic lakes: Diatoms more abundant than Chrysophytes, while the opposite was the case in the oligo-mesotrophic and the mesotrophic lake). In the case of the oligo-mesotrophic lake, the microscopic counts indicated a dominance of Diatoms rather than Chrysophytes. One possible explanation might be the usage of a CHEMTAX ratio matrix established for meso- and eutrophic lakes (Schlüter et al., 2016, Tab. 1.3). However, CHEMTAX calculations for the oligo-mesotrophic lake with the ratio matrix established for oligotrophic lakes (Schlüter et al., 2016, Tab. 1.3) yielded the same results (data not shown). This indicates that a differentiation between Diatoms and Chrysophytes based on their specific fucoxanthin : chlorophyll *a* ratios is not sufficient to accurately discriminate these two phytoplankton groups. We thus suggest the inclusion of other pigments into the CHEMTAX approach for a more accurate differentiation of Diatoms and Chrysophytes, e.g. the inclusion of violaxanthin, which is a commonly used marker pigment for Chrysophytes (Buchaca et al., 2005; Descy et al., 2000; Lauridsen et al., 2011; Schlüter et al., 2016).

Within the CHEMTAX analyses, the pigment : chlorophyll *a* ratios are changed trough a series of iterations until the RMS error is stable (Mackey et al., 1996). This means that depending on the data and parameters chosen, the ratios in the final matrix can be very different from the ones in the input matrix. This has positive aspects, as it indicates that the actual data (HPLC derived pigment concentrations) has the highest effect on the outcome of the CHEMTAX analysis. On the other hand, this means that under certain conditions, independently from the original input matrix, ratios can change in a wide range (here: 1/6 to 6 fold). When it comes to pigments that are shared between phytoplankton groups such as fucoxanthin, such an approach could shift the pigment : chlorophyll *a* ratios between the groups in the opposite direction (input

matrix: higher fucoxanthin : chlorophyll *a* for group A compared to group B, output matrix: the other way around). This may be an additional explanation for the inconsistent relative abundance of Diatoms versus Chrysophytes in the oligo-mesotrophic lake of our study. Similar results were found by Simmons et al. (2016), who compared the phytoplankton community composition via HPLC/CHEMTAX estimates to biovolume estimates derived from microscopic counts for the oligotrophic Lake Michigan. There, CHEMTAX overestimated Chrysophytes versus Diatoms. Interestingly, the input fucoxanthin : chlorophyll *a* ratios for both groups of Simmons et al. (2016) were similar to the final output fucoxanthin : chlorophyll *a* ratios for the oligo-mesotrophic and the mesotrophic lake from our study, which leads to a consistent favoring of Chrysophytes over Diatoms in those CHEMTAX matrices. To overcome the observed mismatch between Diatoms and Chrysophytes, Simmons et al. (2016) suggested to include chlorophyll c_1 and c_2 into CHEMTAX analyses. This is because (freshwater) Diatoms contain both chlorophyll c_1 and c_2, while most Chrysophytes contain only chlorophyll c_2 (Jeffrey et al., 2011). Interestingly, when Simmons et al. (2016) combined the CHEMTAX-derived relative abundances of Chrysophytes and Diatoms, the match with the combined relative abundances of these two groups from the microscopic counts was much better, which was also observed in our study (personal observation, data not shown).

1.4.3 Phytoplankton biodiversity

Independent from the number of groups included into the calculation of the Shannon-Diversity Index, the biodiversity of the phytoplankton community based on CHEMTAX was higher than the biodiversity calculated based on Algae Lab Analyser data.

This indicates an advantage of CHEMTAX over Algae Lab Analyser, as it allows for a higher resolution of the phytoplankton community composition.

The lower biodiversity estimates based on the data from the Algae Lab Analyser may be related to the observation, that in more than 63% of the samples, the Algae Lab Analyser identified only 1 or 2 phytoplankton groups. This seems highly unlikely for samples from natural phytoplankton communities. Thus, for studies on biodiversity of phytoplankton communities, CHEMTAX appears to be more suitable.

Another observation made in our study was the high positive correlation between the pigment-based and the phytoplankton-based Shannon-Diversity. This indicates that even the pigment-based diversity can be used as a good proxy for the biodiversity of the phytoplankton community, without the necessity to perform CHEMTAX calculations.

1.4.4 Effects of trophic status

In some cases, the agreement of the two used methods depended on the trophic status of the lake. For example, the best agreement for TChl *a*, Chlorophytes and Chromophytes was found in the oligo-mesotrophic lake Klostersee (Tab. 1.4), while there were no effects of the trophic status on the agreement between Algae Lab Analyser and CHEMTAX for the biomass estimates of Cryptophytes and Cyanobacteria. This might indicate that the agreement between the two methods depends not only on the trophic status of the lake and phytoplankton group, but also on the overall biomass found in the lakes: too low or too high chlorophyll *a* concentrations might be difficult to allocate accurately to the phytoplankton groups.

When differentiating between the Chrysophytes and Diatoms via CHEMTAX, we found higher abundances of Diatoms in the oligotrophic lake compared to Chrysophytes, while the opposite was the case in the oligo-mesotrophic and the mesotrophic lake. However,

microscopic counts indicated a mismatch between the Chrysophytes and Diatoms in the oligo-mesotrophic and the mesotrophic lake, while the abundances of those two groups, as determined with CHEMTAX, corresponded well to the cell counts in the oligotrophic lake. One explanation for such findings may be the usage of different input ratio matrices for the oligotrophic lake compared to the oligo-mesotrophic and the mesotrophic lake. However, a repetition of CHEMTAX calculations for the oligo-mesotrophic lake with the input ratio matrix for oligotrophic lakes (Schlüter et al., 2016) yielded unaltered results.

Interestingly, with both Algae Lab Analyser and CHEMTAX, we found the lowest average diversity in the oligotrophic lake Brunnsee. This was surprising, as many studies claim that oligotrophic lakes usually harbor more diverse phytoplankton communities compared to mesotrophic or eutrophic lakes (Stanley-Samuelson et al., 1987; Harrison, 1990; Leonard et al., 2004)(Leibold, 1999; Dodson et al., 2000). This is probably due to a strong dominance of chromophytes and in particular Diatoms in lake Brunnsee. Nevertheless, we cannot exclude that despite the low functional diversity observed in Brunnsee, there may be an underlying high species richness within one functional group.

1.4.5 Conclusions

Both the Algae Lab Analyser and HPLC/CHEMTAX are fast and useful tools for the assessment of the phytoplankton community composition. However, the agreement between the methods was not always satisfactory. This is similar to findings by Richardson et al. (2010) and may be due to different marker pigments utilized by the two methods. Also, more pigments should be included in the HPLC analysis, especially to be able to distinguish between Diatoms and Chrysophytes, e.g. violaxanthin and

chlorophylls c_1 and c_2. As both methods have advantages and disadvantages, the method of choice depends on the aim of the study or the field of use.

While the Algae Lab Analyser is more suitable for rapid monitoring, CHEMTAX provides a higher resolution of the biodiversity in the community and better estimates of cyanobacterial abundances. Within the present study, pigment-based method via HPLC and CHEMTAX was used in **Chapter 4** to assess the phytoplankton community composition.

Chapter 2

Equal relevance of omega-3 and omega-6 polyunsaturated fatty acids for the fitness of *Daphnia* spp.

2.1 Introduction

The food quality of phytoplankton for *Daphnia* is frequently determined by the availability of essential polyunsaturated fatty acids (PUFAs), i.e. fatty acids with two or more double bonds in their carbon chain. Similar to other arthropod animals, *Daphnia* are incapable of synthesizing ω3- and ω6-PUFAs *de novo* (Stanley-Samuelson et al., 1987; Harrison, 1990; Leonard et al., 2004). However, previous studies suggest that at least some *Daphnia* species are able to convert ω3- and ω6-PUFAs within the respective PUFA family, albeit at conversion rates insufficient to cover the PUFA demands of the daphnids (Taipale et al., 2011). Thus, certain ω3- and ω6-PUFAs are considered as essential for *Daphnia* and have to be derived from the diet (Weers et al., 1997; von Elert, 2002; Schlechtriem et al., 2006). In several field studies, the juvenile somatic growth of *Daphnia* sp. was shown to correlate with the content of ω3-PUFAs in the seston, in particular α-linolenic acid (α-LA, 18:3ω3; Wacker and von Elert, 2001) and eicosapentaenoic acid (EPA, 20:5ω3; Müller-Navarra, 1995b). As juvenile somatic growth rate is a good proxy for *Daphnia* fitness (Lampert and Trubetskova, 1996), this suggests that the dietary availability of ω3-PUFAs can limit the fitness of *Daphnia* in nature. This view is supported by multiple laboratory studies: *Daphnia galeata* fed with algal food supplemented with single ω3-PUFAs showed increased somatic growth rates when EPA, α-LA and docosahexaenoic acid (DHA, 22:6ω3) were supplemented (von Elert, 2002). In particular the dietary EPA availability was shown to limit not only the somatic growth rate of *Daphnia* (Becker and Boersma, 2003; Sperfeld and Wacker, 2012), but also their reproduction (Ravet et al., 2003; Martin-Creuzburg et al., 2008, 2010) and population growth (Martin-Creuzburg et al., 2010). Hence, EPA has been strongly recognized as a dietary PUFA limiting the fitness of various *Daphnia* species.

Besides EPA, the C_{20}-PUFA arachidonic acid (ARA, 20:4ω6) is assumed to play an important role for *Daphnia*'s fitness and physiology (Kainz et al., 2004; Brett et al., 2006; Schlotz et al., 2014). The main structural difference between these two PUFAs is the position of the first double-bond relative to the ω-end (methyl-end) of the carbon chain (ω3 vs. ω6). Both ARA and EPA serve as precursors for eicosanoids, a family of hormone-like substances such as prostaglandins, which are known to affect the reproduction, the immune system and the ion transport physiology of both vertebrates and invertebrates (Stanley-Samuelson, 1994; Stanley, 2000). Nevertheless, studies investigating the eicosanoid pathway in *Daphnia* were mostly focused on the role of ARA (Heckmann et al., 2008b; Schlotz et al., 2012), while the relevance of EPA for the eicosanoid metabolisms was poorly understood until recently (Schlotz et al., 2016; Fink and Windisch, 2018). The relevance of ARA for *Daphnia* is further supported by the finding that daphnids accumulate significant amounts of ARA, both during starvation and feeding, either by direct uptake from the diet or by bioconversion of other available ω6-PUFAs (Kainz et al., 2004; Schlechtriem et al., 2006; Smyntek et al., 2008; Burns et al., 2011; Taipale et al., 2011).

However, in contrast to EPA, the reports on potential constraints of *Daphnia*'s fitness through ARA availability are rather inconsistent. In a field study, Wacker and von Elert (2001) applied a modified Monod model to describe the relationship between the somatic growth rate of *D. galeata*, which was raised on natural seston of Lake Constance, and the sestonic content of different ω3-and ω6-PUFAs. They found equal proportions of variance explained by the Monod model when the sum of the sestonic content of all ω3-PUFAs was considered compared to the sum of ω6-PUFAs (R^2 = 0.86 for both ω-families). Furthermore, their Monod model describing the relationship between the growth of daphnids and the sestonic concentration of ARA explained 70% of the

variance. The study of Wacker and von Elert (2001) hence provided first evidence that in the field, ω6-PUFAs might play a role for the fitness of *Daphnia*. The first controlled laboratory study on the role of PUFAs for *Daphnia* fitness by von Elert (2002) found *D. galeata* to be limited only by the availability of ω3-, but not of ω6-PUFA (namely ARA) availability. These findings were supported by a later study by Ravet et al. (2012), where an ARA-supplemented diet did (in contrast to EPA supplementation) not increase the somatic growth rate or reproduction of *Daphnia pulex*, although equal amounts of EPA and ARA were used in the respective diet treatments. This leads to the assumption that the effect size of ARA-supplementation (in terms of increased growth or reproduction) is smaller than the effect size of EPA-supplementation. On the other hand, Martin-Creuzburg et al. (2010) reported an increase in reproduction (determined as cumulative number of offspring), but not of somatic growth of *Daphnia magna* fed a cholesterol- and ARA-supplemented cyanobacterium. Furthermore, survival and reproduction of *D. magna* exposed to an opportunistic bacterial pathogen (*Pseudomonas* sp.) were increased when ARA was supplemented to a C_{20}-PUFA-deficient diet (Schlotz et al., 2014). Additionally, Schlotz et al. (2014) also observed an increased growth of *D. magna* when a C_{20}-PUFA-free diet mixture of *Acutodesmus obliquus* (formerly *Scenedesmus*) and *Synechococcus elongatus* was supplemented with ARA, which is in contrast to the previous findings from von Elert (2002), Martin-Creuzburg et al. (2010) and Ravet et al. (2012).

Due to the highly inconsistent findings, the limitation of *Daphnia*'s fitness and in particular its growth and reproduction by the availability of this ω6-PUFA cannot be resolved without the determination of threshold saturation concentrations, as previously established for ω3-PUFAs (Sperfeld and Wacker, 2011a). Such saturation thresholds are defined as minimum dietary PUFA concentrations that are necessary

for a saturated (i.e. unlimited) growth or reproduction (Sperfeld and Wacker, 2011a). Along a nutrient concentration gradient, starting with an infinite availability of a particular PUFA, a higher saturation threshold indicates a stronger and earlier occurring limitation of *Daphnia*'s fitness compared to lower saturation thresholds. The determination of threshold saturation concentrations of dietary PUFAs may therefore not only allow for identification of PUFAs that limit the fitness of *Daphnia*, but also may provide an indication about PUFA-limiting conditions for *Daphnia* in nature (Becker and Boersma, 2005).

To be able to better predict consumer's population dynamics (including consumer's fitness as well as inter- and intraspecific competition), gaining further knowledge on the relevance of ω6-PUFAs for the fitness of *Daphnia* might be crucial. This is of particular importance when shifts in the ratio of dietary availability of ω3- and ω6-PUFAs within the phytoplankton community occur. Additionally, with respect to competitive interactions in the field, potential intra- and interspecific variability in ARA demands within the genus *Daphnia* has to be considered. Such different specific PUFA demands between *Daphnia* species may depend on their body size, as lipid accumulation and assimilation rates of different cladocerans were found to increase with increasing body size (Goulden and Place, 1993). Therefore, it is reasonable to assume that larger sized *Daphnia* species (e.g. *D. magna*) might have higher rates of assimilation and accumulation of ARA and EPA compared to smaller sized *Daphnia* (e.g. *D. pulex, D. pulicaria* and *D. longispina*). Furthermore, it is not yet clear, if and at which rates ARA and EPA can be synthesized due to (retro-)conversion of other available PUFAs by different *Daphnia* species (Weers et al., 1997; von Elert, 2002; Schlechtriem et al., 2006). While specific EPA demands by different *Daphnia* species have been previously reported by Sikora et al. (2016), intra- and interspecific differences in ARA requirements within

the genus *Daphnia* have, to our knowledge, not yet been studied. Although addressing such intra- and interspecific differences would require investigations with more genotypes from multiple species, single clone studies can nevertheless provide first evidence on possible species-specific ARA requirements.

We specifically hypothesize that: (i) insufficient ARA availability limits the fitness of *Daphnia* species; (ii) ARA threshold concentrations that are necessary to allow for saturated (i.e. ARA-unlimited) growth and reproduction of *Daphnia* are lower compared to the respective EPA saturation thresholds. To address these hypotheses, we conducted dose-response growth experiments with two *Daphnia* species of different body size fed with C_{20}-PUFA-free diet supplemented with either EPA or ARA.

2.2 Materials and Methods

2.2.1 Study organisms and cultivation

Two *Daphnia* clones, one from the medium sized species *D. pulex* (clone Gerstel, Koch et al., 2009) and one from the large species *D. magna* (clone B, Lampert and Rothhaupt, 1991) were cultured in clonal lines in aged, aerated and sterile-filtered (0.45 µm) tap water at 20°C and a 16:8 h light:dark cycle. During the preculture phase (at least three generations), the daphnids were fed with the green alga *Acutodesmus obliquus* (strain SAG 276-3a from the Göttingen Algal Culture Collection SAG, Germany), which is rich in C_{18}-PUFAs, but lacks C_{20}-PUFAs such as ARA (C20:4 n-6) and EPA (C20:5 n-3, Windisch and Fink, 2018), at a concentration of 2 mg particulate organic carbon (POC) L^{-1} every other day. *A. obliquus* was cultured in Z/4 medium (Zehnder and Gorham, 1960) in semi-continuous (dilution rate 0.1 d^{-1}) 5 L batch cultures at 100 µE s^{-1} m^{-2} PAR.

2.2.2 Growth experiments

We placed six randomly assigned neonates of the 3rd clutch (hatched within 20 hours) per species into 200 ml (*D. pulex*) or 300 ml (*D. magna*) aged and aerated tap water, respectively. The neonates were fed with *A. obliquus* (2 mg POC L^{-1}). ARA and EPA were supplemented via liposome carriers (Martin-Creuzburg et al. 2008) loaded with of either ARA (122 ng $µl^{-1}$) or EPA (142 ng $µl^{-1}$). Different volumes of the ARA- or EPA-containing liposome suspensions were used, which resulted in a dietary gradient ranging from 0.5 to 10 µg ARA or EPA mg POC^{-1}, respectively. In order to exclude a possible effect of liposome carriers on the growth of the daphnids, we maintained equal concentration of liposomes in all food treatments by adding appropriate amounts of PUFA-free control liposomes to all treatments with < 10 µg PUFA mg POC^{-1}. Further, in the control

treatment, we supplied the same volume of PUFA-free control liposomes as was necessary to achieve the highest (10 µg mg POC^{-1}) PUFA concentrations. In total, we had one control treatment (*A. obliquus* + control liposomes = 0 µg PUFA mg POC^{-1}, which served as the C_{20}-PUFA-free treatment for both experimental lines) and nine PUFA-treatments (0.5, 1, 1.5, 2, 2.5, 3, 5, 7 and 10 µg PUFA mg POC^{-1}) supplemented with either EPA or ARA, resulting in a total of 19 treatments in triplicates. Juveniles were transferred into fresh food + liposome suspensions daily. At the start of the experiments, we placed 3 x 10 neonates into preweighed aluminium boats and dried them at 60°C in the drying oven for at least 24 h. After 6 (*D. pulex*) or 7 days (*D. magna*), we sampled half of the daphnids (all not egg-bearing) and dried them in aluminium boats. The second half (egg-bearing individuals; all eggs were counted to determine the clutch size) was sampled one day later, i.e. after 7 (*D. pulex*) or 8 days (*D. magna*) and dried in aluminium boats. After drying, aluminium boats with daphnids were weighed on a Sartorius microbalance type CP2 P (accuracy 1 µg). The somatic growth rate g (d^{-1}) of the daphnids was calculated as (Eq. 2):

$$g = \frac{\ln(W_t) - \ln(W_0)}{t}$$

where W_0 is the dry mass at the beginning of the experiment, W_t is the dry mass at the end of the experiment and t is the duration of the experiment in days.

2.2.3 Fatty acid analyses

To be able to estimate the volume of the liposome suspensions that had to be added to the treatments in order to achieve a certain concentration gradient, we analyzed the fatty acid content of the respective liposomes via gas chromatography (GC). We first

extracted lipids with 5 ml of dichloromethane/methanol (2:1, v:v) from 100 µl of each liposome suspension (control liposomes, ARA- and EPA-containing liposomes; in triplicates). For subsequent quantification of fatty acids, we added two internal standards to the samples, i.e. 10 µg heptadecanoic acid methyl ester (C17:0 ME) and 5 µg tricosanoic acid methyl ester (C23:0 ME) and sonicated for 1 minute. The samples were evaporated to dryness at 40°C under a stream of nitrogen gas and fatty acids were transesterified at 70°C for 20 min in 5 ml of 10% methanolic HCl. The methanolic HCl was prepared by addition of acetyl chloride (>99%, Acros Organics, Geel, Belgium) to ice-cooled methanol. The resulting fatty acid methyl esters (FAMEs) were extracted twice with approx. 2 ml of isohexane. The isohexane phases were joined and subsequently evaporated at 40°C under a stream of nitrogen gas and the remaining FAMEs were redissolved in 50 µl isohexane per sample. 1 µl of each sample was injected (splitless) into a 6890-N GC System (Agilent Technologies, Waldbronn, Germany) and analyzed using the same method as described by Windisch and Fink (2018). We found ~140 ng μl^{-1} of ARA or EPA in the respective liposomes, while we confirmed the control liposomes to be PUFA-free.

2.2.4 Growth and reproduction saturation thresholds

We determined growth and reproduction saturation thresholds, i.e. PUFA-concentrations at which the growth and reproduction of juvenile daphnids reaches saturation (Sperfeld and Wacker, 2011a). For this, we used the saturation curve procedure described in Sperfeld and Wacker (2011), where a saturation curve is fitted using the growth rates of the daphnids at certain PUFA amounts. We applied the same procedure to our reproduction data. This procedure has two main advantages compared to the conventional statistical method (ANOVA) used to determine growth saturation

thresholds: a) it allows for a calculation of thresholds for different saturation levels; b) combined with a bootstrapping method, it allows for an uncertainty (variance) of the estimated thresholds, which is necessary for statistical comparisons of thresholds between e.g. species or PUFAs. Furthermore, the threshold concentration estimated with ANOVA depends on the distances between the PUFA concentrations within the chosen concentration gradient (for further discussion, see Sperfeld and Wacker, 2011).

Therefore, to estimate the EPA and ARA threshold concentrations for the saturated growth of daphnids, we applied a modified Monod function (Monod, 1950) to describe the growth rate g (d^{-1}) of the daphnids along the EPA or ARA concentration gradient as (Eq. 3):

$$g = g_0 + \frac{(g_\infty - g_0) \times S}{(S + K_S)}$$

where g_0 is the growth rate observed in the PUFA-free treatment (0 µg mg POC^{-1} of EPA and ARA respectively), g_∞ the asymptotic growth rate (d^{-1}), S the amount of EPA or ARA supplemented to the diet (µg mg POC^{-1}) and K_S the half saturation constant (µg mg POC^{-1}; threshold for the 50% growth saturation level $g_{50\%}$). We separately analyzed the growth rates of not egg-bearing vs. egg-bearing mothers. g_0 was calculated as the mean growth rate of the daphnids raised in the C_{20}-PUFA-free treatment (n=3 for *D. pulex* and not egg-bearing *D. magna* and n=2 for egg-bearing *D. magna*), while g_∞ and K_S were predicted from the Monod model. Additionally, we calculated the adjusted R^2 as a measure for the proportion of variance explained by the fitted Monod model.

As asymptotic growth rates can only be reached at an infinite amount of EPA and ARA, we used the predicted parameters for the saturated growth of the two *Daphnia* species from the fitted curves to additionally calculate the EPA and ARA thresholds $S_{75\%}$ (in µg mg POC^{-1}) for the 75% growth saturation level $g_{75\%}$, which corresponds to the reduction

of g_∞ by 25% relative to the baseline g_0. This saturation level was used for two main reasons, which are both further discussed in Sperfeld and Wacker (2011): a) although higher saturation levels (e.g. 90% or 95%) are much closer to the asymptotic growth rate g_∞, calculation of PUFA threshold concentrations for $g_{90\%}$ and $g_{95\%}$ is not recommended as variability in threshold concentrations increases with higher saturation levels and can lead to biased threshold estimates; b) to avoid biased PUFA threshold estimation by neglecting bootstrapped data sets for which regression curves do not intersect with the line of the growth level, which is likely to occur for growth saturation levels ≥ 90%. Using the same model (Eq. 3), we predicted the asymptotic clutch size ($eggs_\infty$), the half saturation constant K_S for the saturated reproduction and the EPA and ARA threshold concentrations $S_{75\%}$ for the 75% reproduction saturation level $eggs_{75\%}$ (equivalent to a reduction of $eggs_\infty$ by 25% relative to the baseline $eggs_0$). The initial clutch size ($eggs_0$) was calculated as the mean number of eggs produced by daphnids raised in the C_{20}-PUFA-free treatment (n=3 for *D. pulex* n=2 for *D. magna*).

2.2.5 Bootstrapping

To be able to statistically compare the estimated growth saturation thresholds between PUFAs and species, we applied the bootstrapping procedure described in Sperfeld and Wacker (2011): we allowed for uncertainty in the calculated thresholds by randomly leaving out one replicate per concentration level > 0 µg PUFA mg POC^{-1} (separately for the EPA- and ARA-limited growth) and thus generating 1000 new data sets per species and PUFA-specific growth response with a sample size of n-9 (as we used 9 different EPA and ARA concentration levels). Those new data sets were used again for curve fitting (using the Eq. 3) resulting in N values for each parameter (g_∞, K_S, $S_{75\%}$ for $g_{75\%}$ and adjusted R^2) per species and PUFA. g_0 was calculated as the mean growth rate of the

daphnids raised in the C_{20}-PUFA-free treatment and was set as a fixed starting point for every curve; thus, there was no variance in g_0 within one species, as we used the same C_{20}-PUFA-free control treatment for both PUFA experimental lines. In some cases, it was not possible to fit the curves through the bootstrapped growth rate data; therefore, these bootstrapped data sets were omitted from further analyses. Additionally, we applied a second data cleaning step by leaving out all data sets were curve fitting resulted in a negative K_S (i.e. $K_S < 0$ µg PUFA mg POC^{-1}), as such results were biological meaningless. The same bootstrapping procedure and the subsequent data cleaning were applied for the reproduction data. The final counts N (max. 1000) of biologically meaningful growth and reproduction saturation curves for both species and both tested PUFAs (and thus the number of bootstrapped values for each parameter) are given in Table 2. Additionally, using the bootstrapped data, we calculated intersection points between 1000 randomly chosen EPA- and ARA-saturation curves for the growth and reproduction of the two *Daphnia* species. We used the function *optimise()* in R, with a fixed maximum of 1 mg PUFA mg POC^{-1}. Although this upper limit is biologically not meaningful and technically not reachable (e.g. via liposome supplementation), it allows for the maximal possible variation within the potential intersection points. All intersection points ≤ 0 µg PUFA mg POC^{-1} and exactly equal to 1 mg PUFA mg POC^{-1} were excluded from the further analysis for following reasons: a) values below zero are not biologically significant; b) values equal zero indicate no intersection of the curves after the initial starting point g_0, which was equal for EPA- and ARA-curves within one species; c) values equal to 1 mg (fixed maximum) indicate that the intersection point is equal to or above 1 mg PUFA mg POC^{-1}, which is biologically not meaningful.

2.2.6 Statistical analyses

Bootstrapping, fitting of the modified Monod function and subsequent statistical analyses were performed in R (version 3.3.2, R Core Team, 2016) and RStudio (version 1.1.383, RStudio Team, 2016). We tested the effect of the factors *PUFA* (EPA vs. ARA), *Species* (*D. pulex* vs. *D. magna*) and their interaction *PUFA* × *Species* on the predicted PUFA threshold concentrations $S_{75\%}$ for 75% growth and reproduction saturation levels as well as the asymptotic growth rate g_{∞} and asymptotic clutch size $eggs_{\infty}$ via two-way ANOVA. For this, we followed a statistical procedure described in Martin-Creuzburg et al. (2014): for each of the predicted parameters, we randomly chose the same number of replicates as in the growth experiments (n=3 for both species and both PUFAs) from the distribution of N bootstrapped parameters. These were compared via two-way ANOVA. The procedure was repeated 400 times and the p-values for the two main factors and the interaction were recorded. As suggested by Martin-Creuzburg et al. (2014), we searched for a critical p-value necessary to hold for a statistical power of 0.8 (equivalent to a type II error of 0.2). Homogeneity of variances was checked with Levene's test. Finally, we compared the estimated intersection points of EPA- and ARA-curves for growth and reproduction between the two *Daphnia* species: we randomly chose 6 replicates (accounting for 3 replicates per EPA and ARA experimental line in the original growth experiments) from the distribution of intersection points and compared these via the non-parametric Wilcoxon-Mann-Whitney-test (due to the lack of residuals normal distribution, checked via Shapiro-Wilk's test). This procedure was repeated 400 times (separately for growth and reproduction) to assess the critical p-value necessary to hold for a statistical power of 0.8.

2.3 Results

2.3.1 C_{20}-PUFA-limited growth of Daphnia *spp.*

Growth rates of egg-bearing *Daphnia pulex* and *Daphnia magna* increased with increasing amounts of ARA supplemented to *Acutodesmus obliquus*, following a typical saturation curve (Fig. 2.1). We observed a similar pattern for the EPA-limited growth of *D. magna* (Fig. 2.1b). Accordingly, 58.34 % and 47.47 % of the variance of *D. magna* growth were explained by the saturation curves fitted along the EPA and ARA concentration gradient, respectively. For *D. pulex* however, the modified Monod model explained only 22.10 % of the variance of the ARA-limited growth of *D. pulex*. Growth rates of *D. pulex* clearly increased at EPA concentrations ≥ 5 µg EPA mg POC^{-1}, but did not show a clear pattern along the experimental EPA concentration gradient (Fig. 2.1a). As a consequence, the modified Monod model explained only 18.74% of the variance of EPA-limited growth of *D. pulex*. In *D. pulex*, the mean growth rate g_0 in the C_{20}-PUFA-free control treatment was 0.370 d^{-1}, while the predicted asymptotic growth rate g_∞ with EPA supply was 0.407 d^{-1} and 0.401 d^{-1} when ARA was supplemented. This accounted for a 9% and 8% growth increase (gain in growth from g_0 in absence of C_{20}-PUFAs to g_∞) by EPA and ARA supply, respectively (Fig. 2.1a, Tab. 2.1). In *D. magna*, the growth rate increased from 0.391 d^{-1} (g_0) to a predicted asymptotic growth rate of 0.461 d^{-1} (~ 15% increase) when EPA was supplemented and to 0.449 d^{-1} when ARA was supplemented (~ 13% increase, Fig. 2.1b, Tab. 2.1). We further used the predicted parameters g_∞ and K_S (Tab. 2.1) from the fitted saturation curves (Fig. 2.1) to calculate EPA and ARA threshold concentration $S_{75\%}$ (given in µg mg POC^{-1}) for the 75% growth saturation level $g_{75\%}$ for both *Daphnia* species. The estimated PUFA threshold concentration $S_{75\%}$ was below 5 µg of PUFA per mg particulate organic carbon for *D. magna* ($S_{75\%\,(EPA)}$ = 4.183 µg

mg POC^{-1}, $S_{75\%\ (ARA)}$ = 2.230 µg mg POC^{-1}, Tab. 2.1). For *D. pulex*, we found similar $S_{75\%}$ for ARA-limited growth ($S_{75\%\ (ARA)}$ = 2.625 µg mg POC^{-1}) and a 3-fold higher $S_{75\%}$ for EPA-limited growth ($K_{S(EPA)}$ = 13.850 µg mg POC^{-1}, Tab. 2.1) compared to *D. magna*. Note that these findings refer to the saturation curves fitted through the raw growth rate data. Interestingly, the growth rate of not egg-bearing *D. pulex* (sampled after 6 days) did not show any clear patterns along the PUFA concentration gradient (Fig. S2a), while the EPA- and ARA-limited growth of *D. magna* followed the saturation curve (Fig. S2b). Similar to egg-bearing *D. magna*, the saturation curves fitted through the growth rate data of not egg-bearing *D. magna* explained 58.93% and 50.12% of variance (adjusted R^2) for the EPA- and ARA-limited growth, respectively (Tab. S1).

Bootstrapping procedure and the subsequent statistical analyses revealed significant differences in the predicted asymptotic growth rates g_∞ among egg-bearing *Daphnia*: we found significantly higher g_∞ for *D. magna* compared to *D. pulex* (two-way ANOVA; p(Species) < 0.001, Fig. 2.2a, Tab. 2.2a and Tab. 2.3). The factors *PUFA* (EPA vs. ARA) and the interaction *PUFA* × *Species* did not have any significant effect on the asymptotic growth rate g_∞ (two-way ANOVA; p(PUFA) = 0.298, p(PUFA × Species) = 0.809, Tab. 2.3). We did not find any significant effects of the two main factors and their interaction on the threshold concentrations $S_{75\%}$ (Fig. 2.2b, Tab. 2.3). Summary of estimated parameters for the growth of *D. pulex* and *D. magna*, derived from the bootstrapping procedure, can be found in Table 2.2a.

2.3.2 C_{20}-PUFA-limited reproduction of Daphnia *spp.*

Similar to the growth, the reproduction (given as number of eggs per individual) of both *Daphnia* species increased upon EPA and ARA supplement (Fig. 2.1c and 2.1d). However, the proportion of variance explained by the fitted saturation curves along the PUFA

concentration gradient was only 21% for EPA- and ARA-limited reproduction of *D. pulex* and 22% and 33% for the reproduction of *D. magna* under EPA and ARA supply, respectively (Tab. 2.1). *D. pulex* and *D. magna* reared in C_{20}-PUFA-free treatment produced ~5 and ~9 eggs per individual, respectively ($eggs_0$, calculated as the mean of n=3 (*D. pulex*) and n=2 (*D. magna*) replicates, Tab. 2.1). Via the Monod model, we predicted an asymptotic clutch size $eggs_\infty$ of 6.456 and 6.442 (Ind^{-1}) for EPA- and ARA-limited reproduction of *D. pulex*, while the predicted asymptotic clutch size was approx. 2-fold higher for *D. magna* (13.791 and 14.284 eggs Ind^{-1} under EPA and ARA supply, respectively). Hence, we predicted a ~29% increase in the number of eggs produced per individual in *D. pulex* in both experimental lines (EPA vs. ARA supply), while *D. magna* was predicted to increase its reproduction by ~33% and ~35% (from $eggs_0$ to $eggs_\infty$) when EPA or ARA are present in infinite amounts, respectively. The estimated threshold concentration $S_{75\%}$ for the 75% reproduction level $eggs_{75\%}$ was below 1.5 µg PUFA mg POC^{-1} for both species and PUFAs (*D. pulex*: $S_{75\%\ (EPA)}$ = 1.141 µg mg POC^{-1}, $S_{75\%\ (ARA)}$ = 0.131 µg mg POC^{-1}; *D. magna*: $S_{75\%\ (EPA)}$ = 1.471 µg mg POC^{-1}, $S_{75\%\ (ARA)}$ = 0.561 µg mg POC^{-1}, Tab. 2.1).

Via the bootstrapping procedure, we found a significantly higher asymptotic clutch size $eggs_\infty$ produced by *D. magna* compared to *D. pulex* (two-way ANOVA; p(Species) < 0.001, Fig. 2.2c, Tab. 2.2b and Tab. 2.3), while there were no significant differences between the two PUFA experimental lines (EPA vs. ARA, p(PUFA) = 0.754). The interaction *PUFA* × *Species* did not have any significant effect on $eggs_\infty$ (p(PUFA × Species) = 0.766). Finally, the threshold concentration $S_{75\%}$ for the 75% reproduction saturation level was neither significantly affected by the two main factors nor by their interaction (Fig. 2.2d, Tab. 2.3). A summary of estimated parameters for the reproduction of *D. pulex* and *D. magna*, derived from the bootstrapping procedure, can be found in Table 2b.

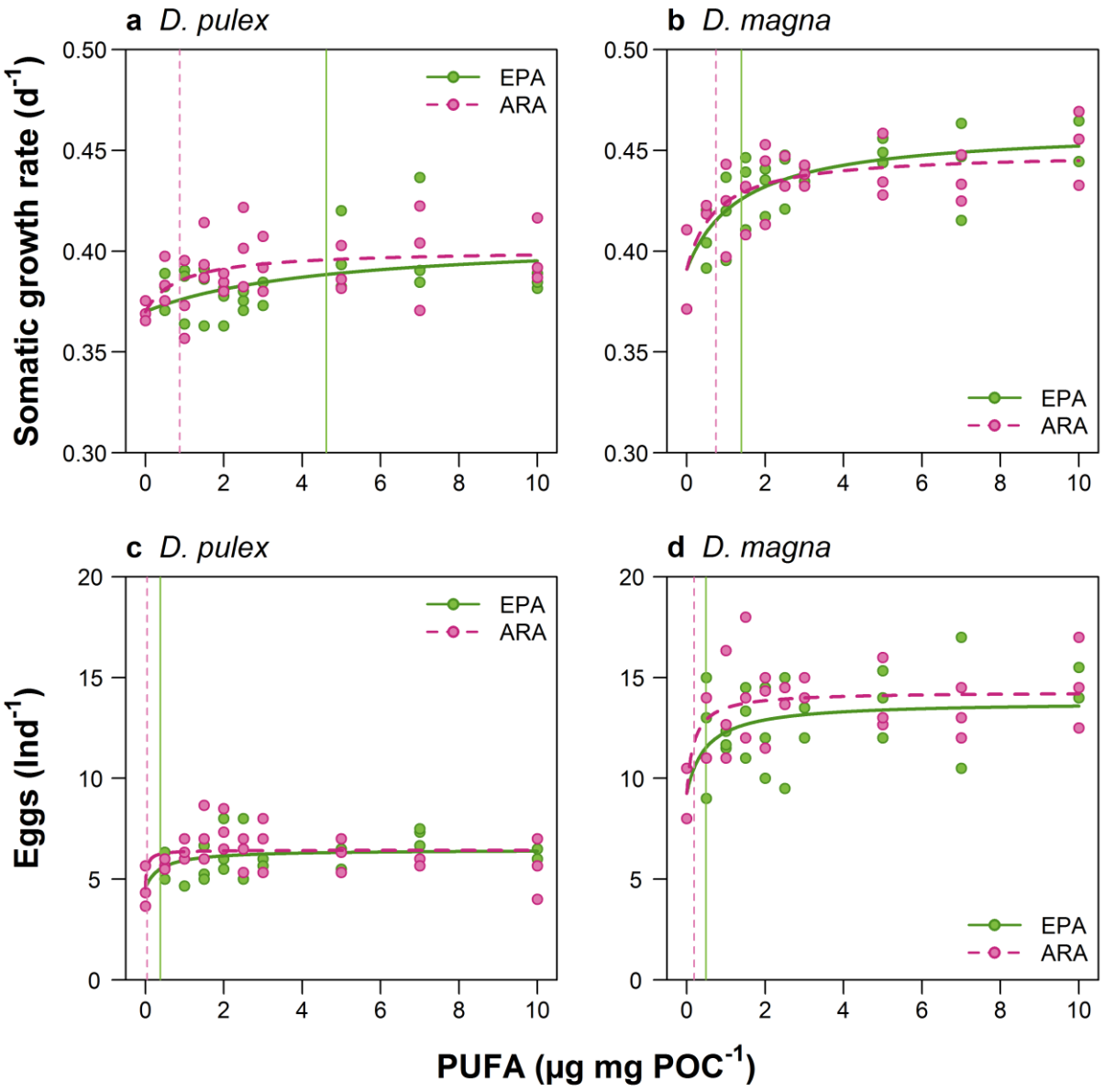

Fig. 2.1: Somatic growth rate g (d^{-1}) and reproduction (clutch size, i.e. number of eggs per individual) of *Daphnia pulex* (egg-bearing, sampled after 7 days; panels a and c, respectively) and *Daphnia magna* (egg-bearing, sampled after 8 days; panels b and d, respectively) grown on *Acutodesmus obliquus* supplemented with different amounts (µg mg POC^{-1}; particulate organic carbon) of eicosapentaenoic acid (EPA, green circles) or arachidonic acid (ARA, pink circles). Solid (EPA) and dashed (ARA) saturation curves are based on modified Monod functions (nonlinear least-square fits, Eq. 3). Vertical lines (green solid: EPA, pink dashed: ARA) indicate half saturation constants K_S (in µg PUFA mg POC^{-1}) of growth and reproduction saturation curves. Summary of the plots can be found in Table 2.1.

2.3.3 Intersection points of EPA- and ARA-curves for growth and reproduction

Finally, we calculated intersection points between 1000 randomly chosen fitted saturation curves describing the EPA- and ARA-limited growth (Fig. 2.3a) and reproduction (Fig. 2.3b) of *D. pulex* and *D. magna*. After data cleaning, 767 for *D. pulex* and 905 for *D. magna* intersection points remained for the growth curves, while the final number of estimated intersection points for the reproduction curves was 515 for *D. pulex* and 603 for *D. magna*. Although we found large variation of the intersection points within each of the two species, the median values were below 10 µg PUFA mg POC^{-1}. The median intersection point for the growth of *D. pulex* was at 9.26 µg PUFA mg POC^{-1}, while the median intersection point for the growth of *D. magna* was much lower (2.64 µg PUFA mg POC^{-1}). Despite this 4-fold difference in median values, the growth-curves intersection points were not significantly different between the two *Daphnia* species (Wilcoxon-Mann-Whitney test, $p = 0.94$). Similarly, we did not find a significant difference among the two *Daphnia* species for the intersection points of the EPA- and ARA-dependent reproduction curves (4.12 and 4.97 µg PUFA mg POC^{-1} (median values) for *D. pulex* and *D. magna*, respectively; Wilcoxon-Mann-Whitney test, $p = 0.82$).

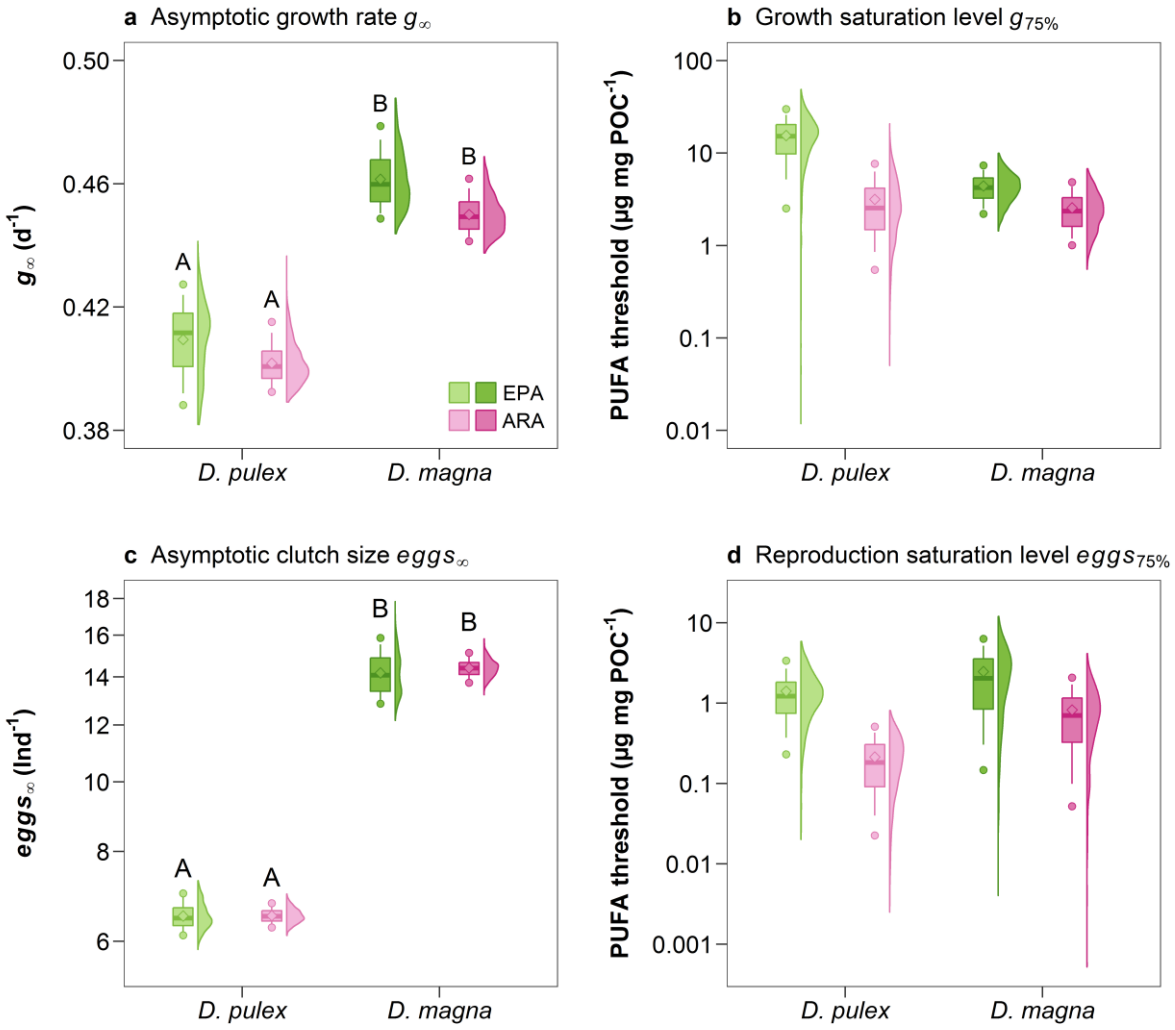

Fig. 2.2: (a) Asymptotic growth rate g_∞ (d^{-1}), (b) PUFA threshold concentration $S_{75\%}$ (in µg mg POC^{-1}) for growth saturation level $g_{75\%}$ (corresponding to reduction of g_∞ by 25% relative to the baseline g_0), (c) asymptotic clutch size $eggs_\infty$ (number of eggs per individual) and (d) PUFA threshold concentration $S_{75\%}$ (in µg mg POC^{-1}) for reproduction saturation level $eggs_{75\%}$ (corresponding to reduction of $eggs_\infty$ by 25% relative to the baseline $eggs_0$) for egg-bearing *D. pulex* and *D. magna* grown on *A. obliquus* supplemented with either EPA (green) or ARA (pink). In all panels, the flat violin plot represents the distribution (density) of the data, while the boxplots represent the median (horizontal lines within each box), 25% and 75% quartiles (box), 10% and 90% percentiles (whiskers), 95% confidence interval (circles) and the mean value (diamond). In the panels a-b, the asymptotic growth rate and the PUFA threshold concentration were derived from nonlinear least-square fits through bootstrapped growth rate data (see Methods). In the panels c-d, the asymptotic clutch size and the PUFA threshold concentration were derived from nonlinear least-square fits through bootstrapped reproduction data (clutch size, i.e. number of eggs per individual; see Methods). Note that the y-axis in the panels b, c and d has a logarithmic scale. Summary of the plots (sample size, median, mean ± standard deviation) and statistical analyses can be found in Table 2.2 and 2.3, respectively. Different letters indicate significantly different groups (two-way ANOVA).

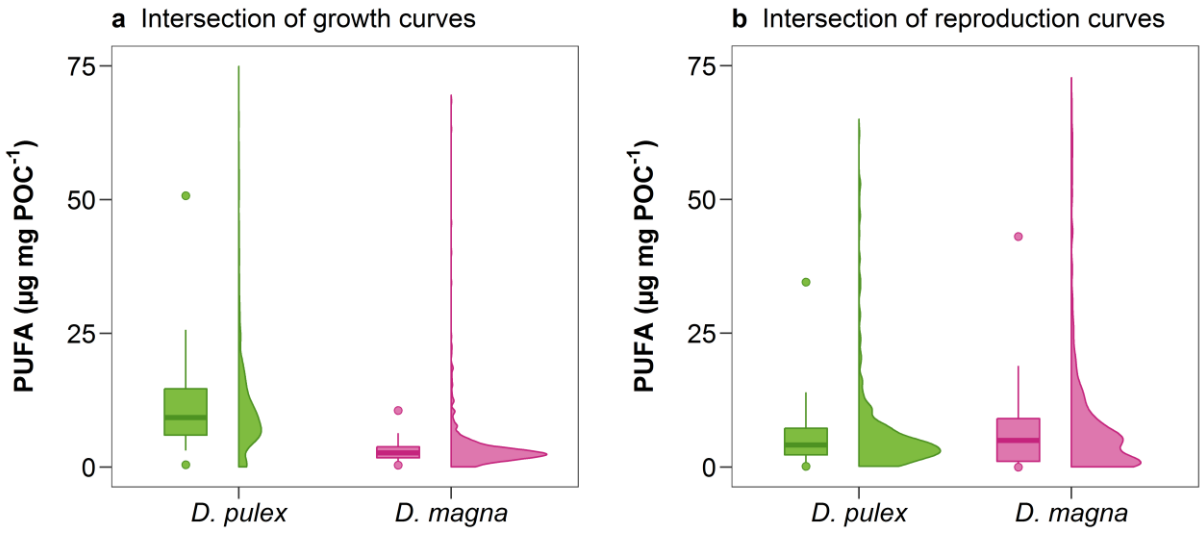

Fig. 2.3: Intersection points (µg PUFA mg POC^{-1}) of EPA- and ARA-limited (a) growth and (b) reproduction saturation curves for egg-bearing *D. pulex* (green) and *D. magna* (pink). In all panels, the flat violin plot represents the distribution (density) of the data, while the boxplots represent the median (horizontal lines within each box), 25% and 75% quartiles (box), 10% and 90% percentiles (whiskers) and 95% confidence interval (circles). All intersection points ≤ 0 µg PUFA mg POC^{-1} were excluded from the data set due to lack of biological significance (i.e. no intersection of the EPA- and ARA-curves). Note that for visualization reasons, the outliers exceeding 75 µg PUFA mg POC^{-1} are not shown in the figure. Number of intersection points for growth curves: n = 767 (*D. pulex*) and n = 905 (*D. magna*); number of intersection points for reproduction curves: n = 515 (*D. pulex*) and n = 603 (*D. magna*). We did not find a significant difference between the species (Wilcoxon-Mann-Whitney-test; Growth: p = 0.94; Reproduction: p = 0.82). For details, see Methods.

Tab. 2.1: Somatic growth rate g_0 (d^{-1}) and clutch size $eggs_0$ (Ind^{-1}) for *D. pulex* (egg-bearing, sampled after 7 days) and *D. magna* (egg-bearing, sampled after 8 days) grown on *A. obliquus* without C_{20}-PUFA supplementation; asymptotic growth rate g_∞ (d^{-1}), asymptotic clutch size $eggs_\infty$ (Ind^{-1}); half-saturation constant K_S (in µg PUFA mg POC^{-1}) for the EPA- and ARA-limited growth and reproduction of the two *Daphnia* species; PUFA threshold concentration $S_{75\%}$ (in µg PUFA mg POC^{-1}) for growth and reproduction saturation levels $g_{75\%}$ and $eggs_{75\%}$ (corresponding to reduction of g_∞ and $eggs_\infty$ by 25% relative to the baseline g_0 and $eggs_0$, respectively). g_0 and $eggs_0$ were calculated prior to curve fitting (mean growth rate and clutch size of *D. pulex* (n=3) and *D. magna* (n=2) while g_∞, $eggs_\infty$, K_S, $S_{75\%}$ (for both growth and reproduction) and the corresponding adjusted R^2 were derived from saturation curves based on modified Monod functions (nonlinear least-square fits, Eq. 3). Total n refers to the total number of data points used to fit the saturation curves (10 different tested concentrations of EPA or ARA in triplicates, i.e. max. 30 data points). This data correspond to the plots in Figure 2.1.

	Somatic growth					**Reproduction**			
	Daphnia pulex		*Daphnia magna*			*Daphnia pulex*		*Daphnia magna*	
	ARA	EPA	ARA	EPA		ARA	EPA	ARA	EPA
Total n	30	30	27	27	Total n	30	30	27	27
g_0 (d^{-1})	0.370	0.370	0.391	0.391	$eggs_0$ (Ind^{-1})	4.556	4.556	9.250	9.250
g_∞ (d^{-1})	0.401	0.407	0.449	0.461	$eggs_\infty$ (Ind^{-1})	6.442	6.456	14.284	13.791
K_S (µg mg POC^{-1})	0.875	4.617	0.743	1.394	K_S (µg mg POC^{-1})	0.044	0.380	0.187	0.490
$S_{75\%}$ for $g_{75\%}$ (µg mg POC^{-1})	2.625	13.850	2.230	4.183	$S_{75\%}$ for $eggs_{75\%}$ (µg mg POC^{-1})	0.131	1.141	0.561	1.471
Adjusted R^2	0.221	0.187	0.475	0.583	Adjusted R^2	0.210	0.212	0.332	0.218

Tab. 2.2: (a) Somatic growth rate g_0 (d^{-1}) for *D. pulex* (egg-bearing, sampled after 7 days) and *D. magna* (egg-bearing, sampled after 8 days) grown on *A. obliquus* without C_{20}-PUFA supplementation; asymptotic growth rate g_∞ (d^{-1}), half saturation constant K_S (in µg PUFA mg POC^{-1}) and PUFA threshold concentration $S_{75\%}$ for growth saturation level $g_{75\%}$ (in µg PUFA mg POC^{-1}) for the EPA- and ARA-limited growth of the two *Daphnia* species. Growth saturation level $g_{75\%}$ corresponds to a reduction of g_∞ by 25% relative to the baseline g_0. (b) Clutch size ($eggs_0$, Ind^{-1}) for *D. pulex* and *D. magna* grown on *A. obliquus* without C_{20}-PUFA supplementation; asymptotic clutch size ($eggs_\infty$, Ind^{-1}), half saturation constant K_S (in µg PUFA mg POC^{-1}) and PUFA threshold concentration $S_{75\%}$ for reproduction saturation level $eggs_{75\%}$ (in µg mg POC^{-1}) for the EPA- and ARA-limited reproduction of the two *Daphnia* species. Reproduction saturation level $eggs_{75\%}$ corresponds to a reduction of $eggs_\infty$ by 25% relative to the baseline $eggs_0$. g_0 and $eggs_0$ were calculated prior to curve fitting (mean growth rate and number of eggs of *D. pulex* (n=3) and *D. magna* (n=2) and used as fixed initial points of the saturation curves during the bootstrapping procedure. g_∞, $eggs_\infty$, K_S, $S_{75\%}$ (for both growth and reproduction) and the corresponding adjusted R^2 were derived from nonlinear least-square fits through bootstrapped growth rate and reproduction data (see Methods) and are given both as median and as mean values ± standard deviation. n refers to the total number of data points used to fit the saturation curves (one replicate per each concentration level > 0 µg PUFA mg POC^{-1} was left out during the bootstrapping procedure), while N refers to the total number of saturation curves fitted after bootstrapping and data cleaning (see Methods). This data corresponds to the plots in Figure 2.2.

(see Table on the next page)

(a) Somatic growth

		Daphnia pulex		*Daphnia magna*	
		ARA	EPA	ARA	EPA
n		21	21	18	18
N		997	974	1000	1000
g_0 (d^{-1})		0.370	0.370	0.391	0.391
g_∞ (d^{-1})	Median	0.401	0.412	0.449	0.460
	Mean ± 1 SD	0.402 ± 0.007	0.409 ± 0.012	0.450 ± 0.006	0.461 ± 0.009
K_S (µg PUFA mg POC^{-1})	Median	0.847	5.070	0.784	1.410
	Mean ± 1 SD	1.050 ± 0.784	5.170 ± 2.72	0.852 ± 0.398	1.470 ± 0.525
$S_{75\%}$ for $g_{75\%}$ (µg PUFA mg POC^{-1})	Median	2.541	15.225	2.353	4.229
	Mean ± 1 SD	3.156 ± 2.352	15.497 ± 8.150	2.556 ± 1.195	4.418 ± 1.576
Adjusted R^2	Median	0.265	0.222	0.529	0.578
	Mean ± 1 SD	0.280 ± 0.077	0.221 ± 0.046	0.531 ± 0.048	0.604 ± 0.074

(b) Reproduction

		Daphnia pulex		*Daphnia magna*	
		ARA	EPA	ARA	EPA
n		21	21	18	18
N		778	984	779	816
$eggs_0$ (Ind^{-1})		4.556	4.556	9.250	9.250
$eggs_\infty$ (Ind^{-1})	Median	6.498	6.461	14.399	14.073
	Mean ± 1 SD	6.510 ± 0.154	6.510 ± 0.271	14.400 ± 0.419	14.200 ± 0.980
K_S (µg PUFA mg POC^{-1})	Median	0.061	0.407	0.233	0.676
	Mean ± 1 SD	0.071 ± 0.050	0.469 ± 0.317	0.274 ± 0.221	0.823 ± 0.675
$S_{75\%}$ for $eggs_{75\%}$ (µg PUFA mg POC^{-1})	Median	0.182	1.220	0.698	2.028
	Mean ± 1 SD	0.213 ± 0.149	1.410 ± 0.952	0.821 ± 0.664	2.470 ± 2.020
Adjusted R^2	Median	0.283	0.243	0.428	0.294
	Mean ± 1 SD	0.293 ± 0.063	0.275 ± 0.080	0.435 ± 0.065	0.304 ± 0.077

Tab. 2.3: Results from two-way ANOVAs: We tested the effect of factors *PUFA* (EPA vs. ARA), *Species* (*D. pulex* vs. *D. magna*) and their interaction *PUFA* × *Species* on the estimated parameters asymptotic growth rate g_∞ (d^{-1}; Fig. 2.2a), $S_{75\%}$ for growth saturation level $g_{75\%}$ (in µg PUFA mg POC^{-1}; Fig. 2.2b), asymptotic clutch size $eggs_\infty$ (Ind^{-1}; Fig. 2.2c) and $S_{75\%}$ for reproduction saturation level $eggs_{75\%}$ (in µg PUFA mg POC^{-1}; Fig. 2.2d) derived from nonlinear least-square fits through bootstrapped growth rate and reproduction data (see Methods). In total, 400 trials of two-way ANOVA were performed for each estimated parameter comparing randomly chosen bootstrapped data (n=3 per group, in total 12 data points). The p-values given in the table correspond to a critical p-value which holds for a statistical power of 0.8 (equivalent to a type II error of 0.2).

	Parameter	Factors	*p*
Somatic growth	g_∞ (d^{-1})	*PUFA*	0.298
		Species	**< 0.001**
		PUFA × *Species*	0.809
	$S_{75\%}$ for $g_{75\%}$ (µg PUFA mg POC^{-1})	*PUFA*	0.096
		Species	0.137
		PUFA × *Species*	0.230
Reproduction	$eggs_\infty$ (Ind^{-1})	*PUFA*	0.754
		Species	**< 0.001**
		PUFA × *Species*	0.766
	$S_{75\%}$ for $eggs_{75\%}$ (µg PUFA mg POC^{-1})	*PUFA*	0.152
		Species	0.528
		PUFA × *Species*	0.805

2.4 Discussion

2.4.1. C_{20}-PUFA-limited fitness of D. magna

In the present study, we show that the dietary availability of both ω3-PUFA (EPA) and ω6-PUFA (ARA) limits the fitness (i.e. both the juvenile somatic growth and reproduction rates) of two different *Daphnia* species. The results obtained from dose-response growth experiments with the large-bodied *D. magna* fed on a green alga supplemented with the ω3-PUFA EPA are in line with previous findings. The estimated threshold concentration $S_{75\%}$ for the 75% saturation level (i.e. reduction of asymptotic growth rate by 25%) of the EPA-limited growth of *D. magna* in our study (4.418 ± 1.576 µg EPA mg POC^{-1} after bootstrapping procedure; mean ± 1 SD) was in the range of previously published 75% growth saturation thresholds for this species found at the same temperature (20°C), e.g. 0.7 µg EPA mg POC^{-1} (Sperfeld and Wacker, 2011) and 5.83 – 7.33 µg EPA mg POC^{-1} (Sikora et al., 2016). Furthermore, we observed an increase in the somatic growth rate of *D. magna* when ARA was supplied. This is in line with the results from Schlotz et al. (2014), who observed higher growth rates of *D. magna* fed with an ARA-enriched food mixture of *A. obliquus* and *S. elongatus* compared to a C_{20}-PUFA-free diet. Becker and Boersma (2005) also observed an increase in somatic growth of *D. magna* when ARA was supplemented to P-sufficient *A. obliquus* and provided an ARA threshold (lowest ARA concentration at which the growth of *D. magna* was not limited) of only 0.06 mg per g dry mass which corresponds to approx. 0.136 µg ARA mg POC^{-1} when a conversion factor of dry mass to carbon of 0.44 is used (Becker and Boersma, 2005, 2010). While Becker and Boersma (2005) found similarly low EPA thresholds (0.02 and 0.25 µg of EPA mg^{-1} dry mass, corresponding to 0.05 and 0.57 µg EPA mg POC^{-1}, respectively), their approach and findings were strongly criticized by

Brett (2010) and thus may need to be interpreted with caution. We here report for the first time reasonable ARA saturation thresholds for the growth of *D. magna*. Furthermore, contrary to expectations, our data show that equal (not statistically different) amounts of EPA and ARA are required to allow for saturated (i.e. unlimited) growth of *D. magna*. At saturating concentrations, EPA and ARA seem to be equally utilized by *D. magna*, which results in similar asymptotic growth rates.

2.4.2 C_{20}-PUFA-limited fitness of D. pulex

Our data suggest that also the growth of *D. pulex* is limited by both dietary EPA and ARA availability. The estimated 75% threshold concentration $S_{75\%}$ for EPA-limited growth of *D. pulex* (2.625 µg EPA mg POC^{-1} estimated from the raw data and 3.156 ± 2.352 µg EPA mg POC^{-1} after bootstrapping procedure) was almost 10-times higher than the previously reported EPA-threshold (0.3 ± 0.3 µg EPA mg POC^{-1}) for the 90% growth saturation (i.e. concentration at which the growth rate is reduced by 10%) for this species grown on the same food organism (Ravet et al., 2012). The estimated EPA-threshold concentration for the 75% reproduction saturation level, however, was in the range of EPA-thresholds reported by Ravet et al. (2012) (0.17 ± 0.06 and 1.5 ± 0.6 µg EPA mg POC^{-1} for the 50% and 90% reproduction saturation level, respectively, compared to 1.141 µg EPA mg POC^{-1} observed in our study). Although the range of ARA supply (0.53 – 9.2 µg ARA mg POC^{-1}) was similar to the one used in our study, Ravet et al. (2012) didn't find any effects of ARA availability on the growth or reproduction of *D. pulex*. In contrast to these earlier findings, we observed similar patterns in the somatic growth rate and clutch size of *D. pulex* when grown on either ARA- or EPA-supplemented *A. obliquus*.

To our knowledge, the results of our study for the first time demonstrate a limitation in growth of *D. pulex* by the availability of a ω6-PUFA such as ARA. Additionally, we provide evidence for an equal relevance of both tested PUFA for the fitness of *D. pulex*.

2.4.3 Interspecific variation

Interspecific variation in growth and reproduction saturation thresholds may affect competition between *Daphnia* species, in particular when essential dietary PUFAs are present in limiting amounts (DeMott, 1989). At such conditions, the species with the lowest growth and reproduction saturation threshold for a particular PUFA is suggested to be superior over other species with higher PUFA requirements (von Elert, 2004; Brzeziński and von Elert, 2007). We expected that the ARA thresholds for saturated growth of the smaller species *D. pulex* will be lower than those for the large-bodied species *D. magna*. However, we only found significant differences regarding the asymptotic growth rate and reproduction (given as clutch size, i.e. number of eggs per individual) among the two *Daphnia* species, while there were no significant difference between the ARA threshold concentrations for saturated growth and reproduction of the daphnids. Other than expected, the somatic growth rate and reproduction of the two *Daphnia* species, differing in their body size, were limited similarly by both the ω3-PUFA EPA and the ω6-PUFA ARA. This indicates that both ω3- and ω6-PUFAs are equally relevant for the growth and reproduction of daphnids. This is in contrast to earlier findings of Sikora et al. (2016), who demonstrated that the EPA growth saturation thresholds increase with increasing body size across different *Daphnia* species. However, we found a significantly higher asymptotic growth rate of *D. magna* compared to *D. pulex* (in both EPA and ARA experimental line), which is in accordance with the positively correlated juvenile growth rate and body size of different *Daphnia* species

reported by Sikora et al. (2016). Likewise, we observed a significantly higher clutch size for the larger *D. magna* compared to *D. pulex*.

The saturation threshold approach used in our study to access possible interspecific differences between *D. pulex* and *D. magna* did not reveal significant results. We assume that the effect sizes depending on EPA- and ARA-supplementation were too similar to reveal any differences and therefore suggest to repeat the experiments at colder temperatures, where possible interspecific differences may be more visible (Masclaux et al., 2012; Pajk et al., 2012) due to a higher requirement for highly unsaturated fatty acids to maintain proper membrane fluidity (Hazel, 1995; Valentine and Valentine, 2004). Nevertheless, we do provide evidence for interspecific differences in the response of the two *Daphnia* species that might be explained exclusively by insufficient amounts of EPA and/or ARA: the growth rate and the clutch size of *D. magna* along the EPA and ARA concentration clearly followed a saturation curve (between 30% and 50% of the variance was explained by the modified Monod model, see Fig. S3), while this was not the case for *D. pulex*. Although the growth rate and clutch size of *D. pulex* increased when EPA or ARA were present, only a small proportion the variance of the two response variables was explained by the fitted saturation curve (20% - 30%, see Fig. S3). It is important to note, however, that our findings are restricted to only one genotype per species. As shown in previous studies, intraspecific differences in response to PUFA-deficiency and in the body content of single ω3-PUFAs (e.g. EPA) might occur (Brzeziński and von Elert, 2007; Sikora et al., 2016; Werner et al., 2018). It is hence necessary to test more genotypes per *Daphnia* species to draw general conclusions on the strength of the effect of insufficient EPA and ARA availability for the fitness of different *Daphnia* species.

2.4.4 Intersection points of the saturation curves and potential co-limitation scenarios

The interpretation of the bootstrapped intersection points of the saturation curves derived from the EPA- and ARA-limited growth and reproduction of *D. pulex* and *D. magna* is limited by the lack of information on intraspecific differences. Nevertheless, we report intersection points of EPA- and ARA-saturation curves with median values between 2.64 and 9.26 µg PUFA mg POC^{-1} for both growth and reproduction of the daphnids. As we found intersection points of the EPA- and ARA-reproduction curves to be above the estimated EPA- and ARA-saturation thresholds, they are probably of minor importance for competitive interactions. In contrast, the intersection points of EPA- and ARA-growth curves were found to be above the estimated ARA-saturation thresholds for the growth, but below the estimated EPA-thresholds. This might indicate that shifts in the relative relevance of ARA and EPA for the growth of *Daphnia* might occur along the PUFA concentration gradient. Furthermore, the positive fitness response to the addition of both PUFAs provides evidence for a potential independent co-limitation, i.e. simultaneous limitation of growth or reproduction by both tested PUFAs (*sensu* Sperfeld et al., 2016). Hence, ARA and EPA might serve as substitutable resources in *Daphnia*'s nutrition. However, the extension of this co-limitation scenario necessary to classify EPA and ARA as substitutable or essential resources would require controlled growth experiments with simultaneous supplementation of the diet with both PUFAs along a concentration gradient, i.e. response surface or matrix experiments (Sperfeld et al. 2012, 2016). In nature, this probably plays a minor role, as the ratio of EPA to ARA found in seston is usually higher than 1 (Ahlgren et al., 1997; Müller-Navarra, 2006).

As both ARA and EPA serve as precursors for eicosanoids (Heckmann et al., 2008a, 2008b; Schlotz et al., 2016; Garreta-Lara et al., 2018), they play an important role for *Daphnia*'s reproduction and the immune system (Martin-Creuzburg et al., 2010; Schlotz

et al., 2014; Fink and Windisch, 2018). For example, ARA was shown to improve the survival and reproduction of *D. magna* exposed to an opportunistic bacterial pathogen (Schlotz et al., 2014). However, in vertebrates, ARA- and EPA-derived eicosanoids have partially opposing effects, where their pro- and anti-inflammatory activity, respectively, serves as the best example (Schmitz and Ecker, 2008; Alcock et al., 2012). The possible inhibition of the synthesis of ARA-derived eicosanoids by EPA (Sargent et al., 1999; Schmitz and Ecker, 2008) led to the assumption that the actions of eicosanoids in fish physiology depend on the ratio between EPA and ARA in the tissue (Koussoroplis et al., 2011). Nevertheless, both ARA and EPA were shown to be important for the development and physiology of fish (Sargent et al., 1999; Bell and Sargent, 2003), which in freshwater systems feed on *Daphnia*. Thus, the dietary availability of EPA and ARA in nature might not only influence *Daphnia*'s performance, but it could also affect higher trophic levels in lakes.

2.4.5 Conclusions

Overall, our study provides clear evidence that arachidonic acid, a ω6-PUFA, limits the fitness of two different *Daphnia* species to an equal extent as the ω3-PUFA eicosapentaenoic acid. We thus suggest that together with the ω3-PUFA EPA, ARA availability needs to be considered in further studies on food quality and trophic transfer efficiency within freshwater ecosystems. Finally, shifts in phytoplankton community composition might result in environmental fluctuations in the dietary availability of ω3- and ω6-PUFAs, as their presence and amount varies among different phytoplankton groups. Therefore, our findings are of particular importance to better predict and understand the consequences of environmental changes and the ongoing global biodiversity loss for the phytoplankton-zooplankton interface.

Chapter 3

Intrapopulation variability in a functional trait: susceptibility of *Daphnia* to limitation by dietary fatty acids

3.1 Introduction

As discussed in **Chapter 2**, certain ω3- and ω6-PUFAs are considered essential for *Daphnia* and have to be derived from the diet. The phytoplankton PUFA-content, however, is found to be taxon-specific and differs strongly between different phytoplankton groups (Ahlgren et al., 1990b; Lang et al., 2011): Cyanobacteria usually lack sterols and polyunsaturated fatty acids (von Elert et al., 2003; Martin-Creuzburg et al., 2005, 2008). Therefore, Cyanobacteria represent a low quality food, while most Chlorophytes contain sterols and are rich in short-chain PUFAs (C_{18}), thus representing a diet of a modest quality for *Daphnia*. Long-chain PUFAs (C_{20}), such as EPA and ARA, are usually found in Cryptophytes, Diatoms and Dinoflagellates (Ahlgren et al., 1990), which often dominate the spring bloom in the lakes, while in the late summer, the phytoplankton community is usually dominated by Cyanobacteria and Chlorophytes (Sommer et al., 1986). Thus, seasonal and annual fluctuations in availability of essential dietary ω3- and ω6-PUFAs can occur, resulting in seasonal changes in food quality of the phytoplankton for *Daphnia* (Müller-Navarra and Lampert, 1996; Ahlgren et al., 1997; Wacker and von Elert, 2001). Such environmental heterogeneity mediated by seasonal fluctuations in food quality is assumed to contribute to the maintenance of genetic variation within natural populations and to promote seasonal succession of different *Daphnia* genotypes due to natural selection based on trait-variation (DeMott, 1983; Hu and Tessier, 1995; Brzeziński and von Elert, 2007).

Based on these observations, we here hypothesized that there is considerable intraspecific variability in susceptibility of coexisting genotypes within a natural *Daphnia* population to the availability of essential dietary PUFAs.

To test our hypothesis, we first isolated clones from a natural *Daphnia longispina* population of the oligo-mesotrophic lake Klostersee (see **Chapter 1**) and assessed the different genotypes present via microsatellites. Using laboratory growth experiments, we estimated the juvenile somatic growth rate of the daphnids as a proxy for the fitness (Lampert and Trubetskova, 1996; Wacker and von Elert, 2001) of each of the isolated genotypes in the presence and absence of ω3- or ω6-PUFAs (ALA/EPA and ARA, respectively). Additionally, we estimated the susceptibility of each of the genotypes to the availability of tested PUFAs (Brzeziński and von Elert, 2007). To our knowledge, this is the first study that addresses the intraspecific susceptibility of coexisting genotypes from a natural *Daphnia* population to the availability of several essential dietary PUFA, a probable basis for intraspecific competition for essential resources.

3.2 Materials and Methods

3.2.1 Daphnia population

In September 2014, a natural *Daphnia longispina* population was sampled with a 200 µm plankton net from the oligo-mesotrophic lake Klostersee (see **Chapter 1**). Subsequently, the *D. longispina* clones were cultivated in clonal lines in a climate chamber at 20 °C and a16:8h light:dark cycle in aged, aerated and sterile-filtered (0.45 µm) tap-water under non-limiting food conditions (2 mg particulate organic carbon (POC) L^{-1}, fed every other day). To ensure that even the most sensitive clones (i.e. the most susceptible to PUFA limitation that are of particular interest here) would survive the acclimation to laboratory conditions, we fed the animals with a 2:1 mixture of *Chlamydomonas klinobasis* (strain 56, culture collection of the Limnological Institute at the University of Konstanz, Germany) and *Cryptomonas* sp. (strain SAG 26.80, Culture Collection Göttingen, Germany). The cryptophyte *Cryptomonas* sp. contains various short- and long-chain ω3-PUFAs, including EPA (Ahlgren et al., 1990b; Windisch and Fink, 2018) and is thus considered to be of particularly high food quality for *Daphnia* sp. The green alga *C. klinobasis* was cultured in Cyano medium (von Elert and Jüttner, 1997) with vitamins (0.3 µmol L^{-1} thiamine hydrochloride, 0.002 µmol L^{-1} biotin and 0.004 µmol L^{-1} cyanocobalamine [vitamin B12]) in semi-continuous (dilution rate 0.1 d^{-1}) 5 L batch cultures at 20 °C and 100 µE s^{-1} m^{-2} PAR. The cryptophyte *Cryptomonas* sp. was cultured at the same conditions, but at a lower light intensity (40 µE s^{-1} m^{-2} PAR).

3.2.2 Microsatellites

Five polymorphic microsatellite primer pairs (Tab. 3.1) were chosen from Brede et al. (2006) to test for genetic differentiation of the *D. longispina* clones. DNA from

D. longispina was extracted by homogenizing 5 individuals per clone in 31.5 µl ultrapure water, 5 µl 10x PCR buffer (from 5'Prime Taq DNA polymerase PCR kit), 3.5 µl dithiothreitol (1 M DTT, Biochemica, AppliChem, dissolved in 0.08 M sodium acetate, MERCK, pH adjusted to 5.2 by adding HCl) and 5 µl proteinase K (20 mg ml^{-1}, peqlab), resulting in 45 µl of extraction mix. Individuals were homogenized with a Teflon pestle and the homogenate was incubated for 1 h at 56 °C and 300 rpm in a heat block (Thermomixer compact, Eppendorf), followed by 10 min at 96 °C (without further mixing). Subsequently, the homogenate was centrifuged at 12000 x g for 10 min (HERAEUS FRESCO 17 Centrifuge, Thermo Scientific). The supernatant containing the extracted DNA was transferred into 1.5 ml tubes and stored at -20 °C. Polymerase chain reactions (PCR) were performed using the 5'Prime Taq DNA polymerase PCR kit. Each PCR reaction contained 1µl 10x PCR buffer, 0.4mM dNTPs, 0.15 µM of each primer (Sigma Aldrich, fluorescence-labeled forward primers, Tab. 3.1), 2 units Taq-Polymerase and 1µl of DNA template in a final volume of 10 µl (modified after Kuster et al., 2013). Cycling parameters were 95 °C for 3 min, followed by 35 cycles of 1 min steps at 95°C, 57.5°C (an average annealing temperature for all microsatellite primers, see Table S1) and 72 °C. The PCR program was completed by a final step at 72 °C for 7 min (Brede et al., 2006). The PCR products were diluted 1:70, mixed with the Gene Scan 500 Rox Size Standard (ABI) and electrophoresed on the ABI 48-capillary 3730 DNA Analyzer at the Cologne Center for Genomics. Allele sizes were analyzed with the software Gene-Marker v1.8 (SoftGenetics). The microsatellite analysis of tested polymorphic loci revealed 25 different genotypes within the 35 clones tested from the lake Klostersee *D. longispina* population, of which twelve were used in this study (Tab. 3.2).

3.2.3 Growth experiments

For the standardized growth assays, precultures of each *D. longispina* genotype were synchronized by feeding them every second day with 2 mg POC $L^{-1}$of the green alga *Acutodesmus obliquus* for at least three generations. *Acutodesmus obliquus*, which is rich in short-chain PUFAs, especially ALA (18:3 n-3), but does not contain any C_{20}-PUFAs such as ARA (C20:4 n-6) and EPA (C20:5 n-3, Windisch and Fink, 2018), was cultured in Z/4 medium (Zehnder and Gorham, 1960) in semi-continuous (dilution rate 0.1 d^{-1}) 5L batch cultures at 20 °C and 100 µE s^{-1} m^{-2} PAR. Per genotype, three to four growth experiments were conducted over a period of 9 months in total. Juvenile *D. longispina* used in the growth experiments originated from synchronized mothers that carried the fourth clutch of parthenogenetic offspring. Neonates (5-10 per jar, within max. 24 hours after release from the mothers' brood pouch) were randomly distributed to jars containing 200 ml aged, aerated and filtered lake water, *A. obliquus* (2 mg POC L^{-1}) and 64 µl (320 µl L^{-1}) of either PUFA-free (control) liposomes or liposomes containing ALA, EPA or ARA. All treatments were prepared in triplicates which resulted in a total of 3 x 4 = 12 jars per experiment and genotype. Liposomes were prepared according to Martin-Creuzburg et al. (2008). The experimental animals were transferred into fresh food + liposome suspensions every second day. At the start of each experiment, a subsample of up to 20 juveniles was taken to determine the dry mass at day 0 (W_0). After 6 days, all individuals from each jar were collected, placed in preweighed aluminium boats and dried at 60°C for at least 24 h. Subsequently, the dried daphnids were weighted to determine the dry mass after 6 days (W_t) which was used to calculate the somatic growth rate g (day^{-1}) given as (Eq. 2, see **Chapter 2**):

$$g = \frac{\ln(W_t) - \ln(W_0)}{t}$$

where W_t is the dry mass at the end of the experiment, W_0 is the dry mass at the beginning of the experiment and t is the duration of the experiment (6 days). Somatic growth rate g has been shown to be a good proxy for the fitness of *Daphnia* (Lampert and Trubetskova, 1996).

Susceptibility S (%) was calculated as the difference between the somatic growth rate (d^{-1}) of the *D. longispina* juveniles fed with *A. obliquus* supplemented with PUFA-free liposomes g_C and the somatic growth rate of juveniles fed with *A. obliquus* supplemented with one of the three PUFAs g_{PUFA}, divided by the PUFA-free somatic growth rate (Eq. 4, Brzeziński and von Elert, 2007):

$$S = \frac{g_C - g_{PUFA}}{g_C} \text{ x } 100$$

Thus, susceptibility describes the reduction in fitness (i.e. somatic growth rate) of *Daphnia* juveniles when fed on PUFA-free diet compared to PUFA-supplemented diet. If *Daphnia* juveniles are limited in their growth by PUFA availability, i.e. show higher somatic growth rate in presence of PUFAs compared to the PUFA-free diet, the value of the susceptibility S will be negative. Thus, the higher the absolute value of S (in other words: the more negative the S value), the greater the susceptibility of the *Daphnia* juveniles to the absence of the respective PUFA.

3.2.4 Data analysis and statistics

Statistical analyses and visual representation of the data were performed with R (version 3.3.2, R Core Team, 2016) and RStudio (version 1.1.383, RStudio Team, 2016). To test the effect of PUFA availability and genotype on somatic growth rate g and susceptibility S, we fitted and validated linear mixed-effects models following Zuur et al.

(2009, Chapters 4 and 5) with fixed effects *PUFA* and *Genotype* and their interaction (*PUFA* × *Genotype*). As a random effect, we used the variable *Day,* describing the day at which every individual growth experiment was started (24 starting dates in a period of 9 months during the year 2017). Note that the fixed factor *PUFA* contained four (PUFA-free, ALA, EPA and ERA) and three levels (ALA, EPA and ARA) when testing the effect of PUFA availability on the somatic growth rate and susceptibility, respectively. Normal distribution of residuals was checked with Shapiro-Wilk's test, while the homogeneity of variances was tested with Levene's test. Because of the heterogeneity of variances for somatic growth rate ($g \sim PUFA$: $p < 0.05$; $g \sim Genotype$: $p < 0.001$; $g \sim PUFA \times Genotype$: $p < 0.001$), variance structure was changed as suggested by Zuur et al. (2009) using the function *varIdent()* from the package "nlme" (version 3.2.128, Pinheiro and Bates, 2000; Pinheiro et al., 2016), which allows for different variances per stratum. The variance structure was changed for *PUFA*, *Genotype* and their interaction and was used in different models (summary of the models and their validation can be seen in Tab. 3.3). For susceptibility, the variances were homogeneous for *PUFA* ($P = 0.88$) and *PUFA* × *Genotype* ($p = 0.15$), but not for *Genotype* only ($p < 0.001$). For this reason, we changed the variance structure for *Genotype*, but also for *PUFA* and *PUFA* × *Genotype* and tested all three variance structures in a set of linear mixed-effects models (Tab. 3.3). All models were fitted using the function *lme()* from the package "nlme". To validate the models, residuals were plotted against fitted values and strata. Final model selection was decided using Aikaike Information Criterion (AIC). For both somatic growth rate and susceptibility, the model with changed variance structure for the interaction *PUFA* × *Genotype* had the lowest AIC value (Tab. 3.3) and was used as the final model. Residuals from the final model for g were not normally distributed (Shapiro-Wilk's test, $p < 0.001$), however, due to a large sample size ($n = 489$), the deviation from the normal (Gaussian)

distribution can be ignored (Underwood, 1997). Residuals from the final model for susceptibility (n = 368) followed a Gaussian distribution (Shapiro-Wilk's test, p = 0.054). The significance of the fixed effects was tested with the function *anova()*. Differences between the groups were tested with Tukey's HSD *post hoc* test ($\alpha < 0.05$) using the *lsmeans()* function from the package "lsmeans" (version 2.24). Additionally, paired t-tests were performed between the somatic growth rate of the daphnids fed supplemented food and the respective controls. Finally, one-sample t-tests against zero (μ=0, Mcdonald, 2009) were performed for the susceptibility of the different genotypes. When $p < 0.05$, the tested genotype was considered "susceptible" to the availability of the respective PUFA.

Tab. 3.1: Locus name, repeat motif, forward (F) and reverse (R) primer sequences, dye (label) used for the forward primer and annealing temperature (T_a) of five microsatellite loci (from Brede et al., 2006) used for the genotyping of the *Daphnia longispina* population from the Lake Klostersee.

Locus	**Repeat**	**Primer sequences [5' - 3']**	**Dye**	**T_a [°C]**
SwiD5	$(GA)_{13}$	**F:** ACTATGCATAACACAGACACACG **R:** GAAGTACGGCAAGGAGCAAC	FAM	61
SwiD8	$(TG)_4(TG)_{13}$	**F:** GATATTCTCTTGGACTGCGTTTG **R:** GATATGACAAGCCGACGTCA	HEX	56
SwiD10	$(TG)_{19}$	**F:** TGTAGATATCAGCCAGCAGCTC **R:** AAGGTTATTCTCTCCGCTCGTC	HEX	60
SwiD11	$(GT)_{20}$	**F:** ACTCGACAAACTTGGAGAGGTC **R:** GGGGTGGCTATAGATAGACTGG	FAM	57
SwiD16	$(CA)_{15}$	**F:** CATCGACAATGTACGGTGGGAG **R:** GGCTGGTGGTGGTCCAGTGGTT	HEX	57

Tab. 3.2: Genotypes of the *Daphnia longispina* population from the Lake Klostersee used in the study. Fragment sizes (bp) of different loci were obtained via microsatellite analysis using microsatellite markers from (Brede et al., 2006). NA: no amplicon obtained.

Loci	**SwiD5**	**SwiD8**	**SwiD10**	**SwiD11**	**SwiD16**
Genotype	**Fragment size (bp)**				
KL3	124	121	185/190	158/173	170
KL8	124	121	185/194	147/173	181
KL11	124	NA	185/190	158/160	170/181
KL13	124	121/130	185	158/173	172
KL14	132/134	121	185/190	158/160	170/172
KL50	124/132	121	177/185	158/173	170
KL53	132	121	175/190	160/173	172
KL54	123/132	121	175/190	158/160	169
KL73	124/132	121	178/185	158	171
KL82	132	121	175/185	158/160	169/181
KL83	124/132	121	177/185	158	169
KL93	NA	121	175/184	158/173	170/172

Tab. 3.3: Summary of linear mixed-effects models describing somatic growth rate and susceptibility. The models were fitted with the function *lme()* from the R-package *"nlme"*. In all models, fixed effect was specified as *PUFA*Genotype*, which includes effects of both main factors *PUFA* and *Genotype* and their interaction *PUFA* × *Genotype*, while random effect was set to *~1|Day*. Variance structure (Weights) was specified as suggested by Zuur et al. (2009), allowing for different variances per stratum by using the function *varIdent()*. Final model selection was decided using Aikaike Information Criterion (AIC). The model with the lowest AIC (in bold) was chosen for further statistical analysis.

Response variable	Weights	AIC
Somatic growth rate		
Model.null	not specified (NULL)	-1761.273
Model.1	varIdent(form= ~ 1 | *PUFA*)	-1787.238
Model.2	varIdent(form= ~ 1 | *Genotype*)	-1784.124
Model.3	**varIdent(form= ~ 1 | *PUFA*Genotype*)**	**-1820.653**
Susceptibility		
Model.null	not specified (NULL)	2403.447
Model.1	varIdent(form= ~ 1 | *PUFA*)	2402.441
Model.2	varIdent(form= ~ 1 | *Genotype*)	2370.820
Model.3	**varIdent(form= ~ 1 | *PUFA*Genotype*)**	**2363.175**

3.3 Results

3.3.1 Somatic growth rate g

We found intraspecific differences in the somatic growth rate *g* of the different genotypes within the *Daphnia longispina* population ($F_{11, 418}$ = 49.13, p < 0.001, Fig. 3.1 and Tab. 3.4). Additionally, the linear mixed-effects model revealed significant effect of the fixed factor *PUFA* ($F_{3,418}$ = 98.87, p < 0.001) on the somatic growth rate *g* of *D. longispina* juveniles as well as a significant interaction between the two factors *PUFA* and *Genotype* ($F_{33,418}$ = 1.54, p = 0.0313).

The average somatic growth rate *g* (± 1 SD) of *D. longispina* juveniles significantly increased when the daphnids were fed *A. obliquus* supplemented with liposomes containing any of the three PUFAs compared to daphnids raised in the PUFA-free (control, C) treatment (g_C = 0.30 ± 0.03 d^{-1}, Tukey's HSD *post hoc* test following LME, p < 0.001 for all pairwise comparisons between the PUFA-free control treatment and PUFA-supplemented treatments). Interestingly, we observed the highest average somatic growth rate *g* of the *D. longispina* subpopulation when *A. obliquus* was supplemented with the ω6-PUFA arachidonic acid (g_{ARA} = 0.35 ± 0.02 d^{-1}), which significantly differed from the somatic growth rate observed when *A. obliquus* was supplemented with liposomes containing ω3-PUFAs (Tukey's HSD *post hoc* test following LME, p < 0.001). The effect on the somatic growth rate did not differ between the two ω3-PUFAs, ALA and EPA (both g_{ALA} and g_{EPA} = 0.33 ± 0.03 d^{-1}; Tukey's HSD *post hoc* test following LME, P = 0.996).

Daphnia juveniles of the genotype KL93 showed the lowest average somatic growth rate (± 1 SD), independent of the supplementation (g_C = 0.27 ± 0.03 d^{-1}; g_{ALA} = 0.28 ± 0.04 d^{-1}, g_{EPA}= 0.29 ± 0.04 d^{-1}, g_{ARA}= 0.30 ± 0.05 d^{-1}), while the highest average somatic growth

rate was observed for KL83 (g_C = 0.35 ± 0.03 d^{-1}; g_{ALA}= 0.38 ± 0.02 d^{-1}, g_{EPA}= 0.38 ± 0.01 d^{-1}, g_{ARA}= 0.38 ± 0.00 d^{-1}). When ALA was supplemented to the diet, somatic growth rates were higher for three genotypes (compared to the daphnids of the same genotypes fed an unsupplemented diet), while in presence of EPA, the somatic growth rate of only one genotype significantly exceeded the growth of the respective controls. Four genotypes grew faster on an ARA-supplemented diet compared to a PUFA-free diet (t-test, $p < 0.05$).

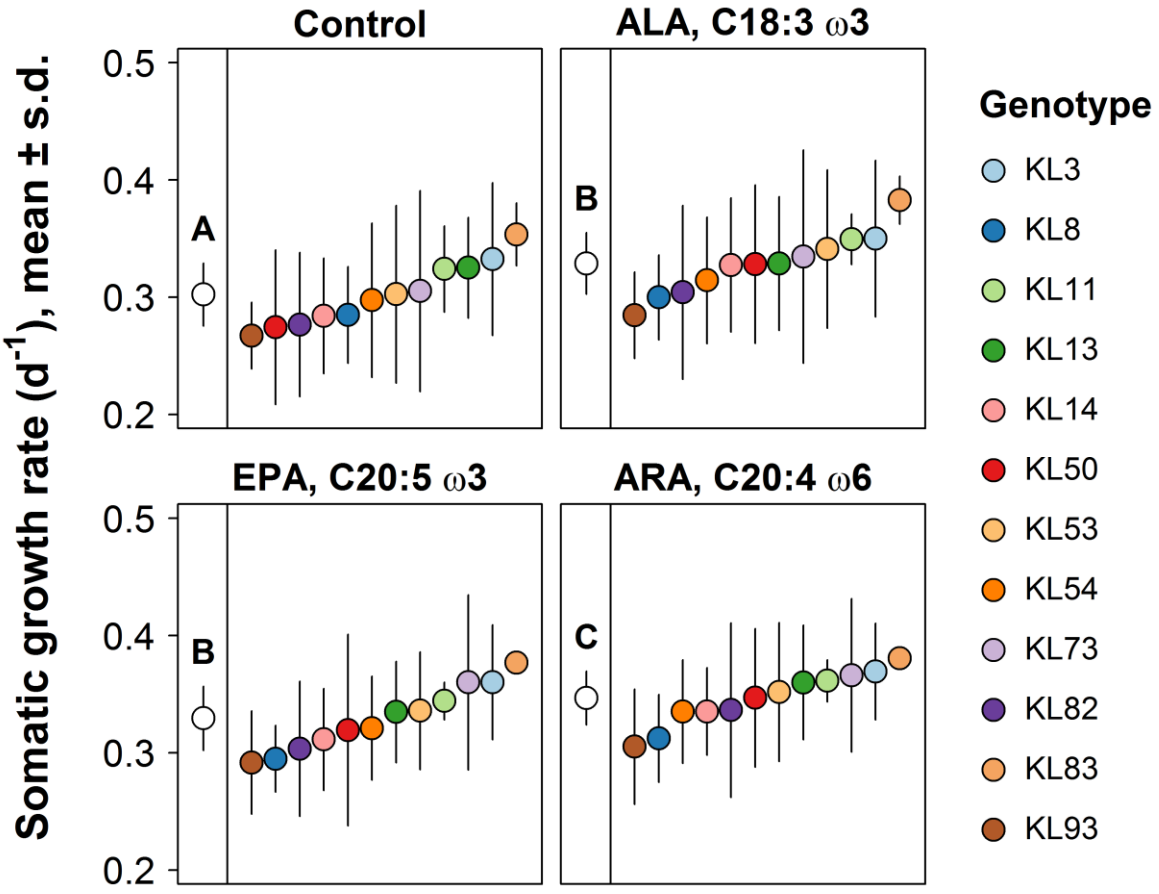

Fig. 3.1: Mean juvenile somatic growth rates (day^{-1}, ± 1SD) of *Daphnia longispina* fed the green alga *Acutodesmus obliquus* supplemented with either PUFA-free (control) liposomes or liposomes containing the PUFAs α-linolenic acid (ALA, C18:3 ω3), eicosapentaenoic acid (EPA, C20:5 ω3) or arachidonic acid (ARA, C20:4 ω6). White circles represent the mean somatic growth rate of the sampled subpopulation (n = 12), while the colored circles represent mean somatic growth rates for each genotype (n = 3 or 4). Different letters indicate heterogeneous groups following Tukey's HSD *post hoc* tests at $p < 0.05$.

3.3.2 Susceptibility S

Similar to the somatic growth rate, the susceptibility to limitations by PUFA availability showed marked variation within the *D. longispina* population (LME, fixed factor *Genotype*: $F_{11,309}$ = 12.91, $p < 0.001$, Fig. 3.2 and Tab. 3.4). Furthermore, we found significant effects of the fixed factor *PUFA* ($F_{2,309}$ = 50.07, $p < 0.001$) on the susceptibility of *D. longispina* juveniles as well as a significant interaction between the two factors *PUFA* and *Genotype* ($F_{22,309}$ = 1.69, p = 0.0284).

We did not find a significant difference in the average susceptibility (± 1 SD) of *D. longispina* juveniles to limitations by the availability of the ω3-PUFAs ALA (S_{ALA} = -7.66 ± 4.00 %) and EPA (S_{EPA} = -8.13 ± 3.51 %, Tukey's HSD *post hoc* test, p = 0.861). However, compared to the two ω3-PUFAs, *D. longispina* population showed a significantly higher average susceptibility (i.e. more negative values) to limitations by the availability of the ω6-PUFA ARA (S_{ARA} = -12.92 ± 4.07 %, Tukey's HSD *post hoc* test, $p < 0.001$).

The most susceptible *D. longispina* genotypes to the availability of the ω3-PUFAs ALA and EPA were KL50 (S_{ALA}= -15.06 ± 18.75 %) and KL73 (S_{EPA} = -15.76 ± 12.62 %), respectively, while the least susceptible genotype was KL13 (S_{ALA} = -0.45 ± 5.13 % and S_{EPA} = -2.77 ± 3.87 %). The strongest susceptibility to the availability of ARA was observed for the genotype KL50 (S_{ARA} = -20.47 ± 14.78 %), while the weakest susceptibility was found for KL83 (S_{ARA} = -6.99 ± 7.30 %). Two out of 12 genotypes were found to be susceptible to the absence of ALA, four out of 12 were susceptible to ARA absence while none of the genotypes was shown to be susceptible to the absence of EPA (t-test, genotype was considered "susceptible" when $p < 0.05$).

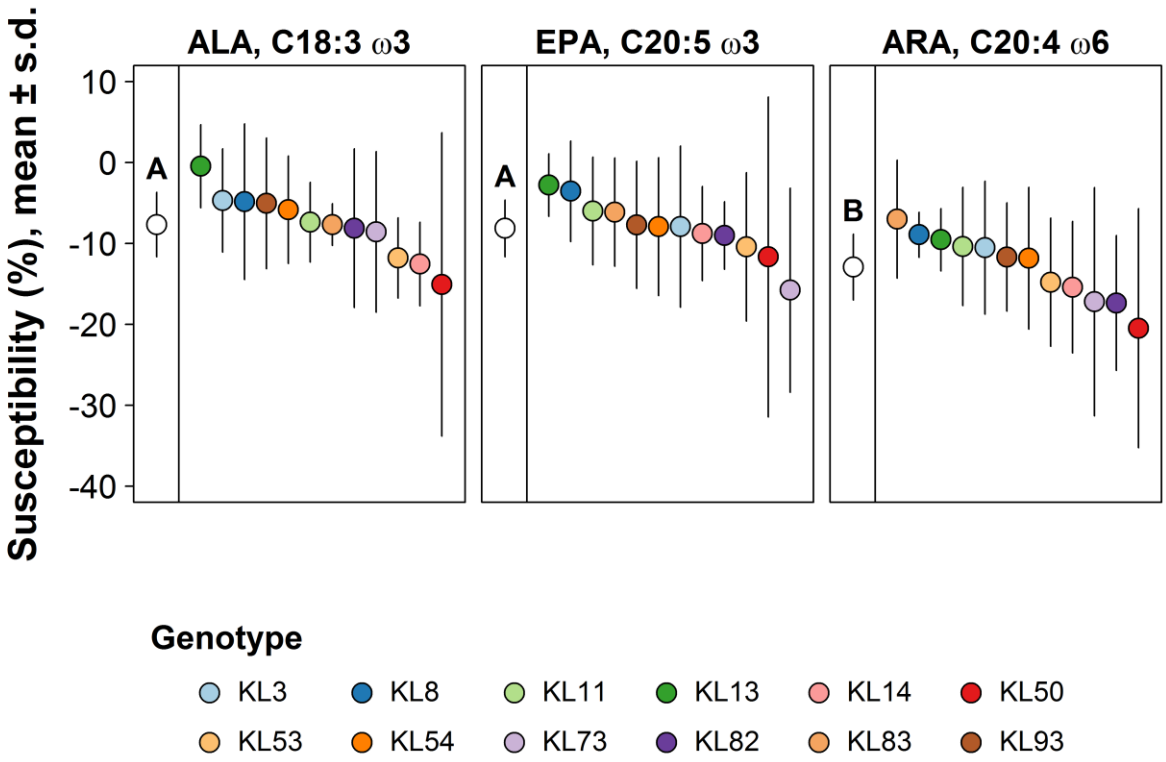

Fig. 3.2: Susceptibility (%, ± 1SD) of *Daphnia longispina* juveniles fed the green alga *Acutodesmus obliquus* supplemented with liposomes containing either of the PUFAs α-linolenic acid (ALA, C18:3 ω3), eicosapentaenoic acid (EPA, C20:5 ω3) or arachidonic acid (ARA, C20:4 ω6). White circles represent the mean susceptibility of the sampled subpopulation (n = 12), while the colored circles represent mean susceptibility of each genotype (n = 3 or 4). Different letters indicate heterogeneous groups following Tukey's HSD *post hoc* tests at $p < 0.05$.

Tab. 3.4: Results from linear mixed-effects models showing effects of fixed factors *PUFA*, *Genotype* and their interaction on the somatic growth rate and susceptibility of *Daphnia longispina* population. For both models, *Day* (given as the day at which every individual growth experiment was started in a period of 9 months; number of levels = 24) was used as a random effect.

Response variable	**Fixed effect**	***df***	***F***	***p***
Somatic growth rate	*PUFA*	3, 418	98.87	< 0.001
	Genotype	11, 418	49.13	< 0.001
	PUFA × *Genotype*	33, 418	1.54	0.0313
Susceptibility	*PUFA*	2, 309	50.07	< 0.001
	Genotype	11, 309	12.91	< 0.001
	PUFA × *Genotype*	22, 309	1.69	0.0284

3.4 Discussion

3.4.1 Intrapopulation variability

We found strong evidence for marked intrapopulation variability in susceptibility to the availability of essential dietary PUFAs within a natural population of *Daphnia longispina*. Trait-variation within and among *Daphnia* species and its role for population dynamics has been frequently addressed, usually in predator-prey (e.g. predator-mediated life-history changes of prey), host-parasite (survival and fecundity) or producer-consumer (food quality and sensitivity to toxicity) systems: Boersma et al. (1998) found intraspecific variation in various traits among four *Daphnia magna* populations in the presence of fish kairomones (simulation of predator presence). Similarly, in the presence/absence of fish kairomones, *Daphnia hyalina* × *galeata* hybrid clones displayed differences in numerous life-history traits, such as size or age at maturity and habitat use (De Meester and Weider, 1999). Several studies reported clonal variation of *Daphnia* in their sensitivity to the toxic cyanobacterium *Microcystis* sp. (Hietala et al., 1997; Chislock et al., 2013). Studying the host-parasite system with *Daphnia dentifera* and the virulent yeast pathogen *Metschnikowia bicuspidate*, Duffy and Sivars-Becker (2007) found highly significant variation in the susceptibility of different *Daphnia* clones and populations to parasite infection. Recently, Werner et al. (2018) reported intraspecific variation in heat tolerance within a natural *Daphnia magna* population. Furthermore, *Daphnia* showed interclonal variation in their response to varying food quality, both in terms of nutrient stoichiometry (Weider et al., 2005) and dietary fatty acid availability, in particular to the dietary availability of EPA (Brzeziński and von Elert, 2007; Brzeziński et al., 2010). Additionally, Sikora et al. (2016) found interspecific variation in somatic growth rates and growth saturation thresholds (saturating EPA-

concentration) between different species of the genus *Daphnia*, depending on the body size of the respective species within the genus *Daphnia*. However, most previous studies used clones with spatial (habitat) and temporal (time point of isolation) differences in their origin. We specifically addressed trait-variation in a natural *Daphnia* community of coexisting genotypes from the same lake, and thus with a shared evolutionary history. Notwithstanding this, we could confirm a general limitation of daphnids' fitness by the absence of EPA in their diet, as previously reported in numerous studies (Müller-Navarra, 1995b; von Elert, 2002; Becker and Boersma, 2003). Furthermore, our results are in line with findings from Wacker and von Elert (2001), who showed that sestonic ALA content is a good predictor of the somatic growth of *D. galeata*, a closely related member of the *D. longispina* species complex. Additionally, Wacker and von Elert (2001) suggested that the ratio of ALA and EPA present in the diet determines which of these two ω3-PUFAs is limiting the daphnids' fitness. In his study, von Elert (2002) suggested that *D. galeata* is capable to convert ALA into EPA. Therefore, one might argue that the increase in somatic growth rate of *D. longispina* fed on ALA-supplemented diet is a result of elevated availability of EPA due to the bioconversion of ALA into EPA. However, it is not yet clear, whether all *Daphnia* species are capable of a bioconversion of ALA into EPA or if this capability is species-specific. Nevertheless, the conversion rate is assumed to be very low (von Elert, 2002; Taipale et al., 2011) so that it is unlikely that *Daphnia* can produce EPA in amounts that are not limiting their growth. Although the availability of EPA was strongly emphasized in previous studies as the PUFA that limits the fitness of *Daphnia* (von Elert, 2002; Martin-Creuzburg et al., 2010; Sperfeld and Wacker, 2011), in our study, the reduction in *Daphnia* genotypes' fitness was even more pronounced when the ω6-PUFA ARA was absent. This corroborates our related findings that ARA is limiting the fitness of two *Daphnia* species to a similar extent as the ω3-PUFA EPA (see

Chapter 2). The even stronger effect of the absence of ARA on the fitness of *D. longispina* in the present study might be due to adaptations of the local *D. longispina* population of the lake Klostersee to the availability of ω3- and ω6-PUFAs.

3.4.2 Susceptibility to limitations by PUFA availability - a functional trait affecting population dynamics in natural Daphnia *populations?*

Natural selection acts on traits through performance in terms of growth, survival and reproductive success of organisms and is often driven by competition for limiting resources (Ghalambor et al., 2007; Boyden et al., 2008; Bolnick et al., 2011). Trait variation, caused by within population genetic variation, is therefore necessary to allow for selection and thus for adaptation of a population to changing environmental conditions (McGill et al., 2006; Violle et al., 2007; Violle and Jiang, 2009). Such environmental variables could e.g. be temporally and spatially variable predation pressure or resource availability. Local adaptation is thus expected to be driven by selection for beneficial alleles. In our particular case, less susceptible clones are less affected by the absence and low availability of essential dietary PUFAs and are therefore assumed to have a higher chance of survival and persistence when PUFA availability in the diet becomes limiting. Therefore, at low food quality (in terms of availability of essential dietary PUFAs), we expect to find changes in clonal composition within the natural *Daphnia* population, where the frequencies of less susceptible clones are expected to increase in comparison to strongly susceptible genotypes (scenario 1). On the other hand, given a temporal environmental heterogeneity which includes periods of intermediate to high food quality and no too rapid resource fluctuations, the observed intraspecific trait-variation is assumed to be the basis for the maintenance of the genotypic variation and thus promote coexistence of different genotypes within the

D. longispina population (scenario 2, Brzeziński and von Elert, 2007; Kremer and Klausmeier, 2013). Nevertheless, we suggest that susceptibility to limitations by the availability of essential dietary PUFAs is one of the functional traits that might affect the outcome of the intraspecific competition within natural *Daphnia* populations, thus affecting the trophic interactions and eco-evolutionary dynamics within freshwater ecosystems.

3.4.3 Conclusions

We here demonstrate significant intrapopulation variation in a functional trait, i.e. susceptibility to limitations by dietary fatty acid availability among coexisting genotypes of a natural *Daphnia longispina* population. While the absence of the two ω3-PUFAs ALA and EPA affected the fitness of different *D. longispina* genotypes to a similar extent, we show the ω6-PUFA ARA to be the most limiting PUFA for the investigated *D. longispina* population. We suggest that such intrapopulation differences in susceptibility to absence of dietary PUFAs might not only contribute to the maintenance of the genetic variation within natural animal populations, but may also affect the outcome of intraspecific competition and thus be the driving force of natural selection and local adaptation in many ecosystems. Guided by these hypotheses, we address the role of susceptibility to limitations by PUFA availability for the intraspecific competition of naturally coexisting *D. longispina* genotypes within a common garden experiment in **Chapter 4**.

Chapter 4

Effects of phytoplankton diversity on intraspecific competition in *Daphnia* – The role of essential dietary fatty acids

4.1 Introduction

In freshwater ecosystems, trophic transfer efficiency between phytoplankton and zooplankton is mainly determined by the dietary quality of the phytoplankton, which depends on several factors, such as stoichiometry of elements C, N and P (Sterner et al., 1993; Urabe et al., 1997) and the biochemical composition in terms of fatty acids and sterols (Müller-Navarra, 1995b; Wacker and von Elert, 2001; Martin-Creuzburg et al., 2005), as previously discussed. In particular the sestonic content of dietary polyunsaturated fatty acids (PUFAs) was found to be crucial for the fitness of the herbivorous grazer *Daphnia,* which is not capable of *de novo* synthesis of such PUFAs (Stanley-Samuelson et al., 1987; Harrison, 1990; Leonard et al., 2004). However, the fatty acid composition of seston was shown to be taxon-specific (Ahlgren et al., 1990a; Lang et al., 2011). Therefore, biodiversity loss on the producer level might decrease its food quality and in turn have cascading effects on multiple trophic levels and affect eco-evolutionary dynamics on population, community or ecosystem level.

In this Chapter, methods and findings from previous Chapters are combined to study the effects of phytoplankton diversity (in terms of food quality) on the intraspecific competition between naturally coexisting *Daphnia longispina* genotypes and to identify phytoplankton and zooplankton functional traits responsible for the observed dynamics.

In **Chapter 1**, two different methods used for a fast assessment of phytoplankton community composition are discussed. The obtained results show that the indirect assessment of phytoplankton community composition via HPLC-derived pigment concentrations provides a higher resolution of phytoplankton diversity compared to a method based on fluorescence spectra, as it includes six different classes, while the spectrofluorometric method provides identification of only four different phytoplankton

classes. Furthermore, the spectrofluorometric method seems to strongly underestimate Cyanobacteria compared to the pigment-based method. Therefore, the pigment-based method is used within the present study to determine the phytoplankton community composition (for details, see *Methods and Materials* below). In **Chapter 2**, ω6-PUFA ARA is shown to limit the fitness (in terms of growth and reproduction) of two different *Daphnia* species in an equal manner as the ω3-PUFA EPA. Therefore, these two long-chain PUFAs, as well as the C_{18}-PUFA ALA, which belongs to the family of ω3-PUFAs, were included into laboratory growth assays, which are discussed in **Chapter 3**. Via these single clone microcosm experiments, we assessed intrapopulation variability of different coexisting *Daphnia longispina* genotypes in their susceptibility to fitness limitations by the availability of essential dietary PUFAs. Here, susceptibility to PUFA availability is defined as a consumer's functional trait, which is suggested to be of crucial importance for the outcome of competitive interactions within a natural *Daphnia* population.

We hypothesize that (i) biodiversity loss within a natural phytoplankton community results in a decreased diversity of fatty acids, in particular PUFAs. Furthermore, we hypothesize that (ii) the intraspecific competition between naturally coexisting *D. longispina* genotypes is affected by several fatty acids related dietary factors (i.e. phytoplankton functional traits), such as PUFA diversity. In particular, the availability of ω3-PUFAs ALA, EPA and DHA and the ω6-PUFA ARA is suggested to affect the outcome of competitive interactions between *D. longispina* genotypes, as these PUFAs were previously shown to limit the fitness of *Daphnia* (von Elert, 2002; **Chapters 2** and **3** of this thesis) or to serve as precursors for other essential PUFAs (e.g. DHA can be bioconverted into EPA; (von Elert, 2002).

These hypotheses were addressed within an outdoor common garden experiment. Such common garden experiments are often performed to study local adaptation and eco-evolutionary dynamics on population or community level (Kawecki and Ebert, 2004). They involve direct comparison of the performance of different genotypes under the same environmental conditions (i.e. in the same habitat) and therefore allow for the assessment of natural complexity on a meso- to macro-scale under controlled conditions. Depending on the aim of the experiment, common garden experiments usually include assessment of fitness-related traits, such as growth or reproduction, which are used as measures of performance of different genotypes.

In order to study effects of biodiversity loss on the phytoplankton-zooplankton interface, we aimed to establish a diversity gradient by manipulating a natural phytoplankton community. We determined the food quality of the phytoplankton in terms of stoichiometry and fatty acid composition. Finally, we introduced *D. longispina* genotypes with pronounced differences in their susceptibility to limitations by PUFA availability and assessed the *Daphnia* community composition in the further course of the experiment (10 weeks) via microsatellite analyses.

4.2 Materials and Methods

4.2.1 Study organism

In **Chapter 3**, twelve different naturally coexisting *D. longispina* genotypes from the oligo-mesotrophic lake Klostersee (see **Chapter 1**), previously identified via microsatellite analysis, were used in standardized growth experiments to access their susceptibility to limitations by the availability of essential dietary polyunsaturated fatty acids. For the common garden experiment, we chose three *D. longispina* genotypes with pronounced differences in their susceptibility to the absence of ω3-PUFAs ALA and EPA and the ω6-PUFA ARA, namely genotypes KL14, KL83 and KL93.

For the laboratory growth assays as well as for the outdoor common garden experiment, precultures of the *D. longispina* genotypes were synchronized by feeding them every second day with 2 mg POC L^{-1} of the green alga *Acutodesmus obliquus* for at least three generations. *A. obliquus* was cultured in Z/4 medium (Zehnder and Gorham, 1960) in semi-continuous (dilution rate 0.1 d^{-1}) 5L batch cultures at 20 °C and 100 µE s^{-1} m^{-2} PAR.

For the common garden experiment, we used 5 days old third clutch juveniles (hatched within 24 hours).

4.2.2 Common garden experiment

4.2.2.1 Experimental design

The outdoor common garden experiment was performed from 18th July until 24th October 2016 in the Botanical garden of the University of Cologne using 15 mesocosm tanks (max. volume 75 L) in total, randomly distributed in two pools (diameter: 2 m, Fig. 1). The pools were filled with ground water to ensure cooling of the tanks during the hot summer days. In every pool, one temperature logger (series EBI 20, ebro, Xylem

Analytics, Germany) was sank and used to continuously measure the temperature. To keep the water temperature constant (at approx. 22 °C), the pool water was replaced with fresh ground water when necessary.

4.2.2.2 Growth phase

To establish a diversity gradient within a natural phytoplankton community, five 20 L canisters were filled with ground water and inoculated with different volumes (1 µl, 10 µl, 100 µl, 1000 µl and 10000 µl) of an initial phytoplankton community (in following abbreviated as IP 1, IP 10, IP 100, IP 1000 and IP 10000), following the dilution method from Hammerstein et al. (2017). For this, the natural seston from lake Klostersee was used (origin of *D. longispina* genotypes used in this study, see also **Chapter 1**), sampled by Maria Stockenreiter (Ludwig-Maximilians-University Munich) on 10th June 2016 and filtered over a 200 µm mesh to exclude zooplankton from the lake. Until the start of the experiment, the canisters were kept at outdoor temperature (22-24 °C) and gently shaken and aerated twice a week.

Four days before the start of the experiment, the 75 L mesocosm tanks were each filled with 60 L tap water and aerated for 24 h on the last day before the experiment was initiated. All mesocosm tanks were covered with mesh to prevent animals and litter from entering.

The common garden experiment was started on 18th July 2016 by inoculating the prefilled mesocosm tanks with 2 L (+ 2 L tap water) or 4 L of the pregrown phytoplankton communities (depending on the phytoplankton biomass measured as chlorophyll *a* content, see section *Chlorophyll* a *content as a biomass proxy* in this Chapter). Each of the pregrown phytoplankton communities (five in total, corresponding to five different volumes of initial phytoplankton community) was filled into three

randomly chosen tanks, resulting in a 5 x 3 experimental design (Fig. 4.1). Additionally, samples from each pregrown phytoplankton community were taken for further analysis (see section *Laboratory analyses* in this Chapter).

The water in the mesocosms was mixed once a day to prevent sedimentation of the phytoplankton. Once per week during the growth phase (4 weeks), 2 L samples were taken from each tank with a custom-built tube-sampler for further analyses and replaced with 2 L tap water, so that the volume in the tanks was kept constant.

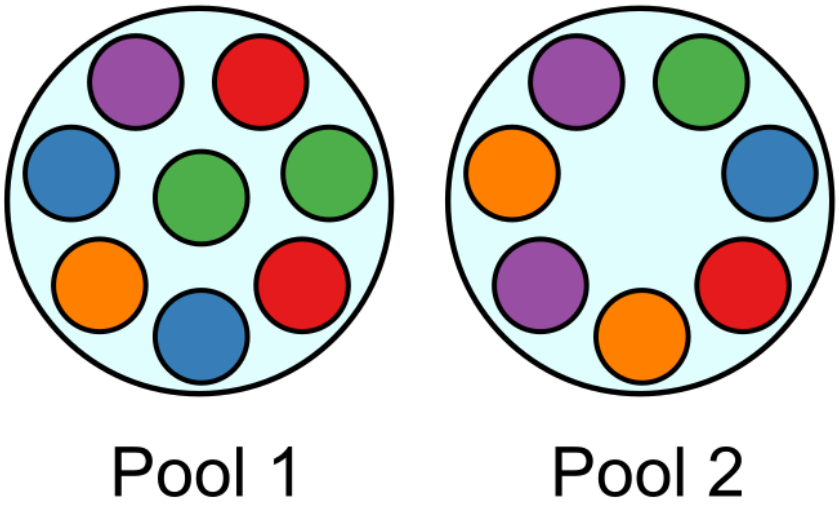

Fig. 4.1: Distribution of mesocosm tanks within the pools. Different colours indicate different treatments (i.e. volume of initial phytoplankton community, given as IP in µl): red: IP 1; blue: IP 10; green: IP 100; purple: IP 1000; orange: IP 10000.

4.2.2.3 Grazing phase

After four weeks of growth phase, on the 15th August 2016, the grazing phase was started by adding 20 juveniles of each *D. longispina* genotype (KL14, KL83 and KL93) to each of the mesocosm tanks, resulting in 60 juvenile daphnids per tank (1 individual L^{-1}). Similar to the growth phase, 2 L samples from each mesocosm were taken once a week for the next 10 weeks (until 24th October 2016) and replaced with 2 L tap water.

The samples were immediately filtered over a 250 µm mesh to isolate and subsequently count the *Daphnia* individuals. All daphnids were kept in 50 ml tubes until further analyses (see section *Microsatellite analysis* in **Chapter 3**). To avoid possible crowding and starvation of the daphnids, which might occur at low food quantity as a result of strong grazing pressure due high *Daphnia* densities, we randomly removed excessive individuals from respective mesocosm tanks when *Daphnia* density exceeded 20 individuals per litre.

4.2.2.4 Environmental parameters

Over the entire duration of the common garden experiment, we collected data on temperature (in °C, measured at 03:00, 09:00, 15:00 and 21:00 h), precipitation (in mm) and sunlight (in hours), provided by weather station Cologne / Bonn Airport on wetteronline.de, the German weather website.

4.2.3 Laboratory analyses

4.2.3.1 Determination of particulate organic carbon (POC) and nitrogen (PON)

To determine POC and PON content of the phytoplankton community, 75 – 250 ml of water sample from every mesocosm tank were filtered on precombusted glass-fibre filters (GF/F, Ø 25 mm, VWR, Germany). The filters were dried at 60 °C for 24 h and subsequently packed into tin capsules (10 x 10 mm, HEKAtech, Germany) and analyzed via Flash 2000 elemental analyzer (Thermo Fisher, Germany).

4.2.3.2 Determination of particulate phosphorus (PP)

To determine PP content of the phytoplankton community, 70 – 250 ml of water samples were filtrated on precombusted GF/F filters, which were previously rinsed with 5% H_2SO_4 solution and ultrapure water. Each filter was transferred into 10 ml of reaction solution (containing potassium peroxodisulphate and 1.5% sodium hydroxide) and autoclaved for 1 h at 120 °C. Subsequently, particulate phosphorus was analyzed with the molybdate-ascorbic acid method (Greenberg, Trussel and Clesceri, 1985) using a DR5000 UV-Vis spectrophotometer (Hach, Germany). Subsequently, the PP content of the respective phytoplankton community was calculated using a previously established calibration curve.

4.2.3.3 Chlorophyll a content as a biomass proxy

Chlorophyll *a* concentrations was measured using a TD-700 Laboratory Fluorometer (Turner Designs, USA). Chlorophyll *a* is a photosynthetic pigment that can be found across all phytoplankton groups and therefore can be used as a biomass proxy.

4.2.3.4 Physiology of the phytoplankton community

To obtain information on the physiological state of the phytoplankton community, the photosynthetic activity (given as the yield of the photosystem II, φ_{PSII}) and the relative electron transport rate (ETR) can be estimated via chlorophyll *a* fluorescence detection. This can be achieved via pulse-amplitude modulation (Schreiber, 1998). Therefore, dark-adapted subsamples of the phytoplankton community (up to 3 ml) were analyzed using a Water-PAM Fluorometer (cuvette version, Walz, Germany) and the WinControl-3 software.

The following parameters were recorded: the chlorophyll *a* fluorescence yield in the quasi-dark state (F_0) and the maximum fluorescence yield measured during the last saturating light pulse triggered (F_m') (Schreiber, 1998). From the obtained values, φ_{PSII} and ETR were calculated by the WinControl-3 software using the following equations (Schreiber, 1998):

$$\varphi_{PSII} = (F_m'-F_0) / F_m' \quad \text{(Eq. 5)}$$

$$ETR = \varphi_{PSII} \times PAR \times 0.5 \times ETR\text{-factor} \quad \text{(Eq. 6)}$$

where PAR is the quantum flux density of photosynthetically active radiation. ETR-factor was set to 0.84 (default settings). The data obtained over the entire course of the experiment are given in Fig. S4, but are not further analyzed or discussed within this thesis.

4.2.3.5 Pigment analysis via high-pressure liquid chromatography (HPLC)

Up to 500 ml of the water samples were filtrated onto GF/F filters, which were fold in half, wrapped in aluminium foil and stored at -20 °C until further analysis. To correct for sample loss during the extraction procedure and evaporation, trans-β-apo-8'-carotenal (Sigma Aldrich) was used as an internal standard (ISTD) and 200 ng of the ISTD were added to the samples prior to extraction. The samples were extracted with 3.5 ml 100% acetone (Alfa Aesar, HPLC grade), sonicated for 2 min and then placed on ice for 1 min. This procedure was repeated 5 times, resulting in a total of 10 min sonication and extraction time. Subsequently, the filters were kept in acetone at 4°C over night to allow for further extraction. On the following day, the filters were removed from the tubes and

the extracts were centrifuged for 15 min at 4500 x g (Eppendorf Centrifuge 5804) to remove cell and filter debris. Finally, 2 ml of the extracts were transferred to new tubes, evaporated to dryness under a gentle stream of nitrogen gas, re-dissolved in 100 µl acetone and transferred to HPLC vials. All extracted samples were stored at -20 °C and measured within 72 hours after extraction.

For the subsequent analysis of phytoplankton pigments, 50 µl per sample were injected into the Prominence HPLC system and separated with a method modified after Garrido and Zapata (1993). For details on the HPLC system, the solvents and the HPLC gradient, see **Chapter 1**. Absorbance was recorded in the PDA from 350 to 700 nm. Pigments were identified by the retention times and the absorption spectra, which were obtained from previous measurements of the pure pigment standards. Peak areas were integrated at 436 nm and corrected for internal standard. The used HPLC gradient enabled separation and identification of 10 pigments (see **Chapter 1**, Fig. 1.1).

4.2.3.6 Pigment-derived phytoplankton community composition via CHEMTAX

The phytoplankton community composition within mesocosm tanks was estimated via CHEMTAX, a matrix factorization programme (Mackey et al., 1996, see **Chapter 1**), using the HPLC-derived pigment concentrations. The pigments diatoxanthin and β-carotene were excluded from the CHEMTAX calculations, as these pigments did not have any effect on the output data (previous CHEMTAX runs, data not shown). As an additional input, CHEMTAX requires a ratio matrix containing taxon-specific pigment : chlorophyll *a* ratios, which can be obtained from the literature. The ratio matrix for mesotrophic lakes given in Schlüter et al. (2016) was used as the initial ratio matrix (see Tab. 4.1), from which 60 different ratio matrices were generated. Finally, 10 % (n=6) of the matrices with the lowest residual root mean square (RMS) were averaged and used as a

final input ratio matrix. The parameters used within CHEMTAX were set as recommended by Mackey et al. (1996) and are given in **Chapter 1**. The final output ratio matrix is given in Tab. S1.

Subsequently, the CHEMTAX-derived biomasses of the phytoplankton groups (given as contribution to the total chlorophyll *a* in units of µg L^{-1}) were used to calculate the Shannon-Diversity Index (in following: $H'_{CHEMTAX}$) as an estimate of phytoplankton diversity (Shannon and Weaver, 1949). For this, the equation (Eq. 1, see **Chapter 1**) was used:

$$H' = -\sum p_i \times \ln(p_i)$$

where p_i is the proportion of the phytoplankton class relative to the total biomass. All six phytoplankton classes (given in Tab. 4.1) were included into the calculation of $H'_{CHEMTAX}$.

Tab. 4.1: Pigment:chlorophyll *a* ratio matrix for mesotrophic lakes from Schlüter et al. (2016), used as initial ratio matrix for CHEMTAX. Allo: alloxanthin, Chl *b*: chlorophyll *b*, Echi: echinenone, Fuco: fucoxanthin, Lut: lutein, Peri: peridinin, Zea: zeaxanthin.

	Allo	Chl *b*	Echi	Fuco	Lut	Peri	Zea
Bacillariophyceae	0	0	0	0.367	0	0	0.005
Chlorophyceae	0	0.271	0	0	0.119	0	0.001
Chrysophyceae	0	0	0	0.283	0	0	0.001
Cryptophyceae	0.156	0	0	0	0	0	0
Cyanobacteria	0	0	0.071	0	0	0	0.427
Dinopyceae	0	0	0	0	0	0.501	0

4.2.3.7 Phytoplankton community composition derived from microscopic counts

During each sampling procedure, 100 ml of the phytoplankton community from each mesocosm were preserved with 3 ml of Lugol's solution (RAL Diagnostics, France) and stored in dark until further analyses. The phytoplankton cells were counted in subsamples of 10 – 50 ml (depending on the biomass given as chlorophyll *a* concentration in µg L^{-1}) under an inverted microscope. Prior to the microscopic analysis, the subsamples were left to settle in the Utermöhl-chamber (Utermöhl, 1931) for at least 24 h. The taxonomic identity of counted phytoplankton was determined on the genus level. The genera of small to intermediate cell size (< 20 µm) were counted at 630x magnification in random fields. The number of fields (between 50 and 100) was chosen depending on the cell density (at least 400 cells were counted). Larger phytoplankton (e.g. *Cosmarium, Peridinium*) was counted at 200x or 400x magnification in horizontal lines along the entire chamber bottom. In order to obtain the total biovolume of the genera in each sample, the average number of cells per taxon was multiplied with the biovolume estimates, based on the typical cell morphology (Hillebrand et al., 1999). The obtained data are not shown or further discussed in this work.

4.2.3.8 Fatty acid analysis via gas chromatography (GC)

In order to address possible effects of food quality on the intraspecific competition between the different *D. longispina* genotypes, the fatty acid content of the respective phytoplankton community was analyzed. Although *Daphnia* is a non-selective filter feeder, the upper size of food particles that can be collected and ingested by *Daphnia* is limited by the size of its carapace gape. Therefore, in order to access the fatty acid content of phytoplankton cells that can be consumed by *Daphnia*, the seston samples were prefiltered over a 30 µm mesh. Subsequently, up to 500 ml of the seston samples

(< 30 µm) were filtered onto GF/F filters, which were transferred into tubes filled with 5 ml of extraction solvent (dichloromethane/methanol, 2:1, v:v) to allow for the extraction of lipids and stored at -20 °C. For subsequent quantification of fatty acids, two internal standards were added to the samples, i.e. 5 µg nonadecanoic acid methyl ester (C19:0 ME) and 5 µg tricosanoic acid methyl ester (C23:0 ME) and the samples were sonicated for 1 minute. The extracts were transferred into new tubes and additional 3 ml of extraction solvent were added to the filters for further extraction. After the samples were again sonicated for 1 min, the extracts were joined and evaporated to dryness at 40 °C under a gentle stream of nitrogen gas. Subsequently, the extracted fatty acids were transesterified at 70 °C for 20 min in 5 ml of 3 N methanolic HCl (Supelco). The resulting fatty acid methyl esters (FAMEs) were extracted three times with approx. 2 ml of isohexane. The isohexane phases were joined and subsequently evaporated at 40 °C under a stream of nitrogen gas and the remaining FAMEs were redissolved in 50 µl isohexane per sample. Finally, 1 µl of each sample was injected (splitless) into a 6890-N GC System (Agilent Technologies, Waldbronn, Germany) and analyzed using the same method as described by (Windisch and Fink, 2018). In total, the GC method enabled separation and identification of 38 different FAMEs. Subsequently, the GC-derived data were used to calculate the fatty acid content in µg per mg POC (phytoplankton biomass).

4.2.3.9 Estimation of Daphnia community composition via microsatellite analysis

To estimate the *D. longispina* community composition within the mesocosm tanks, given as the relative abundance of the three *D. longispina* genotypes KL14, KL83 and KL93, 5 × 2 adult individuals from *Daphnia* collected during the sampling procedure (starting at day 42, i.e. two weeks after *Daphnia* introduction) were randomly picked and stored in 1.5 ml reaction tubes at -80 °C until further analysis. First, the DNA was extracted

following the same procedure as given in **Chapter 3**. The concentration of extracted DNA from each reaction tube was measured using the NanoPhotometer P330 (IMPLEN, Germany) and was between 100 and 150 ng μl^{-1}. For the subsequent microsatellite-PCR, the Multiplex PCR Kit (QIAGEN, Germany) was used. Three of five previously tested polymorphic microsatellite loci (SwiD5, SwiD10 and SwiD11, see **Chapter 3**, Tab. 3.1) were identified as sufficient to distinguish between the three *D. longispina* genotypes used in the common garden experiment. Multiplex Manager v. 1.2 (Holleley and Geerts, 2009) was used to design multiplex PCRs. All three microsatellite primer pairs (see Tab. 3.1 in **Chapter 3**) were combined into a single 10x primer mix (concentration of each primer in the mix: 2 µM). Note that in order to combine all three primer pairs into a single multiplex PCR, the labelling for SwiD5 forward primer was changed to HEX. Each subsequent multiplex PCR reaction (10 µl reaction volume each) contained 5 µl 2x Multiplex PCR-Master Mix (containing 5 units μl^{-1} HotStartTaq DNA Polymerase), 1 µl of the 10x primer mix (concentration of each primer in the PCR reaction: 0.2 µM), 3 µl RNase-free water and 1 µl of template DNA. PCR conditions were: initial heat-activation at 95 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 90 sec and extension at 72 °C for 90 sec and a final single extension step at 72 °C for 10 min. The PCR products were diluted 1:100, mixed with Gene Scan 500 Rox Size Standard (ABI) and electrophoresed on the ABI 48-capillary 3730 DNA Analyzer at the Cologne Center for Genomics. Allele sizes were analyzed with the software Gene-Marker v2.7.0 (SoftGenetics, Pennsylvania).

From microsatellite data, the relative abundances of each *D. longispina* genotype (KL14, KL83 and KL93) were estimated within each mesocosm tank during all 10 weeks of the grazing phase.

Additionally, for each sampling point during the grazing phase, the diversity of the *D. longispina* community was calculated using the Shannon-Diversity Index (in following: $H'_{Daphnia}$) as given in Eq. 1 (see **Chapter 1**).

4.2.3.10. Data analysis and statistics

Statistical analyses and visual representation of the data were performed with R (version 3.3.2, R Core Team, 2016) and RStudio (version 1.1.383, RStudio Team, 2016).

To reveal possible differences in the response of the three *D. longispina* genotypes upon PUFA supplementation, paired t-tests were performed for every genotype and every PUFA tested, comparing the somatic growth rate of the daphnids in the control treatment with the somatic growth rate of daphnids raised on diet supplemented with the ω3-PUFAs ALA or EPA or the ω6-PUFA ARA. Additionally, a two-way analysis of variance (ANOVA) was performed to test for overall differences between the genotypes, with fixed factors *Genotype* (3 levels) and *PUFA* (4 levels, including the control treatment) as well as their interaction *Genotype* × *PUFA*. Note that both statistical procedures were performed using average somatic growth rates estimated within each separate growth experiment. Finally, absolute effect sizes upon PUFA supplementation were compared via one-way ANOVAs, separately for each PUFA. The sample size for each group of data (grouped by genotype and food regime) was equal to the number of growth experiments performed, i.e. $n = 5$ for genotype KL14, $n = 3$ for genotype KL83 and $n = 4$ for genotype KL93. To correct for this unbalanced design, Sum of Squares Type III was used within the two-way ANOVA.

Two main aims of the common garden experiment were (i) to test for possible links between phytoplankton diversity and fatty acid diversity and (ii) to find dietary factors (i.e. phytoplankton traits) that affect the intraspecific competition of the different *D. longispina* genotypes. Therefore, the first part of the statistical analyses focused on day 28, when *D. longispina* individuals were added to the mesocosm tanks (i.e. start of the grazing phase). These initial conditions for *Daphnia* growth and competition (in terms of food quantity and quality) were compared across different treatments (i.e. volume of initial phytoplankton community), by performing single one-way ANOVAs with *Inoculum* as fixed factor (five IP levels), followed by Tukey's HSD *post hoc* test. If the assumptions for ANOVA were violated (i.e. in case of deviation from the normal distribution and heterogeneous variances), Kruskal-Wallis test was applied. The parameters (i.e. dependent variables) tested were: POC (mg L^{-1}), PON (mg L^{-1}), PP (µg L^{-1}), molar C:N, N:P and C:P ratio, chlorophyll *a* (in µg L^{-1}, derived from the fluorometric measurements and used as phytoplankton biomass proxy) and CHEMTAX-derived phytoplankton diversity ($H'_{CHEMTAX}$).

Second, the diversity of dietary fatty acids was calculated as Shannon-Diversity Index (as given in Eq. 1) including all identified fatty acids (in following: H'_{FA}). Similarly, the diversity of dietary polyunsaturated fatty acids was calculated including both ω3- and ω6-PUFAs (in following: H'_{PUFA}). Subsequently, a linear regression analysis was performed to test for a significant relationship between the phytoplankton diversity ($H'_{CHEMTAX}$) and the diversity of fatty acids, including either all identified fatty acids (H'_{FA}) or only polyunsaturated acids (H'_{PUFA}). A one-way ANOVA was performed to reveal possible differences in H'_{FA} and H'_{PUFA} between the treatments.

Third, the relative abundance of the three *D. longispina* genotypes and the genotype diversity ($H'_{Daphnia}$) were used to describe the population dynamics and intraspecific competition of the *D. longispina* population over time. A two-way ANOVA with the fixed factor *Genotype* and the interaction *Genotype* × *Inoculum* was performed to test for overall differences in *Daphnia* community composition between the treatments (i.e. IP levels) on day 56 (four weeks after *Daphnia* introduction, corresponding to approx. 2 generation cycles). Note that *Inoculum* was not included as a fixed factor. Additionally, the relative abundance of the *D. longispina* genotypes within each treatment was compared with single one-way ANOVAs on days 56, 77 (seven weeks after *Daphnia* introduction; here, the average *Daphnia* abundance in the mesocosms dropped below 5 ind L^{-1}) and 98 (ten weeks after *Daphnia* introduction; last day of the experiment). The genotype diversity $H'_{Daphnia}$ was compared among the treatments on each sampling point during the grazing phase (starting on day 42) by performing single one-way ANOVAs.
Finally, to find dietary factors (i.e. phytoplankton traits) that might affect the *Daphnia* community composition and the intraspecific competition of *D. longispina* genotypes, different parameters were tested in a series of linear regressions of the form:

$$y = a + bx \quad \text{(Eq. 7)}$$

where y is the relative abundance of the respective *D. longispina* genotype on day 56, x is the independent variable (dietary factor) on day 28, a is the y-axis intercept and b is the slope of the regression line. The time difference of 4 weeks, in which *Daphnia* individuals undergo approx. 2 generation cycles, was chosen to test for a response of *Daphnia* community to the initial food quality.

The dietary factors tested were: H'_{FA}, H'_{PUFA}, relative sestonic abundance of ω3- and ω6-PUFAs, as well as their sum and ratio, and the relative sestonic abundance of ω3-PUFAs ALA, EPA or DHA and ω6-PUFA ARA.

To check for specific statistical assumptions prior to performing the statistical tests, we checked the normal distribution of the data with Shapiro-Wilk's test, while the homogeneity of variances was tested with Levene's test.

4.3 Results

4.3.1 Environmental factors

During the first 11 weeks of the outdoor common garden experiment, the average temperature was 18.82 ± 3.76 °C (Fig. 4.2). In the last four weeks of the experiment, the average daily temperature dropped below 10 °C and reached a minimum of only 6.25 °C on day 95. Precipitation was the highest in the first four weeks and in the last week of the experiment (up to 17 mm). The sunlight was the highest (on average 8 h) in the middle of the experiment (weeks 5 to 10).

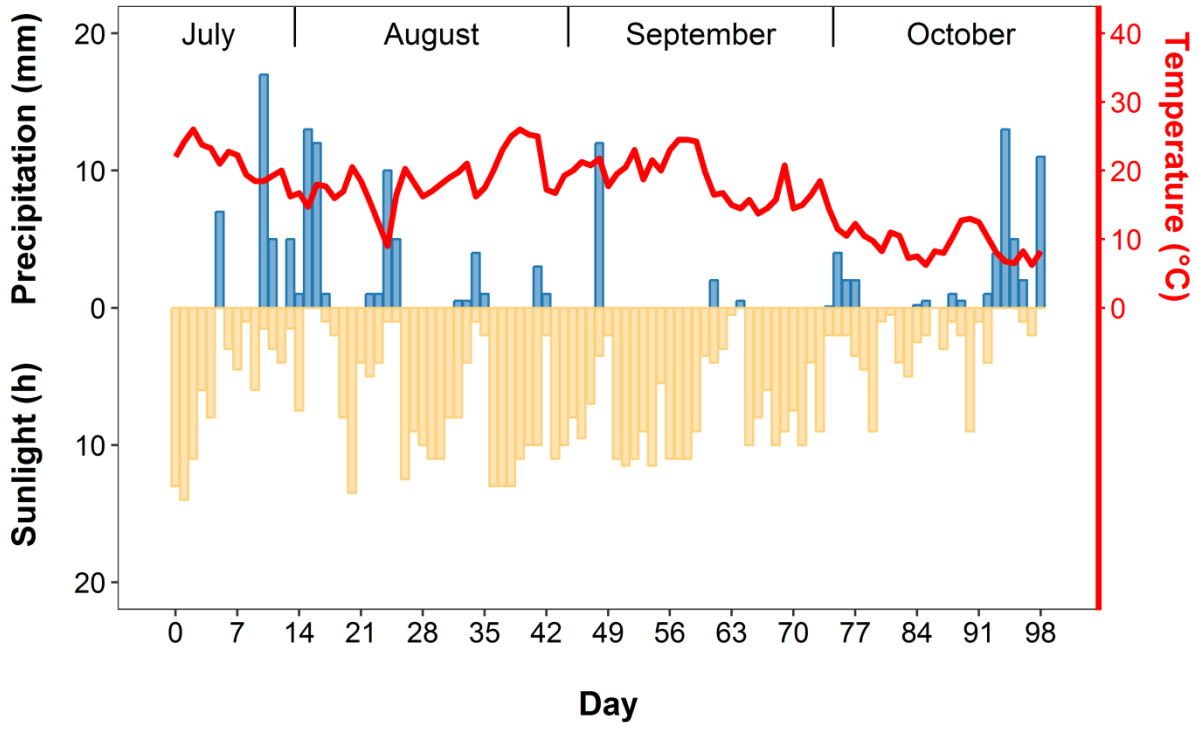

Fig. 4.2: Environmental parameters collected over the entire duration of the common garden experiment (18th July until 24th October 2016): precipitation (mm; blue bars), sunlight (h; orange bars) and average daily temperature (°C; red line).

4.3.2 Laboratory growth experiments with D. longispina genotypes

Upon PUFA-supplementation to their diet, *D. longispina* genotypes KL14, KL83 and KL93 showed pronounced differences in their response (Fig. 4.3). The somatic growth rate of the genotype KL14 significantly increased upon supplementation of any of the three tested PUFAs (paired t-tests, $p < 0.001$ for ALA and ARA, $p < 0.05$ for EPA-enriched diet). The somatic growth rate of the genotype KL83 was significantly increased only upon ALA-supplementation (paired t-test, $p < 0.05$), while EPA- and ARA-supplementation to the diet did not significantly affect its growth (paired t-test, $p = 0.229$ for both EPA- and ARA-enriched food regime). In contrast, the somatic growth rate of the genotype KL93 was not significantly affected by ALA ($p = 0.077$) or EPA supplementation ($p = 0.053$), while daphnids grown on the ARA-enriched diet showed significantly higher growth rates compared to the daphnids in the control treatment (paired t-test, $p < 0.05$).

Overall, a significant effect of the factor *Genotype* on the somatic growth rate was found, while the factors *PUFA* and the interaction *Genotype* × *PUFA* did not significantly affect the somatic growth rate of the daphnids (two-way ANOVA, p(*Genotype*) < 0.001, p(*PUFA*) = 0.083, p(*Genotype* × *PUFA*) = 0.951). As revealed by the Tukey's HSD *post hoc* test, the genotype KL83 daphnids showed the highest somatic growth rate (> 0.38 d^{-1} upon PUFA-supplementation and 0.35 ± 0.03 d^{-1} in the control treatment; mean ± 1 SD), while the smallest growth rate was found when the genotype KL14 and KL93 daphnids were raised in the control treatment (0.27 ± 0.06 d^{-1} and 0.27 ± 0.03 d^{-1}, respectively). While absolute effect size (equivalent to the slope of the reaction norm) upon EPA or ARA supplementation did not differ between the genotypes (one-way ANOVA, EPA: $F_{2,9} = 0.580$, $p = 0.580$; ARA: $F_{2,9} = 1.107$, $p = 0.372$), we found a significantly higher absolute effect size upon ALA supplementation for genotype KL14 compared to genotype KL83 (Tukey's HSD *post hoc* test following one-way ANOVA, $F_{2,9} = 6.274$, $p < 0.005$, Fig. 4.4).

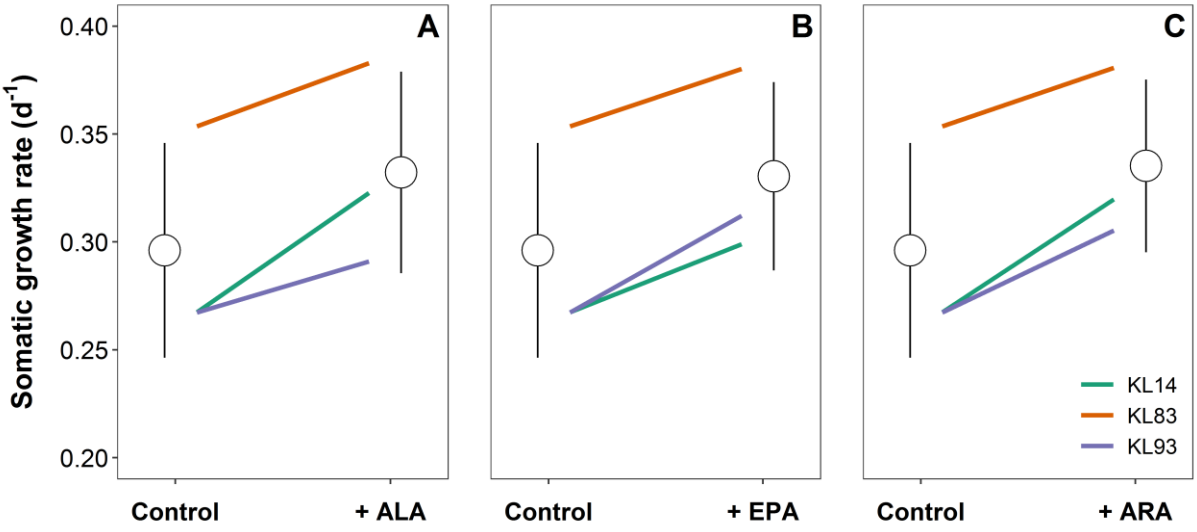

Fig. 4.3: Reaction norms representing the average somatic growth rate of *D. longispina* genotypes KL14 (turquoise), KL83 (orange) and KL93 (purple) raised on *Acutodesmus obliquus* supplemented with either PUFA-free control liposomes or liposomes containing ALA, EPA or ARA. White circles with error bars: average somatic growth rate (± 1 SD) of all three *D. longispina* genotypes combined. *n* = 5 for KL14, *n* =3 for KL83 and *n* = 4 for KL93.

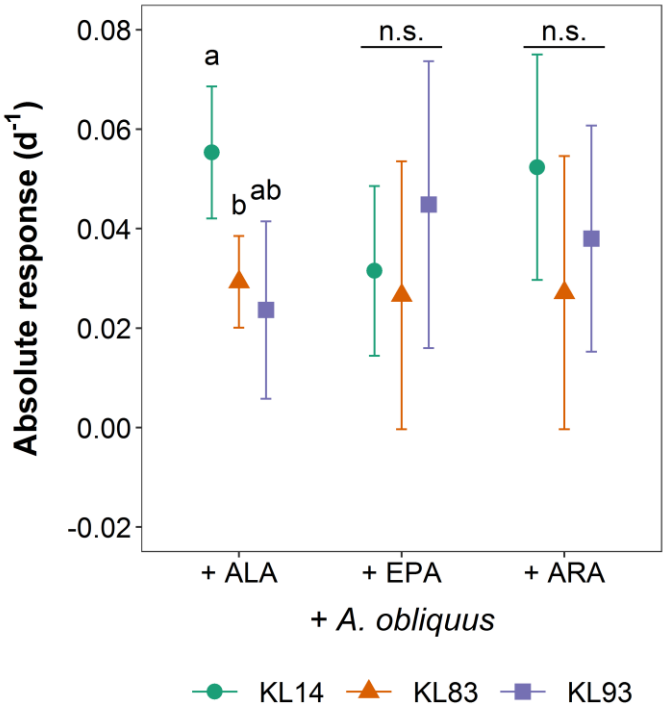

Fig. 4.4: Absolute response (i.e. effect size) of the somatic growth rate of *D. longispina* genotypes KL14 (turquoise), KL83 (orange) and KL93 (purple) upon ALA, EPA or ARA supplementation. Depicted are mean values (equivalent to the slopes of the reaction norms in Fig. 4.3). Different letters indicate significant differences between the means (one-way ANOVA followed by Tukey's HSD *post hoc* test). Error bars represent the standard deviation. n.s. = not significant. Number of experiments per genotype: *n* = 5 for KL14, *n* =3 for KL83 and *n* = 4 for KL93.

4.3.3 Producer and consumer dynamics

Over the entire course of the common garden experiment, typical producer-consumer dynamics were observed in all treatments (Fig. 4.5). During the first four weeks of the experiment (i.e. growth phase), the biomass of the phytoplankton communities (given as chlorophyll *a* in µg L^{-1}, Fig. 4.5A) increased from 0.06 ± 0.06 µg L^{-1} (mean ± 1 SD, all mesocosm tanks considered) to a maximum of 14.80 ± 6.49 µg L^{-1} (found for IP 10). Although the phytoplankton biomass in the mesocosms IP 1 and IP 1000 seemed to develop much slower during the growth phase compared to the other mesocosms, no significant differences between the treatments were found on day 28, when *D. longispina* individuals were introduced (one-way ANOVA, $F_{4,10} = 1.397$, $p = 0.304$). Upon *Daphnia* introduction, the average phytoplankton biomass in the mesocosms IP 100 and IP 10 decreased over time and reached values below 2 µg L^{-1} (day 49 and 63, respectively) and did not recover by the end of the experiment. In contrast, the phytoplankton biomass in mesocosms IP 1 and IP 1000 decreased only slightly upon grazing pressure and started to recover after day 49; the phytoplankton biomass in these mesocosms even increased towards the end of the experiment and reached its maximum on day 91 (9.16 ± 5.73 µg L^{-1} and 15.52 ± 14.08 µg L^{-1} found for IP 1 and IP 1000 mesocosms, respectively).

The abundance of *Daphnia* individuals increased rapidly in the first four weeks of the grazing phase (day 28 to 56, Fig. 4.5B) to a maximum of 52.33 ± 15.04 ind L^{-1} (found for IP 10000). To avoid starvation and crowding, excessive daphnids were removed on days 56, 63, 70, 77 and 84 to keep the abundance of *Daphnia* in the respective mesocosms at max. 20 ind L^{-1}. Due to the removal and the overall decrease in the available food quantity, the abundance of daphnids decreased in four out of five treatments below 10 ind L^{-1} by day 77 and continued to decrease towards the end of experiment below 5 ind L^{-1} (average among all mesocosm tanks).

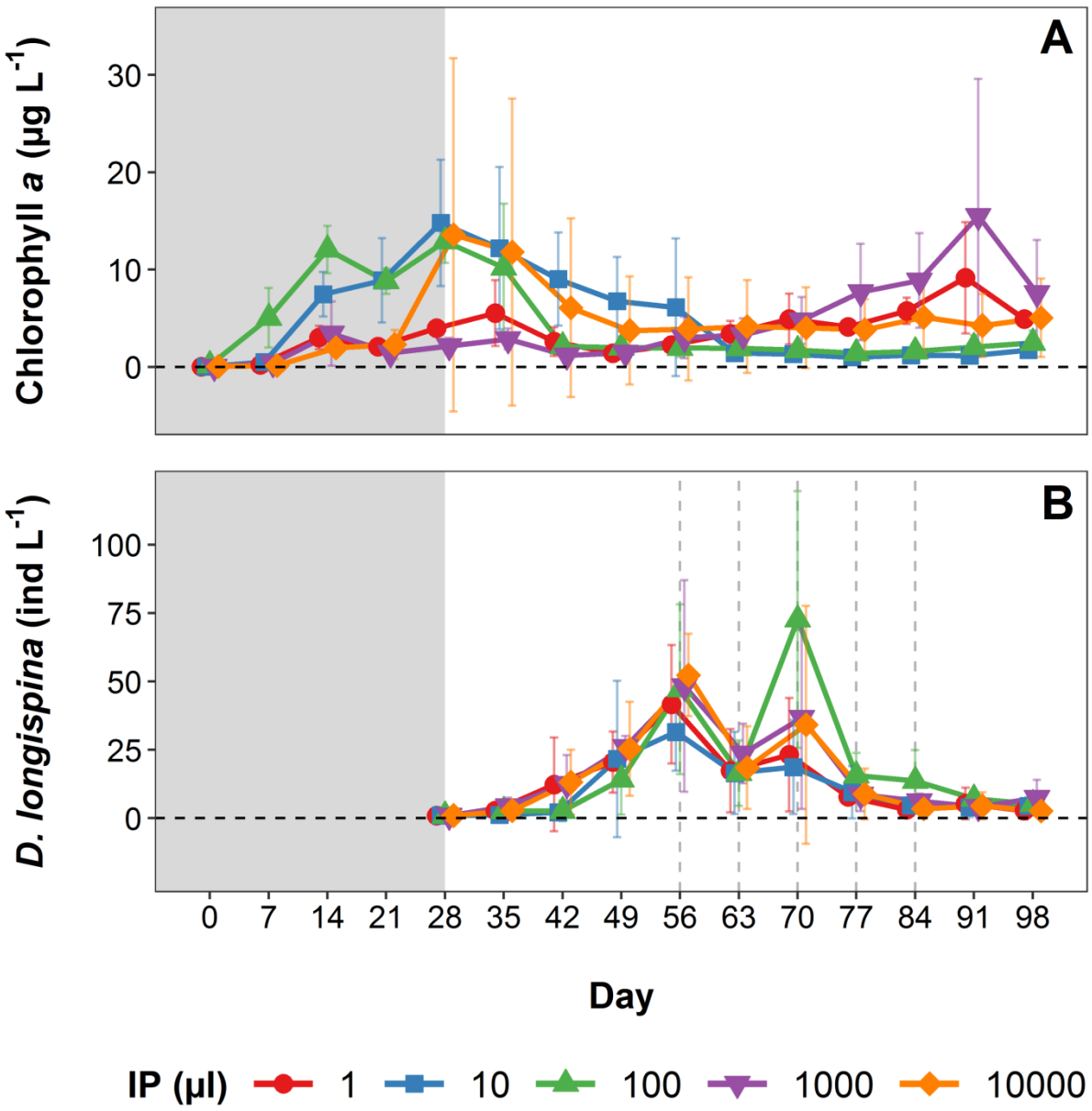

Fig. 4.5: (A) Biomass development of the phytoplankton communities (given as chlorophyll *a* in µg L^{-1}) over the entire duration of the common garden experiment. (B) *Daphnia longispina* abundance (individuals L^{-1}) during the grazing phase of the experiment (10 weeks). Depicted are means per treatment (i.e. volume of initial phytoplankton community, given as IP in µl, see legend in the figure). Error bars represent the standard deviation. The gray area represents the *Daphnia*-free growth phase (4 weeks). Vertical dashed lines indicate days at which excessive *Daphnia* individuals were removed to keep the *Daphnia* abundance in the respective mesocosms below 20 ind L^{-1}, in order to avoid starvation and crowding. For better visualisation of the data, the mean values and error bars are jittered around the respective day of data collection. n = 3 for each IP level.

4.3.4 Stoichiometry

Over the entire duration of the experiment, similar patterns were observed across all treatments regarding the phytoplankton stoichiometry (Fig. 4.6): the concentration of POC, PON and PP increased during the first 4 weeks of the experiment (*Daphnia*-free growth phase) and stayed constant until the end of the experiment. Solely the mesocosms IP 10 showed a different pattern: here, POC and PON reached the maximum by day 49 (1.73 ± 1.50 mg L^{-1} and 0.17 ± 0.10 mg L^{-1}, respectively), while PP reached a maximum during the growth phase, at day 21 (15.73 ± 6.02 µg L^{-1}). The average POC in the other four treatments (along all 14 weeks of experiment) was between 0.01 and 1.20 mg L^{-1}, while PON was between 0.001 and 0.1 mg L^{-1}. The average PP showed the steepest increase during the growth phase (from 0.15 µg L^{-1} up to 5.42 µg L^{-1}) and stayed constant during the grazing phase.

During the growth phase, the molar C:N ratios (Fig. 4.6A) increased in all treatments and reached average values between 27.15 and 40.76 at day 28. Within one week upon *Daphnia* introduction, by day 35, the C:N ratios dropped to a minimum of only 7.32, as observed in IP 10000, but stayed on average above the Redfield C:N ratio of 6.67 over the entire grazing phase. Although the molar N:P ratio oscillated much more compared to the C:N ratio, it increased over the course of 14 weeks from values below 16 (i.e. Redfield N:P ratio) to a maximum of 64.61 ± 22.11 (day 91, IP 10, Fig. 4.6B). Similarly, no clear patterns or differences among treatments were observed regarding the molar C:P ratio, which only partly increased during the experiment (Fig. 4.6C). Except for IP 10000 on day 7 and IP 10 on day 21, the average C:P ratio per treatment was above the Redfield C:P ratio of 106. No significant differences were found across treatments on day 28 (i.e. start of the grazing phase) for any of the tested parameters (two-way ANOVA or Kruskal-Wallis test; for details see Tab. 4.2).

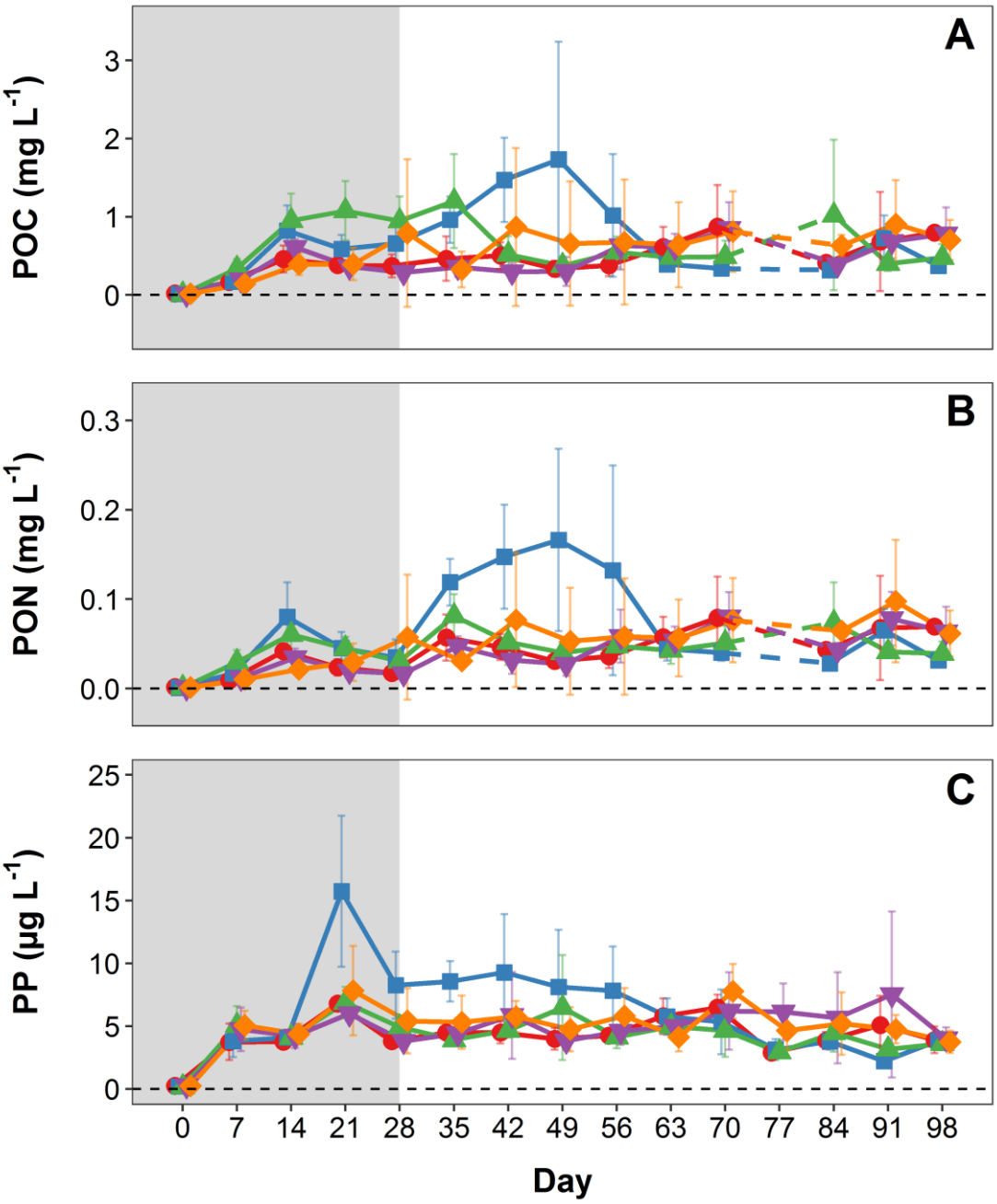

Fig. 4.6: (A) Particulate organic carbon (POC, in mg L^{-1}), (B) particulate organic nitrogen (PON, in mg L^{-1}) and (C) particulate phosphorus (µg L^{-1}) over the entire duration of the common garden experiment. Depicted are means per treatment (i.e. volume of initial phytoplankton community, for legend see Fig. 4.5). Error bars represent the standard deviation. Days 71 and 84 in panels A and B are connected with a dashed line, as samples from day 77 were lost due to technical problems. *n* = 3 for each IP level.

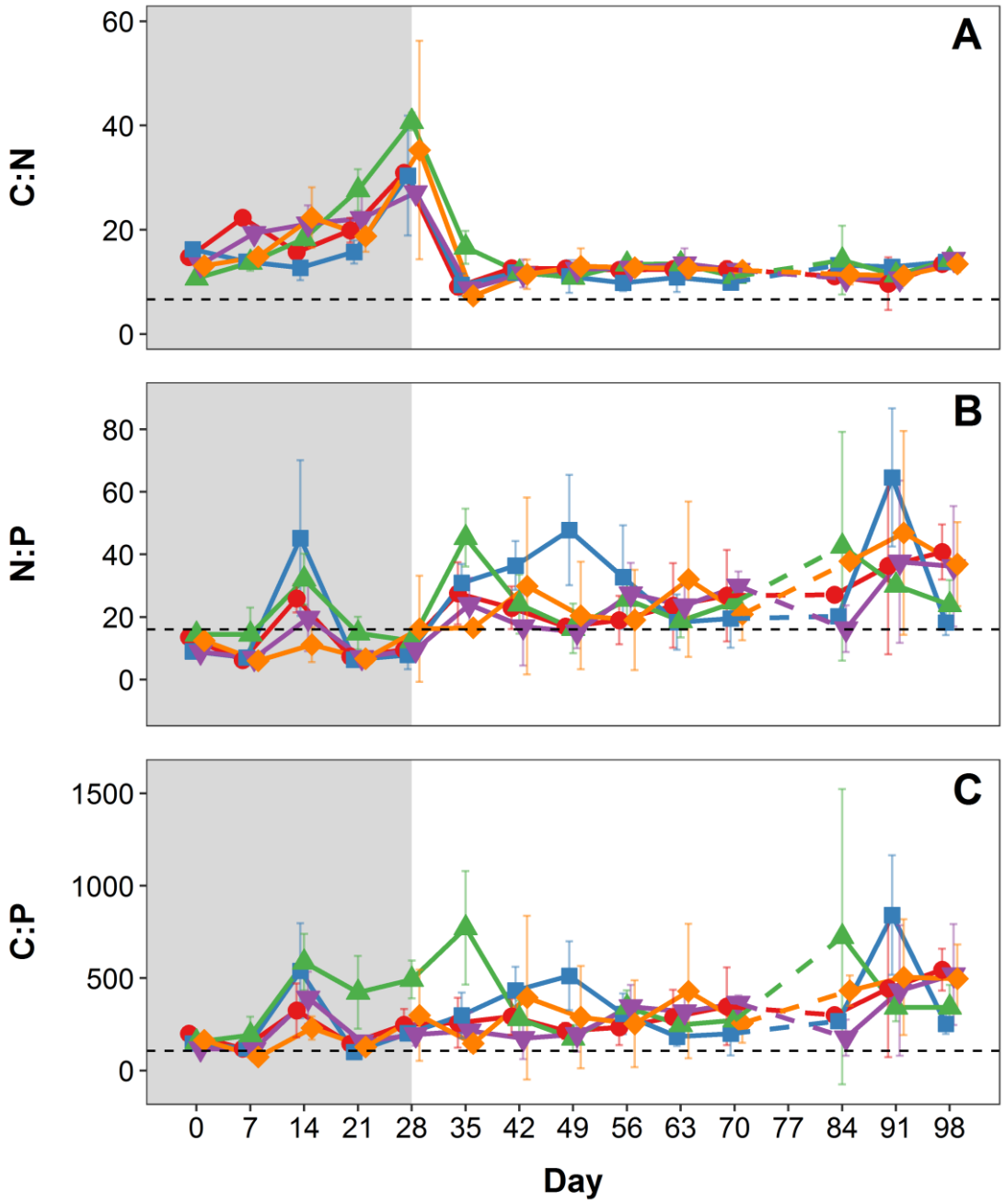

Fig. 4.7: (A) Molar C:N, (B) N:P and (C) C:P ratios over the entire duration of the common garden experiment. Depicted are means per treatment (i.e. volume of initial phytoplankton community, for legend see Fig. 4.5). Error bars represent the standard deviation. Days 71 and 84 in all panels are connected with a dashed line, as POC and PON samples from day 77 were lost due to technical problems. *n* = 3 for each IP level. The horizontal dashed line in each panel represents the Redfield ratios (C:N:P = 106:16:1, Redfield, 1958). *n* = 3 for each IP level.

Tab. 4.2: Results from one-way ANOVA and Kruskal-Wallis test (KW) testing for differences between the treatments (i.e. volume of initial phytoplankton community). Response (dependent) variables are given in the column "Parameter". Only data from day 28 (i.e. start of the grazing phase, Fig. 4.6 and 4.7) were included into the statistical analyses. *: degrees of freedom (*df*) = 4.

Parameter	**Test**	***F* or X^2**	***p***
POC (mg L^{-1})	ANOVA	$F_{4,10}$ = 1.056	0.427
PON (mg L^{-1})	KW*	X^2= 3.000	0.558
PP (µg L^{-1})	ANOVA	$F_{4,10}$ = 3.357	0.055
molar C:N ratio	KW*	X^2= 2.533	0.639
molar N:P ratio	KW*	X^2= 0.867	0.929
molar C:P ratio	ANOVA	$F_{4,10}$ = 2.679	0.094

4.3.5 Start of the grazing phase

4.3.5.1 Pigment-derived phytoplankton community composition

By the end of the *Daphnia*-free growth phase, i.e. after four weeks, among all treatments, Chlorophyceae were the dominating phytoplankton class (with average relative abundance found between 67.22 ± 20.50 % and 96.77 ± 1.27 %; average values in treatments IP 1 and IP 10, respectively), while the classes Cryptophytes and Cyanobacteria were present in very low abundances, i.e. both classes together made up only 2.98 ± 1.62 % of the whole community (average across all mesocosm tanks; Fig. 4.8). According to the CHEMTAX calculations, Bacillariophyceae were found only in two mesocosm tanks from two different treatments: they were highly abundant in one IP 1000 mesocosm (41.06 %), while only a very small proportion of Bacillariophyceae was found in one IP 10000 mesocosm (1.17 %).

The highest average proportion of Chrysophyceae was found in IP 10000 mesocosms (12.38 ± 20.48 %), while less than 1% of Chrysophyceae were found in IP 10 mesocosms (0.18 ± 0.15 %). Dinophyceae were found across all treatments, and were most abundant in the IP 1 mesocosms (20.28 ± 16.52 %).

Interestingly, some variation was found within treatments: in all five treatments, the phytoplankton community in two out of three replicates seemed to develop very similarly, while the third replicate showed a high deviation from this community composition. Finally, no significant differences were found regarding the diversity of phytoplankton communities (calculated as Shannon-Diversity Index, H'_{CHEMTAX}) between the treatments (one-way ANOVA, $F_{4,10} = 1.866$, $p = 0.193$).

4.3.5.2 Seston fatty acid composition

The proportion of SAFAs in the phytoplankton community was between 20.41 % and 41.88 %, while the relative abundance of MUFAs was between 28.43 % and 57.38 %. PUFAs, including both ω3- and ω6-PUFAs, were the least abundant class (29.49 % ± 6.40 %, average across all mesocosms), with an average ratio of ω3- to ω6-PUFAs of 2.08 (Fig. 4.9). The most abundant ω3-PUFA was ALA (67.85 ± 12.14 % of the total ω3-PUFA content; mean ± 1 SD across all mesocosms), while the most abundant ω6-PUFA was linolenic acid (LA; 74.97 ± 11.50 % of the total ω6-PUFA content; mean ± 1 SD across all mesocosms).

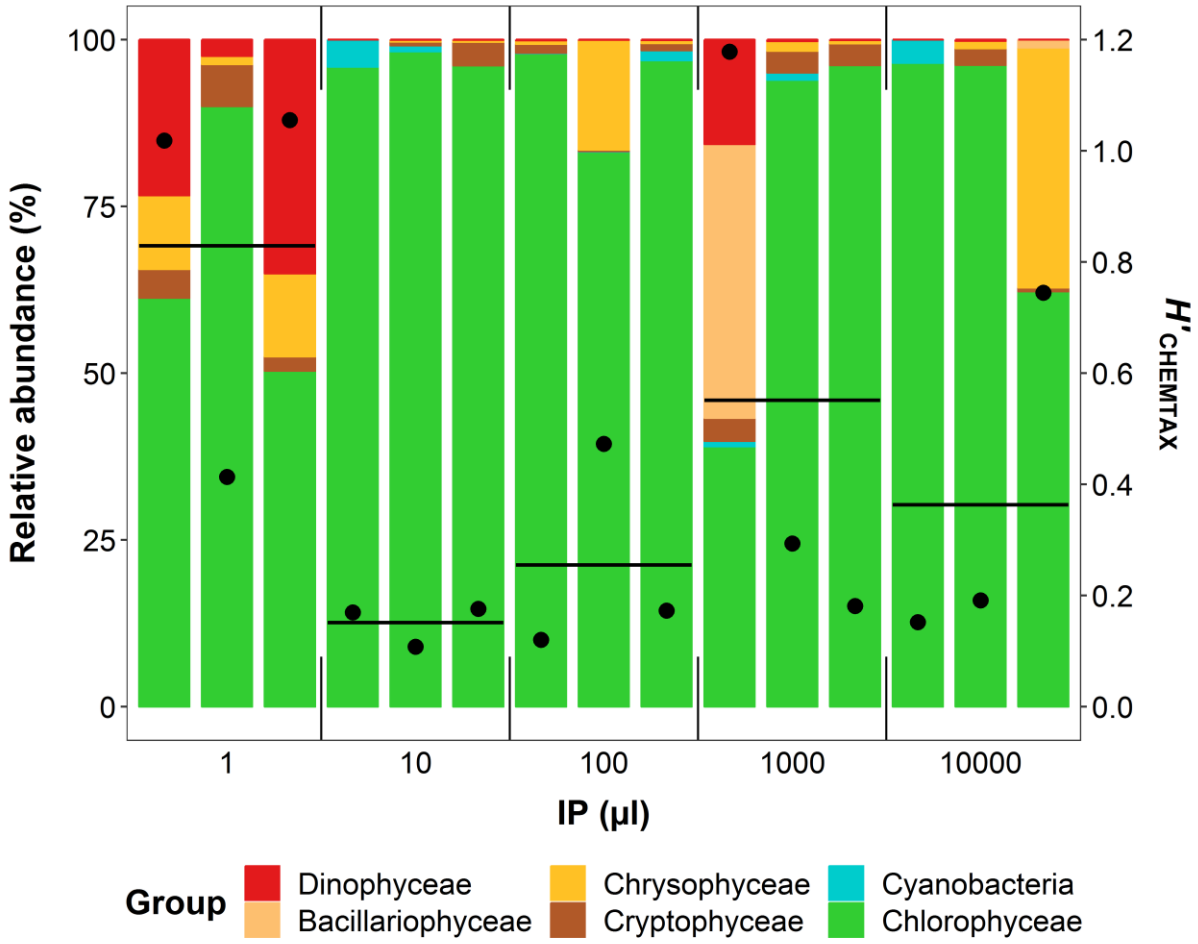

Fig. 4.8: Pigment-derived phytoplankton community composition within all 15 mesocosm tanks at the end of the growth phase (day 28). Black circles: diversity of the phytoplankton community (calculated as Shannon-Diversity Index $H'_{CHEMTAX}$) for each mesocosm; black solid lines: average phytoplankton diversity within one treatment. n = 3 for each IP level.

The overall fatty acid diversity (including all identified fatty acids) was the lowest in IP 100 mesocosms (H'_{FA} = 1.86 ± 0.20), while significantly higher H'_{FA} was observed in mesocosms IP 1 (H'_{FA} = 2.40 ± 0.27), IP 1000 (H'_{FA} = 2.59 ± 0.11) and IP 10000 (H'_{FA} = 2.40 ± 0.17) (one-way ANOVA, $F_{4,10}$ = 7.683, p < 0.01, followed by Tukey's HSD *post hoc* test, α= 0.05). Similarly, the highest diversity of polyunsaturated fatty acids was found in IP 1000 mesocosms (H'_{PUFA} = 1.81 ± 0.08), while the lowest H'_{PUFA} was found in mesocosms IP 10 (H'_{PUFA} = 1.28 ± 0.13) and IP 100 (H'_{PUFA} = 1.22 ± 0.05) (one-way ANOVA, $F_{4,10}$ = 13.97, p < 0.001, followed by Tukey's HSD *post hoc* test, α= 0.05).

4.3.5.3 Relationship between phytoplankton diversity and fatty acid diversity

Although the fatty acid diversity (given as H'_{FA}) increased with increasing phytoplankton diversity ($H'_{CHEMTAX}$; pigment-derived), their relationship was not significant (linear regression, $R^2 = 0.2473$, $p = 0.0593$, Fig. 4.10A). However, a significant relationship was found between phytoplankton diversity and the diversity of polyunsaturated fatty acids (given as H'_{PUFA}; linear regression, $R^2 = 0.3621$, $p < 0.05$, Fig. 4.10B).

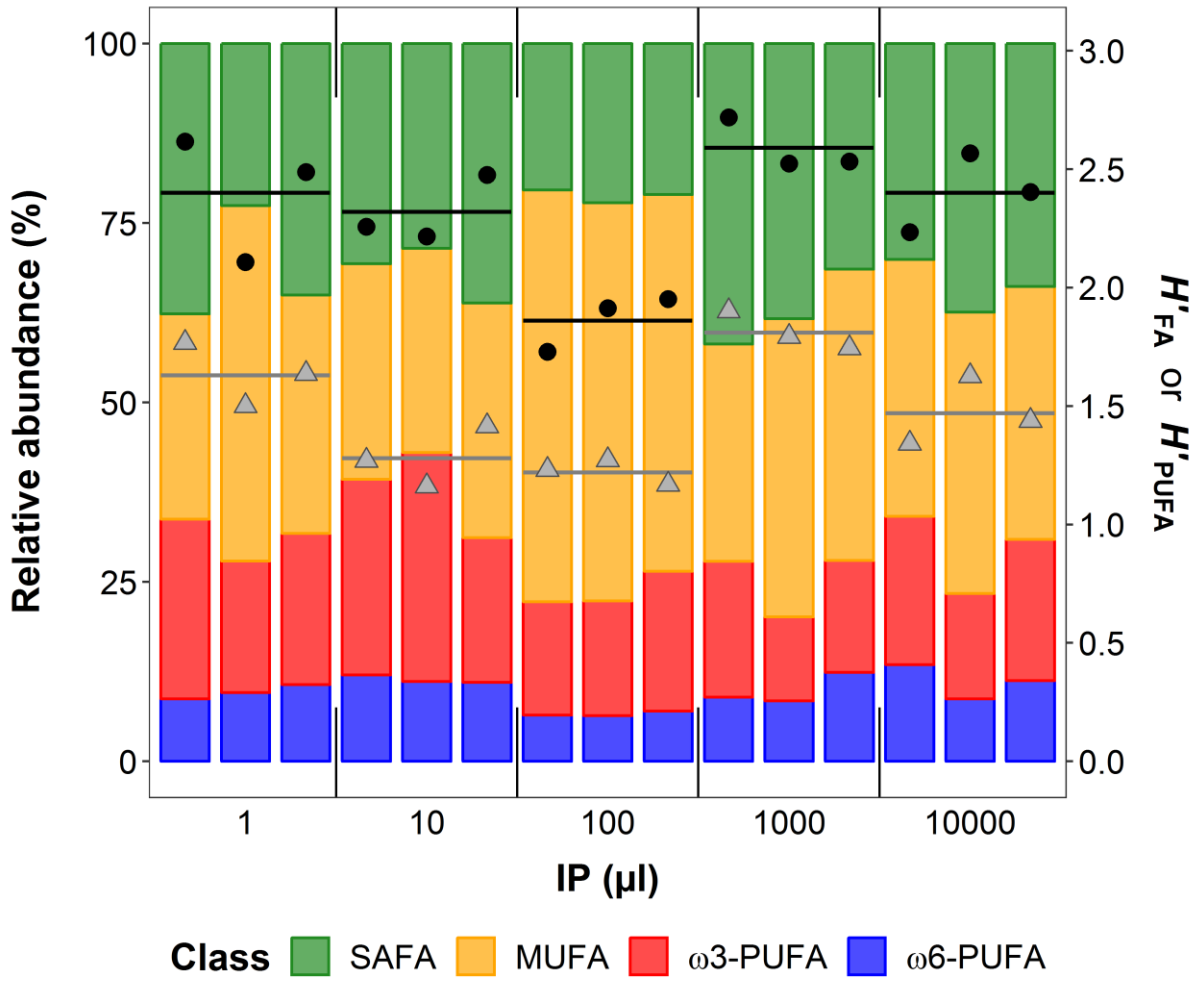

Fig. 4.9: Fatty acid composition of the phytoplankton communities within all 15 mesocosm tanks at the end of the growth phase (day 28), grouped in classes. SAFA: saturated fatty acids, MUFA: monounsaturated fatty acids, ω3- and ω6-PUFAs: polyunsaturated fatty acids. Black circles: fatty acid diversity (calculated as Shannon-Diversity Index; H'_{FA}) for each mesocosm; black solid lines: average fatty acid diversity within each treatment. Gray triangles: polyunsaturated fatty acid diversity (calculated as Shannon-Diversity Index; H'_{PUFA}) for each mesocosm; gray solid lines: average polyunsaturated fatty acid diversity within each treatment. $n = 3$ for each IP level.

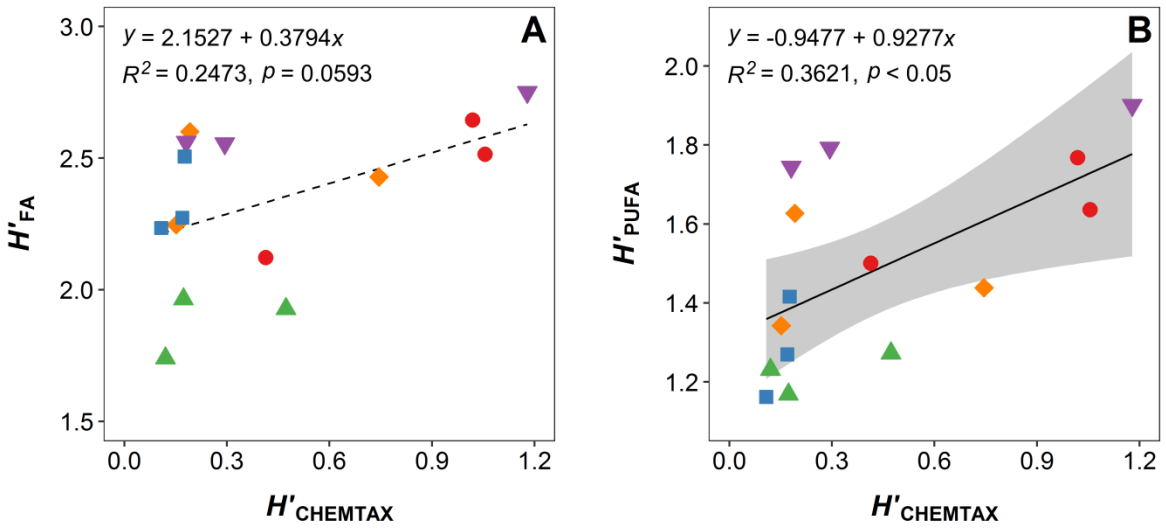

Fig. 4.10: Relationship between the pigment-derived phytoplankton diversity ($H'_{CHEMTAX}$) and (A) the fatty acid diversity (H'_{FA}) or (B) the polyunsaturated fatty acid diversity (H'_{PUFA}) at the end of the growth phase (day 28). Each point represents one mesocosm (for legend, see Fig. 4.5). The solid line represents a significant linear regression, while the dashed line represents a non-significant relationship (see figure for details). The gray area surrounding the solid line represents the 95% confidence interval. n = 3 for each IP level.

4.3.6 Daphnia *population dynamics*

Over the course of the experiment, similar patterns among treatments were observed in the distribution of the three *D. longispina* genotypes KL14, KL83 and KL93 (Fig. 4.11). In all treatments, the relative abundance of the genotype KL93 increased within two weeks upon introduction (day 28 to 42), but decreased stepwise in the further course of the grazing phase and reached a minimum of only 19.17 ± 6.29 % on day 91 (mean ± 1 SD; IP 1000 mesocosms). In contrast, genotype KL83 decreased by day 42 to values below 3 % (average across all mesocosms), but recovered and increased during the grazing phase to a maximum of 80.83 ± 6.29 %, as found in IP 1000 mesocosm on day 91. The maximum proportion of the genotype KL14 was found in IP 100 mesocosms on days 56 and 63 (30 ± 8.66 % and 30 ± 20 %, respectively). After day 63, the relative abundance of the genotype KL14 decreased to average values around 0 % and stayed at low abundances until the end of experiment.

On day 56 (four weeks after *Daphnia* introduction, approx. 2 generation cycles), significant differences were found between the relative abundance of the three genotypes in all treatments except for IP 1 mesocosms (revealed via single one-way ANOVAs, see Tab. S3). Genotype KL93 was significantly the most abundant genotype in IP 10 and IP 1000 mesocosms (85.00 ± 13.23% and 53.33 ± 5.77 %, respectively), while significant differences in the abundance of genotypes KL83 and KL14 were found only in IP 1000 mesocosms, with genotype KL14 being the least abundant genotype (13.33 ± 5.77 %, Fig. 4.12). When all 15 mesocosms were taken together, a significant effect of the fixed factor *Genotype* was found ($F_{2,30}$ = 42.676, p < 0.001), while the interaction *Genotype* × *Inoculum* was not significant ($F_{12,30}$ = 1.688, p = 0.120 ; two-way ANOVA, note that *Inoculum* was not included as a second fixed factor). In this case, significantly higher relative abundance of genotype KL93 was found (63.83 ± 14.62 % across all mesocosms), while no differences were found for genotypes KL83 (19.67 ± 9.55 %) and KL14 (16.50 ± 10.51 %).

Seven weeks after *Daphnia* introduction, on day 77, the average abundance of KL14 genotype dropped to 0% in all treatments (Fig. 4.12). Although no significant differences were found between the relative abundance of the genotypes KL83 and KL93 in any of the treatments (Kruskal-Wallis test, see Tab. S3), the genotype KL93 was on average twice as abundant as the genotype KL83. Therefore, the overall ratio between the genotypes KL14 : KL83 : KL93 was 0 : 1 : 2 in all treatments.

By the end of experiment, on day 98, the average relative abundance of the genotype KL83 increased in all treatments except for IP 1 mesocosms. In fact, in IP 10 mesocosms, the relative abundance of the genotype KL83 was significantly higher compared to genotypes KL93 and KL14 (one-way ANOVA, see Tab. S3). The genotype KL14 was only found in IP 10 mesocosms, while in other mesocosms, it was completely absent.

Over the entire course of the grazing phase, similar patterns in *Daphnia* diversity $H'_{Daphnia}$ across all treatments were found (Fig. 4.13). After a rapid decrease within two weeks upon *Daphnia* introduction, $H'_{Daphnia}$ increased and reached a maximum of 0.96 ± 0.14 on day 49 (IP 1000 mesocosms). In the further course of the experiment, $H'_{Daphnia}$ decreased stepwise in all treatments and showed some oscillations after day 77. At the end of experiment, on day 98, the average *Daphnia* diversity was between 0.22 ± 0.38 (IP 1 mesocosms) and 0.69 ± 0.12 (IP 10 mesocosms). No significant differences were found between the treatments over the entire duration of the grazing phase, except for day 63 (one-way-ANOVA, $F_{4,10} = 4.207$, $p < 0.05$).

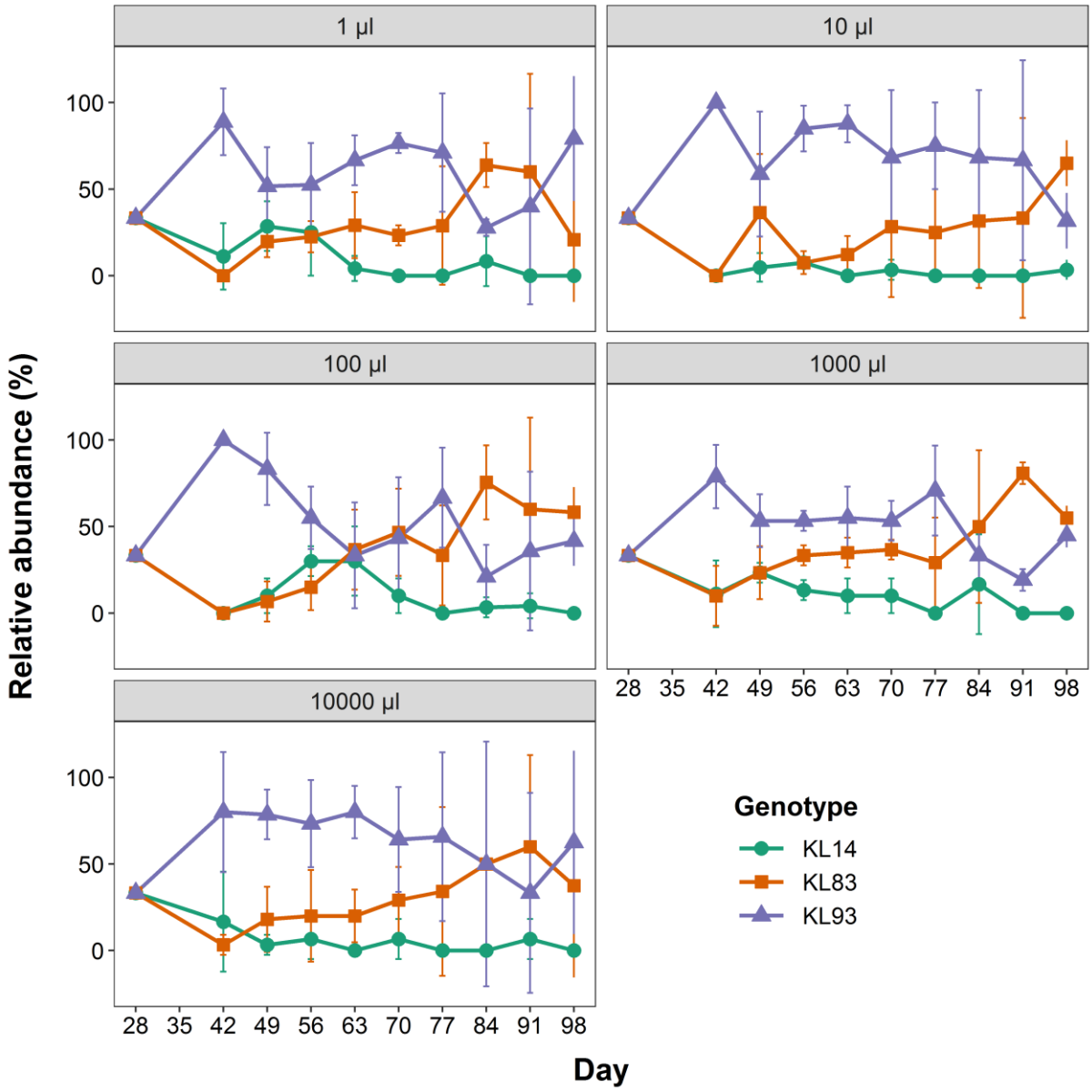

Fig. 4.11: Relative abundance (%) of *D. longispina* genotypes KL14 (turquoise), KL83 (orange) and KL93 (purple) in each treatment (i.e. volume of initial phytoplankton community IP in µl, given in the title of each panel) over the entire duration of the grazing phase (10 weeks in total, day 28 to 98). Depicted are mean values per day. Error bars represent the standard deviation. *n* = 3 for each IP level.

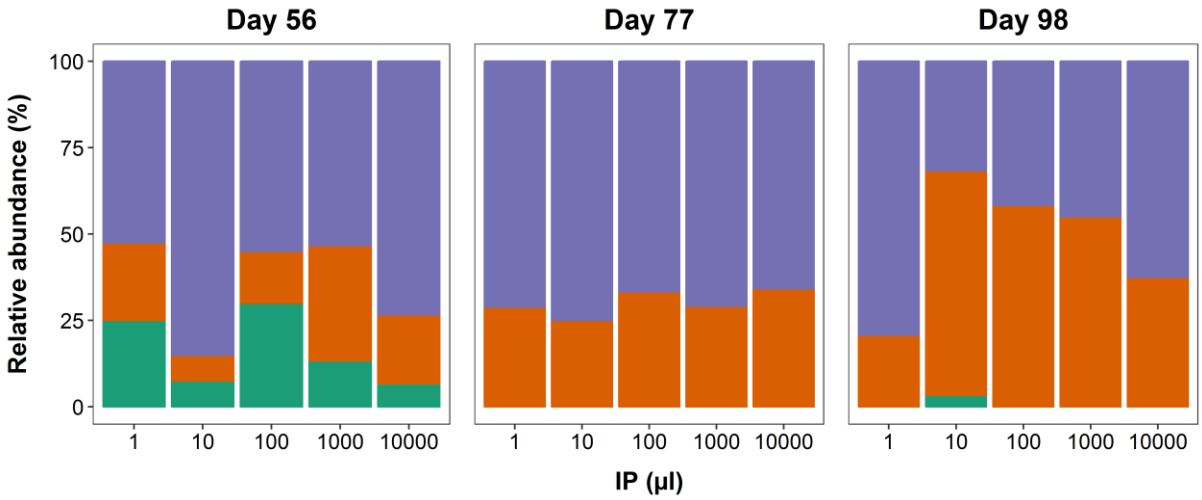

Fig. 4.12: Relative abundance (%) of *D. longispina* genotypes KL14 (turquoise), KL83 (orange) and KL93 (purple) per treatment (i.e. volume of initial phytoplankton community IP in µl) on day 56 (four weeks after *Daphnia* introduction, approx. 2 generation cycles), day 77 (seven weeks after *Daphnia* introduction) and day 98 (end of the experiment). Given are mean values per day and treatment. The data plotted in this figure represents a subset of data plotted in Fig. 4.11.

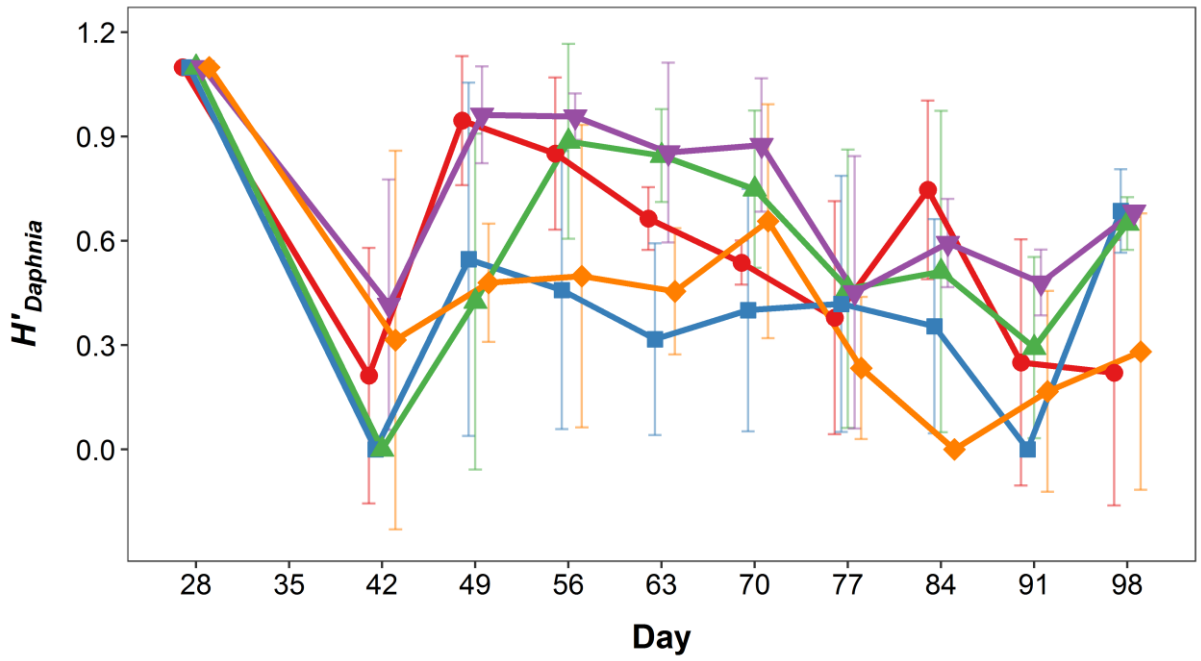

Fig. 4.13: *Daphnia* diversity (given as Shannon-Diversity Index $H'_{Daphnia}$) per treatment (i.e. volume of initial phytoplankton community, for legend, see Fig. 4.5) over the entire course of grazing phase. Depicted are mean values per treatment and day. Error bars represent the standard deviation.

4.3.7 Response of Daphnia *community to the dietary quality of the phytoplankton community*

While none of the tested fatty acid-related dietary factors significantly affected the diversity of *Daphnia* community $H'_{Daphnia}$, some of the dietary factors significantly affected the relative abundance of genotypes KL83 and KL93 (Fig. 4.14 and 4.15, Tab. 4.3 and 4.4): the relative abundance of the genotype KL83 significantly increased with increasing diversity of polyunsaturated fatty acids (given as H'_{PUFA}, $R^2 = 0.31$, $p < 0.05$, Fig. 4.14A), but was negatively correlated with the relative proportion of ω3-PUFAs in the seston ($R^2 = 0.27$, $p < 0.05$, Fig. 4.14C). On the other hand, the relative abundance of the genotype KL93 significantly increased with the relative abundance of PUFAs in the seston ($R^2 = 0.28$, $p < 0.05$, Fig. 4.14B). Furthermore, a significant positive relationship was found between the proportion of the ω3-PUFA ALA in the seston and the relative abundance of the genotype KL93 ($R^2 = 0.32$, $p < 0.05$), while the relative abundance of the genotype KL83 was negatively correlated with the proportion of ALA ($R^2 = 0.31$, $p < 0.05$; Fig. 4.15A). In contrast, the relative abundance of the genotype KL83 significantly increased with increasing proportions of the ω6-PUFA ARA in the seston ($R^2 = 0.32$, $p < 0.05$; Fig. 4.15B). Although the relative abundance of the genotype KL93 seemed to decrease with increasing proportions of ARA, this relationship was not significant ($p = 0.21$). No significant relationships were observed between the relative abundance of the genotype KL14 with any of the tested PUFAs. The relative abundance of ω3-PUFAs EPA (Fig. 4.15C) and DHA (Fig. 4.15D) did not have any significant effects on the *Daphnia* community composition. Results from linear regression analyses can be found in Tab. 4.4.

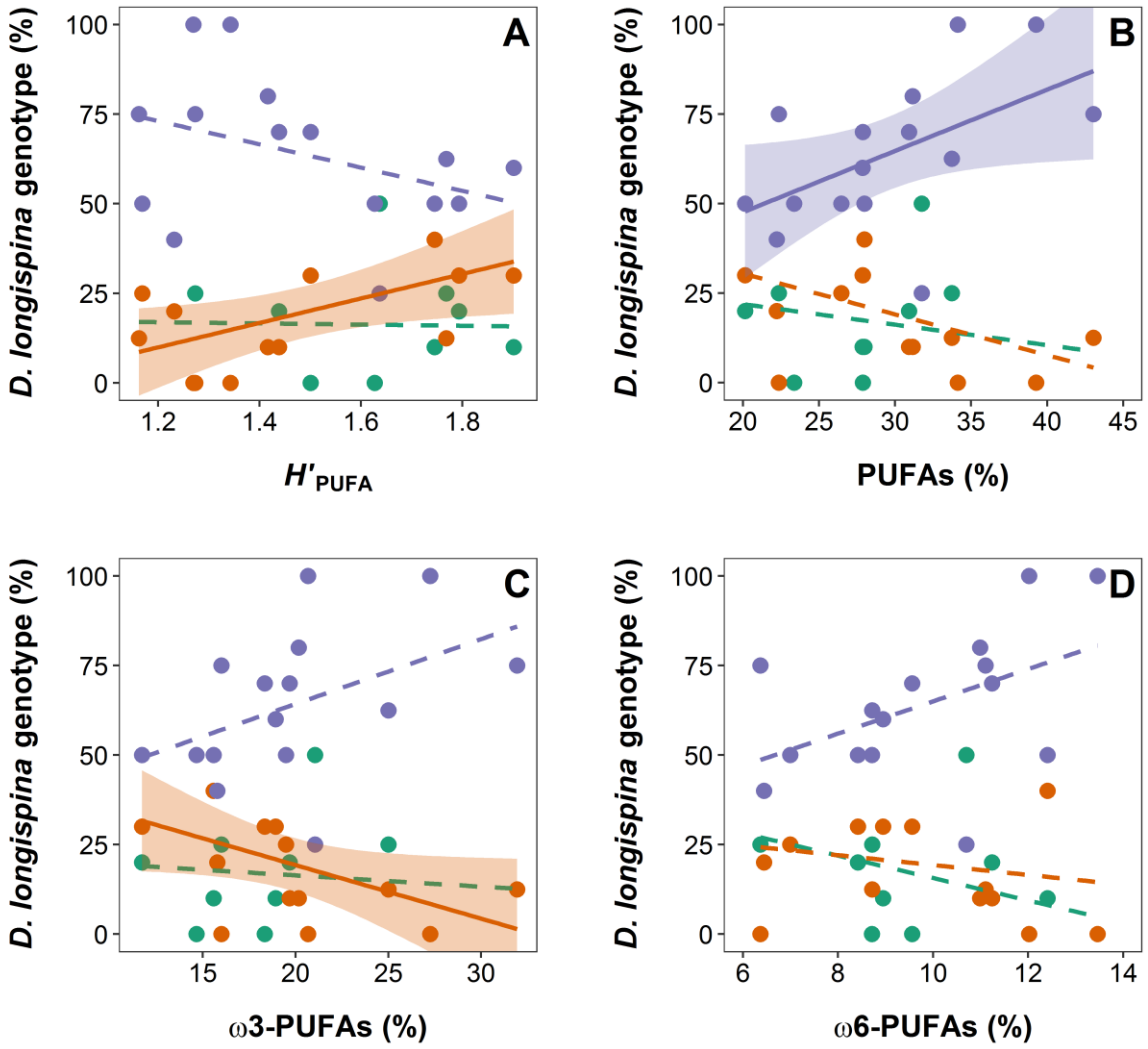

Fig. 4.14: Relative abundance (%) of *D. longispina* genotypes KL14 (turquoise), KL83 (orange) and KL93 (purple) on day 56 plotted against (A) the diversity of polyunsaturated fatty acids (H'_{PUFA}), (B) the relative abundance of ω3- and ω6-PUFAs combined or (C) ω3-PUFAs and (D) ω6-PUFAs separately. All fatty acid related parameters (dietary factors) refer to the day 28 (start of the grazing phase). The time delay of four weeks was chosen to test for a response of the *Daphnia* community to the initial dietary quality of the phytoplankton community at the beginning of the grazing phase. Four weeks correspond to approx. two generations of *Daphnia* life cycle. Solid lines surrounded by colored area (95 % confidence intervals) represent a significant linear relationship, while dashed lines represent a non-significant relationship. Depicted are raw values for every mesocosm (n = 15 for each genotype). Results from the linear regression analyses can be found in Tab. 4.3.

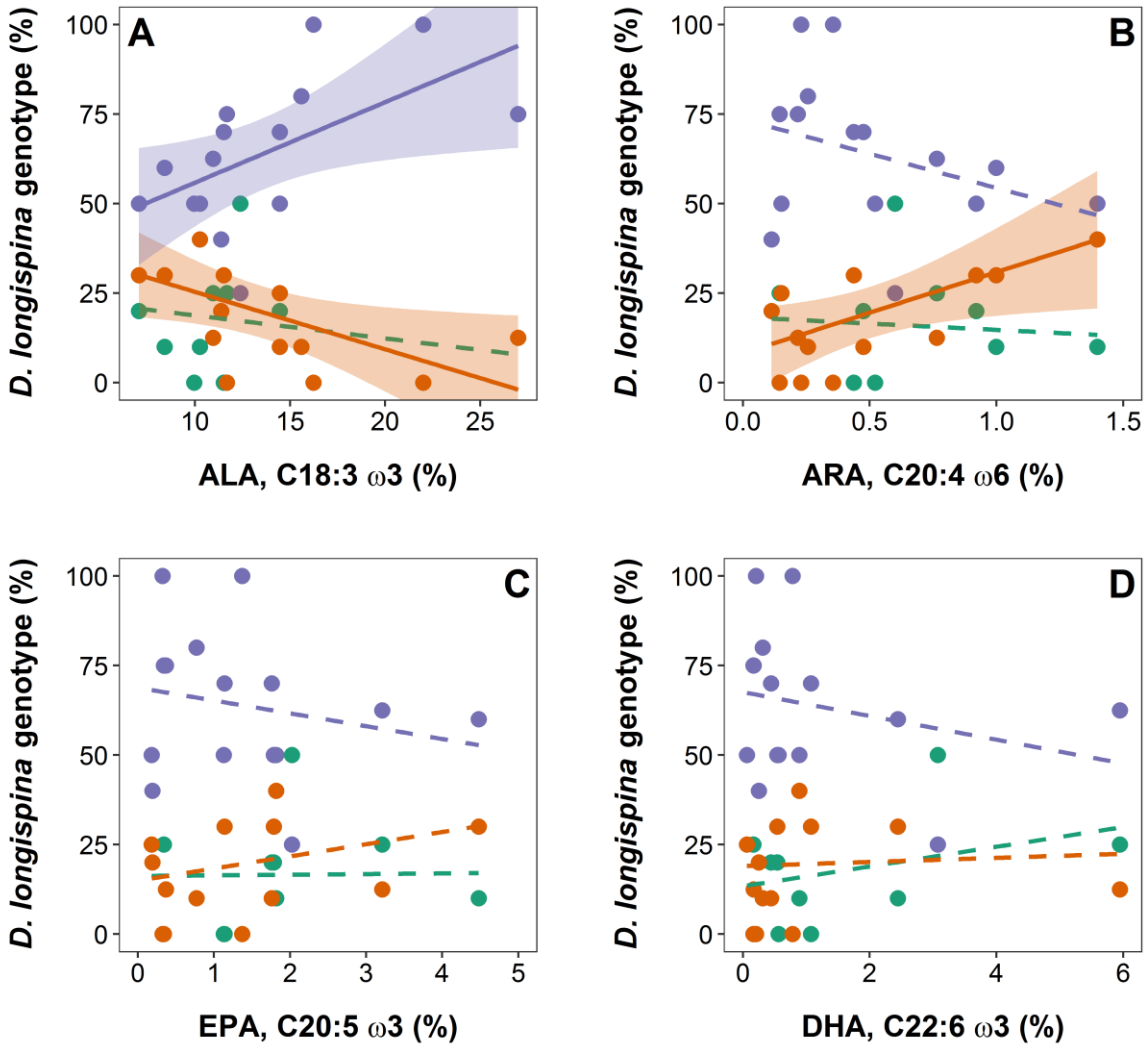

Fig. 4.15: Relative abundance (%) of *D. longispina* genotypes KL14 (turquoise), KL83 (orange) and KL93 (purple) on day 56 plotted against (A) the relative abundance of ω3-PUFAs ALA, (C) EPA or (D) DHA and (B) the relative abundance of ω6-PUFA ARA on day 28 (start of the grazing phase). The time delay of four weeks was chosen to test for a response of the *Daphnia* community to the initial dietary quality of the phytoplankton community at the beginning of the grazing phase. Four weeks correspond to approx. two generations of *Daphnia* life cycle. Solid lines surrounded by colored area (95 % confidence intervals) represent a significant linear relationship, while dashed lines represent a non-significant relationship. Depicted are raw values for every mesocosm (n = 15 for each genotype). Results from the linear regression analyses can be found in Tab. 4.4.

Tab. 4.3: Results from linear regression analyses. Columns: *y*: response variable, i.e. relative abundance (%) of *D. longispina* genotypes KL14, KL83 and KL93 on day 56 (four weeks after *Daphnia* introduction); *x*: independent variable, all parameters refer to day 28 (start of the grazing phase); *a*: intercept; *b*: slope of the regression line; R^2: proportion of variance explained by the linear regression, *p*: *p*-value of the linear regression. Significant relationships are given in bold ($p < 0.05$). Visual representation of the data can be found in Fig. 4.14.

y	***x***	***a***	***b***	R^2	***p***
KL14 (%)	Fatty acid diversity H'_{FA}	44.12	-11.93	0.05	0.41
KL83 (%)		-23.48	18.63	0.13	0.19
KL93 (%)		79.35	-6.70	0.01	0.74
KL14 (%)	PUFA diversity H'_{PUFA}	18.98	-1.67	0.00	0.92
KL83 (%)		-31.01	34.13	0.31	**< 0.05**
KL93 (%)		112.03	-32.46	0.15	0.16
KL14 (%)	PUFAs (%)	33.36	-0.57	0.06	0.38
KL83 (%)		53.26	-1.14	0.24	0.07
KL93 (%)		13.38	1.71	0.28	**< 0.05**
KL14 (%)	ω3-PUFAs (%)	22.70	-0.31	0.01	0.70
KL83 (%)		49.19	-1.49	0.27	**< 0.05**
KL93 (%)		28.11	1.81	0.20	0.09
KL14 (%)	ω6-PUFAs (%)	47.03	-3.14	0.21	0.09
KL83 (%)		33.04	-1.37	0.04	0.47
KL93 (%)		19.92	4.51	0.22	0.07
KL14 (%)	Ratio ω3 : ω6	-2.76	9.26	0.11	0.23
KL83 (%)		41.53	-10.51	0.14	0.17
KL93 (%)		61.22	1.25	0.00	0.91

Tab. 4.4: Results from linear regression analyses. Columns: *y*: response variable, i.e. relative abundance (%) of *D. longispina* genotypes KL14, KL83 and KL93 on day 56 (four weeks after *Daphnia* introduction); *x*: independent variable, i.e. relative abundance of the ω3-PUFAs ALA, EPA and DHA and the ω6-PUFA ARA in the seston on day 28 (start of the grazing phase); *a*: intercept; *b*: slope of the regression line; R^2: proportion of variance explained by the linear regression, *p*: *p*-value of the linear regression. Significant relationships are given in bold ($p < 0.05$). Visual representation of the data can be found in Fig. 4.15.

y	***x***	***a***	***b***	R^2	***p***
KL14 (%)	ALA C18:3 ω3 (%)	25.21	-0.64	0.05	0.43
KL83 (%)		41.43	-1.61	0.31	**< 0.05**
KL93 (%)		33.36	2.25	0.32	**< 0.05**
KL14 (%)	EPA C20:5 ω3 (%)	16.25	0.18	0.00	0.96
KL83 (%)		14.94	3.40	0.07	0.32
KL93 (%)		68.82	-3.58	0.04	0.46
KL14 (%)	DHA C22:6 ω3 (%)	13.40	2.75	0.08	0.29
KL83 (%)		19.02	0.57	0.00	0.83
KL93 (%)		67.58	-3.32	0.06	0.36
KL14 (%)	ARA C20:4 ω6 (%)	18.30	-3.56	0.01	0.75
KL83 (%)		8.20	22.69	0.32	**< 0.05**
KL93 (%)		73.50	-19.13	0.12	0.21

4.4 Discussion

4.4.1 Common garden experiment – General discussion

4.4.1.1 Stoichiometry

In nature, organic carbon (C) is produced from atmospheric CO_2 via photosynthesis by primary producers (i.e. phytoplankton). Therefore, in an isolated experimental setup with no further nutrient supply or grazing, but access to atmospheric CO_2, the amount of particulate organic carbon (POC) will continuously increase with growing phytoplankton population and increasing photosynthetic activity. The availability of nitrogen (N) in nature can be increased either by natural deposition (dry deposition, rain or snow; Schlesinger, 2009) or by additional nitrogen input, e.g. as a result of anthropogenic activities such as fossil fuel combustion and application of agricultural fertilizers (Vitousek et al., 1997; Bergström et al., 2005; Elser et al., 2009a). Similarly, phosphorus (P) can be mobilized by human activities and transported into ecosystems, while naturally, phosphorus has no gaseous atmospheric cycle and is mainly accessible through weathering processes of rocks (Schindler, 1977). Hence, without further addition of nitrogen or phosphorus, their availability in a closed experimental setup is expected to be regulated by recycling processes caused by *Daphnia* grazing and digestion on one hand and the assimilation by phytoplankton on the other hand. Only the presence of cyanobacteria such as *Anabaena* or *Nostoc*, which are capable of N_2-fixation (Allen and Arnon, 2005), could result in a further increase in particulate organic nitrogen, accessible to *Daphnia* upon grazing on these cyanobacteria species.

Over the entire duration of the common garden experiment, the molar ratio of the elements C, N and P within the mesocosms was above the Redfield ratio (C:N:P = 106:16:1, Redfield, 1958). Therefore, the limitation of the phytoplankton growth by the availability of N and/or P cannot be excluded. Furthermore, across all treatments, we found molar C:P ratios above 300, which were previously shown to limit the growth of *Daphnia* (Sterner et al., 1993; Urabe et al., 1997; Becker and Boersma, 2003). Hence, we cannot exclude that these limiting conditions affected the growth and reproduction of *Daphnia* and the intraspecific competition between the different *D. longispina* genotypes. In our previously performed laboratory growth assays, we fed *Daphnia* with P-sufficient *Acutodesmus obliquus*. Therefore, potential intraspecific differences regarding the susceptibility of *Daphnia* to limited P-availability were not assessed prior to the common garden experiment, but might additionally explain the observed patterns in the community composition of the *Daphnia* population.

4.4.1.2 Phytoplankton community composition and fatty acid diversity

To establish a diversity gradient within a natural phytoplankton community, a dilution method was applied as described in Hammerstein et al. (2017). Application of this method is expected to result in a loss of rare species when very small volumes of an initial natural phytoplankton community are used for inoculation (i.e. 1 µl), while in larger inocula (i.e. 1 ml), all species from the respective lake seston are expected to be present. Therefore, the diversity within the manipulated phytoplankton communities is expected to increase with the volume of the initial phytoplankton community. However, in the present study, diversity of the manipulated phytoplankton communities, estimated using the Shannon-Diversity Index, did not differ between the treatments (i.e. volume of initial phytoplankton community). This might have occurred because the

Shannon-Diversity Index was calculated on the class level, including the relative abundance of only six different phytoplankton classes. The proportion of each class within the respective phytoplankton community was assessed indirectly via CHEMTAX, using the previously estimated phytoplankton pigment composition. As shown in **Chapter 1**, this method provides fast assessment of the phytoplankton community composition, but has the disadvantage of low taxonomical resolution, as it only allows for the determination of phytoplankton community composition on class level. Therefore, a higher taxonomical resolution (i.e. on genus or species level) might be necessary to reveal differences in phytoplankton community composition as a result of the applied dilution method, which can be achieved via microscopic counting of e.g. Lugol-fixed seston samples. Although part of the seston samples taken during the common garden experiment was counted, the obtained data was not further analyzed and is therefore not discussed in the present thesis. Nevertheless, although no clear patters in phytoplankton community composition were observed which could be attributed to the applied dilution method, this procedure still resulted in a diversity gradient, which was one of the crucial aims of this study.

Furthermore, a significant relationship was found between the CHEMTAX-derived phytoplankton diversity and PUFA diversity, as revealed via linear regression analysis. This finding is in line with our expectations, as it has been previously shown that phytoplankton classes drastically differ in their fatty acid composition, in particular regarding the presence of PUFAs (Ahlgren et al., 1990a; Lang et al., 2011). For example, Chlorophyceae are rich in the ω3-PUFA ALA, while C_{20}-PUFAs like EPA and ARA are usually found in Cryptophyceae, Bacillariophyceae and Dinophyceae. The observed relationship between phytoplankton diversity on class level and PUFA diversity further indicates that the rather low taxonomical resolution of the phytoplankton community

composition, assessed indirectly via pigment composition, is sufficient enough to predict the nutritional quality (in terms of PUFA availability) of the phytoplankton for the zooplankton. This can further be explained by the fact that in general, although there are some differences in fatty acid profiles between the species belonging to the same taxonomical class, loss of one species is unlikely to result in a loss of a class-specific fatty acid, as long as other species from the same taxonomical class are present. Therefore, in terms of biodiversity loss, the food quality of the phytoplankton is expected to be strongly affected by the loss of a whole class, while the loss of a single genus or species will most probably result in minor food quality changes.

In nature, such food quality changes also occur as a result of seasonal fluctuation of essential dietary PUFAs such as ALA, EPA, DHA and ARA, caused by seasonal changes in the phytoplankton community composition, e.g. dominance of Cryptophyceae, Bacillariophyceae and Dinophyceae in spring blooms in lakes vs. dominance of Cyanobacteria and Chlorophyceae in late summer (Sommer et al., 1986; Müller-Navarra and Lampert, 1996; Ahlgren et al., 1997). In our common garden experiment, we found high amounts of ALA in almost all mesocosms, which can be explained by the strong dominance of Chlorophyceae in the manipulated phytoplankton communities of the respective mesocosm tanks. In contrast, small amounts of EPA, DHA and ARA were found, as phytoplankton classes rich in these long-chain PUFAs, such as Cryptophyceae, were less abundant in the manipulated phytoplankton communities. Therefore, we established a certain variation in the availability of these dietary PUFAs which was crucial in order to study the effects of PUFA availability on the intraspecific competition between the different *D. longispina* genotypes.

4.4.1.3 Producer-consumer dynamics

Within the common garden experiment, typical producer-consumer dynamics were observed: phytoplankton biomass decreased strongly upon *Daphnia* introduction due to grazing. In turn, *Daphnia* reproduced rapidly and reached high abundances within four weeks after introduction (day 56). In order to maintain a sufficient food quantity in the mesocosm tanks, *Daphnia* were manually removed from the experiment on several occasions (starting at day 56 of the experiment). Therefore, the patterns in population development of phytoplankton and *Daphnia* observed after day 56 cannot be completely explained by naturally occurring population dynamics between these two trophic levels. Nevertheless, in some mesocosms, the *Daphnia* population recovered after manual removals, but as the temperature decreased below 10 °C, *Daphnia* reproduced much slower than at warmer temperatures. Therefore, in the last four weeks of the common garden experiment, no further population growth of *Daphnia* was observed. Due to decreased grazing, phytoplankton communities were allowed to recover, but the increase in phytoplankton biomass in the last four weeks of the experiment was observed only in three out of five different treatments (i.e. IP 1, IP 1000 and IP 10000). This could be explained by the potential presence of fast growing and/or reproducing phytoplankton species in the respective phytoplankton communities. However, in order to draw final conclusions on this suggestion, the phytoplankton community composition would have to be determined on the genus or species level.

4.4.2 Intraspecific competition – does it depend on fatty acid availability?

In general, at high (i.e. not limiting) availability of essential dietary ω3- and ω6-PUFAs, the different *D. longispina* genotypes are expected to coexist, as no competition regarding the biochemical food quality (in terms of PUFA availability) should take place.

At such environmental conditions, intraspecific competition is expected to be affected either by the absolute performance of a certain genotype (i.e. the fastest growing genotype is expected to dominate the community) or by other factors, such as P-availability (see section *Stoichiometry* in this Chapter).

At low PUFA availability, several potential scenarios might occur, depending on genotype-specific traits such as susceptibility to limitations by PUFA availability or capability and rate of bioconversion of PUFAs (within the same PUFA family, i.e. ω3 or ω6). For example, at low EPA availability, the genotype with the lowest susceptibility to EPA and/or highest rate of bioconversion of ALA or DHA into EPA is expected to outcompete the other genotypes and dominate the *Daphnia* population, given the high availability of ALA and/or DHA. In general, genotypes with the lowest susceptibility to limited PUFA availability (i.e. showing the least effect size upon PUFA supplementation in laboratory growth assays) are expected to be the least affected by low PUFA availability.

Prior to the common garden experiment, we assessed the overall growth and susceptibility of different *D. longispina* genotypes, while we have no data on potential intraspecific differences in the bioconversion of PUFAs. Therefore, we cannot precisely predict how availability of PUFAs like ALA and DHA, which serve as precursors for EPA (von Elert, 2002), or the availability of LIN, which serves as a precursor for ARA (Kainz et al., 2004; Ravet et al., 2012), will affect the intraspecific competition. Nevertheless, guided by the results obtained from the laboratory growth assays, we expected the *D. longispina* genotype KL83 to outcompete the other two genotypes and dominate the *Daphnia* community or at least persist in the community at various PUFA availabilities, as this genotype showed the highest somatic growth rate, both in dietary treatments with and without PUFA supplementation. On the other hand, genotypes KL14 and KL93

showed similar growth rates and absolute effect sizes upon EPA and ARA supplementation. However, we observed a higher effect size upon ALA supplementation for genotype KL14 compared to genotype KL83 and thus a stronger susceptibility of genotype KL14 to limitations by ALA availability. Therefore, the success of the genotype KL14 and the overall outcome of the intraspecific competition between the three tested *D. longispina* genotypes are expected to be strongly affected by low ALA availability. Taken together, the outcome of the intraspecific competition seems to be rather complex, as several factors have to be taken into account.

In contrast to our expectations, genotype KL14 was almost completely outcompeted by KL83 and KL93 after only four weeks, regardless of PUFA availability, and did not recover in the further course of the experiment. Also, no significant relationship was found between the relative abundance of genotype KL14 and any of the tested fatty acid related dietary factors, such as PUFA diversity and availability of ω3-PUFAs ALA, EPA and DH or ω6-PUFA ARA. Therefore, the success of genotype KL14 in the given *Daphnia* population seems to be affected by other factors rather than by the biochemical food quality in terms of fatty acid availability.

Genotypes KL83 and KL93 were mostly persistent over the entire course of the experiment. While the genotype KL93 was more dominant in the first half of the experiment, the abundance of KL83 increased in the second half of the experiment. Moreover, genotype KL83 partly dominated the *Daphnia* community in the last two weeks of the experiment. The persistence of the genotype KL83 in the community and its recovery from low densities might be explained by high somatic growth rates observed in the laboratory growth assays, both in dietary treatments with and without PUFA supplementation. From the observed dynamics of the genotypes KL83 and KL93, it cannot be excluded that given a longer time period, the density of these two genotypes

will stabilize and allow for their coexistence in the population. Nevertheless, we provide evidence that the outcome of the intraspecific competition between the genotypes KL83 and KL93 depends on several fatty acid related dietary factors: genotype KL83 is found to have higher chances of outcompeting genotype KL93 at higher PUFA diversity or high ARA availability, as the abundance of the genotype KL83 was found to positively correlate with these two dietary factors. In contrast, at high PUFA availability and in particular at high ALA availability, genotype KL93 is more likely to outcompete genotype KL83. It is necessary to mention that ALA was the most abundant PUFA and therefore the relative abundance of PUFAs, and in particular of ω3-PUFAs is most probably autocorrelated with ALA availability. In contrast, diversity of PUFAs is negatively correlated with ALA availability, as increasing amounts of a single, dominating PUFA decreases the overall diversity, at least in terms of Shannon-Diversity Index. Therefore, the opposite effect of H'_{PUFA} and ALA availability on the competition outcome of the two genotypes KL83 and KL93 can also be interpreted as an autocorrelation of these two parameters. Nevertheless, evidence is given that the ω3-PUFA ALA and ω6-PUFA ARA both might play an important role in the intraspecific competition of coexisting *D. longispina* genotypes. In our study, these two PUFAs even had opposite effects on the success of the two genotypes KL83 and KL93. Interestingly, ALA was the only PUFA that played a significant role in a field study by Wacker on von Elert (2001), who found that the sestonic content of ALA correlates with the growth of *D. galeata*, which was raised on natural seston of the Lake Constance. Therefore, it is reasonable to assume that the dietary availability of ALA is important in the field and has a significant effect on *Daphnia* population dynamics.

EPA is highly recognized as a ω3-PUFA that limits the growth of *Daphnia* (Müller-Navarra et al., 2000; von Elert, 2004; Martin-Creuzburg et al., 2010). Although first evidence comes from the field, the significant role of EPA for *Daphnia*'s fitness arises much stronger from the laboratory growth experiments. In this study, EPA did not significantly affect the intraspecific competition between different *D. longispina* genotypes. This might be explained by the fact that no intraspecific differences between the three used *D. longispina* genotypes were found regarding the absolute effect size of the growth rate upon EPA supplementation. However, it cannot be excluded that EPA availability will affect the population dynamics of *Daphnia* in the field. In fact, in **Chapter 3**, we report intraspecific variation in susceptibility to limitations by EPA availability within 12 tested coexisting *D. longispina* genotypes. Therefore, our choice of the genotypes KL14, KL83 and KL93 seems to be rather unfortunate to study effects of EPA availability on intraspecific competition between coexisting *D. longispina* genotypes. Hence, to draw final conclusions on the role of EPA for the competitive interactions within a natural *Daphnia* community, the inclusion of additional genotypes with more pronounced differences in their susceptibility to EPA availability is necessary.

Similarly, we did not find any significant relationship between the availability of the ω3-PUFA DHA and the competition success of the tested *D. longispina* genotypes. However, the observed patterns (although not significant) were very similar to patterns observed between the tested genotypes and EPA availability. This indicates that the role of DHA for the intraspecific competition within a natural *Daphnia* community is similar to the role of EPA, which could be explained by the potential capability of *D. longispina* to bioconvert DHA into EPA. Although previous studies showed that some *Daphnia* species, such as *D. galeata*, which belongs to the *D. longispina* complex (von Elert, 2004; Brzeziński and von Elert, 2007) are capable to bioconvert DHA into EPA (von Elert,

2002), we did not test this ability within our *D. longispina* genotypes. Furthermore, we did not access potential intraspecific differences in susceptibility to limitations by DHA availability. Therefore, we can only speculate on the role of DHA for population dynamics of natural *Daphnia* communities.

Finally, the present study provides further evidence that the ω6-PUFA ARA plays an important role for *Daphnia*'s fitness and population dynamics, as it significantly affected the outcome of the intraspecific competition between the genotypes KL83 and KL93, in particular favoring the genotype KL83 over genotype KL93. Here, the absolute somatic growth rate of the tested genotypes in the presence of ARA seem to play an important role for the competitive interactions, as the somatic growth rate of genotype KL83 in presence of ARA was higher (but not significant) compared to the genotype KL93, as revealed via laboratory growth assays. Therefore, the success of the genotype KL83 at high ARA availability can be explained by its potentially better utilization of ARA and hence higher growth rate, while KL93 seems to be less susceptible to low ARA availability.

It is important to keep in mind that from day 56 on, excessive daphnids were removed from the mesocosms to keep the *Daphnia* abundances below 20 ind L^{-1}. Although this was done randomly, it cannot be excluded that this affected the community composition in the further course of the experiment. Nevertheless, relationships between fatty acid related dietary factors and the individual success of the tested *D. longispina* genotypes, discussed above, are independent from the random removal of excessive daphnids and are therefore based solely on natural population dynamics and the trophic transfer efficiency between phytoplankton and *Daphnia*.

4.4.3 Can results from single clone microcosm experiments be transferred to complex natural communities?

The observed patterns in population dynamics within the *D. longispina* community studied within the outdoor common garden experiment can only partly be explained by the results obtained from the single clone laboratory growth assays. Our study was only focused on the effect of fatty acid related dietary factors on the outcome of intraspecific competition between the tested *D. longispina* genotypes, while we ignored the potential limitation of *Daphnia*'s fitness by low P availability. Furthermore, competitive interactions under more natural conditions are more complex. In general, food quality in terms of biochemical composition of the phytoplankton is not the only force driving the competition between species. In nature, other abiotic and biotic factors, such as temperature and food quantity (Van Doorslaer et al., 2009, 2010; Cuenca Cambronero et al., 2018) or presence of predators and parasites (Decaestecker et al., 2005; Duffy and Sivars-Becker, 2007) are also likely to affect the consumer's population dynamics. Therefore, upscaling results from single clone microcosm experiments to complex natural communities should be treated with care. Nevertheless, we provide evidence that a phytoplankton trait, namely the content of essential dietary PUFAs, in particular the availability of ω3-PUFA ALA and ω6-PUFA ARA, affects the competitive interactions between naturally coexisting *D. longispina* genotypes. Furthermore, we show that the outcome of the intraspecific competition depends at least partly on a consumer's trait, namely the susceptibility of *Daphnia* to limitations by availability of essential dietary PUFAs.

Concluding remarks and perspectives

Global warming and eutrophication represent an ongoing threat for the Earth's biodiversity (Loreau, Cardinale, Hooper). Especially the biodiversity loss on the level of primary producers is of particular interest, as such species loss is expected to have cascading effects on multiple trophic levels. To better predict the consequences of the biodiversity loss for ecosystem functioning and services, trait-based approaches are frequently applied to identify functional traits that might provide further insight into the linkage between biodiversity and ecosystem functioning. In the present study, I investigated the effects of phytoplankton trait diversity in terms of fatty acid availability on the community structure and population dynamics of the herbivorous grazer *Daphnia*.

In **Chapter 1**, I compared two commonly used methods for an indirect estimation of phytoplankton community composition. I show that both the spectrofluorometric method via Algae Lab Analyser and pigment-based method via HPLC/CHEMTAX are fast and useful tools for the assessment of the phytoplankton community composition, although the agreement between the methods was not always satisfactory. This is similar to findings by Richardson et al. (2010) and may be due to different marker pigments utilized by the two methods. Also, I suggest including more pigments in the HPLC analysis, especially to be able to distinguish between Diatoms and Chrysophytes, e.g. violaxanthin and chlorophylls c_1 and c_2. As both methods have advantages and disadvantages, the method of choice depends on the aim of the study or the field of use. However, Algae Lab Analyser should be carefully calibrated prior to use, to achieve more accurate results, especially regarding the estimates of cyanobacterial abundances (Beutler, 2002).

In terms of diversity, CHEMTAX provides a higher resolution and can be modified in a way that it allows for assessment of lake-specific phytoplankton groups, especially when prior knowledge on the phytoplankton community composition of the lake of interest is available (Schlüter et al., 2016). Within the present study, pigment-based method via HPLC and CHEMTAX was used in **Chapter 4** to assess the phytoplankton community composition and revealed satisfactory results.

In **Chapter 2**, I performed dose-response experiments to compare the relevance of two PUFAs, namely ω3-PUFA EPA and ω6-PUFA ARA, for the growth and reproduction of two *Daphnia* species, which differed in their body size. In previous studies, EPA was recognized as an important ω3-PUFA that limits the fitness of *Daphnia*, while findings on the importance of ARA for *Daphnia* were rather inconsistent (von Elert, 2002; Martin-Creuzburg et al., 2010; Ravet et al., 2012; Schlotz et al., 2014). I therefore estimated the concentration thresholds of both PUFAs for saturated growth and reproduction of the two *Daphnia* species. Overall, the study provides clear evidence that ARA, a ω6-PUFA, limits the fitness of two different *Daphnia* species to an equal extent as the ω3-PUFA EPA. This is of particular interest for *Daphnia*'s performance in nature, as shifts in phytoplankton community composition might result in environmental fluctuations in the dietary availability of ω3- and ω6-PUFAs, as their presence and amount varies among different phytoplankton groups (Ahlgren et al., 1990a; Lang et al., 2011). I thus suggest that together with the ω3-PUFA EPA, ARA availability needs to be considered in further studies on food quality and trophic transfer efficiency within freshwater ecosystems.

In **Chapter 3**, I first isolated *Daphnia longispina* individuals from a natural *Daphnia* population of the oligo-mesotrophic lake Klostersee. The different genotypes were identified via microsatellite analyses. The aim of the Chapter was to assess the intraspecific variability in a consumer's functional trait, i.e. susceptibility to limitations by dietary PUFA availability among coexisting genotypes of a natural *Daphnia longispina* population. While the absence of the two ω3-PUFAs ALA and EPA affected the fitness of different *D. longispina* genotypes to a similar extent, I show the ω6-PUFA ARA to be the most limiting PUFA for the investigated *D. longispina* population. Overall, I here demonstrate significant intrapopulation variation in a consumer's functional trait and suggest that such intrapopulation differences in susceptibility to absence of dietary PUFAs might not only contribute to the maintenance of the genetic variation within natural animal populations, but may also affect the outcome of intraspecific competition and thus be the driving force of natural selection and local adaptation in many ecosystems.

Guided by the hypotheses mentioned above, I addressed the role of susceptibility to limitations by PUFA availability for the intraspecific competition of naturally coexisting *D. longispina* genotypes within a common garden experiment in **Chapter 4**. I further manipulated the diversity of a natural phytoplankton community to alter the phytoplankton trait diversity in terms of PUFA availability. I found a correlation between phytoplankton diversity, estimated via CHEMTAX (**Chapter 1**), and PUFA diversity, showing that biodiversity loss on the level of primary producers can result in decreased phytoplankton trait diversity. Furthermore, I show that the availability of single PUFAs, such as ω3-PUFA ALA and ω6-PUFA ARA, directly affects the intraspecific competition of the tested *D. longispina* genotypes, albeit not as predicted from single clone microcosm experiments.

Nevertheless, this study for the first time provides evidence for an interplay of producer's and consumer's functional traits, which both affect the community structure and population dynamics and therefore represent a potential link between biodiversity and ecosystem functioning in aquatic ecosystems.

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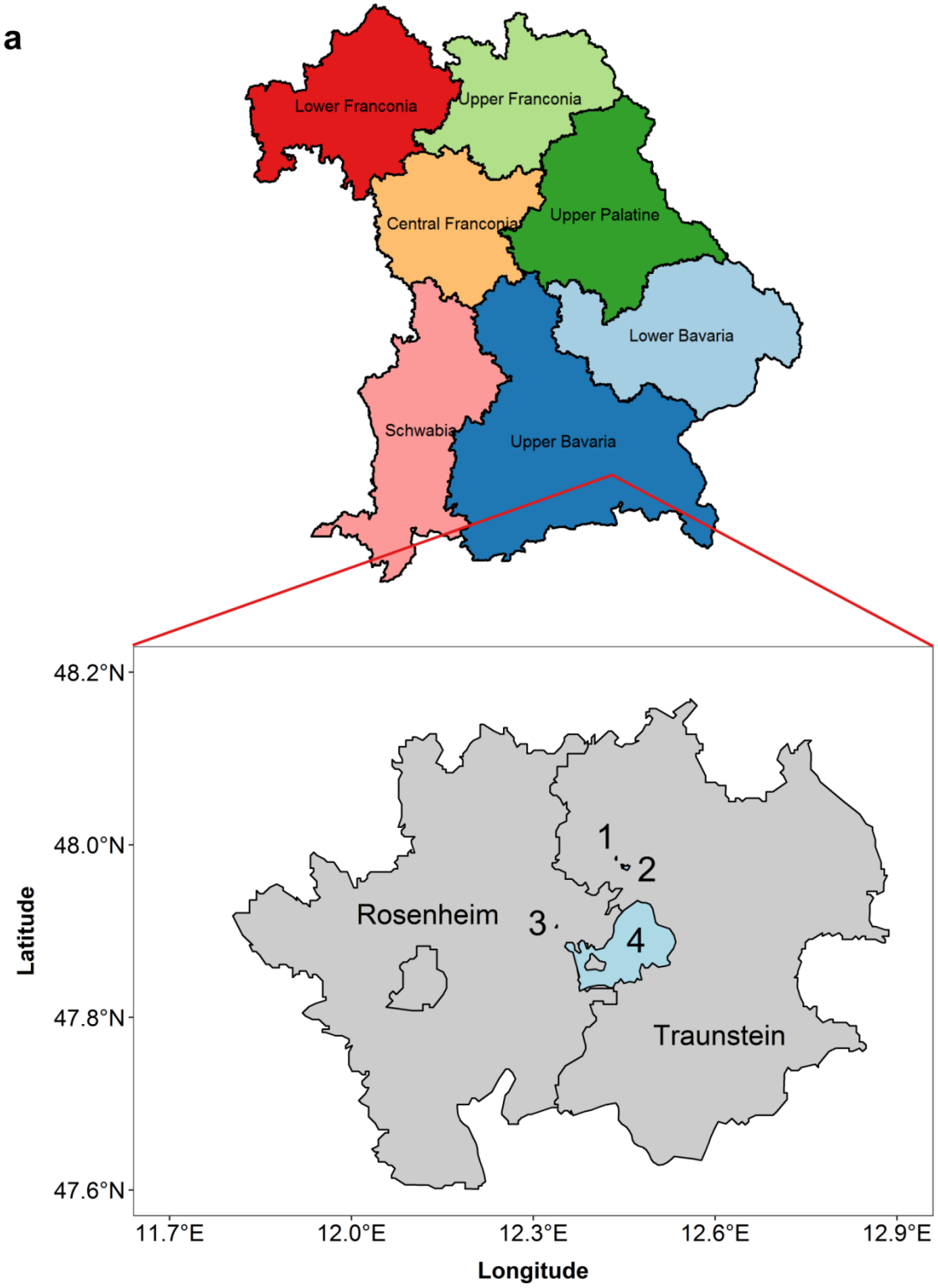
Appendix – Supplementary Information



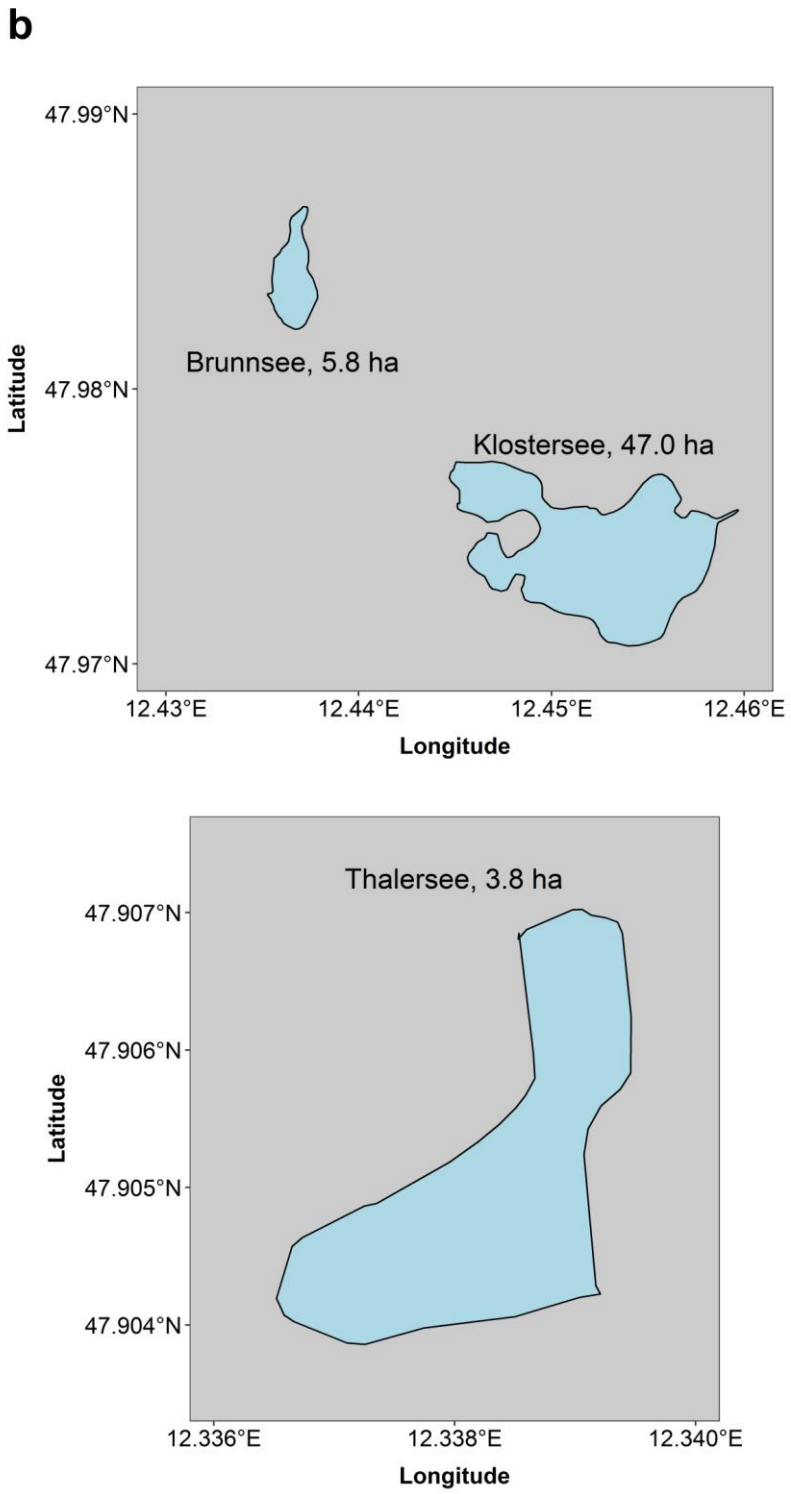


Fig. S1: Map of (a) Bavaria, a federal state in the south of Germany, and counties Rosenheim and Traunstein, which are situated in Upper Bavaria, with (b) location of sampling sites of the present thesis: oligotrophic (1) lake Brunnsee, (2) oligo-mesotrophic lake Klostersee and (3) mesotrophic lake Thalersee. (4) Lake Chiemsee (not part of the thesis) is the largest lake in this region (7990.0 ha).

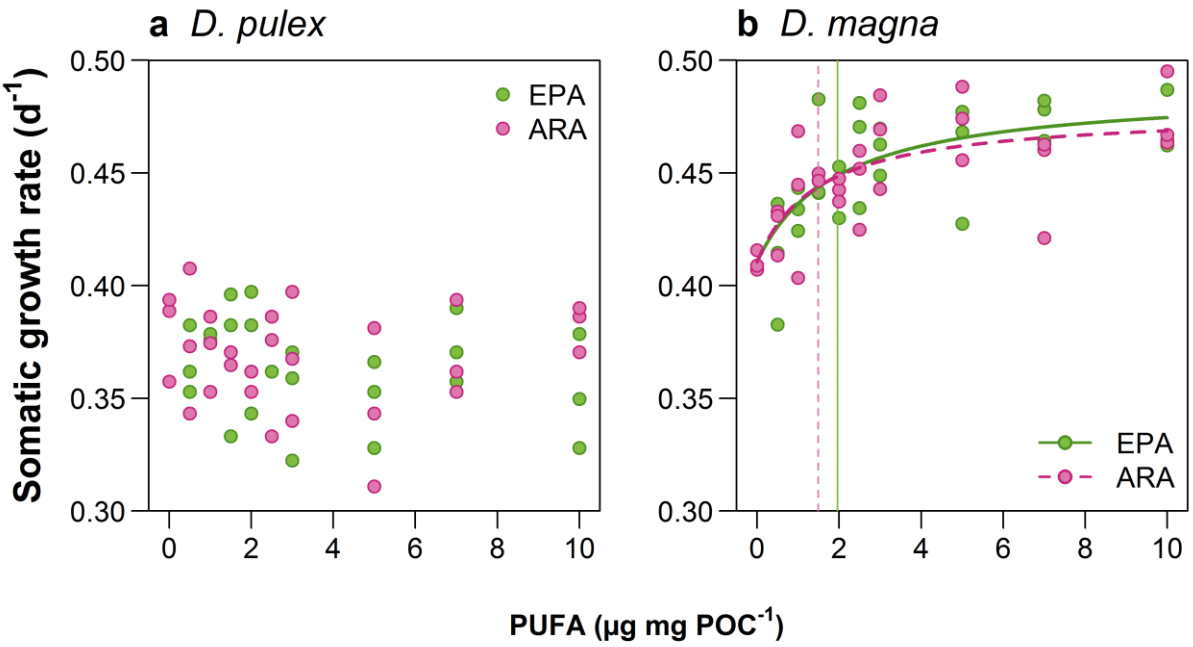
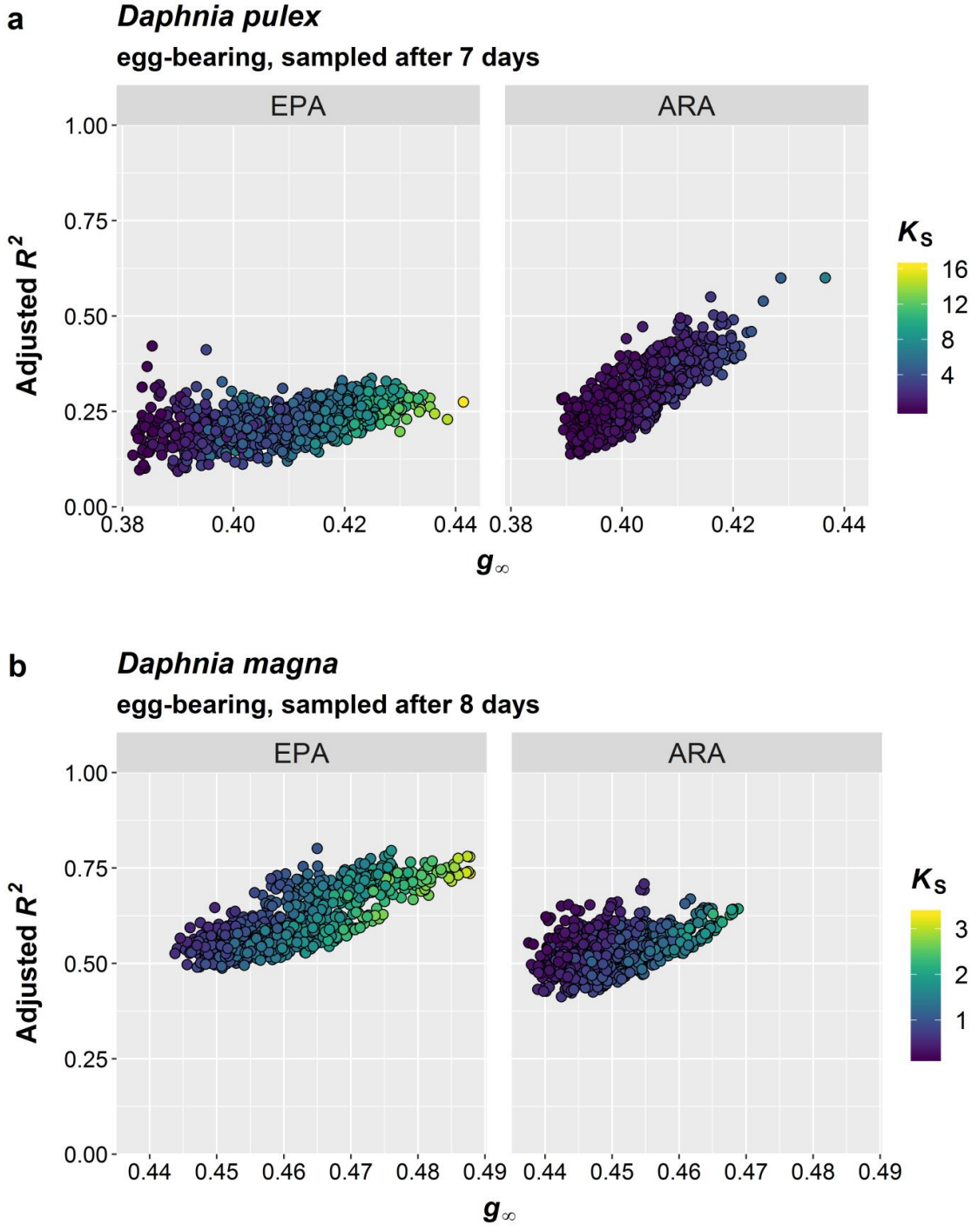


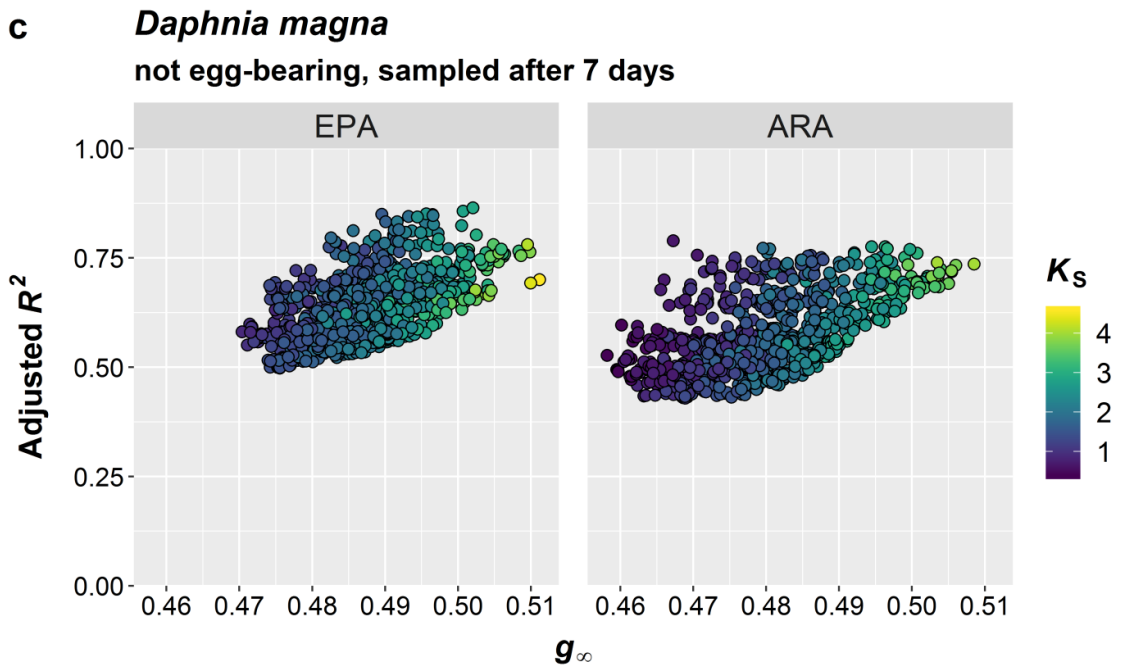
Fig. S2: Somatic growth rate g (d^{-1}) of (a) *Daphnia pulex* (not egg-bearing, sampled after 6 days) and (b) *Daphnia magna* (not egg-bearing; sampled after 7 days) grown on *Acutodesmus obliquus* supplemented with different amounts (µg mg POC^{-1}; particulate organic carbon) of eicosapentaenoic acid (EPA, green circles) or arachidonic acid (ARA, pink circles). Solid (EPA) and dashed (ARA) growth saturation curves for *D. magna* (panel b) are based on modified Monod functions (nonlinear least-square fits, Eq. 3). Vertical lines (panel b; green solid: EPA, pink dashed: ARA) indicate half saturation constant K_S (in µg PUFA mg POC^{-1}) for EPA- or ARA-limited growth of *D. magna*. Saturation curves fitted through the growth rate data of *D. magna* (panel b) explained 58.93% and 50.12% of variance (adjusted R^2) for the EPA- and ARA-limited growth, respectively. The somatic growth rate of *D. pulex* did not show clear patterns along the concentration gradient of EPA and ARA, therefore, saturation curves were not fitted through the data. n =30 for EPA (both species) and n = 29 for ARA (both species). Summary of the panel b can be found in Tab. S1.

Tab. S1: Mean somatic growth rate g_0 (d^{-1}) of *D. magna* (not egg-bearing, sampled after 7 days) grown on *A. obliquus* without C_{20}-PUFA supplementation (n=3); asymptotic growth rate g_∞ (d^{-1}), half-saturation constant K_S (in µg PUFA mg POC^{-1}) and the corresponding adjusted R^2 derived from the saturation curve (nonlinear least-square fit using a modified Monod function) describing the EPA- and ARA-limited growth of *D. magna*. Total n refers to the total number of data points used to fit the saturation curves (10 different experimental concentrations of EPA or ARA in triplicates, i.e. max. 30 data points). This data corresponds to the plot in Fig. S1, panel b.

	Daphnia magna	
	ARA	EPA
Total n	29	30
g_0 (d^{-1})	0.411	0.411
g_∞(d^{-1})	0.477	0.487
K_S (µg PUFA mg POC^{-1})	1.494	1.962
Adjusted R^2	0.501	0.589
Increase from g_0 to g_∞	14 %	16 %

Fig. S3: Adjusted R^2 as a measure for the proportion of variance explained by the saturation curves (nonlinear least-square fits using a modified Monod function, Eq. 3) fitted through the bootstrapped growth rate data of (a) *D. pulex* (egg-bearing, sampled after 7 days, see Fig. 2.1a, (b) *D. magna* (egg-bearing, sampled after 8 days, see Fig. 2.1b) and (c) *D. magna* (not egg-bearing, sampled after 7 days, see Fig. S2b). Adjusted R^2 is plotted against the asymptotic growth rate g_∞ (d^{-1}). The color gradient is representing the half-saturation constant K_S (in µg PUFA mg POC^{-1}). G_∞ and K_S were predicted from the Monod model describing the EPA- and ARA-limited growth of *Daphnia* species.





Tab. S2: Final output pigment : chlorophyll *a* ratio matrix, derived from CHEMTAX runs. Allo: alloxanthin, Chl *b*: chlorophyll *b*, Echi: echinenone, Fuco: fucoxanthin, Lut: lutein, Peri: peridinin, Zea: zeaxanthin.

	Allo	Chl *b*	Echi	Fuco	Lut	Peri	Zea
Chlorophytes	0	0.307	0	0	0.147	0	0.001
Cryptophytes	0.111	0	0	0	0	0	0
Cyanobacteria	0	0	0.066	0	0	0	0.461
Chrysophytes	0	0	0	0.159	0	0	< 0.001
Diatoms	0	0	0	0.317	0	0	0.004
Dinoflagellates	0	0	0	0	0	0.337	0

Tab. S3: Results from one-way ANOVA and Kruskal-Wallis test (KW) testing for differences in *Daphnia* community composition (given as relative abundances of the genotypes KL14, KL83 and KL93) between the treatments (i.e. volume of initial phytoplankton community, IP levels given in µl). Tests were performed for days 56 (four weeks after *Daphnia* introduction, corresponding to approx. 2 generation cycles), 77 (seven weeks after *Daphnia* introduction; here, the average *Daphnia* abundance in the mesocosms dropped below 5 ind L^{-1}) and 98 (ten weeks after *Daphnia* introduction; last day of the experiment). * if ANOVA was performed, the value given in the column *F* or X^2 is *F* with degrees of freedom (*df*) = 2 (effect) and 6 (residuals); if Kruskal-Wallis test was performed, X^2 with *df* = 2 is given. Significant tests are given in bold ($p < 0.05$).

Day of experiment	IP level (µl)	Test*	*F* or X^2	*p*
56	1	ANOVA	1.94	0.224
	10	ANOVA	68.64	**< 0.001**
	100	ANOVA	6.39	**< 0.05**
	1000	ANOVA	36.00	**< 0.001**
	10000	ANOVA	7.64	**< 0.05**
77	1	KW	5.84	0.054
	10	KW	6.34	**< 0.05**
	100	KW	5.63	0.060
	1000	KW	6.34	**< 0.05**
	10000	KW	4.58	0.101
98	1	KW	5.60	0.061
	10	ANOVA	18.38	**< 0.01**
	100	KW	6.74	**< 0.05**
	1000	KW	4.32	0.115
	10000	KW	2.90	0.234

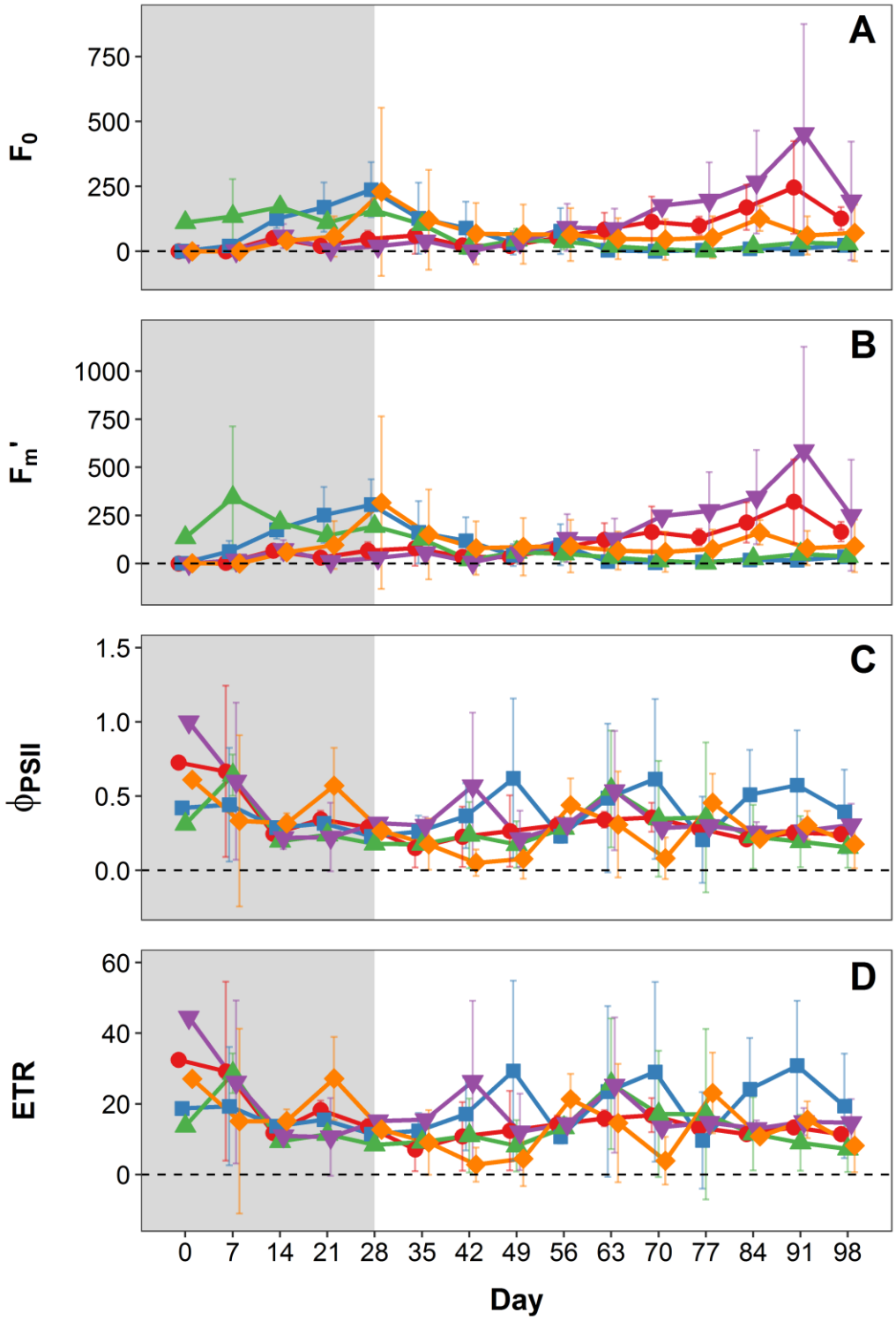


Fig. S4: Parameters describing the physiological state of the phytoplankton communities within the mesocosm tanks over the entire duration of the experiment: (A) chlorophyll *a* fluorescence yield in the quasi-dark state (F_0), (B) maximum fluorescence yield measured during the last saturating light pulse triggered (F_m'), (C) yield of the photosystem II (φ_{PSII}) and (D) relative electron transport rate (ETR). Given are means per treatment (i.e. volume of initial phytoplankton community, for legend see Fig. 4.5). Error bars represent the standard deviation.

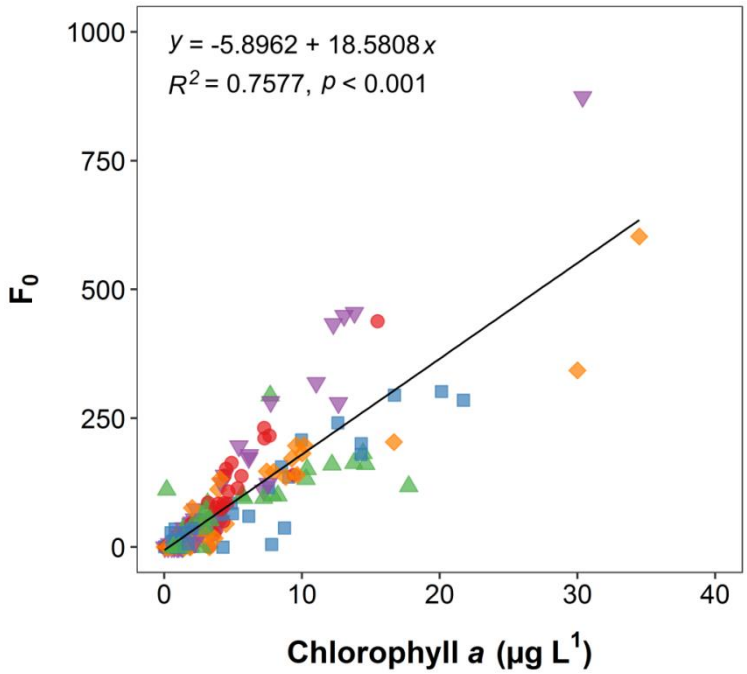


Fig. S5: Correlation between chlorophyll *a* fluorescence yield in the quasi-dark state (F_0), measured with Water-PAM, and chlorophyll *a* concentration (µg L^{-1}), measured with the TD-700 Laboratory Fluorometer. We found a highly significant linear relationship (solid line) between these two variables (R^2 = 0.7577, p < 0.001).

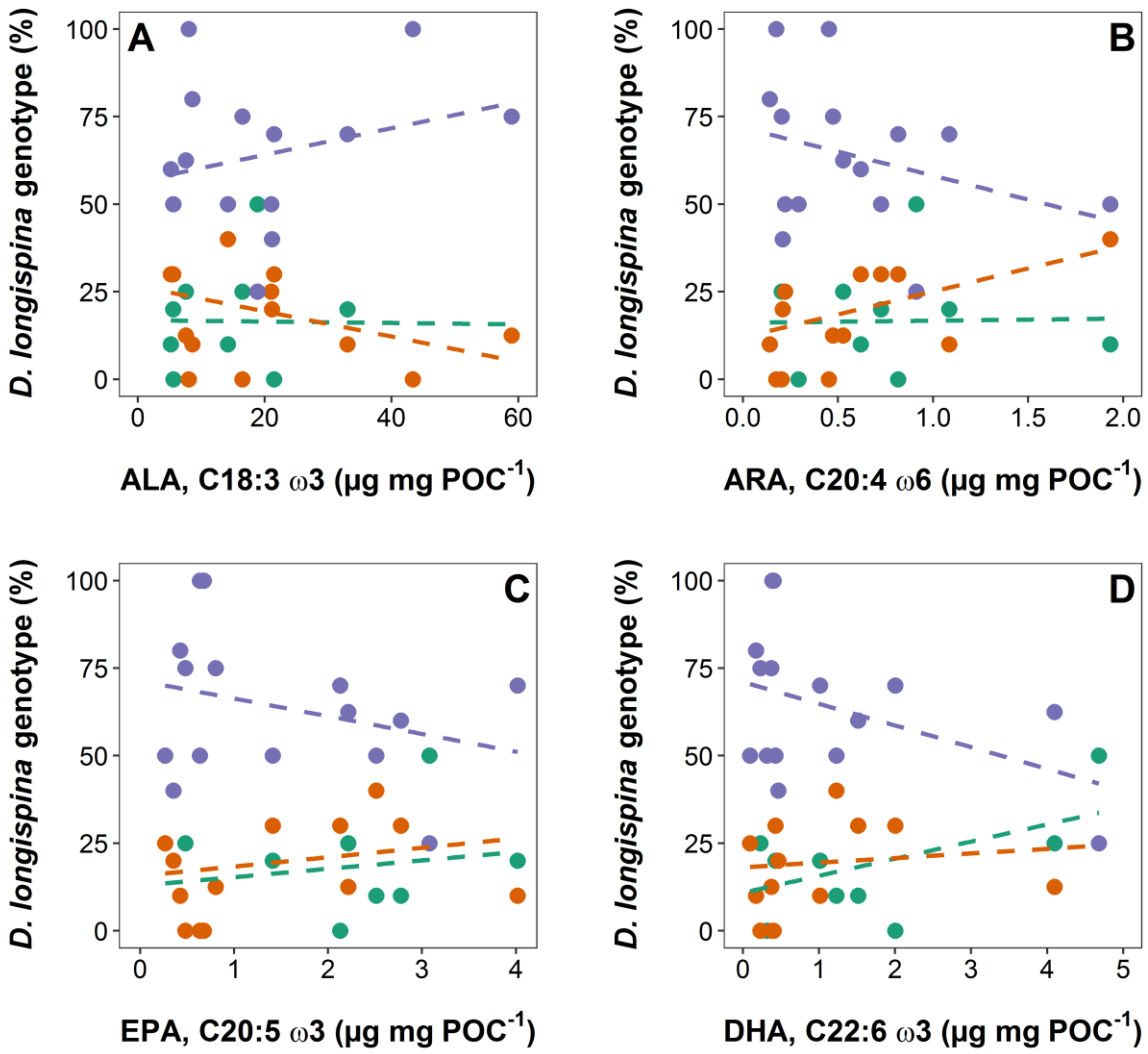


Fig. S6: Relative abundance (%) of *D. longispina* genotypes KL14 (turquoise), KL83 (orange) and KL93 (purple) on day 56 plotted against the absolute amounts (in µg mg POC^{-1}) of ω3-PUFAs ALA (panel A), EPA (panel C) and DHA (panel D) and of ω6-PUFA ARA (panel B) on day 28 (start of the grazing phase). The time delay of four weeks was chosen to test for a response of the *Daphnia* community to the initial fatty acid content at the beginning of the grazing phase. Four weeks correspond to approx. two generations of *Daphnia* life cycle. Dashed lines represent a non-significant relationship. Depicted are raw values for every mesocosm (n = 15 for each genotype). Results from the linear regression analyses can be found in Tab. S4.

Tab. S4: Results from linear regression analyses. Abbreviations: *y*: response variable, i.e. relative abundance (%) of *D. longispina* genotypes KL14, KL83 and KL93 on day 56 (four weeks after *Daphnia* introduction, approx. 2 generation cycles); *x*: independent variable, i.e. absolute abundance of the ω3-PUFAs ALA, EPA and DHA and the ω6-PUFA ARA in the seston on day 28 (start of the grazing phase); *a*: y-axis intercept; *b*: slope of the regression line; R^2: proportion of variance explained by the linear regression, *p*: *p*-value of the linear regression. Significant relationships are given in bold ($p < 0.05$). Visual representation of the data can be found in Fig. S6.

Y	***x***	***a***	***b***	***R²***	***p***
KL14 (%)	ALA C18:3 ω3 (µg mg POC^{-1})	16.88	-0.02	0.00	0.94
KL83 (%)		26.60	-0.36	0.14	0.17
KL93 (%)		56.51	0.38	0.08	0.31
KL14 (%)	EPA C20:5 ω3 (µg mg POC^{-1})	12.93	2.39	0.04	0.50
KL83 (%)		15.68	2.67	0.05	0.45
KL93 (%)		71.39	-5.06	0.08	0.29
KL14 (%)	DHA C22:6 ω3 (µg mg POC^{-1})	10.83	4.88	0.21	0.08
KL83 (%)		18.14	1.32	0.02	0.66
KL93 (%)		71.03	-6.20	0.18	0.12
KL14 (%)	ARA C20:4 ω6 (µg mg POC^{-1})	16.15	0.60	0.00	0.95
KL83 (%)		12.00	13.09	0.17	0.13
KL93 (%)		71.85	-13.69	0.10	0.26

Record of achievement

Chapter 1: Determination of phytoplankton biodiversity in lakes of different trophic state through pigment and fluorescence proxies

The mesocosm experiment described in this chapter was performed by me and Sara K. Hammerstein (PhD student at the Ludwig-Maximilians-University (LMU), Munich, Germany) as a part of the collaboration project "DYNATLOSS" within the priority programme "DynaTrait". My supervisor Patrick Fink, Maria Stockenreiter (LMU, Munich, Germany), Herwig Stibor (LMU, Munich, Germany) and Sara K. Hammerstein were involved in the design of the experiment. Sara K. Hammerstein was responsible for the usage of Algae Lab Analyzer, while all other laboratory analyses, including the development of a method for pigment extraction and identification via HPLC, were performed at the University of Cologne by me or under my direct supervision. Statistical analyses were performed exclusively by me.

Chapter 2: Equal relevance of omega-3 and omega-6 polyunsaturated fatty acids for the fitness of *Daphnia* spp.

The experiments described in this chapter were performed exclusively by me or under my direct supervision. Patrick Fink was involved in the design of the experiments. Christian Werner (PhD student, University of Cologne, Germany) was involved in the development of the R-code for the Monod model. All other statistical analyses were performed exclusively by me.

Chapter 3: Intrapopulation variability in a functional trait: susceptibility of *Daphnia* to limitation by dietary fatty acids

The experiments described in this chapter were performed exclusively by me or under my direct supervision. Statistical analyses were performed exclusively by me. Patrick Fink was involved in the design of the experiment.

Chapter 4: Effects of phytoplankton diversity on intraspecific competition in *Daphnia* – The role of essential dietary fatty acids

The experiment and the laboratory analyses described in this chapter were performed exclusively by me or under my direct supervision. Statistical analyses were performed exclusively by me. Patrick Fink was involved in the design of the experiment.

List of publications

[1]Ilić, M., Walden, S., Hammerstein, S. K., Stockenreiter, M., Stibor, H. and Fink, P. (2019): Determination of phytoplankton biodiversity in lakes of different trophic state through pigment and fluorescence proxies. *In preparation.*

[2]Ilić, M., C. Werner and Fink, P. (2019): Equal relevance of omega-3 and omega-6 polyunsaturated fatty acids for the fitness of *Daphnia* spp.. *Limnology and Oceanography.*

[3]Ilić, M. and Fink, P. (2019): Intrapopulation variability in a functional trait: susceptibility of *Daphnia* to limitation by dietary fatty acids. *Freshwater Biology (under review).*

[4]Ilić, M. and Fink, P. (2019): Effects of phytoplankton diversity on intraspecific competition in *Daphnia* – The role of essential dietary fatty acids. *In preparation.*

[1]Chapter 1

[2]Chapter 2

[3]Chapter 3

[4]Chapter 4

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Köln, 11.07.2019

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen –, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von PD Dr. Patrick Fink betreut worden.

_____

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Curriculum vitae

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Berufliche Erfahrung

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Publikationen (Peer-Reviewed)

Ilić, M., Werner, C. and Fink, P. (2019): "Equal relevance of omega 3 and omega-6 polyunsaturated fatty acids for the fitness of Daphnia spp." *Limnology and Oceanography*. https://doi.org/10.1002/lno.11201

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Präsentationen

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Ilić, M., Hammerstein, S. K., Stockenreiter, M., Stibor, H. und Fink, P. (2017): "*Pigment composition of natural phytoplankton communities – possible link between biodiversity loss and ecosystem functioning?"* (Vortrag, in Englisch); ASLO Tagung ("Mountains to the Sea") in Honolulu, Hawai'i

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Ilić, M., Hammerstein, S. K., Stockenreiter, M., Stibor, H. und Fink, P. (2015): *"Cascading effects of biodiversity loss in freshwater plankton"* (Vortrag, in Englisch)**;** 108. Jahrestagung der Deutschen Zoologischen Gesellschaft (DZG) in Graz, Österreich