Mixotrophs in aquatic ecosystems

Linking physiology to food web dynamics

Thesis committee: Prof.dr. S. Diehl
Prof.dr. G.A. Kowalchuck
Dr. J.C. Kromkamp
Prof.dr. J.J. Middelburg
Prof.dr. K. Rengefors

This thesis should be cited as:
Wilken S (2013) Mixotrophs in aquatic ecosystems: linking physiology to food web dynamics. PhD thesis. Utrecht University, Utrecht, The Netherlands.
ISBN: 978-90-6464-646-1

Mixotrophs in aquatic ecosystems

Linking physiology to food web dynamics

Mixotrofen in aquatische ecosystemen

Van fysiologische processen tot voedselwebdynamiek

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge
het besluit van het college voor promoties in het openbaar te verdedigen
op woensdag 27 maart 2013 des middags te 2.30 uur

door

Susanne Wilken

geboren op 4 mei 1981 te Pinneberg, Duitsland

Promotoren: Prof.dr. E. van Donk
Prof.dr. J. Huisman

This thesis was accomplished with financial support from the Research Council for Earth and Life Sciences (ALW) of the Netherlands Organization for Scientific Research (NWO) and the Netherlands Institute of Ecology (NIOO-KNAW).

Content

Chapter 1

General introduction

Aquatic ecosystems play a major role in global biogeochemical cycles. The net effect of an ecosystem on the carbon cycle will depend on its balance between primary production and respiration. Primary producers convert inorganic carbon into their biomass, ultimately removing CO_2 from the atmosphere. The organic carbon is subsequently transferred through the food chain and with each trophic level part of it is respired and released as CO_2 back into the environment. The two key processes in this cycle, primary production and consumption of organic material followed by its use for respiration, are usually thought to be performed by different groups of organisms, autotrophs and heterotrophs. However, some organisms can perform both of these processes simultaneously and these mixotrophs are the topic of this thesis. Mixotrophs are increasingly recognized as key players in the microbial food web of aquatic ecosystems and an understanding of their physiology and ecology is therefore crucial for understanding biogeochemical cycles.

But can we understand mixotrophs based on our knowledge on autotrophic and heterotrophic organisms? In other words, can mixotrophs and their impact on ecological processes be described as the sum of the parts? Or conversely, does the combination of two opposing nutritional pathways within a single organism allow different insights into the parts than can be acquired by studying them in separation? This thesis aims at contributing to answers to these questions and thereby, ultimately, to gain a better understanding of carbon cycling and food-web dynamics in aquatic ecosystems.

Mixotrophy – The combination of two extremes

All living organisms require energy to sustain their metabolic activity, and carbon to build up biomass for their growth. Organisms differ, however, in their ability to utilize different sources of both carbon and energy. Autotrophic organisms use inorganic carbon to build up their biomass, while heterotrophic organisms rely on the uptake of organic carbon sources. The energy that fuels the metabolism can originate from inorganic substances (lithotrophy), from organic carbon (organotrophy), or from the light (phototrophy). Although these different nutritional strategies require completely different sets of enzymes and cellular structures, some organisms are able to utilize several sources of carbon and energy at the same time. Such mixotrophic nutrition can be found among taxonomic groups ranging from prokaryotes to a huge diversity of eukaryotic protists as well as higher plants and animals (Pringsheim 1958, Selosse and Roy 2009, Johnson 2011). Mixotrophy is often represented as the combination of photoautotrophy and organoheterotrophy, and this is what the term mixotrophy will be used for in this thesis. Contrary to the common tendency to categorize species into autotrophs or heterotrophs, mixotrophy is widespread among diverse taxonomic groups and can be found in numerous different habitats. Furthermore, it has implications for our understanding of both the evolution of photosynthetic eukaryotes and of biogeochemical cycles, especially carbon and nutrient cycling in aquatic ecosystems.

Ancestors of all the immensely diverse eukaryotic photosynthetic organisms we are surrounded by today, ranging from microscopic unicellular algae to the higher plants producing our daily food, have acquired their ability to perform photosynthesis by ingesting another organism. The ancestral eukaryote was a heterotroph that engulfed a cyanobacterial cell, which escaped digestion and was maintained as an endosymbiont instead to provide its host cell with organic carbon derived from photosynthesis. The endosymbiont was subsequently brought under the control of the host and turned into a chloroplast (Mereschkowski 1905, Sagan 1967). From this ancestor several lineages of eukaryotic phototrophs evolved including red algae, green algae and plants. The plastids were subsequently passed on to other eukaryotes via secondary and even tertiary endosymbiosis events (Gibbs 1978, Gibbs1981, McFadden 2001, Archibald and Keeling 2002). The ability to ingest food particles was therefore crucial for the evolution of photosynthetic eukaryotes and was present in their original ancestors (Raven 1997). While it was lost during the evolution of many species, most taxonomic groups still contain representatives that combine the ability to grow photoautotrophically utilizing light energy and inorganic carbon with the ability to grow heterotrophically on organic carbon sources (Pringsheim 1958, Flynn et al. 2013).

Mixotrophs are increasingly recognized to significantly contribute to ecosystem processes. As they can dominate the total phytoplankton biomass (Safi and Hall 1999), with up to 90% recorded for a humic lake (Jansson et al. 1996), mixotrophs are expected to significantly contribute to primary production in some ecosystems. On the other hand, mixotrophs can also be important as consumers of both bacteria and phytoplankton. In aquatic systems bacterivory by mixotrophs is often of equal or even higher importance than bacterivory by heterotrophic flagellates (Bird and Kalff 1987, Isaksson et al. 1999, Unrein et al. 2007, Zubkov and Tarran 2008, Hartmann et al. 2012). Furthermore, herbivory by mixotrophs can be an important loss process for pico- and nanophytoplankton (Havskum and Hansen 1997, Sanders et al. 2000, Callieri et al. 2006). The simultaneous role as primary producers and consumers of other microorganisms represents a challenge for the quantification of the net contribution of mixotrophs to ecosystem processes like carbon and nutrient cycling.

Ecophysiology of mixotrophs

The net impact of mixotrophs on both carbon flow and nutrient cycling will depend on their nutritional strategy (Fig. 1.1). Understanding the nutritional balance of mixotrophs and the factors by which it is affected is therefore crucial for a mechanistic understanding of food-web dynamics and biogeochemical cycles in aquatic ecosystems. The relative importance of photosynthesis and food uptake to the nutrition of mixotrophs depends on both species identity and resource availability. Jones (1997) classified mixotrophs based on their nutritional behaviour. He distinguished between primarily heterotrophic species, which employ phototrophy only when prey concentrations are limiting, and primarily autotrophic species. For the latter a further distinction can be made based on the need for phagotrophy to provide essential substances for growth, to supplement phototrophy during light limitation, or to allow maintenance during prolonged periods of darkness. Although there might be some overlap between the different strategies, an equal ability for both autotrophic and heterotrophic nutrition is remarkably rare (Jones 1997, Stoecker 1998). This is usually explained by the trade-off caused by the extra costs involved in sustaining the cellular structures and biochemical machinery needed for two different nutritional modes (Raven 1997).

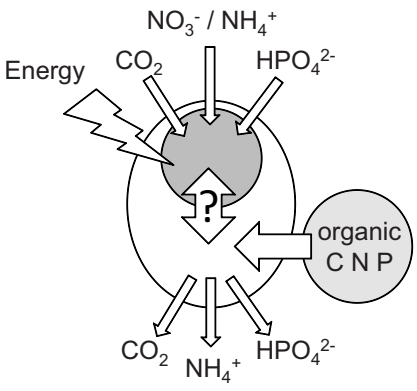

Figure 1.1: Alternative routes for the acquisition of carbon, energy and nutrients in mixotrophs.

These different strategies illustrate that mixotrophy is not purely the sum of two independent nutritional modes, but the acquisition of different resources along different pathways is closely regulated and integrated on the subcellular level. Therefore, the relative importance of photosynthesis and the ingestion of prey might differ for the acquisition of different resources. Phagotrophy can, for instance, provide the major fraction of nutrients, while photosynthesis provides both energy and carbon for growth (Caron et al. 1993). Furthermore, the nutritional balance of a given mixotrophic species is by no means fixed, but can vary considerably with resource availability. For instance, ingestion rates might increase with light intensity, when the prey mainly provides nutrients for autotrophic growth (Caron et al. 1993, Li et al. 2000, Kim et al. 2008). But ingestion rates might also decrease with light intensity, when photosynthesis replaces the ingestion of prey as source of energy and carbon (Keller et al. 1994, Skovgaard 1996).

Mixotrophs in the food web

The ecological benefits of combining two nutritional strategies seem obvious, as mixotrophs can make use of alternative and substitutable sources of carbon, energy, and nutrients needed for growth (Fig 1.2A; Rothhaupt 1996a). This gives them a competitive advantage over specialists, when resource availability is low. Mixotrophs therefore typically occur in oligotrophic waters, where the ingestion of food particles provides an alternative source of nutrients (Nygaard and Tobiesen 1993, Rothhaupt 1996b) or photosynthesis allows to cope with low prey abundances (Tittel et al. 2003). Also under low light conditions the ability to utilize alternative sources of carbon and energy gives mixotrophs a competitive advantage (Flöder et al. 2006) and allows them to dominate the phytoplankton biomass in deep chlorophyll maxima (Bird and Kalff 1989) or under the ice (Berninger et al. 1992, Wiedner and Nixdorf 1998).

Due to the trade-off between nutritional modes, mixotrophs are expected to have a disadvantage under growth conditions enabling specialists to achieve high growth rates, as for instance during the phytoplankton spring bloom. This does not generally exclude them from eutrophic systems, however, as there are many examples of mixotrophs reaching high abundances in eutrophic environments (Bennett et al. 1990, Olrik and Nauwerck 1993), with the formation of harmful algal blooms by mixotrophic dinoflagellates being a prominent example (Burkholder et al. 2008).

Interestingly, because of their simultaneous action as primary producers and consumers, mixotrophs can often feed on their competitors such as other phytoplankton species, with which they compete for dissolved nutrients or light (Fig. 1.2B). Feeding upon their competitors might allow mixotrophs to coexist with or even dominate over

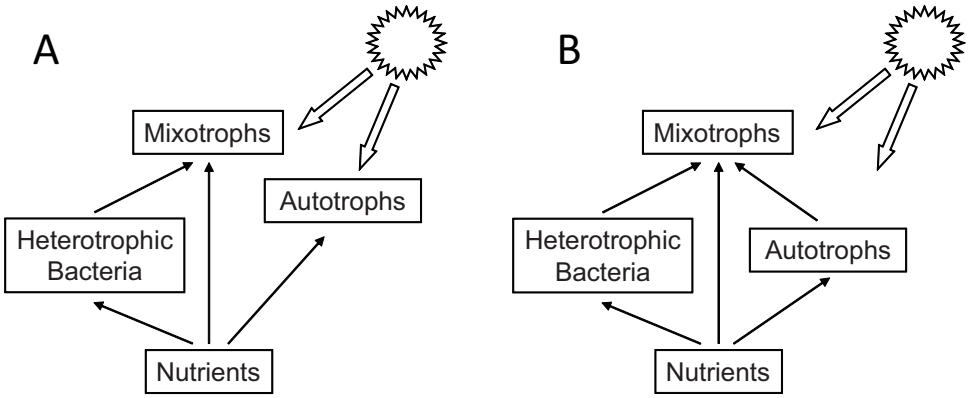

Figure 1.2: The position of mixotrophs in a food web, when feeding mainly on heterotrophic bacteria (A) or also on autotrophic organisms (B).

superior competitors for inorganic resources (Thingstad et al. 1996). This combination of competition and grazing in the interaction of two species is called intraguild predation and is common in many ecosystems at higher trophic levels (Polis et al. 1989). Intraguild predation theory has been developed for three-species systems, where the resource is a shared prey species of both the intraguild prey and predator (Holt and Polis 1997). In this case the intraguild predator is predicted to strongly benefit from an increasing resource carrying capacity, while the intraguild prey is increasingly suppressed by the predator and can even be excluded from the system at high carrying capacity (Diehl and Feissel 2000). When acting as intraguild predators, mixotrophs might therefore benefit from nutrient enrichment, as the high carrying capacity might allow them to exclude competing species.

Interaction with harmful cyanobacteria

High nutrient loads caused by anthropogenic eutrophication can stimulate the formation of harmful algal blooms in both fresh and marine waters (Anderson et al. 2002, Huisman et al. 2005, O'Neil et al. 2012). In marine waters, harmful algal blooms are often dominated by mixotrophic species, especially dinoflagellates (Burkholder et al. 2008), while cyanobacteria are the most frequent bloom-forming species in freshwater. Cyanobacteria are especially favoured by a combination of eutrophication and high temperatures, both through a strong direct effect on their growth rate and indirectly through increased stratification (Paerl and Huisman 2008), as their ability to regulate their buoyancy allows some cyanobacteria to accumulate at the surface during periods of low turbulence (Reynolds and Walsby 1975, Huisman et al. 2004). Several cyanobacterial species can produce neuro- and hepatotoxins,

and their proliferation causes risks for birds, cattle, pets and human health and hampers the use of water for recreational purposes or drinking water production (Chorus and Bartram 1999, Codd et al. 1999, Falconer 1999).

A cosmopolitan species commonly dominating the phytoplankton community in eutrophic lakes is *Microcystis aeruginosa* (Visser et al. 2005). *M. aeruginosa* seems to be well defended against zooplankton grazing for several reasons. First, the formation of large colonies deters small grazers (Fulton and Paerl 1987) and also the mucilage layer around the cells has been suggested to inhibit ingestion by zooplankton (Rohrlack et al. 1999a). Also its biochemical composition is of low food quality to many grazers, as it contains rather low amounts of polyunsaturated fatty acids and sterols, which are essential for most heterotrophs (Müller-Navarra et al. 2000, von Elert et al. 2003). Furthermore, *Microcystis* produces a wide array of cyanotoxins, many of which are toxic to potential grazers (Rohrlack et al. 1999b, Rohrlack et al. 2003, Welker and von Dohren 2006, Tooming-Klunderud et al. 2007, Portmann et al. 2008). The best studied of these are the microcystins, a family of cyclic non-ribosomal peptides that inhibit eukaryotic protein phosphatases (MacKintosh et al. 1990), cause oxidative stress, and act as hepatotoxins in higher animals (Dittmann et al. 2006).

Several mixotrophic chrysophytes of the genera *Ochromonas* and *Poterioochromonas* are able to efficiently feed on toxic cyanobacteria and have already been suggested as potential biological control agents (Fig. 1.3; Cole and Wynne 1974, Burkert et al. 2001, Zhang and Watanabe 2001, Zhang et al. 2008). As primarily heterotrophic mixotrophs, these genera typically show high grazing and growth rates when prey is available (Caron et al. 1990, Holen 1999, Sanders et al. 2001). The combination of grazing and competition for inorganic resources might allow mixotrophic chrysophytes to strongly suppress their cyanobacterial prey and this mechanism could be useful for the suppression of nuisance cyanobacterial species in eutrophic lakes.

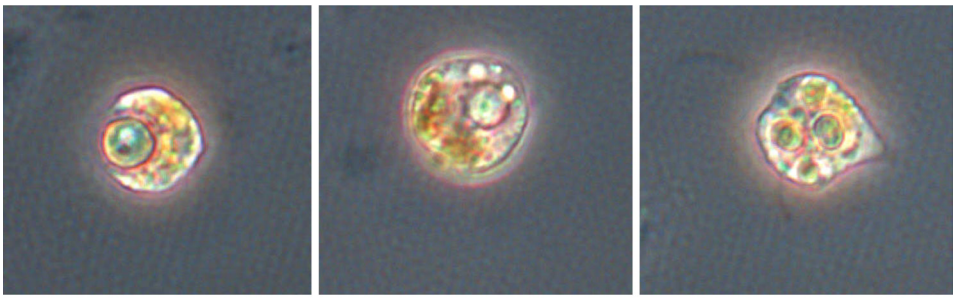

Figure 1.3: *Ochromonas* sp. ingesting *M. aeruginosa*.

Outline of this thesis

This thesis focuses on the nutritional strategy of mixotrophs and their functional role in food webs. Furthermore, it addresses the question whether mixotrophs might serve as biological control agents against toxic cyanobacterial blooms. Our approach combines physiological and ecological lab experiments with mathematical models to test the applicability of several general ecological concepts to mixotrophs.

In food webs, mixotrophs can act as both primary producers and consumers, and hence have opposing effects on biogeochemical cycles. Understanding the intricate interaction and regulation of autotrophic and heterotrophic processes within mixotrophs is a prerequisite for understanding their functional role for ecosystem processes. **Chapter 2** and **3** therefore focus on the integration and balance of the two nutritional pathways in mixotrophs. In **Chapter 2**, we investigate one of the key predictions of the Metabolic Theory of Ecology, that heterotrophic process rates increase more strongly with temperature than autotrophic processes (Allen et al. 2005). An important implication of this prediction, if correct, is that mixotrophic organisms may tend to suppress their autotrophic growth and shift towards more heterotrophy with rising temperature. In **Chapter 3**, we study the composition of the photosynthetic machinery during autotrophic and mixotrophic growth with the aim to elucidate whether the function of photosynthesis shifts from providing both carbon and energy during autotrophy towards mainly providing energy during mixotrophy. In other words, we test whether mixotrophs shift from photoautotrophy to photoheterotrophy after prey is provided.

Several mixotrophic chrysophytes are capable of grazing on toxic cyanobacteria and have been suggested as biological control agents. **Chapter 4** and **5** address the interaction of the chrysophyte *Ochromonas* with toxic cyanobacteria. In **Chapter 4** we test the ability of *Ochromonas* to graze on several species of toxic cyanobacteria varying in particle size and microcystin content. We furthermore assess the co-occurrence of *Ochromonas* spp. with *Microcystis* spp. using a dataset from Scandinavian lakes. Despite the ability of *Ochromonas* to graze on toxic cyanobacteria, the specific effects of microcystins in its food remain unknown. Do microcystins affect the growth of *Ochromonas*? And if so, does *Microcystis* respond to grazers by increasing its microcystin content? These questions are addressed in **Chapter 5** by comparing the grazing and growth of *Ochromonas* on a microcystin-containing strain of *Microcystis* and its microcystin-deficient mutant.

In **Chapter 6** and **7** the nutritional flexibility of mixotrophs is set into a food-web context. The ability to simultaneously feed on and compete with other autotrophs distinguishes mixotrophs from purely heterotrophic grazers. The implications of this intraguild predation for the population dynamics of the mixotroph and its cyanobacterial prey are studied in

chemostat experiments and compared to predictions by mathematical models. In **Chapter 6** we show that *Ochromonas* can utilize ammonium but not nitrate as nitrogen source. This allows altering the food-web structure by altering the nitrogen source, as the mixotroph *Ochromonas* can compete for nitrogen with its autotrophic prey if ammonium is provided, but not if nitrate is provided as nitrogen source. In **Chapter 7** we use the mixotroph *Ochromonas* and the cyanobacterium *Microcystis* to test several of the key predictions of intraguild predation theory (Holt and Polis 1997, Diehl and Feissel 2000). In particular, we are interested in the question how intraguild predation of mixotrophs upon cyanobacteria is affected by nutrient enrichment, a question that is not only of fundamental interest but also of practical relevance because cyanobacterial blooms are generally associated with eutrophic and hypertrophic waters.

Finally, in **Chapter 8**, I synthesize the results presented in this thesis. In particular, the implications of the physiology of mixotrophs for their role in food webs and impact on ecosystem processes will be discussed. Furthermore, the potential use of *Ochromonas* as a biological control agent against cyanobacterial blooms will be assessed.

Chapter 2

Mixotrophic organisms become more heterotrophic with rising temperature

Abstract

The Metabolic Theory of Ecology predicts that temperature affects heterotrophic processes more strongly than autotrophic processes. We hypothesized that this differential temperature response may shift mixotrophic organisms towards more heterotrophic nutrition with rising temperature. The hypothesis was tested in experiments with the mixotrophic chrysophyte *Ochromonas* sp., grown under autotrophic, mixotrophic and heterotrophic conditions. Our results show that (i) grazing rates on bacterial prey increased more strongly with temperature than photosynthetic electron transport rates, (ii) heterotrophic growth rates increased exponentially with temperature over the entire range from 13 to 33 °C, while autotrophic growth rates reached a maximum at intermediate temperatures, and (iii) chlorophyll contents during mixotrophic growth decreased at high temperature. Hence, the contribution of photosynthesis to mixotrophic growth strongly decreased with temperature. These findings support the hypothesis that mixotrophs become more heterotrophic with rising temperature, which alters their functional role in food webs and the carbon cycle.

This chapter is based on the paper: Wilken S, Huisman J, Naus-Wiezer S, and Van Donk E (2013) Mixotrophic organisms become more heterotropic with rising temperature. Ecology Letters 16: 225-233.

Introduction

Recent theory and meta-analysis indicate that heterotrophic metabolism responds more strongly to rising temperature than autotrophic metabolism (Allen et al. 2005; Lopez-Urrutia et al. 2006; Rose & Caron 2007). This fundamental difference in temperature response of autotrophic and heterotrophic processes may have major implications for communities and ecosystems. In food webs, rising temperatures may strengthen top-down control of primary producers by their heterotrophic consumers (Rose & Caron 2007; O'Connor et al. 2009; Kratina et al. 2012). At the ecosystem level, the balance between primary production and community respiration may shift from net autotrophy to net heterotrophy with increasing temperature (Hoppe et al. 2002; Lopez-Urrutia et al. 2006; Yvon-Durocher et al. 2010), which could potentially reduce global carbon sequestration (Wohlers et al. 2009).

So far, most studies investigating the temperature response of autotrophic and heterotrophic processes have compared a wide range of different autotrophic and heterotrophic species. Some species, however, can combine both autotrophic and heterotrophic nutrition. These mixotrophic organisms are represented by, for instance, microalgae that acquire inorganic carbon by photosynthesis but can also utilize dissolved organic carbon or ingest prey species by phagocytosis. Based on increasing reports of phagotrophy in a wide range of phytoplankton species previously believed to be obligate phototrophs, recent studies have even argued that mixotrophy is the rule rather than the exception in plankton communities (Flynn et al. 2013; Hartmann et al. 2012). Several higher plants are also able to acquire organic carbon, by trapping insects and other invertebrates, parasitizing on other plants, or through symbiosis with mycorrhizal fungi (Selosse & Roy 2009). Furthermore, heterotrophic organisms can gain the ability to photosynthesize by harbouring photosynthetic endosymbionts or by retaining the chloroplast of their photosynthetic prey organisms (i.e., kleptochloroplastidy). The most prominent examples of endosymbiosis might be lichens in terrestrial systems and scleractinian corals with their photosynthetic zooxanthellae in marine systems, but also other cnidaria, sea slugs, ciliates, radiolaria and foraminifera can contain photosynthetic endosymbionts (Stoecker 1998; Johnson 2011).

The diversity of taxa in which mixotrophy can be found is reflected in their diverse effects on ecosystem functioning, ranging from the development of harmful algal blooms by mixotrophic dinoflagellates (Burkholder et al. 2008) to large-scale reef formation by scleractinian corals. In both marine and freshwater ecosystems mixotrophs can dominate the plankton community. In some cases, they even represent > 90 % of the total phytoplankton (Jansson et al. 1996), suggesting a major contribution of mixotrophs to aquatic primary production. Conversely, mixotrophs can also dominate grazing on the

microbial community, accounting for 40-95 % of the bacterivory in oligotrophic habitats (Bennett et al. 1990; Sanders et al. 2000; Zubkov & Tarran 2008; Hartmann et al. 2012). Hence, mixotrophs can play an important functional role as primary producers but also as consumers, which results in strikingly different effects on food-web structure and ecosystem carbon sequestration depending on their nutritional strategy.

Mixotrophic organisms offer a unique opportunity to compare the temperature response of autotrophic versus heterotrophic metabolism within the same species. Most mixotrophic species favour one nutritional pathway above the other, and due to trade-offs between the two opposing nutritional pathways an equal contribution of both autotrophy and heterotrophy is seldom observed (Jones 1997; Stoecker 1998). Nevertheless, the relative contribution of autotrophic versus heterotrophic nutrition varies widely among mixotrophs, depending not only on species identity but also on the environmental conditions (Jones 1997; Hansen 2011). In particular, if the difference in temperature response of autotrophic versus heterotrophic processes is indeed a generic phenomenon, as argued by the metabolic theory of ecology (Allen et al. 2005), then we may hypothesize that mixotrophic organisms will shift towards more heterotrophic nutrition with increasing temperature.

In this study, we test this intriguing hypothesis by investigating the temperature dependence of the growth rate and energy acquisition of mixotrophic organisms during autotrophic, mixotrophic and heterotrophic nutrition. We focus on the mixotrophic chrysophyte *Ochromonas*, which is a widespread genus commonly abundant in plankton communities of both freshwater and marine habitats (Estep et al. 1986; Bennett et al. 1990; Jansson et al. 1996). *Ochromonas* is a predominantly heterotrophic mixotroph possessing chloroplasts and feeding on microbes by phagocytosis (Andersson et al. 1989; Sanders et al. 2001). It can grow both purely photoautotrophically on a mineral medium and purely heterotrophically on organic carbon sources in the dark. Therefore, it is an ideal candidate to compare the temperature response of both nutritional pathways separately as well as in combination during mixotrophic growth. Our findings show that, indeed, *Ochromonas* reduces its photosynthetic capacity and shifts towards more heterotrophic nutrition at high temperatures.

Theoretical Background

The temperature dependence of metabolic processes can often be approximated by the Arrhenius equation:

$$R = R_0 e^{\frac{-E_a}{kT}} \tag{2.1}$$

where R is a metabolic rate, R_0 is a normalization constant, E_a is the activation energy, k is Boltzmann's constant (8.62×10^{-5} eV K^{-1}) and T is absolute temperature in Kelvin. Hence, the activation energy is a quantitative measure of the temperature dependence. Activation energies vary among different processes. Comparison across a wide variety of different species showed an overall activation energy of the heterotrophic resting metabolism of organisms of, on average, $E_a = 0.65$ eV (Gillooly et al. 2001; Allen et al. 2005). This value corresponds to a 16-fold increase in heterotrophic metabolism over a temperature range from 0 to 30 °C.

The temperature dependence of photosynthesis depends on the light conditions, as already pointed out by the early work of Blackman (1905). At low light, the photosynthetic rate is limited by the light-dependent reactions of photosynthesis (i.e., light absorption and photosynthetic electron transfer). These photochemical reactions themselves are in essence temperature independent, although there can be some minor temperature effects on the light reactions. For instance, the diffusivity of the electron carriers (e.g., plastoquinone) and the viscosity of the photosynthetic membranes in which these electron carriers are embedded both increase with temperature (Falkowski & Raven 2007). Overall, however, we do not expect a major effect of temperature on photosynthetic rates under light-limited conditions ($E_a \approx 0$ eV).

At light-saturating conditions, the photosynthetic rate is limited by the dark reactions of photosynthesis, and these enzymatic reactions are clearly temperature dependent. The rate limiting step is primarily the activity of the Rubisco enzyme. Rubisco reacts with carbon dioxide (CO_2 fixation) and oxygen (photorespiration), and photorespiration tends to increase faster with temperature than carboxylation. This results in a relatively low overall activation energy of light-saturated photosynthesis, which Allen et al. (2005) estimated at $E_a = 0.32$ eV for terrestrial C_3 plants exposed to ambient concentrations of atmospheric CO_2 and O_2 based on data of Bernacchi et al. (2001). This activation energy yields only a 4-fold increase in light-saturating photosynthesis when temperature is raised from 0 to 30 °C. Although CO_2 and O_2 concentrations vary much more widely in aquatic than terrestrial ecosystems and the Rubisco kinetics of aquatic and terrestrial autotrophs tend to be different (Tcherkez et al. 2006), the relatively weak temperature dependence of photosynthesis has also been confirmed for aquatic organisms (Rose & Caron 2007).

Hence, the resting metabolism of organisms has a higher activation energy than the photosynthetic rate. This difference is at the heart of the hypothesis to be tested in this paper. It suggests that, all else being equal, mixotrophic organisms will display a stronger temperature response for heterotrophic than for autotrophic growth, and thus become more heterotrophic with increasing temperature. However, despite the plausibility of this

hypothesis, it does not necessarily hold. For instance, mixotrophic organisms may suppress (or strengthen) the thermodynamic tendency towards more heterotrophy, by adjusting the relative size of their photosynthetic and heterotrophic machinery in response to changes in temperature.

Materials and Methods

Species and culture conditions

Our experiments were carried out with a freshwater strain of the mixotrophic chrysophyte *Ochromonas* sp. (probably *Ochromonas globosa* Skuja, determined by Dr R. Bijkerk, Koeman & Bijkerk B.V., Ecological Research and Advice, Haren, The Netherlands). This strain was detected as infection in a large-scale mesocosm experiment with a laboratory culture of the cyanobacterium *Microcystis aeruginosa* (Van Donk et al. 2009). It was isolated using micro-needle techniques and subsequently maintained in uni-algal but not axenic cultures under autotrophic growth conditions. It had an average cell size of 298 μm^3 (s.d. = 73 μm^3; n = 46). *Ochromonas* sp. was grown autotrophically on a nutrient-rich mineral medium containing 500 µM NH_4Cl, 500 µM K_2HPO_4, 150 µM $MgSO_4$, 250 µM $CaCl_2$, 600 µM $NaHCO_3$, 250 µM Na_2SiO_3, 3.7 µM $FeCl_2$, 11.7 µM Na_2EDTA, 43.3 µM H_3BO_3, 9.15 µM $MnCl_2$, 0.77 µM $ZnSO_4$, 0.32 µM $CuSO_4$, 0.27 µM $CoCl_2$, 1.62 nM $NaMoO_4$, 0.4 nM Vitamin B12, 2 nM Biotin, and 0.3 µM Thiamin. Mixotrophic and heterotrophic growth were studied using the bacterium *Pseudomonas fluorescens* strain Pf 0-1 (Compeau et al. 1988) as prey. *P. fluorescens* was grown on DSMZ 7 medium (1 g L^{-1} glucose, 1 g L^{-1} yeast extract, 1 g L^{-1} peptone). Stock cultures of both species were maintained at 23 °C with constant illumination of 90 µmol quanta m^{-2} s^{-1}.

Experimental setup

We investigated the growth characteristics of *Ochromonas* at 6 different temperatures ranging from 13 to 33 °C. *Ochromonas* was acclimated to the experimental temperatures for at least 2 weeks, but up to 4 weeks for the lowest temperature. The experiments were performed in 100 mL Erlenmeyer flasks incubated in temperature-controlled waterbaths and illuminated continuously by white fluorescent tubes (Philips TL-D 30W/33-640, Philips Lighting, Eindhoven, The Netherlands).

For autotrophic growth, at each temperature 16 Erlenmeyer flasks were provided with 65 mL of the nutrient-rich mineral medium and were wrapped into different neutral density foils to create different light conditions ranging from 1 to 170 µmol quanta m^{-2} s^{-1}. Light intensity was measured using a quantum photometer (LI-250; LI-COR Biosciences,

Lincoln, NE, USA). The Erlenmeyer flasks were inoculated with *Ochromonas* to a final density of 2×10^4 cells mL^{-1} and incubated for 11 days. Samples for cell counts were taken every second day and photosynthetic parameters were measured once during exponential growth.

Mixotrophic and heterotrophic growth rates of *Ochromonas* sp. were determined with saturating prey abundances of *P. fluorescens* (1×10^7 cells mL^{-1}). Before being used as prey, bacterial cultures were centrifuged and resuspended in the algal mineral medium. This washing procedure was performed twice to remove traces of DSMZ 7 medium. Cultures for heterotrophic growth were incubated in the dark, while mixotrophic growth was determined at a constant light intensity of 100 µmol quanta m^{-2} s^{-1}. *Ochromonas* sp. was precultured with the prey species at the described light conditions for 4 days prior to the start of the experiment. At each temperature, a grazing treatment consisted of an Erlenmeyer flask with saturating prey abundance that was inoculated with *Ochromonas* sp. at an abundance of 2×10^4 cells mL^{-1}, while a treatment with only the prey species served as control. All grazing treatments and controls were run in triplicates and samples were taken after 0, 3, 6, 9, 12, and 20 h.

Population dynamics

Samples for cell counts were fixed with a mixture of glutaraldehyde and paraformaldehyde (final concentration of 0.025 and 0.0037 percent by mass, respectively) and stored at 4 °C. Population abundances were counted with a particle counter (CASY-counter; Schärfe-System GmbH, Reutlingen, Germany) for the autotrophic growth experiment and with a flow cytometer (MoFlo XDP Cell Sorter, Beckman Coulter, Miami, FL, USA) for the grazing experiment. Flow-cytometer samples were stained with PicoGreen (Life Technologies, Paisley, UK) prior to counting and *Ochromonas* sp. could be distinguished from *P. fluorescens* based on its pigmentation and higher DNA-content.

Specific growth rates (µ) were calculated during exponential growth as:

$$\mu = \frac{\ln(N_2) - \ln(N_1)}{t_2 - t_1} \tag{2.2}$$

where N_1 and N_2 denote the abundances at time steps t_1 and t_2, respectively. Grazing rates (G) during mixotrophic and heterotrophic growth were calculated from the difference in prey growth rate in the control (μ_{PC}) versus the grazing treatment (μ_{PT}) (Heinbokel 1978):

$$G = \frac{(\mu_{PC} - \mu_{PT})N_P}{N_O} \tag{2.3}$$

where N_O and N_p are the mean abundances of *Ochromonas* and its prey, respectively, in the grazing treatment. For the determination of prey carbon content, the cultures were filtered onto pre-combusted glassfibre filters (Whatman GF/F, Maidstone, UK), dried at 60 ºC overnight and measured in an organic elemental analyzer (Flash 2000; Thermo Fisher Scientific, Waltham, MA, USA).

Photosynthesis

Chlorophyll *a* (Chl *a*) concentrations and the quantum yield of photosystem II (ΦPSII) were measured with a Phyto-PAM (Walz, Effeltrich, Germany). Chl *a* concentrations provide an estimate of the amount of photosynthetic machinery, while ΦPSII gives the fraction of the absorbed quanta that are used for photosynthetic electron transport and thus provides a measure of photosynthetic efficiency (e.g., Maxwell & Johnson 2000). Traditional methods to measure photosynthetic rates, such as the ^{14}C method, are likely to underestimate the carbon fixation rate, because internally recycled carbon dioxide derived from respiration can account for more than 50 % of the photosynthetically fixed carbon in mixotrophic organisms (Stoecker & Michaels 1991). Therefore, we quantified the rate of photosynthesis during autotrophic and mixotrophic growth using the photosynthetic electron transport rate (*ETR*), which can be calculated from the quantum yield of photosystem II and the cellular chlorophyll content as (Falkowski & Raven 2007):

$$ETR = 0.5 \times \overline{a^*} \times \frac{Chla}{cell} \times I \times \Phi_{PSII} \tag{2.4}$$

Here, I is the light intensity, the factor 0.5 takes into account that two electron excitations (at photosystem II and photosystem I) are required to transport one electron through the Z-scheme of photosynthesis, and $\overline{a^*}$ is the spectrally averaged (400 to 700 nm) chlorophyll-specific absorption cross section. The value of $\overline{a^*}$ was estimated according to Falkowski & Raven (2007), from absorption spectra of *Ochromonas* measured with a spectrophotometer (Lambda 800; Perkin Elmer, Waltham, MA, USA) equipped with an integrating sphere. This yielded $\overline{a^*}$ = 26.86 m^2 (g Chl *a*)$^{-1}$ as a representative value for all cultures. Potential rates of carbon fixation were estimated from electron transport rates, assuming a maximum electron yield of 0.25 mol C (mol electrons)$^{-1}$.

Photosynthesis-irradiance (P-I)-curves were fitted to the *ETR* data and specific growth rates measured during autotrophic growth using the equation of Platt et al. (1980):

$$P = P_{sat}\left(1 - e^{\frac{-\alpha I}{P_{sat}}}\right) e^{\frac{-\beta I}{P_{sat}}} - P_0 \tag{2.5}$$

where P stands for either the electron transport rate (ETR) or specific growth rate (μ), P_{sat} is the light-saturated rate without photoinhibition, α is the initial slope of the P-I curve, ß is a measure of photoinhibition, and P_0 represents rates in the dark. Fits were based on non-linear regression using the iterative least-squares procedure in SigmaPlot 11.0. We note that P_{sat} is a theoretical value that may greatly exceed the actual maximum rate of photosynthesis. Therefore, we calculated the maximum electron transport rate (ETR_{max}) and maximum specific growth rate (μ_{max}) by setting the first derivative of eqn (2.5) equal to zero.

Statistical analysis

For the model parameters derived from the photosynthesis-irradiance curves (α, ETR_{max}, μ_{max}), we estimated activation energies from Arrhenius plots using linear regression of the natural logarithm of these parameters against $1/(kT)$.

For the measured variables (cellular Chl *a* content, quantum yield of PS II, potential carbon fixation rate, grazing rate and growth rate), we tested whether their temperature dependence differed between autotrophic, heterotrophic and mixotrophic growth using analysis of co-variance (ANCOVA). Natural logarithms of the measurements were used as response variable, nutritional mode (autotrophy, heterotrophy, mixotrophy) as categorical factor, and temperature as continuous factor. We tested both linear and quadratic temperature effects (T and T^2). For all rate measurements (carbon fixation rate, grazing rate, growth rate), we replaced T and T^2 by $1/kT$ and $(1/kT)^2$ in accordance with the Arrhenius equation (eqn 2.1). A significant quadratic temperature effect is indicative of a unimodal temperature response (e.g., Englund et al. 2011). In this case, the temperature dependence cannot be accurately described by the Arrhenius equation and an activation energy cannot be given. If the quadratic term was not significant, it was removed from the model. In this case, the activation energy was obtained from the slope of the linear temperature response. In all cases, homogeneity-of- regression models were used to test for significant interactions between temperature and nutritional mode. If interaction terms were significant, we ran a new ANCOVA using separate regression parameters for the different nutritional modes.

Results

During autotrophic growth, photosynthetic electron transport rates and specific growth rates of *Ochromonas* sp. increased with light intensity and in most cases reached saturating values at light intensities > 80 μmol quanta m^{-2} s^{-1} (Fig. 2.1). Some photo-inhibition was observed in the temperature range from 13 to 25 °C, especially for the electron transport rates (Fig. 2.1A-D). The light-response curves could be well described by eqn (2.5) (black

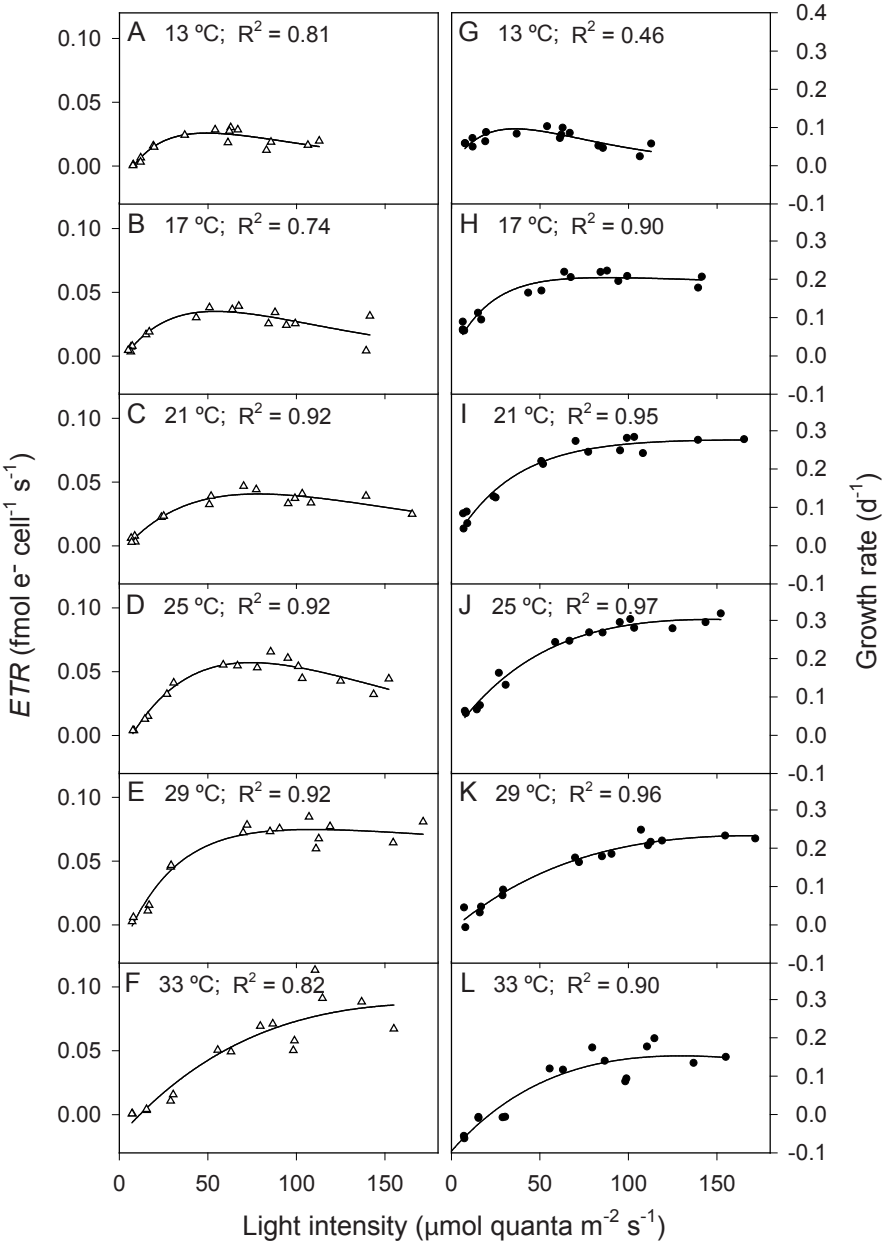

Figure 2.1: (A-F) Electron transport rates and (G-L) specific growth rates of *Ochromonas* sp. during autotrophic growth as function of light intensity at 6 different temperatures. Lines show light-response curves described by eqn (2.5) fitted to the data points.

lines in Fig. 2.1; parameter estimates are provided in Table A1 in Appendix A). The initial slopes α of the light-response curves were not significantly affected by temperature, neither for the electron transport rates (α_{ETR}) nor for the specific growth rates (α_{growth}) (Fig. 2.2A; linear regression of ln-transformed data, for α_{ETR}: $R^2 = 0.05$, n = 6, p = 0.322; for α_{growth}: $R^2 = 0.47$, n = 6, p = 0.081). Maximum electron transport rates increased with temperature over the whole temperature range (Fig. 2.2B) with an estimated activation energy of 0.47 eV (linear regression of ln-transformed data: $R^2 = 0.99$, n = 6, p < 0.001). Maximum autotrophic growth rates increased with temperature up to 0.3 d^{-1} at 25 °C, and decreased with a further increase in temperature (Fig. 2.2B). Hence, the temperature dependence of the maximum autotrophic growth rate was better described by an optimum curve than by the Arrhenius equation.

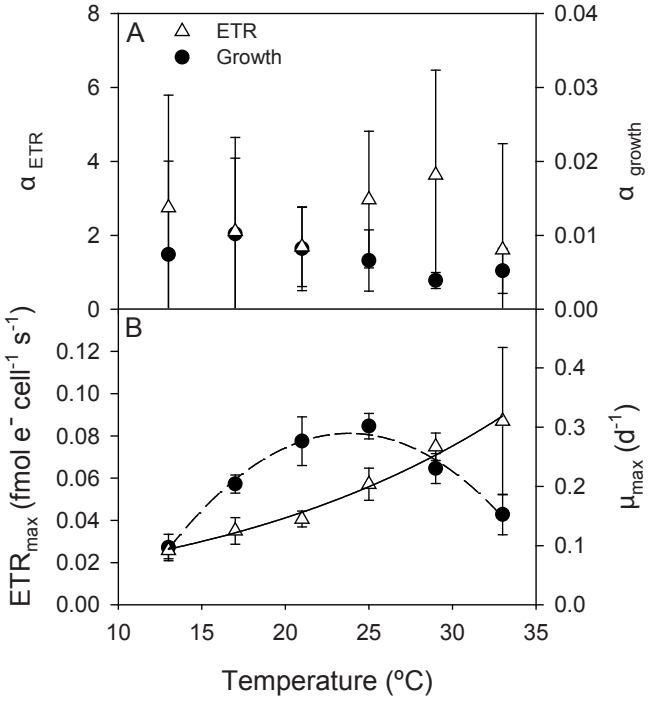

Figure 2.2: (A) Initial slopes of the light-response curves of the electron transport rates (α_{ETR}) and specific growth rates (α_{growth}) as function of temperature. (B) Maximum electron transport rates (ETR_{max}) and maximum specific growth rates (μ_{max}) as function of temperature. The values are the parameter estimates obtained from the light-response curves for autotrophic growth in Fig. 2.1. Error bars indicate 95% confidence intervals of these parameter estimates. The solid line shows the Arrhenius equation fitted to the ETR_{max} data, while the dashed line shows a second-order polynomial fitted to the μ_{max} data. Units for α_{ETR} are 10^{-3} fmol electrons $cell^{-1}$ (μmol quanta $m^{-2})^{-1}$ and for α_{growth} are d^{-1} (μmol quanta m^{-2} $s^{-1})^{-1}$.

Cellular Chl *a* contents were significantly lower during mixotrophic than during autotrophic growth, and much lower during heterotrophic growth (Fig. 2.3A; Table 2.1). In autotrophically growing cells the Chl *a* content increased with temperature up to 29 °C, while it decreased at temperatures above 21 °C in mixo- and heterotrophically growing cells. Cells growing heterotrophically in the dark maintained a positive quantum yield of photosystem II, which shows that these cells were still capable of photosynthesis if they had been exposed to light. The quantum yield of photosystem II remained high during autotrophic and mixotrophic growth, but decreased above 25 °C during heterotrophic growth (Fig. 2.3B).

Potential rates of photosynthetic carbon fixation, calculated from electron transport rates, were significantly lower for mixotrophic than for autotrophic cells (Fig. 2.4A; Table 2.1). Potential carbon fixation rates increased with temperature during autotrophic growth, with an activation energy of 0.50 eV (Table 2.2). During mixotrophic growth, however, potential carbon fixation rates decreased above 25 °C (Fig. 2.4A), in line with the decreasing cellular Chl *a* content (Fig. 2.3A). Grazing rates were not significantly different between mixotrophic and heterotrophic growth (Table 2.1), and increased with temperature

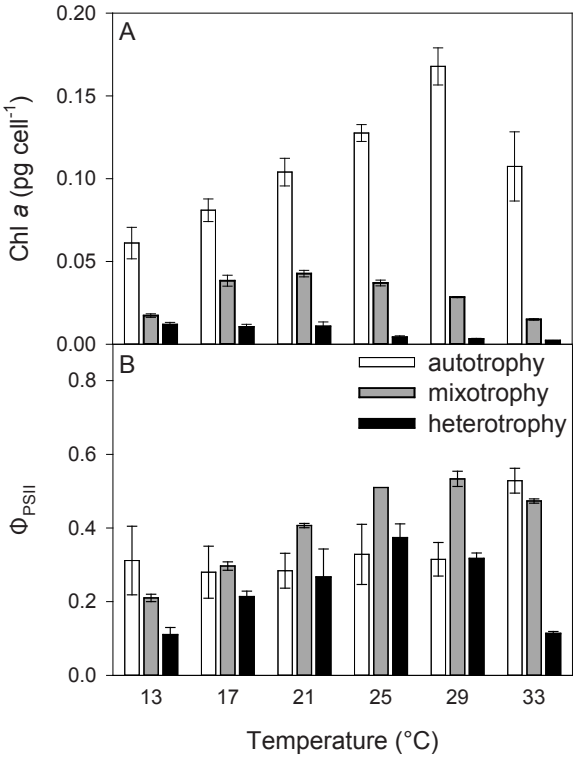

Figure 2.3: (A) Chl *a* content and (B) quantum yield of photosystem II (ΦPSII) during autotrophic, mixotrophic, and heterotrophic growth. Error bars indicate the standard deviation.

Table 2.1: Results of quadratic ANCOVAs applied to several measured variables, with nutritional mode (autotrophic, mixotrophic, or heterotrophic growth) as categorical factor and temperature (T and T^2) as continuous factor. For the rate measurements, we used ($1/kT$) and $(1/kT)^2$ as continuous factors in accordance with the Arrhenius equation.

Variable	Effect	df1	df2	F	p
Cellular Chl *a* content	Nutrition	2	60	1614.8	<0.0001
	T	1	60	82.7	<0.0001
	T x Nutrition	2	60	17.3	<0.0001
	T^2	1	60	101.4	<0.0001
	T^2 x Nutrition	2	60	13.1	<0.0001
Quantum yield of PSII	Nutrition	2	60	50.50	<0.0001
	T	1	60	51.69	<0.0001
	T x Nutrition	2	60	48.58	<0.0001
	T^2	1	60	39.75	<0.0001
	T^2 x Nutrition	2	60	50.35	<0.0001
Carbon fixation rate[a)]	Nutrition	1	45	401.76	<0.0001
	$1/kT$	1	45	104.69	<0.0001
	$1/kT$ x Nutrition	1	45	77.51	<0.000
	$(1/kT)^2$	1	45	107.18	<0.0001
	$(1/kT)^2$ x Nutrition	1	45	76.80	<0.0001
Grazing rate[b)]	Nutrition	1	32	0.622	0.436
	$1/kT$	1	32	97.33	<0.0001
	$1/kT$ x Nutrition	1	32	0.628	0.434
Specific growth rate	Nutrition	2	60	1349.95	<0.0001
	$1/kT$	1	60	46.55	<0.0001
	$1/kT$ x Nutrition	2	60	14.41	<0.0001
	$(1/kT)^2$	1	60	48.26	<0.0001
	$(1/kT)^2$ x Nutrition	2	60	14.15	<0.0001

[a)] Carbon fixation rates were determined only during autotrophic and mixotrophic growth.
[b)] Grazing rates were determined only during mixotrophic and heterotrophic growth.

with an activation energy of 0.85 eV (Fig. 2.4B; Table 2.2). Carbon uptake rates by grazing were much higher than the carbon fixation rates by photosynthesis (compare Fig. 2.4A and 2.4B). The relative contribution of photosynthesis to the total carbon uptake rate during mixotrophic growth decreased with temperature, from 5-8 % at 13 to 21 °C to less than 0.7 % at 33 °C.

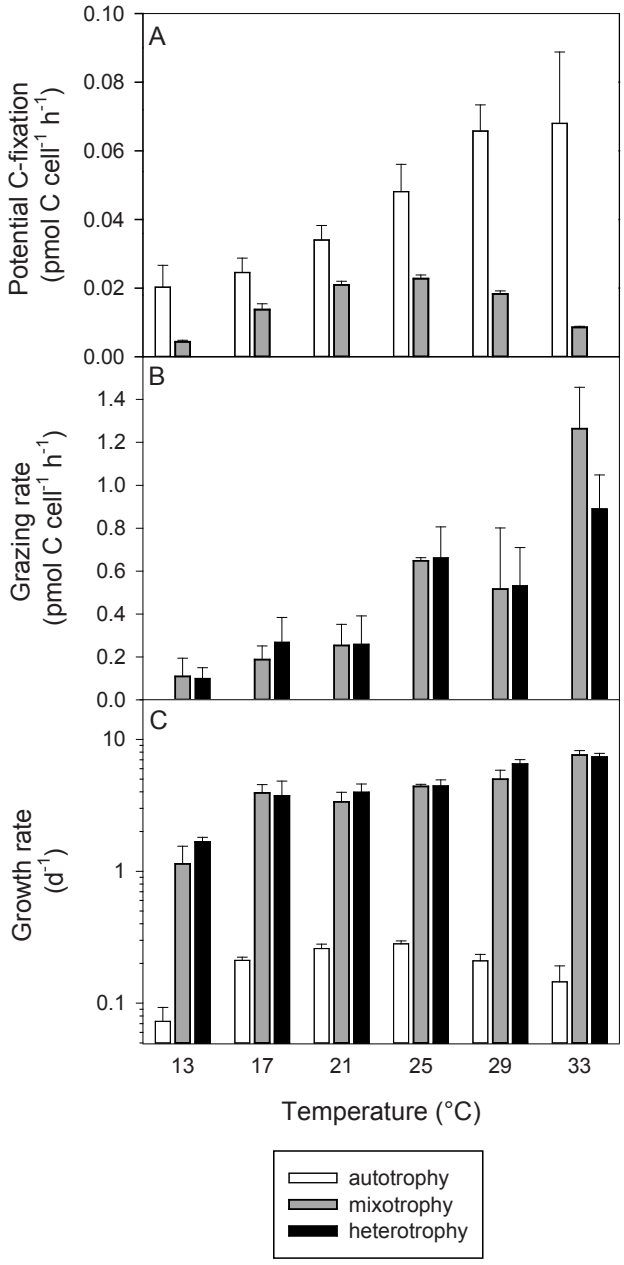

Figure 2.4: (A) Potential rates of carbon fixation by photosynthesis, (B) grazing rates, and (C) specific growth rates during autotrophic, mixotrophic and heterotrophic growth as function of temperature. Error bars indicate the standard deviation.

Table 2.2: Results for the quadratic term in the ANCOVA-model with separate regression parameters for the nutritional modes. Nutritional modes were grouped if their responses were not significantly different. For rate measurements without a significant quadratic temperature response ($p > 0.001$), activation energies (E_a ± 95% confidence intervals) were estimated from the slope of the linear temperature dependence.

		Quadratic ANCOVA			Activation energy
Parameter	**Nutritional mode**	**Quadratic term**	**Value**	**p**	**E_a (eV)**
Cellular Chl *a* content	Autotrophy	T^2	-0.004 ± 0.002	<0.001	
	Mixotrophy	T^2	-0.010 ± 0.002	<0.001	
	Heterotrophy	T^2	-0.003 ± 0.002	0.011	
Quantum yield of PSII	Autotrophy	T^2	0.003 ± 0.002	<0.001	
	Mixotrophy	T^2	-0.004 ± 0.002	0.002	
	Heterotrophy	T^2	-0.011 ± 0.002	<0.001	
Carbon fixation rate	Autotrophy	$1/(kT)^2$	-0.06 ± 0.09	0.182	0.50 ± 0.18
	Mixotrophy	$1/(kT)^2$	-0.76 ± 0.13	<0.001	-
Grazing rate	Mixo- and heterotrophy	$1/(kT)^2$	-0.11 ± 0.23	0.323	0.85 ± 0.17
Growth rate	Autotrophy	$1/(kT)^2$	-0.61 ± 0.12	<0.001	-
	Mixo- and heterotrophy	$1/(kT)^2$	-0.16 ± 0.12	0.010	0.54 ± 0.11

In line with the carbon uptake rates, the specific growth rates were much higher during mixotrophic and heterotrophic growth than during autotrophic growth conditions (Fig. 2.4C). Mixotrophic and heterotrophic growth rates were not significantly different from each other (post-hoc comparison using Tukey's HSD test; MSE_{60}= 0.062; p = 0.28), and increased with temperature with an activation energy of 0.54 eV (Fig. 2.4C; Table 2.2). The autotrophic growth rate decreased above 25 °C. Consequently, the relative magnitude of the autotrophic growth rate decreased with temperature, from ~6 % of the mixotrophic and heterotrophic growth rates between 13 and 21 °C to less than 2 % at 33 °C (Fig. 4C).

Discussion

Inspired by the metabolic theory of ecology (Gillooly et al. 2001; Allen et al. 2005), we investigated to what extent mixotrophic organisms would adjust their autotrophic and heterotrophic metabolism in response to rising temperature. Our results illustrate the tremendous nutritional flexibility of mixotrophic organisms, which has also been observed for other mixotrophic flagellates and endosymbiont-bearing protists (Johnson & Stoecker 2005; Hansen 2011). Moreover, our results demonstrate that the relative contribution of autotrophic versus heterotrophic nutrition to the overall metabolism of mixotrophic organisms varies systematically with temperature.

The initial slopes of the light-response curves for photosynthetic electron transport rates and autotrophic growth rates were not significantly affected by temperature (Fig. 2.2A). This is consistent with the general notion that, at low light levels, photosynthetic electron transfer is limited by light availability rather than by temperature (e.g., Blackman 1905). Conversely, under light-saturated conditions, maximum electron transport rates reflect downstream enzymatic reactions involved in carbon fixation, which are temperature sensitive. Indeed, our results show that maximum electron transport rates under light-saturated conditions increased with temperature during autotrophic growth (Fig. 2.2B), with an activation energy of 0.47 eV. We note that this activation energy exceeded the activation energy for carbon fixation of 0.32 eV predicted for terrestrial C_3 plants by Allen et al. (2005), but is in good agreement with activation energies of 0.41-0.74 eV for phytoplankton growth and primary production reported elsewhere in the literature (Eppley 1972; Coles & Jones 2000; Yvon-Durocher et al. 2010; activation energies were estimated from Q_{10}-values according to Vasseur & McCann (2005)).

Grazing rates on bacterial prey during mixotrophic and heterotrophic conditions increased more strongly with temperature, with an activation energy of 0.85 eV, than photosynthetic electron transport rates. This activation energy falls between the activation energies of 0.97 eV (Caron et al. 1986) and 0.48-0.62 eV (Choi & Peters 1992) estimated

for the grazing rate of the heterotrophic chrysophyte *Paraphysomonas imperforata*, and are also well within the range of activation energies reported for the grazing rates of other metazoan taxa (Englund et al. 2011).

During mixotrophic conditions, the estimated contribution of photosynthesis to the total carbon uptake of *Ochromonas* never amounted to more than 8%. This confirms earlier findings that, for *Ochromonas*, heterotrophic growth tends to dominate over autotrophic growth (Andersson et al. 1989; Sanders et al. 2001). The stronger temperature dependence of grazing rates than of photosynthetic rates was not compensated by enhanced investments in photosynthetic pigments at higher temperature. On the contrary, *Ochromonas* reduced its chlorophyll content at high temperature (Fig. 2.3A), which resulted in a reduced potential for photosynthetic carbon fixation (Fig. 2.4A). The combination of a reduced photosynthetic capacity and enhanced grazing rates led to a strong decrease of the relative contribution of photosynthesis to the total carbon uptake rate with rising temperature (a 10-fold reduction from 21 to 33 °C). This pattern is consistent with the growth rate data, which showed that mixotrophic and heterotrophic growth rates increased with temperature, while the autotrophic growth rate decreased above 25 °C (Fig. 2.4A). These findings support the hypothesis that mixotrophs become more heterotrophic at high temperature, at least for the chrysophyte *Ochromonas*.

One might argue that our study is limited to a single mixotrophic species, and it is therefore not clear to what extent our findings can be generalized to other mixotrophs. However, most phototrophic organisms use largely the same photosynthetic machinery and also the biochemical repertoire for heterotrophic growth is rather conserved across a wide range of different species. Hence, there is good reason to expect that other mixotrophic species will also become more heterotrophic with rising temperature. Nevertheless, mixotrophs employ a wide spectrum of nutritional strategies, ranging from largely heterotrophic species like *Ochromonas* to largely autotrophic species like the marine flagellate *Chrysochromulina brevifilum* and freshwater flagellates of the *Dinobryon* genus (Jones 1997). These different mixotrophic species may vary in their ability to regulate the balance between autotrophic and heterotrophic nutrition in response to changing environmental conditions, which might cause differences in their temperature response. It would therefore be interesting to investigate whether other mixotrophic species, particularly those with a stronger autotrophic component or those relying on either endosymbionts or kleptochloroplasts for their photosynthesis, will respond qualitatively similar to rising temperature as *Ochromonas*.

Although our results are largely in agreement with the metabolic theory of ecology, there are some insightful discrepancies. In particular, several studies have suggested that the temperature dependence of population growth rates can be captured by the Arrhenius equation (Brown et al. 2004; Savage et al. 2004). Our results show that this is not the case for autotrophic growth (Fig. 2.2B). Maximum electron transport rates increased with temperature, as described by the Arrhenius equation, but maximum autotrophic growth rates decreased at high temperature. Autotrophic growth depends on the balance between carbon fixation, photorespiration and respiration. Each of these processes show different temperature dependencies (Bernacchi et al. 2001) and autotrophic growth as a whole can therefore not be accurately described by a single Arrhenius equation. In particular, the oxygenase activity of the Rubisco enzyme (i.e., photorespiration) increases with temperature at the expense of its carboxylase activity (Jordan & Ogren 1984; Bernacchi et al. 2001). This increase in photorespiration in combination with enhanced carbon losses by respiration may explain the observed decline of the autotrophic growth rate at high temperature, even for temperatures that are still within the physiological range for all processes. While this growth response should be rather general for autotrophs, it might be more visible in the chrysophyte *Ochromonas*, because the absence of carbon concentrating mechanisms in chrysophytes (Maberly et al. 2009) makes them potentially more sensitive to photorespiration than other species.

A shift towards more heterotrophic nutrition changes the functional role of mixotrophs from primary producers towards consumers. To what extent this will quantitatively affect the ecosystem processes of primary production and ecosystem respiration will ultimately depend on the contribution of mixotrophs to these processes. Due to the technical difficulties of distinguishing mixotrophs from specialist autotrophs in natural ecosystems and measuring carbon fixation separately for both groups, there is very little quantitative information on the contribution of mixotrophs to total primary production. Likewise, there is little information on the relative distribution of autotrophy versus heterotrophy among mixotrophs in natural systems. However, mixotrophic species are known to be widespread in many freshwater and marine ecosystems, where they can dominate the plankton community (Jansson et al. 1996; Burkholder et al. 2008; Flynn et al. 2013; Hartmann et al. 2012). Hence, our results provide reason to expect that a shift towards more heterotrophy with rising temperature may reduce the overall primary production of these ecosystems, while enhancing the grazing pressure on the microbial community.

Mixotrophs compete with autotrophic and heterotrophic species for limiting resources, but may also prey upon them. Hence, mixotrophic species can act as intraguild predators (sensu Polis & Holt 1992). Attacking species from two sides, through both competition and predation, is a very efficient strategy to suppress intraguild prey (Polis & Holt 1992;

Diehl & Feissel 2000). Our results indicate, however, that these food-web interactions may vary with temperature. For instance, our study organism, *Ochromonas*, is an intraguild predator on toxic cyanobacteria (Van Donk et al. 2009; Wilken et al. 2010). Toxic cyanobacterial blooms are of major concern in water management, and are likely to be favored by global warming (Paerl & Huisman 2008; Jöhnk et al. 2008). If mixotrophic species like *Ochromonas* become more heterotrophic in response to global warming, they will shift their trophic position. They will become more effective predators but less effective competitors of other autotrophic species. Whether the net result is an increase or decrease in the efficiency at which mixotrophs can control their intraguild prey (e.g., toxic cyanobacteria) is a highly relevant question that deserves further investigation.

In conclusion, our findings provide experimental support for the novel hypothesis that mixotrophs become more heterotrophic with increasing temperature. Interestingly, this shift in nutritional strategy at the level of individual mixotrophs is based on the same underlying metabolic processes as large-scale observations indicating that the metabolic balance of entire ecosystems may shift from net autotrophy to net heterotrophy with increasing temperature (Lopez-Urrutia et al. 2006; Yvon-Durocher et al. 2010). Given the key position of mixotrophs in plankton communities (Flynn et al. 2013) and the microbial loop (Zubkov & Tarran 2008), changes in their functional role from primary producers to consumers may cascade through food webs, altering species interactions as well as the magnitude and direction of the carbon flux.

Chapter 3

From photoautotrophy to photoheterotrophy: Does the photosynthetic apparatus provide relatively more energy than carbon during mixotrophic growth?

Abstract

Mixotrophy is increasingly recognized as an important and widespread nutritional strategy in various taxonomic groups. We hypothesize that the availability of alternative carbon and energy sources during mixotrophy allows a switch to photoheterotrophic growth, where the photosynthetic apparatus mainly provides energy but not carbon.

Because such a change in the function of the photosynthetic machinery is likely reflected in its composition, we compared the photosynthetic machinery in *Ochromonas danica* Pringsheim during autotrophic and mixotrophic growth.

Compared to autotrophic growth, the total pigmentation of *O. danica* was lowered during mixotrophic growth. Furthermore, the Photosystem I/Photosystem II-ratio increased, and the cellular content of Rubisco decreased not only absolutely, but also relative to the Photosystem II content.

The changing composition of the photosynthetic apparatus indicates a shift in its function from providing both carbon and energy during photoautotrophy, to mainly providing energy during mixotrophy. This preference for photoheterotrophic growth has interesting implications for the contribution of mixotrophs to carbon cycling in aquatic ecosystems.

This chapter is based on the manuscript: Wilken S, Schuurmans JM, and Matthijs HCP. From photoautotrohy to photoheterotrophy: Does the photosynthetic apparatus provide relatively more energy than carbon during mixotrophic growth?

Introduction

All living organisms need carbon to build up their biomass and energy to fuel their metabolism. Accordingly, species can be classified based on the carbon and energy sources they utilize. Autotrophic organisms fix inorganic carbon to build organic biomass, while heterotrophic organisms rely on the uptake of organic carbon from the environment. In terms of energy source, a distinction can be made between phototrophs that utilize light energy and organotrophs that derive their energy from oxidation of organic substances. Most eukaryotes combine either the utilization of light energy with the fixation of inorganic carbon (photoautotrophy) or rely on the uptake of pre-existing organic carbon as source of both carbon and energy (organoheterotrophy). However, in a wide variety of different taxonomic groups there are organisms that can utilize several sources of carbon and energy simultaneously. This mixotrophic nutrition can be found among diverse groups of protists (Pringsheim 1958; Stoecker 1998; Flynn et al. 2013), but also in higher plants (Gebauer & Meyer 2003; Selosse & Roy 2009) or animals that harbor photoautotrophic endosymbionts (Johnson 2011). Especially, in various aquatic ecosystems mixotrophic microalgae capable of ingesting prey items can both dominate bacterivory (Berninger et al.,1992; Unrein et al. 2007; Zubkov & Tarran 2008) and contribute significantly to primary production (Jansson et al. 1996; Olrik 1998). Depending on the balance between photoautotrophy and organoheterotrophy, the impact of mixotrophs on carbon cycling can take opposite directions and hence, understanding the regulation of their nutritional balance is crucial for the understanding of carbon cycling in aquatic ecosystems.

While the utilization of two alternative carbon sources might bring a competitive advantage (Rothhaupt 1996a,b; Tittel et al. 2003; Flöder et al. 2006), autotrophic and heterotrophic growth each require their own biochemical machinery. Especially investments into the photosynthetic machinery are costly and have been estimated to account for up to 50% of the nutrient and energy budget of photoautotrophic organisms (Raven 1997). To reduce these costs mixotrophs can reduce the amount of their photosynthetic machinery when prey is available (Sanders et al. 1990; Pålsson & Daniel 2004). An interesting alternative might be a division of labor between the two nutritional pathways. The combination of photoautotrophic and organoheterotrophic growth provides access to two alternative carbon sources (inorganic versus organic carbon) and two alternative energy sources (light versus organic substrate). This might allow carbon fixation to cease during mixotrophic growth, while the photosynthetic apparatus continues to provide energy. In other words mixotrophs might tend to grow as photoheterotrophs, with light-driven ATP-synthesis substituting respiration. While photoheterotrophy is well known for diverse groups of prokaryotes (Béjà et al. 2001; Kolber et al. 2001; Gómez-Consarnau et al. 2007), this possibility received much less attention in eukaryotic bacterivorous microalgae, where

the contribution of light and prey to growth is usually measured in terms of carbon uptake (Bird & Kalff 1989; Caron et al. 1990; Adolf et al. 2006). During photoheterotrophy, however, the contribution of photosynthetic processes to carbon cycling consists of the preservation of preexisting organic carbon, rather than carbon fixation, and would therefore escape standard measurements.

A shift in the nutritional strategy towards photoheterotrophy is expected to be reflected in a changing composition of the photosynthetic machinery. During photoautotrophic growth, oxygenic photosynthesis uses light energy to oxidize water. The resulting reductive and energetic power is used to fix inorganic carbon. This process is based on several connected reactions that are performed by different macromolecular structures. In the Photosystem II (PSII) light reaction, electrons are derived through the oxidation of water and are transferred via a chain of electron carriers to the reaction center of Photosystem I (PSI). Here, additional photons drive electron transfer to $NADP^+$, producing NADPH as a stock of reductive power. This linear photosynthetic electron transfer is coupled to the formation of a proton motive force across the thylakoid membrane, which can be used to generate ATP as a stock of energy. In this way, the light reaction provides both ATP and NADPH that are subsequently used for the fixation of CO_2 in the dark reaction with involvement of Rubisco. In contrast to photoautotrophic organisms, photoheterotrophs do not require carbon fixation because they already obtain organic carbon. Hence, they would only need to perform PSI-driven cyclic photosynthetic electron transport, as it contributes to proton transport and therefore synthesis of ATP via so-called cyclic photophosphorylation, without creating reductive power. Hence, a shift towards photoheterotrophy would require less of the structures involved in linear electron transfer and carbon fixation, in particular less of PSII and Rubisco. Such adaptations could strongly reduce the costs for maintenance of the photosynthetic machinery.

To test the hypothesis that the photosynthetic machinery will contain relatively less PSII and Rubisco during mixotrophy, we compared the composition of the photosynthetic machinery of the mixotrophic chrysophyte *Ochromonas danica* during photoautotrophic and mixotrophic growth.

Materials and Methods

Strains and culture conditions

Axenic cultures of the mixotrophic chrysophyte *Ochromonas danica* strain SAG 933-7 were grown photoautotrophically on a mineral medium (Wilken et al. 2013) at 21 °C with continuous illumination at 60 µmol quanta m^{-2} s^{-1}. The heterotrophic bacterium

Pseudomonas fluorescens strain Pf 0-1 (Compeau et al. 1988) was used as prey for *O. danica*. Stock cultures of *P. fluorescens* were grown on DSMZ7-medium (1g L^{-1} glucose, 1g L^{-1} peptone, 1g L^{-1} yeast extract) at 21 °C. Prior to use as prey in the experiment, cultures were centrifuged twice, each time followed by resuspension in the mineral medium and then heat killed at 80 °C for 30 min.

Experimental set-up

O. danica was inoculated into 500 mL Erlenmeyer flasks filled with fresh mineral medium to a final abundance of 1.3×10^5 cells mL^{-1} and a culture volume of 150 mL. Mixotrophic cultures were supplied daily with 1.5×10^7 cells mL^{-1} of *P. fluorescens* over a period of 8 days, while autotrophic cultures did not receive any prey. Both treatments were performed in triplicate. Because of their higher growth rates the mixotrophic cultures were diluted to approximately the same cell abundance as the autotrophic cultures on day 4, based on cell counts performed with a CASY-Counter (Schärfe System GmbH, Reutlingen, Germany). On day 8, samples for the photosynthetic parameters were taken and processed as described below.

Cellular abundance and biomass

For measurements of the cellular biovolume fresh samples were counted at a CASY-Counter. For flow-cytometric counts of both bacteria and *O. danica* samples were fixed with a mixture of formaldehyde and glutaraldehyde (final concentration of 0.025 and 0.0037 percent by mass, respectively) and stored at 4 °C. Prior to counting at a flow cytometer (MoFlo XDP Cell Sorter, Beckman Coulter, Miami, FL, USA) samples were stained with PicoGreen (Life Technologies, Paisley, UK) to allow distinction between bacteria and *O. danica*, based on the chlorophyll fluorescence and higher DNA content of the latter. To determine the cellular carbon and nitrogen content cultures were filtered onto pre-combusted glassfibre filters (Whatman GF/F, Maidstone, UK). Filters were dried overnight at 60 °C and the C and N contents were measured with an organic elemental analyzer (Flash 2000, Thermo Fisher Scientific, Waltham, MA, USA). Because this procedure gave the combined carbon and nitrogen content of both *O. danica* and *P. fluorescens*, the bacterial contribution was derived from flow cytometer counts and carbon content measurements of heat killed bacteria, and was subtracted from the total measurement.

Pigment analysis

For pigment analysis cultures were filtered onto glassfibre filters and stored at -20 °C for a maximum of 4 days. Filters were freeze-dried and extracted in 400 µL 90 % acetone and 200 µL ion pair mix containing 7.5 % ammonium acetate and 1.5 % tetrabutyl

ammonium acetate. Pigment concentrations were measured with a Shimadzu Prominence HPLC (Shimadzu, Columbia, MD, USA) equipped with a Hypersil ODS column (Thermo Fisher Scientific, Waltham, MA, USA) and a diode array detector. A gradient of eluent A (50 % methanol, 25 % acetonitrile, 25 % 1M ammonium acetate) and eluent B (20 % methanol, 60 % acetonitrile, 20 % acetone) was used starting with 100 % A for 18 min, followed by 60 % A and 40 % B for 4 min, and 100 % B for 18 min. Pigment standards for calibration were acquired from DHI LAB Products (Hørsholm, Denmark).

Photosynthetic activity

Freshly harvested samples of cultures were incubated in darkness for 10 min and the maximum quantum yield of PSII (ΦPSII) was measured using a Phyto-PAM instrument (Walz, Effeltrich, Germany) following established protocols. Oxygen production and consumption rates were measured in temperature controlled 3 mL respiration chambers using oxygen microsensors (PreSens, Regensburg, Germany). Net oxygen production was measured at a photon flux density of 60 µmol quanta m^{-2} s^{-1} provided by white fluorescent tubes and gross oxygen production was derived by correction for oxygen consumption measured in the dark.

77 K fluorescence spectroscopy

To detect changes in the relative content of PSI and PSII, low temperature fluorescence spectra were recorded at 77 K using an Olis DM45 spectrofluorometer (Olis, Bogart, GA, USA). For this purpose samples were diluted with the mineral growth medium to give similar pigment concentrations based on their chlorophyll absorbance at 680 nm at room temperature. Glycerol was added to the samples to a final concentration of 33 % by volume and samples were frozen in liquid nitrogen. Three emission spectra were recorded and averaged per sample using an excitation wavelength of 439 nm. The resulting spectra contained up to 3 fluorescence peaks and for quantification of peak areas a Gaussian curve was fitted to the data:

$$F(\lambda) = \sum_{i=1}^{3} h_i e^{-0.5\left(\frac{\lambda-\lambda_i}{b_i}\right)} \tag{3.1}$$

where the fluorescence F at the emission wavelength λ is given by the sum of 3 peaks. The shape of peak i is described by its peak wavelength λ_i, and the parameters h_i and b_i determining the height and width of the peak, respectively. The area A_i of peak i is then given by its integral:

$$A_i = \int_{-\infty}^{\infty} h_i e^{-0.5\left(\frac{\lambda-\lambda_i}{b_i}\right)^2} d\lambda = 2h_i b_i \sqrt{\pi} \quad (3.2)$$

and a change in the ratio of PSI to PSII can be quantified by a change in the ratio of their respective peak areas. Note, however, that the ratio of their peak areas does not give the absolute ratio of both photosystems, as they may differ in their fluorescence quantum yield.

Immuno-detection of proteins from PSI, PSII and Rubisco

Semi-quantitative Western blots were based on immuno-identification of the D1 protein of PSII (PsbA), the PsaC subunit of PSI (PsaC), and the large subunit of Rubisco (RbcL). To this end, 2 mL samples were freeze dried and stored at -20 °C prior to dissolving in Laemmli loading dye (Laemmli 1970). Protein samples equivalent to 5.6, 3.7, and 1.9 ng Chl *a* were loaded onto a pre-casted 12 % SDS-urea polyacrylamide gel (Biorad, Hercules, CA, USA) and separated at a constant voltage of 60 V. Proteins were blotted onto a nitrocellulose membrane using Towbin buffer (0.025 M TRIS, 0.192 M glycine, 20 % methanol) and semi-dry transfer (Biorad). PsbA, PsaC, and RbcL were targeted by primary anti-bodies from rabbit (Agrisera, Vännäs, Sweden) and visualized using anti-rabbit IgG horseradish-peroxidase conjugate along with the Amersham ECL Plus chemiluminescence detection kit (Thermo Fisher Scientific). For three biological replicates ran on three independently performed blots, relative band intensities were quantified with the software ImageJ. Band intensities of PsaC and RbcL were normalized to PsbA for semi-quantitative comparison among treatments.

Student's t-tests were performed for pair-wise comparisons between photoautotropic and photoheterotropic treatments. Where necessary, data were log transformed to improve homoscedasticity.

Results

The low autotrophic growth rate of *O. danica* of 0.1 d^{-1} and the much higher mixotrophic growth rate of 0.4 d^{-1} demonstrate the predominantly heterotrophic lifestyle of this species (Table 3.1). While autotrophic and mixotrophic cells did not differ significantly in their cellular biovolume, autotrophic cells had a higher content of both carbon and nitrogen than mixotrophic cells (Table 3.1). During mixotrophic growth the Chl *a* content was decreased by a factor of 2.8 (Fig. 3.1A; Student's t-test: $t_4 = 3.26$, $p = 0.031$). While all pigments decreased in their absolute amounts, the relative pigment composition changed slightly

during mixotrophy compared to photoautotrophic growth, with significantly lower contributions of chlorophyll *c*2 (Fig. 3.1B; Student's t-test: $t_4 = 3.74$, $p = 0.033$) and zeaxanthin (Fig. 3.1B; Student's t-test: $t_4 = 3.28$, $p = 0.030$).

The photosynthetic quantum yield of PSII was relatively low and not significantly different between autotrophic and mixotrophic growth (Fig. 3.2; Student's t-test: $t_4 = 0.45$; $p = 0.68$). In agreement with the low growth rates, the net oxygen production of *O. danica* was close to 0, even during autotrophic growth, but a significant difference could be observed relative to mixotrophic cultures that showed net oxygen consumption in the light (Fig 3.3A). Autotrophic and mixotrophic cells did not differ significantly in their rates of dark respiration (Fig. 3.3B). Gross oxygen production was much higher in autotrophic than in mixotrophic cells (Fig. 3.3C), yet, when normalized to their Chl *a* contents no difference in gross oxygen production was observed between the different nutritional modes (Fig. 3.3D).

Table 3.1: Measurements of growth rates, cellular biovolume, cellular C- and N-content, and C/N-ratios during autotrophic and mixotrophic growth. Given are treatment means and standard errors. Results of Student's t-tests are given for comparison between treatments.

Variable	Unit	autotroph	mixotroph	t_4	p
Growth rate	d^{-1}	0.10 ± 0.02	0.39 ± 0.01	-21.5	<0.0001
Cellular biovolume	μm^3 $cell^{-1}$	207 ± 30	214 ± 39	-0.26	0.8081
Cellular C-content	pmol $cell^{-1}$	4.03 ± 0.30	2.02 ± 0.32	7.94	0.0014
Cellular N-content	pmol $cell^{-1}$	0.30 ± 0.04	0.12 ± 0.06	4.41	0.0116
C/N-ratio		13.4 ± 1.2	19.5 ± 9.9	-1.05	0.3516

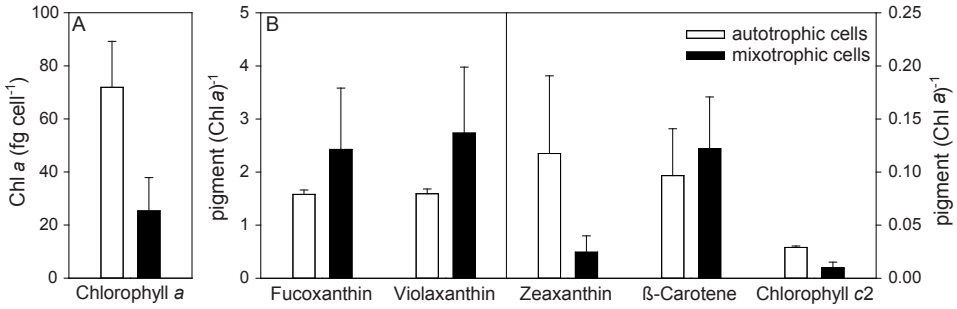

Figure 3.1: Cellular Chl *a* content (A) and ratio of accessory pigments to Chl *a* (B) of *O. danica* during autotrophic and mixotrophic growth. Error bars represent one standard deviation.

The 77K fluorescence spectra reflected appreciable differences between samples from autotrophic and mixotrophic cultures (Fig. 3.4). While autotrophic cells showed a single peak at 674 nm, mixotrophic cells showed two clearly distinct peaks at 670 nm and 684 nm. The 670 nm to 674 nm fluorescence is attributed to antenna fluorescence and the 684 nm fluorescence is emitted by the PSII reaction center. Antenna and reaction centers are closely connected in autotrophic cells, but seem to be disconnected in mixotrophic cells, causing both structures to appear in slightly separate peaks. The peak at 707.7 nm represents the fluorescence from PSI, as was confirmed by its absence from spectra recorded at room temperature (data not shown). This peak was larger in the mixotrophic cultures, while it was only represented as a shoulder in autotrophic cells, suggesting that mixotrophic cells contain relatively more PSI. When including both peaks in the 670 to 685nm wavelength

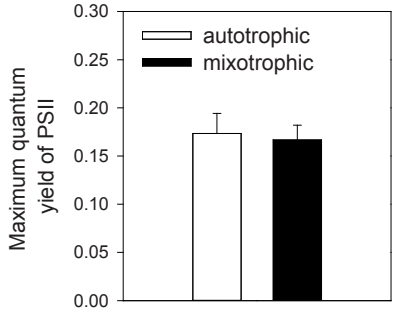

Figure 3.2: Maximum quantum yield of photosystem II during autotrophic and mixotrophic growth of *O. danica*. Error bars indicate one standard deviation.

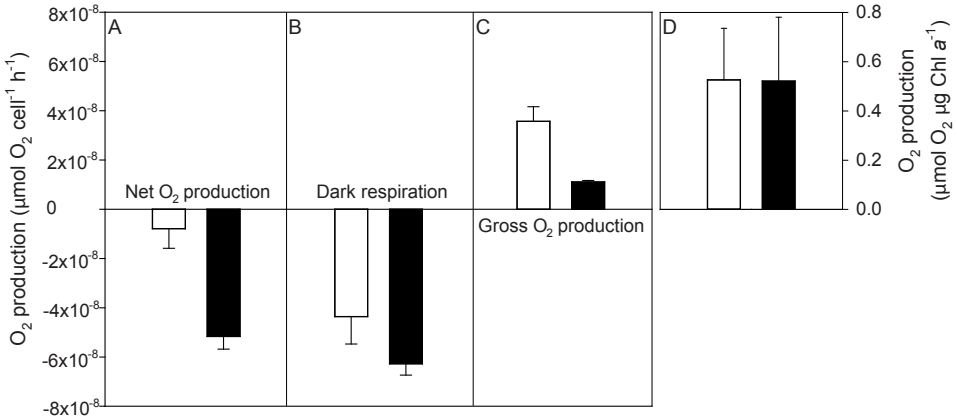

Figure 3.3: Net cellular oxygen evolution in the light (A), oxygen consumption in the dark (B), gross oxygen evolution (C), and gross oxygen evolution normalized to Chl *a* (D) in *O. danica* grown photoautotrophically and mixotrophically. Error bars represent one standard deviation. Results of Student's t-tests for comparison between treatments are given.

domain into the fraction representing PSII, an increase of the PSI/PSII-ratio by a factor of 1.96 (± 0.59 SD) during mixotrophic growth relative to autotrophic growth was calculated. However, it is noted that several secondary effects such as a weaker connection of the antenna to PSII and state transitions (Gibbs & Biggins 1989) might change the strength of the fluorescence signal. For this reason, quantitative comparison must be considered with care and a completely different technique was used to corroborate the results derived by fluorescence measurements.

Proteins of cell pellets from the two different culture conditions were separated by SDS polyacrylamide gel electrophoresis, followed by Western blotting and detection of proteins with specific antibodies. These results quite nicely confirmed a higher content of PsaC relative to PsbA, indicative of a higher PSI/PSII ratio, in mixotrophic cells (Fig. 3.5). Furthermore, a prominently lower RbcL/PsbA ratio, indicative of a lower Rubisco content relative to PSII, was observed for the mixotrophic cultures (Fig. 3.5). Image analysis of the Western blot results confirmed the visual impression of the results on each separate blot. The data indicated an increase in the PSI/PSII-ratio of 73% (± 28% SD) and a decrease in the Rubisco/PSII ratio by 43% (± 35% SD) during mixotrophic compared to photoautotrophic growth.

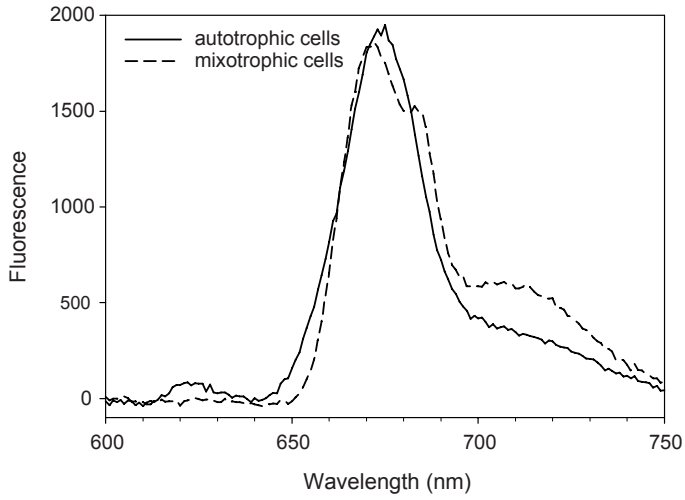

Figure 3.4: Fluorescence spectra of photoautotrophically and mixotrophically grown cells of *O. danica* recorded at 77 K with an excitation wavelength of 439 nm. Shown are averages of spectra from 3 replicates per nutritional treatment.

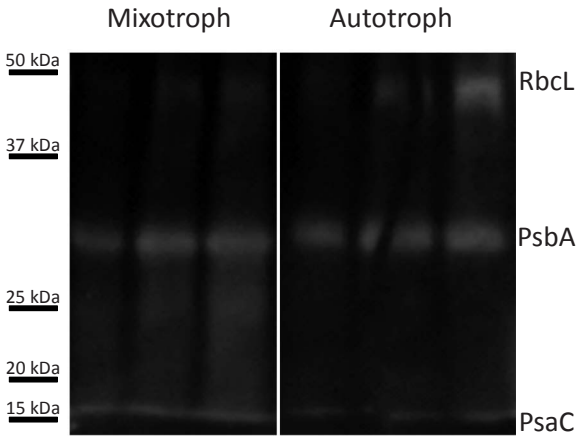

Figure 3.5: Western blot of PsaC, PsbA and RbcL in autotrophic and mixotrophic cultures. The 3 lanes per treatment represent different protein loadings equivalent to 1.9, 3.7, and 5.6 ng Chl *a*.

Discussion

We hypothesized that mixotrophic organisms might tend towards photoheterotrophic growth, primarily utilizing light as their source of energy and organic carbon as their source of carbon. The observed changes in the amount and composition of the photosynthetic apparatus in *O. danica* during mixotrophic growth generally support this hypothesis.

To illustrate the consequences of the modifications to the photosynthetic apparatus the potential integration of photoautotrophic and heterotrophic processes in mixotrophs are depicted in a putative metabolic map (Fig. 3.6). The ingestion of prey and assimilation of organic carbon allows down-regulation of carbon fixation in the Calvin-Benson-Cycle (Wan et al. 2011) and therefore also a reduction in the content of biochemical structures involved in the light reaction. In the primarily heterotrophic mixotroph *O. danica* this was reflected in a reduced pigment content during mixotrophic growth, in agreement with earlier studies (Sanders et al. 1990; Adolf et al. 2006; Wilken et al. 2013). The lower cellular C and N content during mixotrophic growth might be a consequence thereof, as the thylakoid membranes of phototrophs are considered to feature amongst the most protein-rich membranes and a reduced amount of thylakoid membranes therefore results in a lower cellular protein content. The lower photosynthetic capacity resulted in a reduced gross oxygen production per cell compared to autotrophic growth, and a rather low contribution of photosynthesis to the 4 times faster mixotrophic than autotrophic growth. The constant photosynthetic yield of PSII, and identical net oxygen production normalized to Chl *a* demonstrate that the photosynthetic machinery remains fully functional also during mixotrophy.

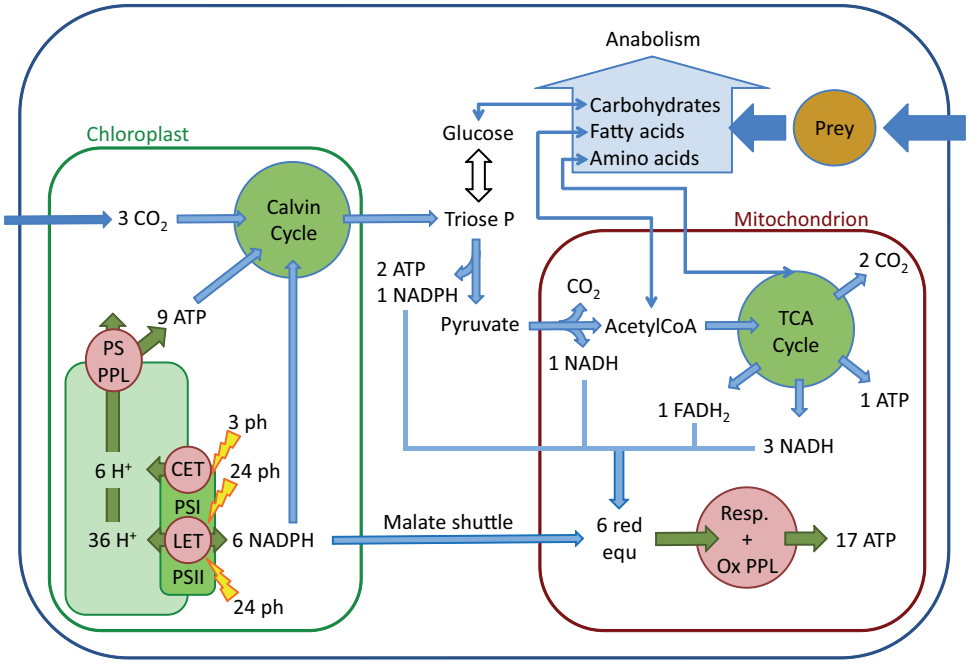

Figure 3.6: Putative integration of photosynthesis and respiration in mixotrophs. The numbers give the stoichiometry of the pathways. Green arrows represent cross-membrane transport of electrons and protons, while blue arrows represent metabolic pathways. There still is debate about the exact stoichiometry of protons required for photosynthetic phosphorylation (ATP synthesis). We used the ratio of H+/ ATP = 14/3 based on the stoichiometry acquired for spinach ATPase (Seelert et al. 2000). Abbreviations: CET, cyclic electron transport; LET, linear electron transport; ph, photon; PS PPL, photosynthetic phosphorylation; Ox PPL, oxidative phosphorylation; Resp., respiration.

In addition to the reduced total pigment content we observed changes in pigment composition and modifications of the photosynthetic apparatus during mixotrophic growth. In particular, the higher content of PSI relative to PSII during mixotrophic growth results in a higher capacity for PSI driven cyclic photophosphorylation compared to the potential for linear electron transport and NADPH production. Hence, our data suggest a tendency towards photoheterotrophy, with the photosynthetic apparatus primarily providing energy in the form of ATP. An increase in PSI/PSII-ratio has been reported during photoheterotrophic growth of the cyanobacterium *Synechocystis* (Bernát et al. 2009) and during growth on acetate in *Chlamydobotrys stellata*, where it was explained by the need to sustain ATP-consumption during acetate metabolism in the glyoxylate cycle (Kovacs et al. 2000). However, our results indicate that it might be a general response during mixotrophic growth that is also observed during utilization of the carbon from bacterial prey representing a complex and natural mixture of different organic compounds.

Interestingly, we observed an even stronger decrease in Rubisco than in PSII content during mixotrophic growth, and hence potentially lower capacity for carbon fixation relative to the supply of reductive power via linear photosynthetic electron transfer. To avoid damage of the photosynthetic machinery due to over-reduction, either the capacity for linear electron transport needs to be reduced or alternative sinks of NADPH need to become more important. The lower Chl *c* content of mixotrophic cells and the relatively lower fluorescence emission in the 670 to 674 nm domain of 77 K spectra indicate a decrease in antenna content. This results in a lower functional capacity of PSII and thereby decreased rates of linear electron transfer during mixotrophic growth. In addition, reductant may be exported from the chloroplast via the malate shuttle (Fig. 3.6; Scheibe 2004; Allen et al. 2008) and might be used in processes involved in organotrophic carbon assimilation. Furthermore, this route could allow the use of photosynthetically derived reductants for oxidative phosphorylation in the mitochondrion and thus represents a second route of photosynthetic ATP-production in addition to cyclic photophosphorylation (Scheibe 2004). While our data do not allow any conclusion about the importance of this route, the early work of Weis & Brown (1959) suggested a competition of photosynthetic reductant with reductant derived from organic substrates in *Poterioochromonas malhamensis* and a use of both in oxidative phosphorylation and ATP-generation. It is noticed that the malate shortcut is furthermore a more efficient route of ATP-production than the simultaneous performance of carbon fixation and respiration, as it prevents the loss of ATPs that are required for carbon fixation and that cannot be recovered during the breakdown of triose phosphate (Fig. 3.6).

Photosynthetic ATP-production might allow mixotrophs to down-regulate rates of respiration via the tricarboxylic acid (TCA) cycle, as also reported for higher plants (Tcherkez et al. 2012). Furthermore, the importance of the TCA cycle for nitrogen assimilation via the glutamine synthetase - glutamate synthase pathway will be lower during mixotrophic than during autotrophic growth, as amino acids can be derived from the prey. Down regulation of both carbon fixation and the TCA-cycle thus results in a preferential use of organic material from the prey as source of fixed carbon and light as energy source, and hence, results factually in photoheterotrophy. Such a nutritional strategy does not require the complete enzymatic machineries for both nutritional pathways, and thereby saves valuable resources for growth. One might speculate, for instance, that the absence of carbon concentrating mechanisms in chrysophytes (Maberly et al. 2009) is related to the preferential use of organic carbon sources. In contrast to the general assumption that mixotrophs face extra costs for the maintenance of two nutritional pathways, the combination of phototrophy

and heterotrophy could even reduce the overall investment required for the enzymatic machineries. This could result in a highly economic metabolism, which is especially advantageous in oligotrophic ecosystems.

While the distinction between mixotrophy and photoheterotrophy might seem meticulous at first, the consequences for our understanding of the carbon cycle can be tremendous. Primary production is usually assessed by measurements of carbon fixation and strict photoheterotrophs do not contribute to this process. Nevertheless, the utilization of light energy substantially enhances their growth rates by increasing assimilation efficiencies of organic carbon and the contribution of light energy to microbial growth could therefore be strongly underestimated when the role of light is only measured in conjunction with carbon fixation. This potential bias is known for photoheterotrophic prokaryotes (Kolber et al. 2001; Zehr et al. 2008; Zubkov 2009), but could be equally severe in some eukaryotes. Given the occurrence of mixotrophy in photosynthetic eukaryotes ranging from diverse microalgae to higher plants, a potential preference for photoheterotrophic growth in mixotrophs could affect carbon cycling in many ecosystems. Especially in aquatic habitats, mixotrophic nutrition is increasingly recognized as a widespread and quantitatively important strategy of nutrient acquisition in oligotrophic environments (Jansson et al. 1996; Hartmann et al. 2012). The present study points at the potential importance of photoheterotrophy in eukaryotic mixotrophs and highlights the need for quantitative measurements of both carbon and energy acquisition by mixotrophs in future research.

Chapter 4

The impact of a mixotrophic chrysophyte on toxic and colony-forming cyanobacteria

Summary

In order to test the effect of *Ochromonas* sp., a mixotrophic chrysophyte, on cyanobacteria, grazing experiments were performed under controlled conditions. We studied grazing on three *Microcystis aeruginosa* strains, varying in toxicity and morphology, as well as on one filamentous cyanobacterium, *Pseudanabaena* sp. Furthermore, we analysed the co-occurrence of *Ochromonas* and *Microcystis* in natural systems in relation to various environmental parameters (TP, TN, DOC, temperature, pH), using data from 460 Norwegian lakes. *Ochromonas* was able to feed on all four cyanobacterial strains tested, and grew quickly on all of them. The chrysophyte caused net growth reductions in all three *Microcystis* strains. The effect of *Ochromonas* was strongest on the Spring CJ strain. Although the effect of *Ochromonas* grazing on the growth of *Pseudanabaena* was relatively smaller, it also reduced the net growth of this cyanobacterium significantly. After 4 days of incubation with *Ochromonas* the total amount of cyanotoxins in the three *Microcystis* strains was reduced by 91.1–98.7% compared with the controls. *Ochromonas* occurred in similar densities across all 460 Norwegian lakes. *Microcystis* occurred only at higher TN, TP, temperature and pH values, although its density was often several orders of magnitude higher than that of *Ochromonas*. *Ochromonas* co-occurred in 94% of the samples in which *Microcystis* was present. From our study it is not clear whether *Ochromonas* could control *Microcystis* blooms in natural lakes. However, our study does demonstrate that *Ochromonas* usually occurs in lakes with *Microcystis*, and our small scale experiments show that *Ochromonas* can strongly reduce the biomass of *Microcystis* and its toxin content.

This chapter is based on the paper: Van Donk E, Cerbin S, Wilken S, Helmsing NR, Ptacnik R, and Verschoor AM (2009) The effect of a mixotrophic chrysophyte on toxic and colony-forming cyanobacteria. Freshwater Biology 54: 1843-1855.

Introduction

Cyanobacteria have increasingly become a nuisance in aquatic ecosystems due to bloom formation and toxin production (Chorus & Bartram 1999). They dominate many aquatic ecosystems due to increases in nutrient loading, water temperatures, duration of summer stratification, increased water residence time, and salination (Paerl & Huisman 2008). Cyanobacteria may cause mass mortality of aquatic organisms in lakes (e.g. Krienitz et al. 2003) and also human illness and even death have been linked to toxic cyanobacteria (Carmichael et al. 2001). One of the most notorious freshwater cyanobacteria that may form dense surface scums in temperate regions during late summer is the colony-forming species *Microcystis aeruginosa*.

The first and most crucial step in mitigating cyanobacterial blooms is to decrease the nutrient load to the ecosystems. An additional measure may be to promote the growth of filter-feeders by food-web manipulation, a strategy known as biomanipulation (Shapiro & Wright 1984; Gulati & van Donk 2002). In earlier studies, the emphasis of biomanipulation lay on taking measures that lead to an increase of the biomass of large cladocerans like daphnids (Shapiro & Wright 1984). However, cyanobacteria may be difficult for cladocerans to ingest, since their size and shape interfere with the filtering system of cladocerans (Lampert 1987). Also the production of microcystins or other cyanotoxins by cyanobacteria contributes to their inedibility (Rohrlack et al. 1999b; Ghadouani et al. 2004; Hansson et al. 2007). Furthermore, cyanobacteria may lack long-chained polyunsaturated fatty acids, which are essential for cladocerans (Müller-Navarra et al. 2000; Wacker & von Elert 2001).

Cole & Wynne (1974) were among the first to report the feeding of *Ochromonas danica*, a mixotrophic chrysophyte, on single-celled *Microcystis aeruginosa*. They suggested that *Ochromonas* might serve as a possible biological control agent against *Microcystis* blooms. Recently, we observed a negative effect of *Ochromonas* sp. on a bloom of colony-forming *M. aeruginosa*. *Ochromonas* invaded a preliminary experiment on scum-layer formation of *M. aeruginosa* in large-scale laboratory mesocosms.

Although mixotrophy among planktonic nanoflagellates has been well documented in the literature (Sanders 1991; Isaksson 1998; Jones 2000), most studies have been conducted in relation to heterotrophic bacteria. Despite several studies that showed the ability of mixotrophic chrysophytes to graze on various phytoplankton species (Cole & Wynne 1974; Zhang et al. 1996; Zhang & Watanabe 2001; Zhang et al. 2008), very little attention has been given to the ecological importance of these organisms as primary consumers in aquatic ecosystems (but see: Tittel et al. 2003; Ptacnik et al. 2004).

The genus *Ochromonas* has been reported from a broad range of habitats in both marine and freshwaters (Sanders 1991). It has been successfully cultured under autotrophic, mixotrophic and heterotrophic conditions (Rothhaupt 1996b; Sanders et al. 2001). This flexibility in metabolic modes has been considered a successful evolutionary strategy to overcome stress situations, like low nutrient concentrations (Stibor & Sommer 2003) or low light conditions (e.g. Holen 1999; Jones 2000; Flöder et al. 2006). Under such conditions mixotrophy may offer a competitive advantage over specialist autotrophy on one hand and specialist heterotrophy on the other hand (Katechakis & Stibor 2006).

Based on these studies, and our own mesocosm observations, we conducted laboratory experiments to analyze the ability of *Ochromonas* to feed on different strains of cyanobacteria, varying in toxicity and the degree of colony formation. Further, very little is known about the natural occurrence of *Ochromonas* in eutrophic lakes, its effects on cyanobacterial blooms and the mechanisms controlling its abundance and grazing rates. To see whether there is a possibility for the interaction between these two organisms to play a role in natural systems, we also analysed data from 460 lakes in Norway. *Microcystis* and *Ochromonas* are both common in Scandinavian lakes, making the latter suitable field sites for the study of the coexistence of *Ochromonas* and *Microcystis* under a wide range of environmental conditions.

Materials and Methods

Grazing experiment

Phytoplankton pre-cultures

Four cyanobacteria strains were used in the grazing experiment (Table 4.1). We selected three *Microcystis aeruginosa* strains that varied in toxicity and morphology; (i) the very toxic single-celled strain PCC 7806; (ii) the less toxic colony-forming Bear AC and (iii) the less toxic single-celled Spring CJ. We also used in the grazing experiment a filamentous cyanobacterium, *Pseudanabaena* sp. CCY 9704, which has relatively long filaments and low toxin content. The mixotrophic chrysophyte *Ochromonas* sp. (probably *Ochromonas globosa* Skuja, determined by Dr. R. Bijkerk, Koeman & Bijkerk B.V., Ecological Research and Advice, Haren, The Netherlands) has been isolated from the mesocosms, in which it occured as a contaminant, using glass micropipettes. Stock cultures were maintained autotrophically in COMBO-medium using ammonia as a nitrogen-source. All species were pre-cultured under experimental conditions in Erlenmeyer flasks containing 200 mL of COMBO medium.

Table 4.1: Cyanobacteria strains used in the grazing experiments. The mean particle volumes (MPV) of the strains are given in μm^3, filament length of *Pseudanabaena* is in µm (average filament diameter 1.2 µm). The microcystin content of the species is given in amount per biovolume ($\mu g\ \mu m^{-3}$).

Phytoplankton strain	**MPV** (μm^3)		**Microcystin content** ($\mu g\ \mu m^{-3}$)	
	Mean	SD	Mean	SD
M. aeruginosa PCC 7806a	8.48	6.87	4.12×10^{-6}	9.81×10^{-7}
M. aeruginosa Spring CJb	153	59.9	6.06×10^{-7}	6.09×10^{-8}
M. aeruginosa Bear ACb	810	425	9.81×10^{-7}	5.58×10^{-7}
	Filament length (µm)			
Pseudanabaena sp. CCY 9704c	78.1	98.0	7.89×10^{-8}	9.11×10^{-9}

Experimental conditions

The grazing experiment was performed in 250 mL Erlenmeyer flasks filled with 50 mL of cyanobacteria, growing exponentially on COMBO medium. The *Ochromonas* treatment flasks were then inoculated with 50 mL of *Ochromonas* culture, while the controls received another 50 mL of COMBO medium. Initial nutrient concentrations in both the controls and treatments were high, supporting the growth of cyanobacteria without nutrient limitation.

The initial biovolume of the cyanobacteria was ca. $1.5\times10^7\ \mu m^3\ mL^{-1}$ and of *Ochromonas* ca. $2.4\times10^6\ \mu m^3\ mL^{-1}$ (1.33×10^4 cells mL^{-1}). Both the treatments and controls were performed in triplicates. The flasks were placed in an incubator on a rotating table (50 rpm) for four days with a 14 h: 10 h light: dark cycle, a light intensity of 125 µmol quanta $m^{-2}\ s^{-1}$ and a temperature of 25 °C.

To further investigate whether *Ochromonas* could have an impact on *Microcystis* cell yield and microcystin concentrations at densities as found in the Norwegian lakes, we did a second grazing experiment with *Ochromonas* grazing on strain PCC 7806. For this experiment we inoculated an initial biovolume of $6.2\times10^6\ \mu m^3\ mL^{-1}$ of *Microcystis* and $3.2\times10^4\ \mu m^3\ mL^{-1}$ of *Ochromonas* into 500 mL Erlenmeyer flasks filled with 300 mL COMBO-medium and a control with *Microcystis* only. Both treatments were done in four replicates. The flasks were incubated at 23 °C under 14 h: 10 h light: dark cycle with a light intensity of 90 µmol quanta $m^{-2}\ s^{-1}$. Cell densities were determined using a CytoSense flow cytometer (CytoBuoy BV, Woerden, The Netherlands). The remaining settings were as described for the previous experiment.

Sampling and measurements

Samples were fixed with glutaraldehyde and formaldehyde, final concentrations being 0.025 mass % and 0.0037 mass % respectively. The changes in cell numbers and biovolumes of the cyanobacteria and *Ochromonas* were determined daily using a Flow Cytometer (MoFlo XDP Cell Sorter, Beckman Coulter, Miami, FL, USA). From these measurements we calculated the growth rates of the cyanobacteria and *Ochromonas* in the controls and the treatment flasks over a period of four days. Daily specific growth rates (μ) for the 4-day period were calculated for each flask as:

$$\mu = \frac{\ln(N_t) - \ln(N_0)}{t} \tag{4.1}$$

where N_0 and N_t are the cell densities at the start and the end. The net growth reductions of the cyanobacteria, due to *Ochromonas* grazing, were calculated from the differences in growth rates between controls and *Ochromonas* treatments.

Changes in size distribution of the cyanobacteria, due to grazing by *Ochromonas*, were studied using an image analyser connected to an inverted microscope measuring the greatest linear dimension of 100 cells or colonies in both controls and treatments flasks at the end of the grazing experiment.

Microcystin analyses

The effect of *Ochromonas* on the microcystin concentrations (both dissolved and particulate microcystin) in the treatment as well as in the control flasks was measured at the end of the grazing experiments (day 4). For measuring the microcystin content of the four different cyanobacteria strains, samples of the cultures were filtered through glass fiber filters (Whatman GF/F; pressure 10 kPa) to separate the cyanobacteria cells from the medium. The remaining filtrate was used for analysis of extracellular microcystins. The GF/F filters were freeze-dried and cell-bound microcystin on these filters was extracted in 75 % methanol, using three extraction rounds as described in Fastner et al. (1998) with an extra step for grinding of the filters in a Mini Bead beater (BioSpec Products Inc., Bartlesville, Oklahoma, USA) with 0.5 mm silica beads (Tonk et al. 2005). The extracts were blow-dried with N_2 at 40 °C and then redissolved in 50 % methanol for analysis of microcystin using an HPLC with photodiode array detection (Kontron Instruments Ltd., Bletchley, UK). Successively the extracts were separated using a LiChrospher 100 ODS 5 μm LiChorCART 250-4 cartridge system (Merck, Darmstadt, Germany) and 30 to 70 % gradient of acetonitrile with 0.05 % trifluoroacetic acid at a flow rate of 1 mL min^{-1}. The different microcystin variants were identified on the basis of their characteristic UV spectra and were quantified by means of microcystin-LR and microcystin-RR gravimetric

standards (provided by Prof. G.A. Codd, University of Dundee, UK). The concentration of extracellular microcystins and the microcystin concentration in the second grazing experiment, with densities of *Ochromonas* as found in the Norwegian lakes, were detected and quantified using an enzyme-linked immunosorbent assay (ELISA) according to the protocol of a Microcystin Plate Kit (SDI EnviroGard®, Portland, Maine, USA). To extract the cellular microcystins for ELISA-quantification, cells were subjected to three freeze-and-thaw cycles each followed by sonification (Gustafsson et al. 2005).

Statistical analyses

Most statistical analyses were carried out with the STATISTICA data analysis software system (StatSoft, Inc., 2007, version 8.0. www.statsoft.com). Data were first tested for homoscedasticity (Levene's test for ANOVA). Net growth reductions and growth rates of *Ochromonas* on different cyanobacteria were compared by one-way ANOVA, followed by post hoc comparisons (Tukey HSD test). Because of their heteroscedasticity, the data of the final biovolumes and cyanobacteria sizes were analyzed using the nonparametric Mann-Whitney U-test. The data on microcystin concentrations (particulate, dissolved and total) were also heteroscedastic, even after transformation. Therefore, the interactive effects of strain identity and *Ochromonas* treatment on microcystin concentrations were analyzed manually using a spreadsheet to perform the Scheirer-Ray-Hare extension of the Kruskal–Wallis test (Sokal & Rohlf 1995), followed by pair-wise comparisons within strains using the Mann-Whitney U-test.

Field data analysis

For the analysis of field data, we used data from a total of 1395 phytoplankton summer samples collected in 460 Norwegian lakes between 1988 to 2000 (Moe et al. 2008). Phytoplankton composition and biovolumes were analyzed by light microscopy and physical and chemical parameters by standard methods (Moe et al. 2008). Data on water temperature were not available, thus we derived estimates of local air temperature from a grid of long-year averages (Mitchell & Jones 2005). We compared the biovolume concentrations of *Microcystis* and *Ochromonas* along several environmental gradients: total phosphorus (TP), total nitrogen (TN), TN: TP ratio, temperature, dissolved organic carbon (DOC: colour/ mg Pt L^{-1} was used as measure for the DOC concentration), pH and total phytoplankton biovolume concentration.

Results

Grazing experiment

Ochromonas caused a net biovolume reduction within all strains (all strains: Mann-Whitney U-test: $Z_{adj} = 1.964$, $p = 0.0495$, $n = 2\times3$; Fig 4.1A). Within the different *Microcystis* strains this reduction was strongest for the less toxic single-celled strain (Spring CJ: 99.8%), whereas the influence on the other two strains of *Microcystis*, i.e. the more toxic (PCC 7806) and the one building colonies (Bear AC) was less strong (98.6% resp. 96.5%). The effect of *Ochromonas* on the biovolume of *Pseudanabaena* was relatively weaker, although still substantial (92.2%).

The net growth reductions were significantly different among the different strains (one-way ANOVA, $F_{2,9} = 29.397$, $p = 0.0001$; Fig. 4.1B). The highest net growth reduction was found for the less toxic single-celled strain *M. aeruginosa* Spring CJ ($\Delta\mu = 1.71 \pm 0.22$ d^{-1}), followed by the very toxic single-celled *Microcystis* PCC 7806 ($\Delta\mu = 1.07 \pm 0.11$ d^{-1}), the colony-forming *Microcystis* Bear AC ($\Delta\mu = 0.834 \pm 0.11$ d^{-1}), and finally *Pseudanabaena* ($\Delta\mu = 0.648 \pm 0.12$ d^{-1}). The growth rates of *Ochromonas*, feeding on different cyanobacterial strains, varied between 0.19 ± 0.025 d^{-1} and 0.47 ± 0.012 d^{-1}. The growth rate was significantly lower on *Pseudanabaena* (one-way ANOVA, $F_{2,9} = 10.218$, $p = 0.0041$; Fig. 4.1C) .

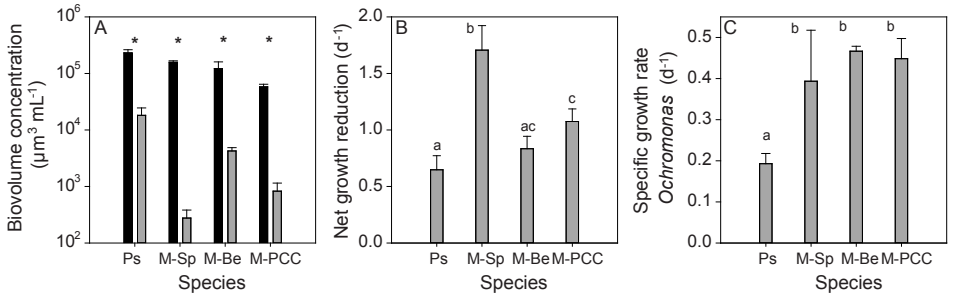

Figure 4.1: Growth parameters of the phytoplankton species used in the grazing experiments. A: Biovolume concentrations of the different cyanobacteria on day 4, in the absence (black bars) or presence (grey bars) of *Ochromonas*. Asterisks indicate significant differences between *Ochromonas* treatments and controls within the different strains. Note the logarithmic ordinate. B: net growth reduction of the different cyanobacteria due to the presence of *Ochromonas*. C: growth rate of *Ochromonas* when cultured with different cyanobacteria. Symbols (a,b,c) indicates homogeneous groups, i.e. groups that do not differ significantly after nonparametric multiple comparisons. Cyanobacteria strain codes: Ps = *Pseudanabaena* sp. CCY 9704; M-Sp = *Microcystis aeruginosa*, Spring CJ; M-Be = *M. aeruginosa*, Bear AC; M-PCC = *M. aeruginosa*, PCC 7806. Error bars indicate ± 1SD.

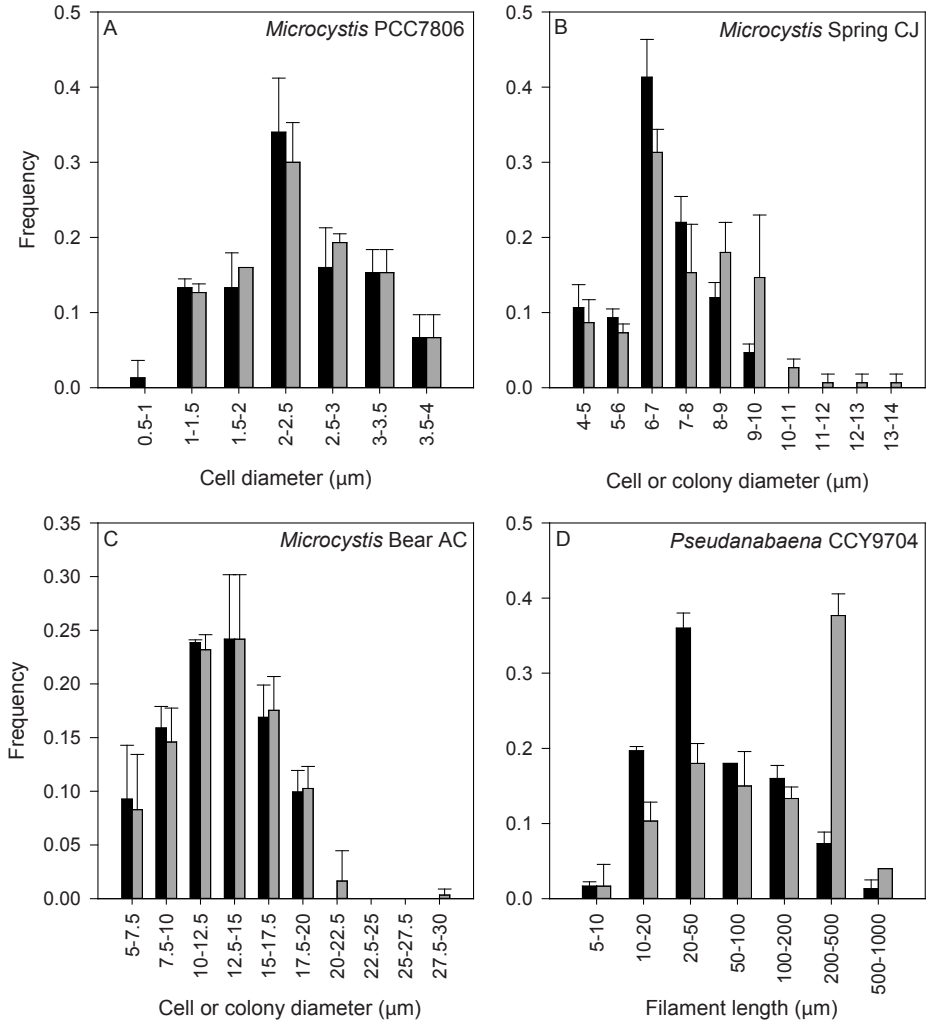

Figure 4.2: Effect of grazing by *Ochromonas* on the size distributions of the different cyanobacteria used in the grazing experiment. To compare the different shapes, we used the greatest linear dimension of each species, i.e. greatest cell or colony diameter for *Microcystis*, and *Pseudanabaena*. Black bars depict the fraction of the control population in a particular size class on day 4, whereas the grey bars depict the fraction of the population exposed to *Ochromonas* in that particular size class on day 4. Bars give averages over 3 replicates, error bars indicate ± 1SD.

The size distribution of the small *Microcystis* PCC7806 (2.35 µm mean cell diameter; Fig. 4.2) did not differ significantly between both treatments. However, for the larger *Microcystis* species and for *Pseudanabaena*, the mean greatest linear dimensions of the cells and colonies increased in the *Ochromonas* treatment (Fig. 4.2). In the presence of *Ochromonas*, for the average-sized *Microcystis* Spring CJ, the mean particle diameter changed from 6.7 to 7.4 µm (Mann-Whitney U- test: $U = 8736.5$, $p = 0.0008$, $n = 2\times150$), and for the larger *Microcystis* Bear AC, from 12.7 to 13.2 µm ($U = 16862.5$, $p = 0.0026$, $n = 2\times202$). For *Pseudanabaena* the difference was the largest, with the mean filament length increasing from 78.1 µm to 192.8 µm in the presence of *Ochromonas* ($U = 26574.5$, $p < 0.0001$, $n = 2\times300$). Visual observations showed that *Ochromonas* seemed to prefer the more readily ingestible smaller filaments, even though it was also attacking the ends of larger filaments.

For all *Microcystis* strains, we observed a dramatic decrease in total microcystin concentrations in the presence of *Ochromonas* (Fig. 4.3). We did not find differences between strains, but did find a significant *Ochromonas* treatment effect on total microcystin concentrations (Scheirer-Ray-Hare extension of Kruskal-Wallis ANOVA, $H_{1,24} = 12.40$, $p < 0.001$). This was mainly due to the cell-bound (particular) microcystins, which decreased significantly in concentration due to *Ochromonas* grazing ($H_{1,24} = 14.52$, $p < 0.001$). Total and particulate microcystin concentrations in the treatments with the weakly toxic *Pseudanabaena* did not decrease significantly. However, for all *Microcystis* strains,

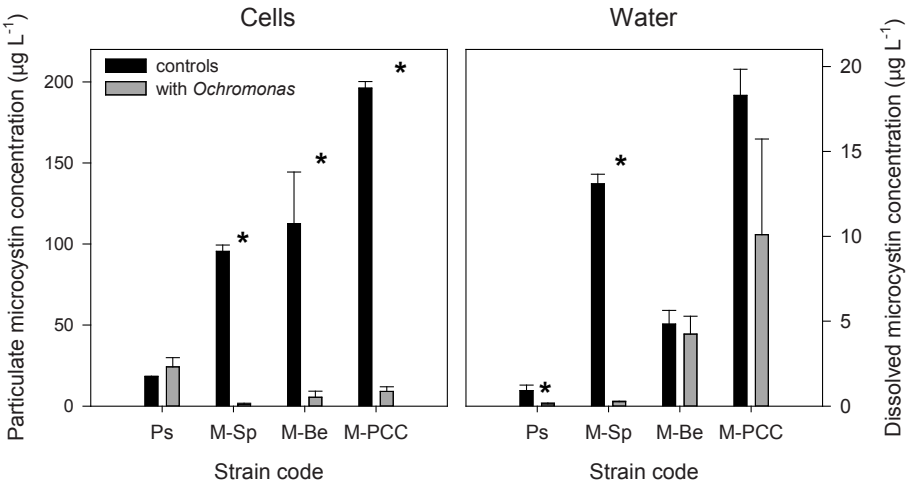

Figure 4.3: Average total microcystin concentrations measured in treatments without (black bars) and with *Ochromonas* (grey bars). Left panel: particulate microcystin concentrations; right panel: dissolved microcystin concentrations. Error bars indicate ± 1SE. Asterisks indicate significancy within-strain differences. Strain codes are as in Fig. 4.1. Note the difference in scales.

particulate microcystin concentrations decreased significantly due to *Ochromonas* grazing (all *Microcystis* strains: Mann-Whitney U-test: $Z_{adj} = 1.964$, $p = 0.0495$, $n = 2\times3$). For the dissolved microcystins we also found a significant strain effect ($H_{3,24} = 13.07$, $p = 0.0045$) and a significant treatment effect ($H_{1,24} = 4.81$, $p = 0.028$), but no significant interaction.

In the second grazing experiment with abundances of *Ochromonas* as found in the Norwegian lakes there was still a significant effect of *Ochromonas* on *Microcystis* biovolume concentration on day 4 ($t = 26.53$, $df = 6$, $p < 0.0001$) and microcystin concentration ($t = 8.42$, $df = 6$, $p < 0.001$). While the net growth reduction of *Microcystis* due to *Ochromonas* was 0.28 d^{-1}, the total microcystin concentration had been reduced by 42 % compared to that in the control on day 4 (Fig. 4.4).

Field data analysis

Ochromonas occurred in 1377 out of 1395 lake samples (99%). *Microcystis* was found in 97 samples (7%), of which 91 also contained *Ochromonas* (94% of the *Microcystis* locations). *Ochromonas* occurs in almost all Norwegian lakes, while the distribution of *Microcystis* is restricted to lowland lakes in the more densely populated southern Norway, where nutrients, temperature and pH are generally higher than in northern and central Norway (Fig. 4.5). *Ochromonas* had more or less similar biovolume concentrations (within one order of magnitude) across a wide range of nutrient concentrations (phosphorus, nitrogen) and ratios (N:P), temperature, dissolved organic carbon and pH (Fig. 4.5). In contrast, *Microcystis* biovolumes varied over 5 orders of magnitude, having higher abundances at higher nutrient concentrations and temperatures and at neutral to alkaline pH

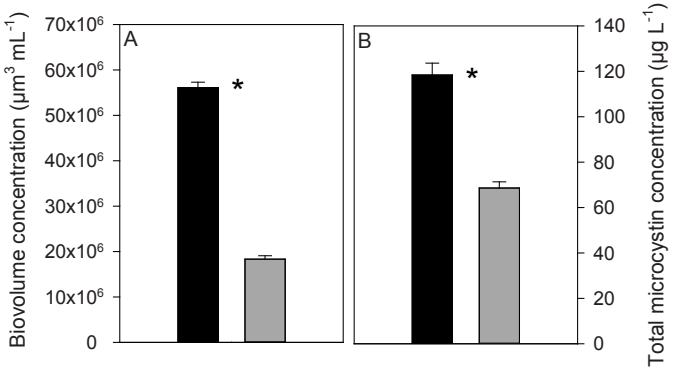

Figure 4.4: Results of the grazing experiment with densities of *Ochromonas* similar to those found in the Norwegian lakes. Black bars show controls and gray bars *Ochromonas* treatments. A: biovolume concentrations of *Microcystis* PCC 7806 on day 4; B: total microcystin concentration on day 4. Asterisks indicate significant differences between treatments. Error bars indicate ± 1SD.

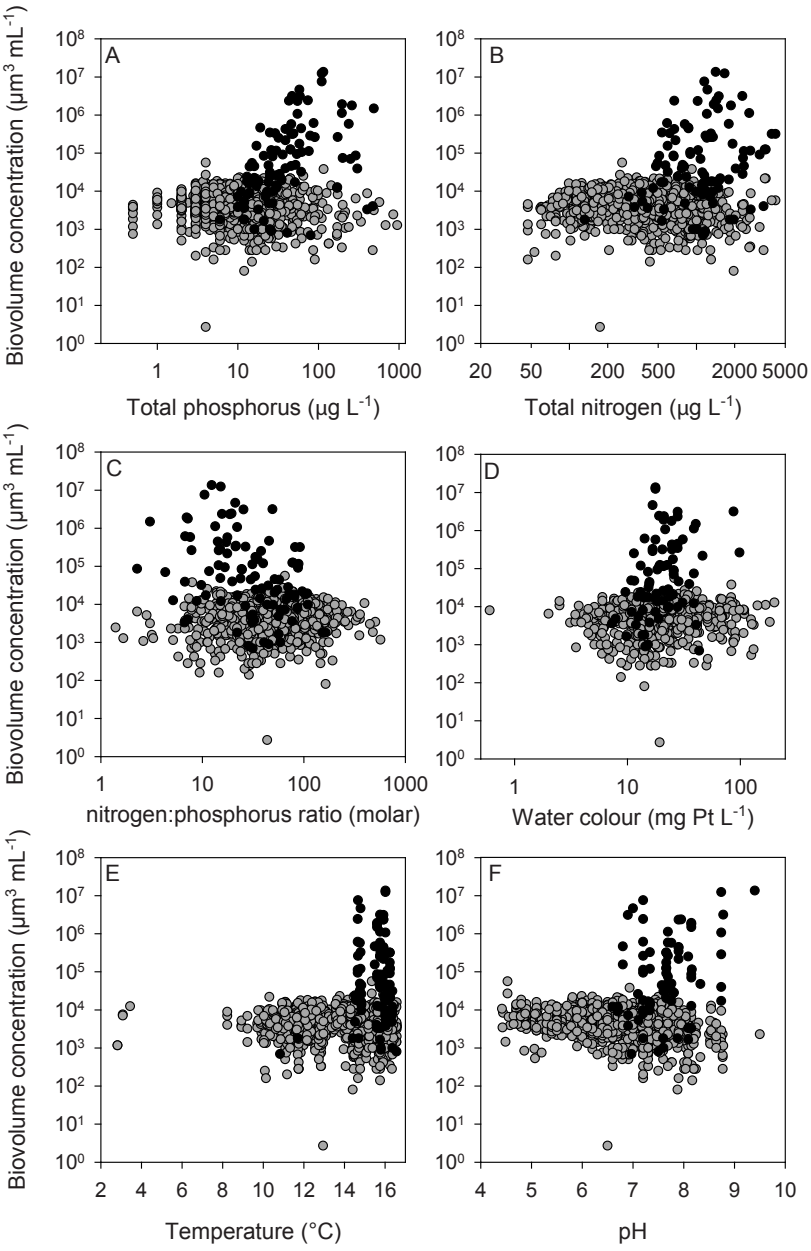

Figure 4.5: Biovolume concentrations of *Microcystis* (black) and *Ochromonas* (grey) in 460 Norwegian lakes (1395 summer samples in the period from 1988-2000) in relation to total phosphorus concentration (A), total nitrogen concentration (B), the total nitrogen: total phosphorus ratio (C), water colour (D) temperature (E), and pH (F).

(Fig. 4.5). Because of the relatively similar biovolume concentration of *Ochromonas* over a wide range of nutrient concentrations and the increasing total phytoplankton concentration with increasing nutrient concentration the relative abundance of *Ochromonas* decreased with increasing total phytoplankton biovolume concentration (Fig. 4.6).

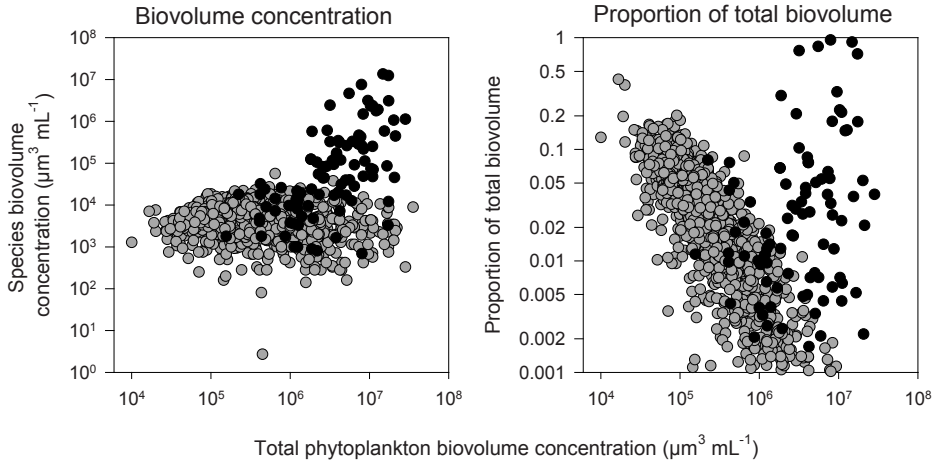

Figure 4.6: Abundance of *Ochromonas* (grey) and *Microcystis* (black) in Norwegian lakes in relation to the total phytoplankton biovolume. Left panel: biovolume concentrations of the two species; right panel: proportion of total phytoplankton biovolume of the two species.

Discussion

Effect of Ochromonas *on growth rates of cyanobacteria*

An important observation of this study is that *Ochromonas* can feed on all *Microcystis* strains tested, including the very toxic *Microcystis* PCC 7806 and the colony-forming *Microcystis* Bear AC. Moreover, *Ochromonas* achieved similar growth rates on all the *Microcystis* strains. The net growth rate reduction that *Ochromonas* caused in *Microcystis* was strongest for the less toxic strain Spring CJ of *Microcystis*, whereas the impact on the other two *Microcystis* strains, i.e. the more toxic strain PCC 7806 and the colony-forming Bear AC, was also high but less strong. Even if *Ochromonas* was inoculated in concentrations more comparable to natural concentrations, it caused a significant net growth reduction of the toxic strain PCC 7806. Furthermore, our mesocosm experiments showed that *Ochromonas* is able to invade a *Microcystis*-culture and grow to high abundances starting from a small inoculum. Although the impact of *Ochromonas* on *Pseudanabaena* was smaller compared to the *Microcystis* strains, the effects on biovolume and growth were still substantial (Figs. 4.1A, B).

Since we did not have a treatment with only the filtrate of an *Ochromonas* culture added, we can not completely rule out a negative effect of chemicals released by *Ochromonas*. However, in experiments performed recently to study the impact of substances excreted by *Ochromonas*, we observed no significant effects of filtrates from *Ochromonas* cultures on the growth and toxin production of these cyanobacteria. The per capita clearance rates of *Ochromonas* ranged from 23 ± 3.1 nL cell^{-1} day^{-1} for *M. aeruginosa* Bear AC as prey, to 47 ± 12 nL cell^{-1} day^{-1} for *M. aeruginosa* Spring. These rates are similar to those of 29–418 nL cell^{-1} day^{-1} for *Ochromonas* sp. grazing on *Synechococcus* (Boenigk et al., 2001), and 24 nL cell^{-1} day^{-1} for *O. minima* in a Norwegian Fjord (Nygaard & Hessen, 1990). It seems likely, therefore, that the negative effect of *Ochromonas* on cyanobacterial growth in our experiment is mainly due to grazing and not by other interactions like allelopathy.

Effect of* Ochromonas *on microcystin concentrations

After four days of incubation with *Ochromonas*, the total microcystin content in the cultures with *Microcystis* was reduced by 91.1- 98.7% compared with those in the controls (Fig. 4.3). These reductions can mainly be attributed to the decrease in particulate microcystin, which varied from 95.4 to 98.8 % compared with the controls and are within the same range as the reduction in biovolume. The decrease seems to be caused by both the degradation of ingested microcystins by *Ochromonas* and the reduction in net growth of *Microcystis*. In the second grazing experiment, with abundances of *Ochromonas* similar to those found in the Norwegian lakes, the net growth reduction of *Microcystis* due to *Ochromonas* was 66 % and the total microcystin concentration had been reduced by 42 % compared to that in the control. Only for *Pseudanabaena* the particulate microcystin concentration was not reduced significantly, probably because *Ochromonas* grazes less efficiently on this species. Even though the calculation of microcystins per unit biomass resulted in slightly higher values at the end than at the start of the experiment (data not shown), we cannot draw any conclusion about an increased intracellular concentration of microcystins in the cyanobacteria, because we did not distinguish between microcystins in the cyanaobacteria and microcystins in *Ochromonas*.

Degradation of microcystin from ingested single-celled *Microcystis* PCC 7806 has recently also been found for the closely related chrysophyte *Poterioochromonas* sp. (Zhang et al. 2008). In contrast to the results of Zhang et al. (2008) we also found a reduction in dissolved microcystin concentrations in the grazing treatments. This again could have been caused indirectly by the reduction in biovolume of the cyanobacteria, resulting in a lower amount of cyanobacterial cells that may release microcystins due to lysis. Moreover, the reduction could have partly been caused by the uptake and degradation of dissolved microcystins by *Ochromonas*, as the degradation of dissolved microcystins

has been described to occur in *Poterioochromonas* (Ou et al. 2005). Regardless of which mechanism is more important in reducing the microcystin concentrations, we show that the amount of total microcystins present can be reduced by *Ochromonas*, even when it is present in low abundances. By detoxifying the ingested microcystins it might even make the cyanobacterial carbon available to other grazers, which are not able to graze directly on microcystin containing cyanobacteria.

Size of particles ingested

The mean colony volume of the largest *Microcystis* strain (*Microcystis* Bear AC) was around 810 μm^3 (Table 4.1) and these colonies could apparently still be ingested easily. These results are in agreement with the work of Zhang et al. (1996) who found the volume of particles ingested by a closely related mixotrophic chrysophyte *Poterioochromonas malhamensis*, to range from about 0.52 μm^3 (bacteria) to 3178 μm^3 (chlorophyte *Carteria inverse*). The wide range of prey sizes ingested by *Ochromonas* in our experiments might be explained by the fact that it encloses its prey by pseudopodia (Boenigk & Arndt 2000) instead of engulfing food particles by invagination of its cell surface like many other phagotrophs do. Even though *Ochromonas* grazed efficiently on all *Microcystis* strains tested, it caused a small but significant shift in size distribution to larger sizes for the strains Spring CJ and Bear AC, whereas this did not occur for the smaller strain PCC7806 (Fig. 4.3). Thus *Ochromonas* seems to prefer the smaller sized particles over the larger ones. This agrees with the results of Pfandl et al. (2004), showing a food-size preference of *Ochromonas* sp. for particles between 0.9 and 1.2 µm. Although *Ochromonas* grazed on the filamentous cyanobacteria *Pseudanabaena*, it showed a lower growth rate on this alga than on the *Microcystis* strains. *Ochromonas* fed mainly on the *Pseudanabaena* filaments < 50 µm long, thereby skewing the filament length distribution of *Pseudanabaena* towards longer filament lengths. Similar shifts in length distribution as shown for *Pseudanabaena* have been reported by Wu et al. (2004) for *Ochromonas* grazing on the filamentous bacterium *Spirochaeta aurantium* ranging from 5 to 30 µm in filament length. Because *Pseudanabaena* has a smaller mean particle volume than, for example, the readily ingestible *Microcystis* Spring CJ, it seems that the greatest linear dimension of a particle rather than its volume determines whether a particle can still be ingested. The mean diameter of the *Microcystis* colonies used in our study lies within the filament length range that could be ingested by *Ochromonas*.

Food-web relationships

Mixotrophic nanoflagellates are important members of planktonic food webs in both marine and freshwater systems (Sanders 1991; Isaksson 1998; Tittel et al. 2003). Phagotrophy by predominantly autotrophic mixotrophs is an important mechanism for obtaining energy and nutrients, and it gives mixotrophs a competitive advantage over autotrophs during nutrient-limiting conditions. Similarly, predominantly heterotrophic mixotrophs have an advantage over purely heterotrophic organisms because they can use light as an energy source when prey abundance is low (Jones 2000). In both cases, mixotrophs will be favoured by oligotrophic conditions and they are traditionally thought to be inferior competitors in eutrophic systems, because of the higher energy costs for maintaining both nutritional metabolisms (Raven 1997). However, the predominantly mixotrophic genus *Poterioochromonas* has been shown to achieve growth rates comparable with those of heterotrophic nanoflagellates (Pålsson & Daniel 2004) and should therefore be able to compete with heterotrophs even under eutrophic conditions. Although mixotrophs in general are important grazers in oligotrophic lakes (Bergström et al. 2003) and can also dominate the plankton community during winter (Wiedner & Nixdorf 1998), their role in eutrophic lakes during summer is largely unknown (Tittel et al. 2003). This does not necessarily mean that they are not important in those systems, as many investigations distinguish only between pigmented and non-pigmented flagellates, and mixotrophs are likely to be mistaken for autotrophs in those studies.

We tested data from 460 Norwegian lakes for the co-occurrence of *Microcystis* and *Ochromonas*. The data show that *Ochromonas* occurred in 94 % of the samples in which *Microcystis* was present and the interaction of both species might therefore be important in natural systems. *Ochromonas* is abundant over a broad range of phosphorus and nitrogen concentrations in Norwegian lakes, including the higher concentrations under which also *Microcystis* becomes more important. While the total abundance of *Ochromonas* is generally low and seems to be unaffected by the environmental factors tested, its relative contribution to the plankton community decreases with increasing nutrient load and pH. Our data confirm that *Ochromonas* is a good competitor especially at nutrient poor conditions and that it may tolerate low pH, but it also shows that *Ochromonas* occurs over a wide gradient of environmental conditions, including eutrophic lakes (Figs. 4.5 and 4.6). Under meso- to eutrophic conditions, *Ochromonas* could potentially be controlled by grazers, e.g. daphnids. Arvola & Salonen (2001) showed that the abundance of *Ochromonas* and other flagellates increases after removal of daphnids from a natural zooplankton community. On the other hand the closely related *Poterioochromonas* has been shown to be toxic to rotifers and daphnids in lab experiments (Boxhorn et al. 1998; Boenigk & Stadler 2004). Even though competition with specialists and grazing on *Ochromonas* might play a role in

reducing the abundance of *Ochromonas* in eutrophic lakes, this chrysophyte can maintain population densities similar to those under oligotrophic conditions (Fig. 4.5). Due to its high grazing rates, *Ochromonas* may have a strong impact on the phytoplankton composition and abundance even when its density is lower than other grazer species

Because mixotrophs can sustain relatively high abundances by photosynthesis, they do not rely on the density of their prey and are therefore less prone to strong fluctuations in population densities. Hammer & Pitchford (2005) showed theoretically that a system, in which a small fraction of the herbivorous plankton is capable of photosynthesis, as for e.g. *Ochromonas*, is less likely to show strong oscillations and to exhibit phytoplankton blooms. By this mechanism the presence of *Ochromonas* might decrease the chance of a *Microcystis* bloom formation.

From our study it is not clear whether *Ochromonas* may control *Microcystis* blooms in natural lakes. Yet our study does demonstrate that *Ochromonas* can strongly reduce the *Microcystis* biomass and toxins in both small-scale and large-scale experiments. This study also shows that *Ochromonas* occurs in lakes where *Microcystis* is present. However, more work is needed on the natural occurrence of *Ochromonas* and its trophic relationships in eutrophic lakes. Light intensity, nutrient and organic matter concentration, bacterial and picophytoplankton abundance, as well as grazing on *Ochromonas* by higher trophic levels are potentially important factors regulating its natural abundance. More field work is therefore needed to clarify which of these factors are essential in determining *Ochromonas* distribution and seasonal dynamics in nature.

Chapter 5

Microcystins do not provide anti-herbivore defence against mixotrophic flagellates

Abstract

While most experiments investigating zooplankton grazing on harmful cyanobacteria have been carried out with metazoan plankton, several protozoa can also feed efficiently on cyanobacteria. We investigated grazing by the mixotrophic flagellate *Ochromonas* sp. on the toxic cyanobacterium *Microcystis aeruginosa*. *Ochromonas* grew rapidly on *M. aeruginosa*, and had a strong impact on the population density of its prey. However, specific growth rates of *Ochromonas* decreased over time, possibly indicating a negative impact on *Ochromonas* mediated by *M. aeruginosa*. Grazing did not have any effect on the intracellular microcystin content of *M. aeruginosa* and the ingested microcystins did not accumulate within *Ochromonas*. We studied the functional and numerical response of *Ochromonas* grazing on the microcystin-producing strain *M. aeruginosa* PCC 7806 and its microcystin-deficient mutant. *Ochromonas* showed a type 3 functional response of very similar shape on both the toxic and non-toxic *M. aeruginosa* strain. Ingestion rates of *Ochromonas* were even slightly higher on the toxic *M. aeruginosa* strain. We therefore found no indication for microcystins acting as defence against mixotrophic flagellates.

This chapter is based on the paper: Wilken S, Wiezer S, Huisman J, and Van Donk E. 2010. Microcystins do not provide anti-herbivore defence against mixotrophic flagellates. Aquatic Microbial Ecology 59: 207-216.

Introduction

Cyanobacterial blooms have increasingly become a nuisance in many freshwater and brackish ecosystems (Chorus & Bartram 1999, Carmichael 2001, Huisman et al. 2005). They are facilitated by high water temperatures and nutrient load, and therefore benefit from anthropogenic influences like eutrophication and climate warming (Codd 2000, Jöhnk et al. 2008, Paerl & Huisman 2008). Microcystin-producing cyanobacteria of the *Microcystis* genus are widely distributed bloom-forming species. The hepatotoxic microcystins inhibit protein phosphatases in eukaryotes (MacKintosh et al. 1990), and are therefore toxic to many potential grazers of *Microcystis* (Fulton & Paerl 1987b, DeMott et al. 1991). Other factors contributing to the low edibility of *Microcystis* are the formation of colonies leading to interference with the feeding behaviour of many zooplankton species (Fulton & Paerl 1987a, Yang et al. 2006) and the low content of long-chain polyunsaturated fatty acids and sterols, which are essential for many herbivores (Wacker & von Elert 2001, von Elert et al. 2003).

While most experiments investigating effects of toxic cyanobacteria on pelagic grazers have been done with metazoans, several protozoa are able to maintain high growth rates when feeding on cyanobacteria including *Microcystis* (Cole & Wynne 1974, Nishibe et al. 2002, Kim et al. 2006). Especially mixotrophic chrysophytes, such as *Ochromonas* spp. and *Poterioochromonas* spp., can graze efficiently on *Microcystis* and even degrade the cyanotoxins (Ou et al. 2005, Zhang et al. 2008, Van Donk et al. 2009). The genus *Ochromonas* is abundant in freshwaters and has been reported to frequently co-occure with *Microcystis* (Van Donk et al. 2009). *Ochromonas* has therefore been proposed as a potential biological control agent against *Microcystis* blooms (Cole & Wynne 1974, Zhang et al. 2008), although little is thus far known about its interaction with toxic cyanobacteria. As mixotrophs, these organisms differ considerably from purely heterotrophic grazers by their photosynthetic machinery and the presence of several biochemical pathways typical of autotrophic organisms. For instance, in contrast to many heterotrophs, *Ochromonas* is able to synthesize long-chain polyunsaturated fatty acids (Boëchat et al. 2007), and thus does not depend on the presence of these substances in its prey. However, *Ochromonas* might be susceptible to the other two mechanisms of grazer deterrence in *Microcystis*, e.g., colony formation and microcystin production. Colony formation in *Microcystis* can be induced by the presence of *Ochromonas* sp. (Burkert et al. 2001, Yang et al. 2006). Even though *Ochromonas* is able to ingest surprisingly large prey items there must certainly be an upper limit of suitable prey size and inducible colony formation is likely to be an efficient way of defence against grazing by flagellates.

In contrast to colony formation, the role of microcystin production as defence against flagellate grazers is less clear. The original function of microcystins is still under debate and its role as a defence against grazers has been questioned, because the genes for microcystin biosynthesis seem to be older than metazoan grazers such as copepods and cladocerans (Rantala et al. 2004). However, since the earliest protozoa probably already preyed upon cyanobacteria long before the metazoans evolved, the early evolution of microcystins does not necessarily exclude its original role as a defence against protozoa. Furthermore, the function of these secondary metabolites might have changed during their evolution and regardless of the original function of microcystins they are currently toxic for many species. *Ochromonas* could pay a metabolic cost for its ability to grow on toxic cyanobacteria and to degrade the toxins, which might be reflected in lower growth rates compared to growth on non-toxic food. Ou et al. (2005) described an initially stimulating effect of dissolved microcystins on growth of *Poterioochromonas* sp., but decreased growth rates after prolonged exposure to the toxins. This suggests that microcystins might indeed act as a defence against grazing by protozoan flagellates. Grazer-induced defences are well known for several phytoplankton taxa, such as the induction of colony formation in the green alga *Scenedesmus* (Lürling & Van Donk 1997). Jang et al. (2003) reported an increased microcystin content in *Microcystis* as a response to *Daphnia* grazing, suggesting the microcystin production of cyanobacteria to be induced by info-chemicals released by the grazer. Whether the microcystin production of *Microcystis* responds to flagellate grazing is not known yet, however.

Here, we investigate the functional and numerical response of the mixotrophic flagellate *Ochromonas* sp. grazing on the microcystin-producing cyanobacterium *Microcystis aeruginosa* PCC 7806 and its microcystin-deficient mutant. Conversely, we also investigate whether flagellate grazing induces an enhanced microcystin production by *M. aeruginosa*.

Materials and Methods

Phytoplankton strains

We investigated the microcystin-producing cyanobacterium *Microcystis aeruginosa* PCC 7806, its microcystin-deficient mutant (Dittmann et al. 1997), and the microcystin-producing strain *M. aeruginosa* HUB 524. All three *M. aeruginosa* strains were single celled and did not show any colony formation in the stock cultures. Our *Ochromonas* sp. strain was detected as contamination in large-scale mesocosm experiments with *M. aeruginosa*. It was isolated by micro-needle techniques. Stock cultures of all strains were maintained on COMBO-medium (Kilham et al. 1998). For *Ochromonas* ammonium was

used as the nitrogen source in the stock cultures, because it grew rather poorly on nitrate. All cultures were unialgal but not axenic. Abundances of heterotrophic bacteria were low, however, and never exceeded 1% of the total biovolume. Cellular microcystin contents and cell volumes of the strains are summarized in Table 5.1.

Table 5.1: Microcystin content (n = 16) and biovolume (n = 3) of the *M. aeruginosa* strains and *Ochromonas* sp. used in the experiments. Standard deviations are given in parentheses.

Strain	**Microcystin content** (fg cell^{-1})	**Biovolume** (µm^3 cell^{-1})
M. aeruginosa PCC 7806 Wildtype	53.32 (± 6.66)	25.02 (± 1.64)
M. aeruginosa PCC 7806 Mutant	-	28.10 (± 3.07)
M. aeruginosa HUB 524	27.16 (± 3.00)	32.08 (± 0.14)
Ochromonas sp.	-	146.57 (± 30.91)

Effect of grazing on toxin production

We investigated effects of grazing on the microcystin production of *M. aeruginosa* PCC 7806 and *M. aeruginosa* HUB 524. The experiments were done in batch cultures using 500 mL Erlenmeyer flasks filled with 300 mL COMBO-medium (Kilham et al. 1998). We applied four experimental treatments to each *M. aeruginosa* strain: (1) monocultures of *M. aeruginosa* that served as controls, (2) monocultures of *M. aeruginosa* to which we added the filtrate of an *Ochromonas* culture pre-fed with the same *M. aeruginosa* strain, (3) monocultures of *M. aeruginosa* to which we added the filtrate of an *Ochromonas* culture pre-fed with a different prey species, the Eustigmatophyte *Nannochloropsis limnetica*, and (4) mixed cultures of *M. aeruginosa* and *Ochromonas* that served as grazing treatment. All four treatments were performed in four replicates. The experiments were incubated at 23 °C with a low shaking frequency of 20 rpm and an incident light intensity of 90 µmol photons m^{-2} s^{-1} under a light: dark cycle of 14 h: 10 h.

In all treatments, we inoculated *M. aeruginosa* to a final population density of 3×10^5 cells mL^{-1}. In the species mixture of the fourth treatment, *Ochromonas* was added to a final population density of 500 cells mL^{-1}. These rather low population densities were chosen to prevent *M. aeruginosa* from reaching the stationary phase in the control, while it was still growing exponentially in the grazing treatment. Such a situation might lead to differences in microcystin concentration caused by differences in growth rates, nutrient status (Long et al. 2001), or light intensity (Wiedner et al. 2003) between the grazing treatment and the control that would not be directly related to the grazing itself. Our inoculation densities were lower than *Microcystis* abundances in dense surface blooms, but comparable to the

abundances of both species in natural waters during non-bloom conditions (Van Donk et al. 2009). The filtrates originated from rather dense cultures of *Ochromonas* prefed with *N. limnetica* and *M. aeruginosa*. The filtrates were added daily in volumes corresponding to the density of *Ochromonas* in the grazing treatment, adding up to 25 % of the culture volume over the course of the experiment. Equal volumes of fresh medium were added to the control and grazing treatments each time. Based on preliminary experiments, samples for population densities and microcystin analysis were taken every 2 to 3 days over a period of 9 days.

In the first set of experiments, with *M. aeruginosa* PCC 7806, population densities were measured directly after sampling using a CytoSense flow cytometer (CytoBuoy b.v., Woerden, The Netherlands). In the second set of experiments, with *M. aeruginosa* HUB 524, samples were fixed with a mixture of glutaraldehyde and formaldehyde (final concentrations of 0.025 and 0.0037 mass %, respectively) and measured at a flow cytometer (MoFloTM XDP Cell Sorter, Beckman Coulter, Inc., Fullerton, USA) after a few days of storage at 4 °C. Fresh samples were regularly checked for colony formation by light microscopy, but *M. aeruginosa* remained single-celled in both experiments.

Microcystin concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) (QuantiPlateTM Kit for Microcystins; EnviroLogix, Portland, Maine, USA). For measurement of particulate microcystins, the samples were centrifuged and the pellet was resuspended in MilliQ water. Samples were diluted to a final cell density of about 5×10^4 cells mL^{-1} to ensure that the microcystin concentration fell within the measurable range of the ELISA. Microcystins were extracted from the cells by three freeze and thaw cycles, each of which was followed by sonification for 15 min using a sonic bath (Gustafsson et al. 2005). For the measurement of dissolved microcystins, samples were filtered through glassfibre filters (Whatman GF/C) and the filtrate was analysed for microcystins. To distinguish between microcystins in *M. aeruginosa* cells and *Ochromonas* cells, we sorted 2.5×10^5 *M. aeruginosa* cells from the mixed cultures using a flow cytometer (MoFloTM XDP Cell Sorter, Beckman Coulter, Inc., Fullerton, USA). The sorted fraction containing only *M. aeruginosa* cells was diluted, and population density and microcystin content were measured using the same methods as for the other samples (CytoSense for the first experiment and MoFlo for the second experiment). Because population densities of *Ochromonas* were too low for flow cytometer sorting throughout most of the experiment, it could only be sorted at the last day.

When variances were homogenous, the data were tested for a treatment effect by repeated measures ANOVA, followed by a Tukey-test as post-hoc comparison. When variances remained heterogeneous even after logarithmic transformation (microcystin data of PCC 7806) the nonparametric Kruskal-Wallis ANOVA was used instead. All statistical analyses were done using STATISTICA 8 software.

Functional and numerical response experiment

To investigate effects of microcystins on *Ochromonas* sp., the functional and numerical response of *Ochromonas* grazing on the toxic strain *M. aeruginosa* PCC 7806 and its microcystin-deficient mutant were compared. *Ochromonas* was precultured for one week on the two strains at prey concentrations used in the experiment. During preculturing, prey concentrations were measured daily using a CytoSense flow cytometer and readjusted to the desired concentrations by either dilution or addition of more prey. Stock cultures were harvested by centrifugation and resuspended in fresh COMBO-medium to prevent any potential effect of 'spent' medium. To assess the functional and numerical response of *Ochromonas*, we used eight different concentrations of *M. aeruginosa* (Table 5.2). This included a treatment without any *M. aeruginosa* as food, to estimate the specific growth rate of *Ochromonas* when grown photo-autotrophically. At each *M. aeruginosa* concentration, we had a treatment with *Ochromonas* grazing and a control for *M. aeruginosa* growth without *Ochromonas*. All treatments were run in triplicate. The experiment was performed in 100 mL Erlenmeyer flasks under continuous illumination of 90 µmol photons m^{-2} s^{-1}. Cultures were incubated at 23 °C for 3 days and samples were taken at 0, 3, 6, 12, 24, 48, and 72 hours after inoculation, fixed with a mixture of glutaraldehyde and formaldehyde as described above, and stored at 4 °C until measurement at a flow cytometer (MoFlo XDP Cell Sorter, Beckman Coulter, Miami, FL, USA).

Because *Ochromonas* might ingest heterotrophic bacteria as well, and our cultures were not axenic, we performed bacterial counts on several samples. We picked the samples of treatments 3, 5 and 7 (Table 5.2) taken at 0 and 12 hours after inoculation, for both the wildtype and the mutant of *M. aeruginosa* PCC 7806. Bacteria were stained with SYBR Green (Invitrogen, Breda, The Netherlands) prior to counting at the MoFlo flow cytometer.

Population densities were plotted against time on a semi-log scale. During periods in which a linear relation was observed, the specific growth rate, µ, was calculated as:

$$\mu = \frac{\ln(N_2) - \ln(N_1)}{t_2 - t_1} \tag{5.1}$$

Table 5.2: Initial population densities of *M. aeruginosa* and *Ochromonas* sp. used in the functional and numerical response experiment.

Treatment Nr.	*M. aeruginosa* (cells mL^{-1})	*Ochromonas* sp. (cells mL^{-1})
1	0	3.9×10^{4}
2	2.6×10^{3}	5.0×10^{2}
3	8.0×10^{3}	8.4×10^{2}
4	2.4×10^{4}	2.9×10^{3}
5	8.0×10^{4}	7.3×10^{3}
6	2.5×10^{5}	2.5×10^{4}
7	8.2×10^{5}	7.3×10^{4}
8	2.7×10^{6}	1.2×10^{5}

where N_1 and N_2 denote population densities at time t_1 and t_2, respectively. Clearance rates were calculated over the first 3 hours of incubation, except for the lowest two prey concentrations for which changes in concentrations were low enough to use a longer time span. The following formula was used (Heinbokel 1978):

$$Cl = \frac{\mu_{Mc} - \mu_{Mt}}{N_O} \tag{5.2}$$

where Cl is the clearance rate, μ_{Mc} and μ_{Mt} are the specific growth rates of *M.aeruginosa* in the control and the grazing treatments, respectively, and N_O is the mean *Ochromonas* abundance over the period used for calculation. Grazing rates are then given by:

$$G = N_M Cl \tag{5.3}$$

where G denotes the grazing rate, and N_M denotes the mean *M. aeruginosa* abundance.

Ochromonas showed a slightly sigmoid increase of the grazing rate and an initial increase of the clearance rate with increasing *M. aeruginosa* abundance. This is indicative of a type 3 functional response, where *Ochromonas* increases its feeding rate with increasing prey density (Holling 1959, Kiørboe 2008). Therefore, we fitted a type 3 functional response model to the grazing rate data (Real 1977):

$$G = \frac{G_{max} N_M^b}{K^b + N_M^b} \tag{5.4}$$

where G_{max} is the maximum grazing rate reached at saturating prey concentrations, the power b describes the curvature of the sigmoid functional response (we note that, if $b = 1$, the model simplifies to a type 2 functional response), and K is the half-saturation constant, representing the prey density at which half the maximum grazing rate is reached. The model parameters were estimated by nonlinear regression, using the iterative least-squares method of STATISTICA 8. The grazing-rate data were log-transformed prior to the model fit to improve the homogeneity of variance.

Results

Effect of grazing on toxin production

Both *M. aeruginosa* strains showed positive growth rates in all treatments, reaching population densities of 4 to 10×10^6 cells mL^{-1} at the end of the experiments (Fig. 5.1). Nevertheless, the presence of *Ochromonas* sp. had a strong effect on *M. aeruginosa* densities, reducing *M. aeruginosa* PCC 7806 by 44% and *M. aeruginosa* HUB 524 by

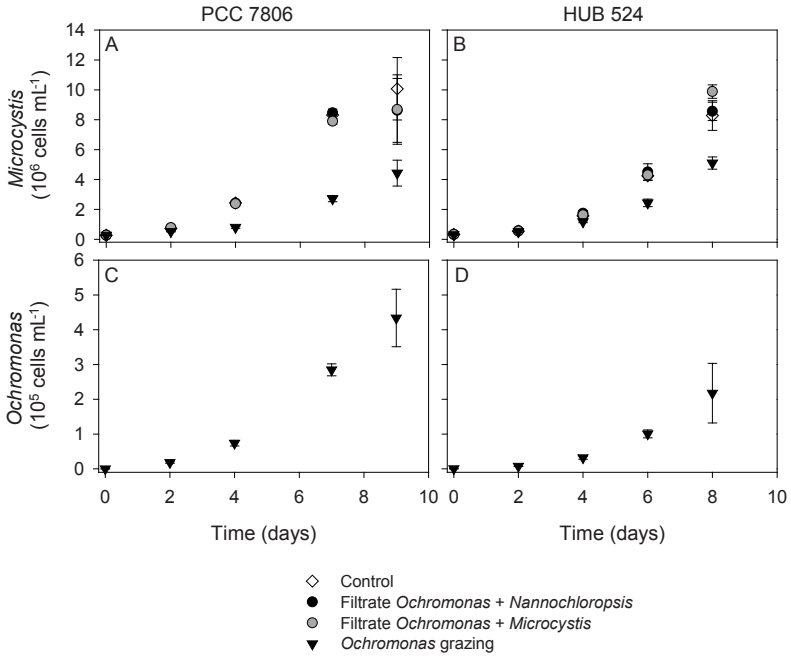

Figure 5.1: Population densities over the course of the grazing experiments with *Ochromonas* sp. as predator and *M. aeruginosa* PCC 7806 (A, C) and HUB 524 (B, D) as prey. Error bars indicate standard deviation.

39% relative to the controls (repeated measures ANOVA, for PCC 7806: $F_{3,12} = 36.37$, $p < 0.001$; for HUB 524: $F_{3,12} = 48.08$, $p < 0.001$). The addition of *Ochromonas* filtrates did not show any effect on *M. aeruginosa* abundances. *Ochromonas* grew rapidly on both strains, increasing its population density by almost 1000-fold over the course of the experiments (Fig. 5.1C,D). It reached its highest specific growth rates of 2.1 d^{-1} (on PCC 7806) and 1.5 d^{-1} (on HUB 524) at the beginning of the experiment, followed by a decrease of its specific growth rates to 0.21 d^{-1} (on PCC 7806) and 0.36 d^{-1} (on HUB 524) at the end even though prey densities increased (Fig. 5.2).

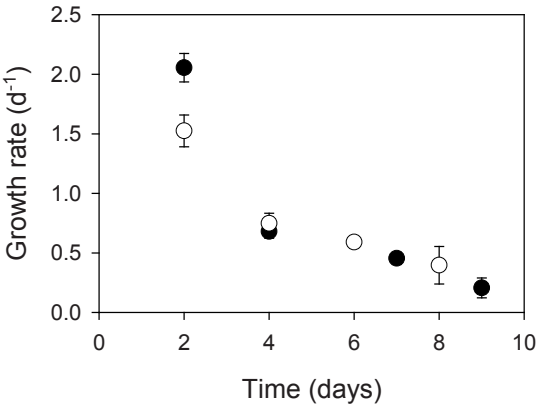

Figure 5.2: Specific growth rates of *Ochromonas* sp. on *M. aeruginosa* PCC 7806 (filled circles) and HUB 524 (open circles) over the course of the grazing experiment. Error bars indicate standard deviation.

The total particulate microcystin concentrations showed a similar increase as the population densities of *M. aeruginosa*, reaching concentrations of 344 µg L^{-1} (PCC 7806) and 175 µg L^{-1} (HUB 524) at the last day in the controls, and about 32% (PCC 7806) and 18% (HUB 524) lower values in the grazing treatment with *Ochromonas* (Fig. 5.3A,B). The treatment effect was significant for *M. aeruginosa* HUB 524 (repeated measures ANOVA, $F_{3,12} = 8.03$, $p < 0.01$) and for *M. aeruginosa* PCC 7806 on day 4 and day 7 (Kruskal-Wallis ANOVA, $H_3 = 9.53$, $p < 0.01$ and $H_3 = 8.49$, $p < 0.05$, respectively). The intracellular microcystin content of *M. aeruginosa* PCC 7806 was about twice as high as that of *M. aeruginosa* HUB 524 (Fig. 5.3C,D). The intracellular microcystin contents of both strains remained constant across all treatments (Fig. 5.3C), except at the end of the experiment with *M. aeruginosa* PCC 7806, where it fell to significantly lower values in the grazing treatment at day 9 (Kruskal-Wallis ANOVA, $H_3 = 8.74$, $p < 0.05$). Dissolved microcystin concentrations were low compared to the particulate concentrations,

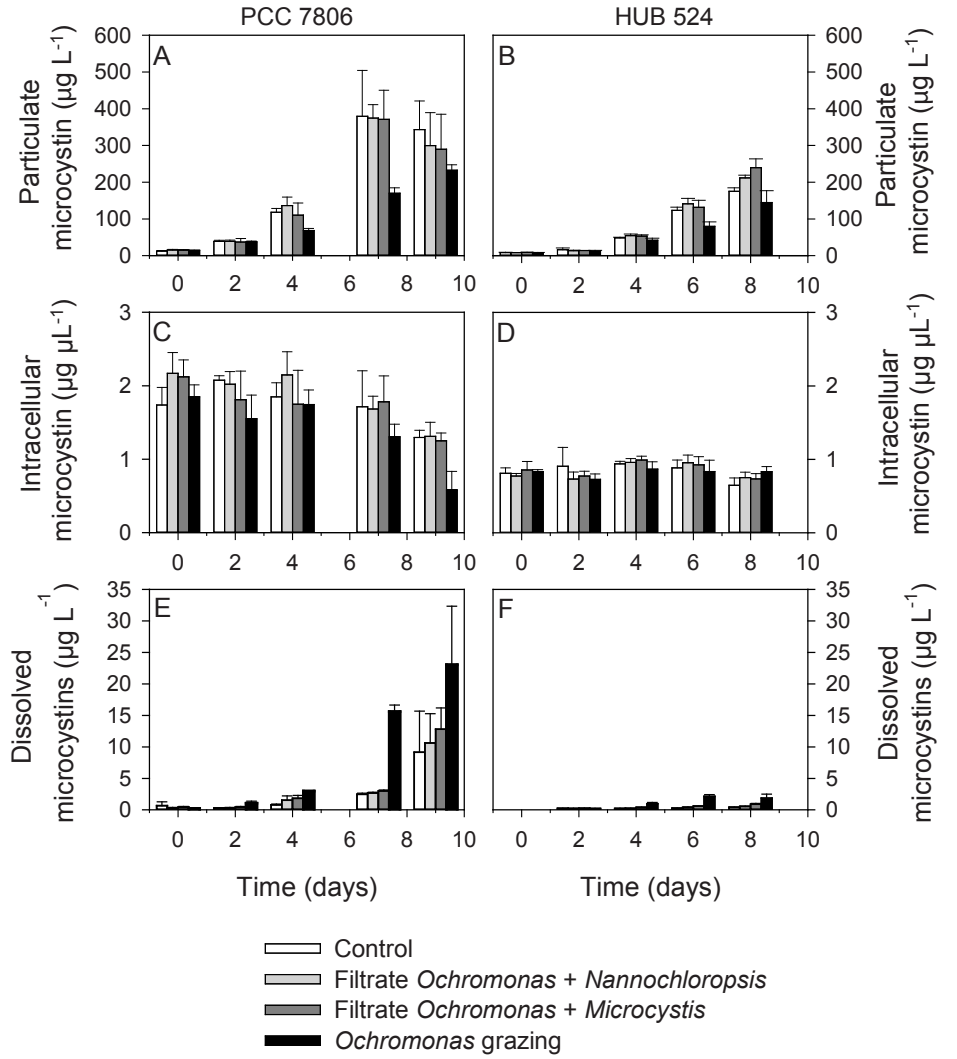

Figure 5.3: Changes in particulate microcystin concentration (A and B), intracellular microcystin content (C and D), and dissolved microcystin concentration (E and F) over the course of the grazing experiments with *Ochromonas* sp. as predator and *M. aeruginosa* PCC 7806 (left panels) and HUB 524 (right panels) as prey. Error bars indicate standard deviation.

exceeding 5 % of the total microcystins only for *M. aeruginosa* PCC 7806 on day 9. Dissolved microcystin concentrations increased with increasing population densities of *M. aeruginosa*, and reached considerably higher concentrations for *M. aeruginosa* PCC

7806 than for *M. aeruginosa* HUB 524 (Fig. 5.3E,F). From day 4 onwards, the dissolved microcystin concentrations were significantly higher in the presence than in the absence of *Ochromonas* (repeated measures ANOVA, for PCC 7806: $F_{3,12} = 16.90$, $p < 0.001$; for HUB 524: $F_{3,12} = 61.78$, $p < 0.001$). The intracellular microcystin content of *Ochromonas* could be measured only at the end of the experiments, when population densities of *Ochromonas* were high enough to be sorted by the flow cytometer. *Ochromonas* contained 3.91 (± 3.19 SD) fg microcystin $cell^{-1}$ after 9 days of grazing on *M. aeruginosa* PCC 7806, and 5.23 (± 1.18 SD) fg microcystin $cell^{-1}$ after 8 days of grazing on *M. aeruginosa* HUB 524. Hence, the intracellular microcystin contents of *Ochromonas* were about one order of magnitude lower than the intracellular microcystin contents of the *M. aeruginosa* cells upon which they fed (Table 5.1).

Functional and numerical response

Ochromonas sp. showed a very similar functional response irrespective of whether it was feeding on the microcystin-producing wildtype or on the microcystin-deficient mutant of *M. aeruginosa* PCC 7806 (Fig. 5.4A). The grazing rate of *Ochromonas* was well described by the type 3 functional response model of Eqn. 5.4. We note that the sigmoid shape of the type 3 functional response is not visible in Fig. 5.4A, because both axes are plotted on logarithmic scales. The estimated model parameters are summarised in Table 5.3. The power *b* had a similar value on both *M. aeruginosa* strains and was significantly larger than 1, confirming the type 3 like shape of the functional response of *Ochromonas*. The maximum grazing rate G_{max} was slightly but significantly higher on the microcystin-producing wildtype than on the microcystin-deficient mutant (Table 5.3). The initial increase of the clearance rate with the *M. aeruginosa* concentration is typical for a type 3-like functional response (Fig. 5.4B). The specific growth rate of *Ochromonas* showed a very similar dependence on *M. aeruginosa* abundance irrespective of whether it was feeding on the microcystin-producing wildtype or the microcystin-deficient mutant (Fig. 5.4C). The specific growth rate of *Ochromonas* was much lower when grown photo-autotrophically than when feeding on *M. aeruginosa* (Fig. 5.4C).

Abundances of heterotrophic bacteria remained below 1% of the total biovolume, and did not decrease in the grazing treatments relative to the control. The contribution of heterotrophic bacteria to the nutrition of *Ochromonas* was therefore regarded as negligible in our experiments.

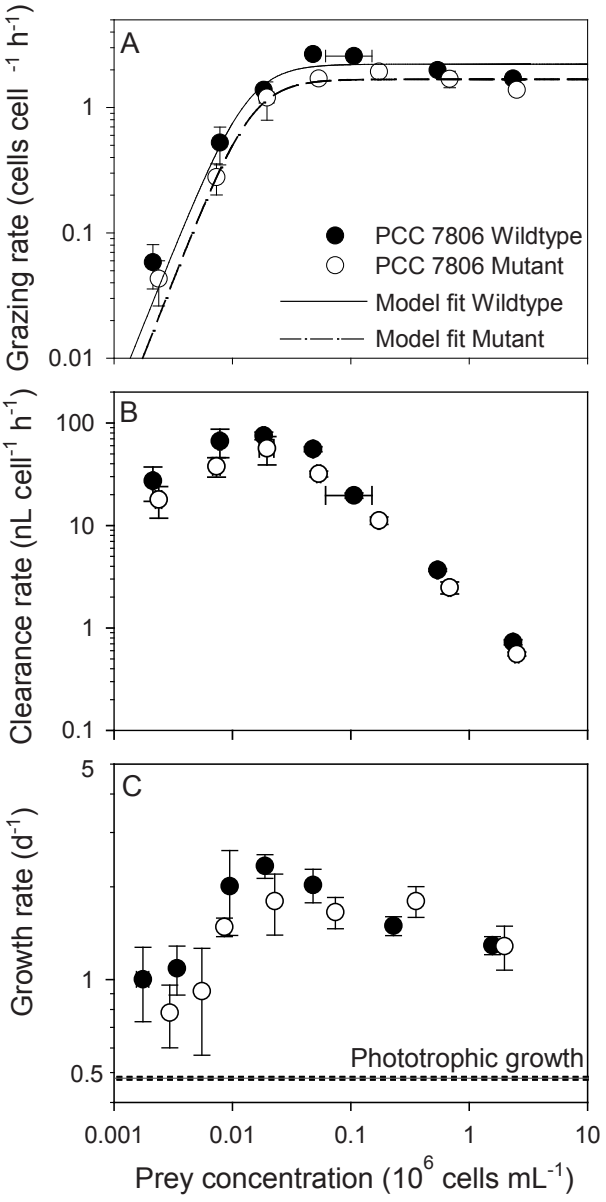

Figure 5.4: Grazing rates (A), clearance rates (B) and specific growth rates (C) of *Ochromonas* sp. grazing on the microcystin-producing wildtype and microcystin-deficient mutant of *M. aeruginosa* PCC7806. Error bars indicate standard deviation. Lines in (A) show the fit of the type 3 functional response model to the ingestion rate data. The grey horizontal line in (C) shows the specific growth rate of *Ochromonas* sp. when grown photo-autotrophically (i.e., without *M. aeruginosa*).

Table 5.3: Estimated parameter values for the type 3 functional response model fitted to the grazing rate data of *Ochromonas* sp. grazing on the microcystin-producing wildtype and microcystin-deficient mutant of *M. aeruginosa* PCC 7806.G_{max} is the maximum grazing rate of *Ochromonas*, *K* is the half-saturation constant, and *b* describes the curvature of the sigmoid functional response (Eqn 5.4). 95% confidence intervals are given in parentheses.

Strain	G_{max} (cells cell^{-1} h^{-1})	*K* (10^6 cells mL^{-1})	*b* (-)
Wildtype	2.21 (± 0.25)	0.013 (± 0.025)	2.37 (± 0.97)
Mutant	1.68 (± 0.14)	0.014 (± 0.025)	2.43 (± 0.85)

Discussion

Genetic evidence has shown that microcystins have a long evolutionary history, indicating that cyanobacteria could probably produce microcystins before metazoan grazers such as copepods and cladocerans entered the evolutionary record (Rantala et al. 2004). This makes it unlikely that microcystins have evolved as defence against metazoa. However, protozoa arrived at the scene much earlier, and microcystins could therefore have evolved as anti-herbivore defence against protozoan grazers. Our laboratory experiments confirm the strong impact of protozoan grazing by the flagellate *Ochromonas* sp. on the cyanobacterium *M.aeruginosa*. The population of *Ochromonas* grew quickly to large densities, and suppressed the abundance of *M. aeruginosa* substantially. This implies that *Ochromonas* has the potential to impact *M. aeruginosa* populations in natural waters, where *Ochromonas* is often present at relatively low background densities but occasionally dominates the phytoplankton (Olrik & Nauwerck 1993, Van Donk et al. 2009).

Grazing by *Ochromonas* did not cause an increase in intracellular microcystin content. Therefore, microcystins do not seem to act as an inducible defence against flagellate grazing, as has been reported for daphnids (Jang et al. 2003). Since we did not observe any colony formation in the presence of grazers, we cannot totally exclude the possibility that the population densities of *Ochromonas* in our experiment were too low to cause a response in *M. aeruginosa*. Colony formation in *M. aeruginosa* can be induced by *Ochromonas* grazing (Burkert et al. 2001, Yang et al 2006). However, these studies used different strains of *M. aeruginosa* and we could not ascertain that the strains used in our experiments had the ability to form colonies. Our inoculation densities were comparable to population densities observed in natural waters (Van Donk et al. 2009) and *Ochromonas* grew rapidly to higher densities over the course of the experiment. Therefore, if these population densities were too low to induce higher intracellular microcystin contents in our experiments,

Ochromonas can neither be expected to induce higher intracellular microcystin in natural waters. Dissolved microcystin concentrations did increase significantly due to grazing by *Ochromonas*, probably due to lysis of *M. aeruginosa* cells or excretion of consumed microcystin by *Ochromonas*.

While *Ochromonas* grew with very high specific growth rates at the start of the experiment, and increased its population densities 500-1000 fold, its specific growth rates decreased markedly during the course of the experiment (Fig. 5.2), even though prey densities remained high. This deceleration of the growth rate could not be observed in earlier studies using *Ochromonas* or *Poterioochromonas* (Cole & Wynne 1974, Zhang et al. 2008), because these earlier studies used very high inoculation densities of the flagellates resulting in the disappearance of *M. aeruginosa* within short periods. What might have caused the reduction of the specific growth rate cannot be answered from our experiments. One possible reason might be the excretion of allelopathic substances by *Microcystis* (e.g. Sukenik et al. 2002). Another explanation might be the toxic effect of microcystins ingested by *Ochromonas*. This possibility has been further investigated in the functional response experiment.

We used the microcystin-producing wildtype and microcystin-deficient mutant of *M. aeruginosa* PCC 7806 to investigate the effect of microcystins on the functional and numerical response of *Ochromonas* sp., since the only difference between these two strains is their ability to produce microcystins. The content of other potentially toxic peptides like cyanopeptolins and microviridins (Jungmann 1992, Tonk et al. 2009) should be the same in both of them, while strains of different origin would probably have differed in their contents of many peptides, fatty acids, and other cellular constituents.

The grazing and clearance rates of *Ochromonas* showed a type 3 functional response. This shape is explained by an increased foraging effort with increasing prey concentration. In the mixotroph *Ochromonas* such a pattern is most likely caused by a trade-off between phototrophic and heterotrophic growth. At low prey densities, mixotrophs will mainly benefit from investments into phototrophic growth, while foraging efforts on prey will contribute less to their total carbon metabolism. Conversely, at high prey densities, the rewards of heterotrophic growth will be high and photosynthesis therefore becomes less important. The growth rate achieved under purely autotrophic conditions was much lower than the maximum growth rate of *Ochromonas* (Fig. 5.4C), indicating that saturating densities of *M. aeruginosa* covered at least 80% of the carbon demand of *Ochromonas*.

Quantitatively, our estimates of the grazing rates compare well to those of 1.7 cells $cell^{-1}$ h^{-1} reported for *Ochromonas danica* grazing on *M. aeruginosa* (Cole & Wynne 1974). The same is true for the clearance rates measured at high prey concentrations. However,

the maximum clearance rates of 55 – 75 nL cell^{-1} h^{-1} measured in this study at prey concentrations of about 3×10^4 cells mL^{-1} are much higher than those of 0.6 – 1.9 nL cell^{-1} h^{-1} reported for *Poterioochromonas* sp. (Zhang et al. 2008) or of 0.7 – 4.1 nL cell^{-1} h^{-1} for the heterotrophic flagellate *Collodictyon triciliatum* grazing on *M. aeruginosa* (Nishibe et al. 2002). The lower clearance rates found in the literature can be explained by the higher prey concentrations used in most other studies and the strong decline of clearance rates with increasing prey concentrations (Fig. 5.4B).

The intracellular microcystins did not have a negative effect on *Ochromonas*. On the contrary, *Ochromonas* even showed slightly higher maximum ingestion rates on the microcystin-containing wildtype than on the microcystin-deficient mutant. Whether this is due to a stimulating effect of microcystins (Ou et al. 2005), or to the slightly smaller size of the wildtype strain (Table 5.1) cannot be answered with certainty. In any case, differences in grazing rates between the wildtype and its microcystin-deficient mutant were small, and intracellular microcystin does therefore not seem to act as defence against these mixotrophic flagellates.

In addition to mixotrophic flagellates such as *Ochromonas*, several heterotrophic flagellates have been reported to graze on *Microcystis* (Nishibe et al. 2002, Park et al. 2003, Kim et al. 2006). Microcystins inhibit the eukaryotic protein phosphatases type 1 and 2A (MacKintosh et al. 1990), and are therefore toxic to most eukaryotes. However, since the protein phosphatases are located in the cytoplasm, microcystins have to be transported into the cell to cause a toxic effect. Active transport into mammalian hepatocytes has been shown (Dawson 1998), while most other organs remain unaffected, presumably because they do not take up microcystins. A possible explanation for the absence of microcystin toxicity in *Ochromonas* might therefore be the lack of an uptake system from the lysosomes into the cytoplasm. Furthermore, *Ochromonas* and *Poterioochromonas* are both able to degrade microcystins (Zhang et al. 2008, Van Donk et al. 2009), and we did not find any bioaccumulation of microcystins within *Ochromonas*. A rapid degradation of the toxins is important in preventing a negative effect on growth. Due to degradation, only a small fraction of the microcystins ingested by *Ochromonas* was released into the dissolved pool, which is contrary to what would be expected if microcystins were egested directly. Because our cultures were not axenic, there might have been some degradation of dissolved microcystins by bacteria as well.

While our results show that the mixotrophic flagellate *Ochromonas* sp. is not affected by microcystins, other studies indicate that several species of heterotrophic flagellates are susceptible to microcystins (Christoffersen 1996, Moustaka-Gouni et al. 2006). These contrasting results suggest great variability in the response of different protozoa

to microcystin-producing cyanobacteria. In addition to microcystin production, colony formation in *Microcystis* can also act as a defence against grazers and has been shown to affect *Ochromonas* clearance rates (Yang et al. 2009). Because *Microcystis* often forms large colonies, the impact of *Ochromonas* grazing on single-celled *Microcystis* strains in our small-scale laboratory experiments cannot be easily extrapolated to natural waters. Further experiments are needed to elucidate the role of *Ochromonas* grazing on colonial *Microcystis* populations.

In conclusion, protozoa such as the mixotrophic flagellate *Ochromonas* can be very effective grazers suppressing the population development of *Microcystis*. *Ochromonas* is not negatively affected by the ingested microcystins, nor is microcystin production by *M. aeruginosa* enhanced by *Ochromonas* grazing. While microcystins are a very powerful defence against many metazoan grazers (DeMott et al. 1991, Lürling 2003), they seem to be less useful against mixotrophic flagellates and colony formation might be the more effective protection against these very small protozoan grazers.

Chapter 6

Experimental comparison of predator-prey interactions with and without intraguild predation

Abstract

Theory predicts that intraguild predation leads to different community dynamics than the trophic cascades of a linear food chain. However, experimental comparisons of these two food-web modules are rare. Mixotrophic plankton species combine photoautotrophic and heterotrophic nutrition by grazing upon other microorganisms. We found that the mixotrophic chrysophyte *Ochromonas* can grow autotrophically on ammonium, but not on nitrate. This offered a unique opportunity to compare predator-prey interactions in the presence and absence of intraguild predation, without changing the species composition of the community. With ammonium as nitrogen source, *Ochromonas* can compete with its autotrophic prey for nitrogen and therefore acts as intraguild predator. With nitrate, *Ochromonas* acts solely as predator, and is not in competition with its prey for nitrogen. We parameterised a simple intraguild predation model based on chemostat experiments with monocultures of *Ochromonas* and the toxic cyanobacterium *Microcystis*, and subsequently tested the model predictions by inoculating *Ochromonas* into the *Microcystis* monocultures. The results showed that *Microcystis* was a better competitor for ammonium than *Ochromonas*. In agreement with theoretical predictions, *Microcystis* was much more strongly suppressed by intraguild predation on ammonium than by top-down predation on nitrate. Yet, *Microcystis* persisted at very low population densities, because it was the better competitor for ammonium, and because the type III functional response of *Ochromonas* implied that the grazing pressure upon *Microcystis* became low when *Microcystis* was rare. Our results provide experimental support for intraguild predation theory, and indicate that intraguild predation may enable biological control of microbial pest species.

This chapter is based on the manuscript: Wilken S, Verspagen JMH, Naus-Wiezer S, Van Donk E, and Huisman J. Experimental comparison of predator-prey interactions with and without intraguild predation. submitted

Introduction

Predators can suppress prey abundances to levels far below their carrying capacity. This top-down control features prominently in classic linear food chains, where foraging by predators cascades downwards to lower trophic levels (Hairston et al. 1960, Oksanen et al. 1981, Carpenter et al. 1985, Power 1990, Shurin et al. 2002). However, many food webs do not obey this classic linear pattern, but show more intricate relationships between the species. For instance, predators often feed upon multiple trophic levels (Polis and Strong 1996, Thompson et al. 2007). This may lead to intraguild predation, in which predators feed upon prey species but also compete against these prey species by utilizing the same basal resources (Polis et al. 1989, Mylius et al. 2001, Arim and Marquet 2004). Theory predicts that attacking prey from two sides, through both competition and predation, can be an efficient strategy to suppress intraguild prey to even lower levels than can be achieved by predation alone (Polis and Holt 1992, Diehl and Feissel 2000). Intraguild predation can therefore be very effective in, for instance, biological control (Bampfylde and Lewis 2007).

Yet, although this key prediction of intraguild predation theory seems highly plausible, it is difficult to test experimentally. The comparison of different experimental food-web modules generally supports the stronger suppression of prey, when suffering from both competition and predation (Lawler and Morin 1993, Holyoak and Sachdev 1998). However, such experiments are usually confounded by variation in species composition, because the transformation of a standard predator-prey system into an intraguild predator-prey system would typically involve a change in the identity of either the predator or the prey species.

Here, we present a direct experimental comparison of top-down predation versus intraguild predation that is not confounded by differences in species composition. Our experiments make use of a mixotrophic organism as intraguild predator. Mixotrophs combine both photoautotrophic and heterotrophic nutrition, and are increasingly recognized as important components of plankton communities and microbial food webs (Zubkov and Tarran 2008, Flynn et al. 2013, Hartmann et al. 2012). While the photosynthetic machinery of mixotrophs enables their survival as primary producers utilizing mineral nutrients and light, mixotrophs can also dominate grazing on phytoplankton communities (Havskum and Hansen 1997, Callieri et al. 2006, Hansen 2011). Hence, mixotrophs eat their competitors (Thingstad et al. 1996) and therefore act as intraguild predators.

In pilot experiments, we found that the mixotrophic chrysophyte *Ochromonas* sp. can utilize ammonium as nitrogen source, but not nitrate. This offers a unique opportunity to manipulate the trophic position of *Ochromonas* without changing the species composition of the community. When grown on ammonium, *Ochromonas* acts as an intraguild predator that feeds upon other phytoplankton species and competes with them for ammonium (Fig.

6.1A). In contrast, when grown on nitrate, *Ochromonas* acts as a top-down predator in a linear food chain but does not compete for nitrogen with the other phytoplankton species (Fig. 6.1B).

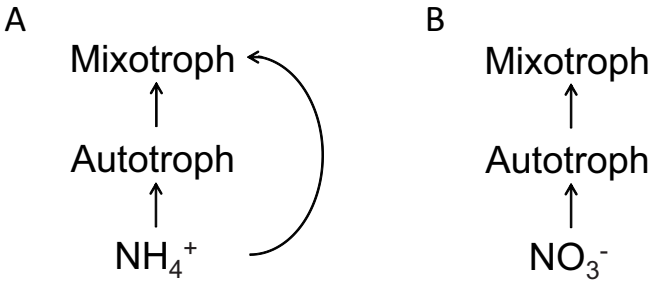

Figure 6.1: Comparison of the two predator-prey interactions: (A) With ammonium as nitrogen source, the mixotroph *Ochromonas* acts as an intraguild predator that not only feeds upon its autotrophic prey but also competes with it for limiting amounts of ammonium. (B) With nitrate as nitrogen source, the mixotroph *Ochromonas* acts as a top-down predator.

We first formulate a mathematical model describing the population dynamics of a mixotroph feeding on an autotrophic prey organism. Subsequently, we run monoculture experiments with *Ochromonas* sp. and the harmful cyanobacterium *Microcystis aeruginosa* on ammonium and on nitrate to estimate the model parameters. Finally, we test the theoretical predictions in species mixtures with and without intraguild predation.

Theory

Our model is an extension of earlier intraguild predation models developed by Holt and Polis (1997) and Diehl and Feissel (2000). These authors assumed that the basal resource was a living organism (e.g., a bacterium) obeying logistic growth, and the intraguild predator and prey were heterotrophic organisms. In our application, however, the basal resource is an inorganic nutrient, the intraguild prey is an autotroph, and the intraguild predator is a mixotroph combining autotrophic and heterotrophic nutrition. This necessitates a model formulation that accounts for (i) competition for nutrients between the autotroph and mixotroph, and (ii) a predator-prey interaction in which the mixotroph assimilates organic nutrients from ingested prey. Furthermore, our experiments showed dynamic changes in cellular nutrient content of the autotrophic and mixotrophic species, which were also incorporated into the model (Droop 1973, Sterner and Elser 2002).

Let P_A and P_M denote the population densities of the autotroph and mixotroph, respectively, let Q_A and Q_M denote their cellular nutrient contents, and let N denote the ambient concentration of dissolved inorganic nutrient. Our model then reads:

$$\frac{dP_A}{dt} = \mu_A(Q_A)P_A - m_A P_A - f_M(P_A)P_M \tag{6.1}$$

$$\frac{dP_M}{dt} = \mu_M(Q_M)P_M - m_M P_M \tag{6.2}$$

$$\frac{dQ_A}{dt} = u_A(N, Q_A) - \mu_A(Q_A)Q_A \tag{6.3}$$

$$\frac{dQ_M}{dt} = u_M(N, Q_M) + f_M(P_A)Q_A - \mu_M(Q_M)Q_M \tag{6.4}$$

$$\frac{dN}{dt} = D(N_{in} - N) - u_A(N, Q_A)P_A - u_M(N, Q_M)P_M \tag{6.5}$$

Equations (6.1) and (6.2) describe the population dynamics of the autotroph and mixotroph, respectively, where $\mu_A(Q_A)$ and $\mu_M(Q_M)$ are their specific growth rates as functions of cellular nutrient status, m_A and m_M are their specific mortality rates, and $f_M(P_A)$ is the functional response of the mixotroph feeding on the autotroph. Equations (6.3) and (6.4) describe changes in cellular nutrient content of the autotroph and mixotroph, respectively, where $u_A(N,Q_A)$ and $u_M(N,Q_M)$ are their uptake rates of inorganic nutrient, the term $f_M(P_A)Q_A$ describes the assimilation of nutrients from ingested prey, while the last terms in equations (6.3) and (6.4) describe dilution of the cellular nutrient content by population growth. Equation (6.5) describes changes in ambient nutrient concentration, where D is the nutrient turnover rate (also known as the 'dilution rate' in chemostats), N_{in} is the nutrient supply concentration, and the last two terms describe nutrient uptake by the autotroph and mixotroph. If the mixotroph is not able to consume nutrients (i.e., $u_M(N,Q_M) = 0$), then this intraguild predation model reduces to a linear food chain.

We assume that the specific growth rates of the autotroph and mixotroph are increasing saturating functions of their cellular nutrient status:

$$\mu_i(Q_i) = \mu_{max}\left(1 - \frac{Q_{max,i} - Q_i}{Q_{max,i} - Q_{min,i}}\right) \qquad i=A,M \tag{6.6}$$

Here, $\mu_{max,i}$ is the maximum specific growth rate of species *i*, and $Q_{max,i}$ and $Q_{min,i}$ are its maximum and minimum cellular nutrient contents, respectively. This formulation differs slightly from Droop's (1973) classic growth equation by the explicit incorporation of a maximum cellular nutrient content.

The model further assumes that nutrient uptake rates of the species increase with the ambient nutrient concentration according to Michaelis–Menten kinetics, and are suppressed when cells become satiated with nutrient (Morel 1987, Ducobu et al. 1998).

$$u_i(N, Q_i) = \frac{u_{max,i} N}{K_i + N}\left(\frac{Q_{max,i} - Q_i}{Q_{max,i} - Q_{min,i}}\right) \qquad i=A,M \qquad (6.7)$$

Here, $u_{max,i}$ is the maximum nutrient uptake rate of species *i* and K_i is its half-saturation constant for nutrient uptake.

We assume that the predation rate, $f_M(P_A)$, of the mixotroph feeding on the autotroph follows a Holling type III functional response in accordance with the experimental data of Wilken et al. (2010):

$$f_M(P_A) = \frac{f_{max}(P_A)^b}{(k_M)^b + (P_A)^b} \qquad (6.8)$$

where f_{max} is the maximum ingestion rate, k_M is the half-saturation constant, and *b* is a parameter defining the curvature of the type III functional response.

Materials and Methods

Our experiments were based on a freshwater strain of the mixotrophic chrysophyte *Ochromonas* sp. as intraguild predator, the harmful cyanobacterium *Microcystis aeruginosa* PCC 7806 as its prey, and inorganic nitrogen as the limiting nutrient. Our *Ochromonas* strain (probably *Ochromonas globosa* Skuja, determined by Dr R. Bijkerk, Koeman & Bijkerk B.V., Ecological Research and Advice, Haren, The Netherlands) was originally detected as infection in a large-scale mesocosm experiment with a laboratory culture of *Microcystis* (Van Donk et al. 2009). It was isolated with micro-needle techniques, and subsequently maintained in uni-algal but non-axenic stock cultures under autotrophic growth conditions using a nutrient-rich mineral medium (Wilken et al. 2013). Earlier studies have shown that *Ochromonas* can grow well autotrophically, but reaches much higher growth rates when feeding upon bacteria and microalgae by phagocytosis (Sanders et al. 2001, Wilken et al. 2013). *Ochromonas* can graze efficiently on the harmful cyanobacterium *Microcystis* spp, and even degrade its cyanotoxins (Van Donk et al. 2009, Wilken et al. 2010).

Batch experiments

We first used simple batch experiments to compare the growth performance of *Ochromonas* on ammonium versus nitrate under autotrophic growth conditions. Prior to the experiments, *Ochromonas* was inoculated from the stock culture into 250 mL Erlenmeyer flasks containing nutrient-rich mineral medium described by Wilken et al. (2013) with either 500 µM ammonium or 500 µM nitrate as nitrogen source. These precultures were transferred to fresh medium three times to remove possible traces of ammonium from the cultures provided with nitrate, and vice versa. Subsequently, the experiments were started by inoculating *Ochromonas* at a final abundance of 2×10^4 cells mL^{-1} in 100 mL of fresh medium containing either ammonium or nitrate. Both nitrogen treatments were performed in triplicate. The batch cultures were incubated at 23 °C and a continuous illumination of 90 µmol photons m^{-2} s^{-1}. Samples were taken every other day and counted with a particle counter (CASY-counter; Schärfe-System GmbH, Reutlingen, Germany). Specific growth rates (µ) were calculated over periods of exponential growth, using linear regression of ln-transformed population abundances versus time.

Chemostat experiments

To estimate the model parameters under autotrophic growth conditions, *Ochromonas* and *Microcystis* were grown in monoculture chemostats provided with ammonium or nitrate as nitrogen source. The monoculture chemostats were run until a steady state was reached. Subsequently, we tested the model predictions in species mixtures created by inoculating a small amount of *Ochromonas* taken from their steady-state monoculture into the steady-state monocultures of *Microcystis*.

The chemostat experiments were performed in flat culture vessels as described in Huisman et al. (1999), with a dilution rate of $D = 0.12$ d^{-1}. The chemostats were provided with a nutrient-rich mineral medium (Wilken et al. 2013) but a low concentration of 20 µM $NaNO_3$ or 20 µM NH_4Cl to ensure nitrogen-limited conditions. Light was supplied by white fluorescent tubes (Philips PL-L 24W/840/4P, Eindhoven, The Netherlands) at a constant incoming irradiance (I_{in}) of 50 ± 2 µmol photons m^{-2} s^{-1}. The temperature was kept constant at 23 °C by a metal cooling finger connected to a water bath. The chemostats were aerated with filter-sterilized air at a flow rate of 20 L h^{-1} and additionally mixed by a magnetic stirrer. To prevent introduction of ammonium by air, the air flow was cleaned by leading it through a 0.5 N phosphoric acid solution before entering the chemostat cultures. Wall growth was removed daily by scraping with the magnet of the magnetic stirrer.

Sampling and measurements

The chemostats were sampled at least every other day. Samples for cell counts were fixed with a mixture of glutaraldehyde and formaldehyde (final concentration of 0.025 and 0.0037 percent by mass, respectively) and stored at 4 °C until counting at a flow cytometer (MoFlo XDP cell sorter, Beckman Coulter, Miami, FL, USA). The flow cytometer distinguished between *Ochromonas* and *Microcystis* based on their size and pigmentation. Samples for determination of the dissolved nitrogen concentration were centrifuged for 10 min at 4,000 rpm and the supernatant was stored at -20 °C until further analysis. Ammonium concentrations were measured using the fluorometric method described in Holmes et al. (1999). Nitrate concentrations were analysed using a Skalar SA400 autoanalyser (Skalar Analytical, Breda, The Netherlands). For the monoculture experiments, cellular nitrogen contents were calculated from the difference between the nitrogen supply concentration and the dissolved nitrogen concentration using mass balance considerations (i.e., $Q_i=(N_{in}-N)/P_i$).

Model parameterisation

The model was parameterised based on our monoculture chemostat experiments and earlier grazing experiments by Wilken et al. (2010). Several model parameters were under direct experimental control, including the dilution rate (D) and the nitrogen concentration in the mineral medium (N_{in}). We assume that the specific mortality rates of the species were governed by the dilution rate of the chemostat (i.e., $m_i = D$ for all i). The functional response of *Ochromonas* grazing on *Microcystis* was based on the grazing experiments of Wilken et al. (2010), adopting their values for the maximum ingestion rate (f_{max}) and the curvature of the functional response (b), but using the half-saturation constant (k_M) as a tuning parameter to account for possible differences in grazing efficiency between the batch experiments of Wilken et al. (2010) and the much more turbulent conditions in our chemostat experiments. The remaining model parameters were estimated by fitting the time courses predicted by the model to the time courses of the experimental variables measured in our monoculture experiments. These experimental variables included the population densities of *Ochromonas* and *Microcystis* (P_M and P_A, respectively), their cellular nutrient contents (Q_M and Q_A), and the dissolved nitrogen concentration (N). The model fits were based on minimization of the residual sum of squares between observed and predicted values using the Gauss-Marquardt-Levenberg algorithm in the software package PEST (Watermark Numerical Computing, Brisbane, Australia), following the same approach as in earlier studies (Huisman et al. 1999, Passarge et al. 2006, Van de Waal et al. 2011).

Results

The batch experiments showed a striking difference in growth rate of *Ochromonas* on ammonium versus nitrate. *Ochromonas* achieved a specific growth rate of 0.30 ± 0.01 (SE) d^{-1} on ammonium (Fig. 6.2A; linear regression on ln-transformed abundances versus time: $R^2 = 0.97$, n = 15, $p < 0.0001$). In contrast, on nitrate it achieved only a marginal growth rate of 0.03 ± 0.01 (SE) d^{-1} (Fig. 6.2B; linear regression: $R^2 = 0.49$, n = 18, $p < 0.001$).

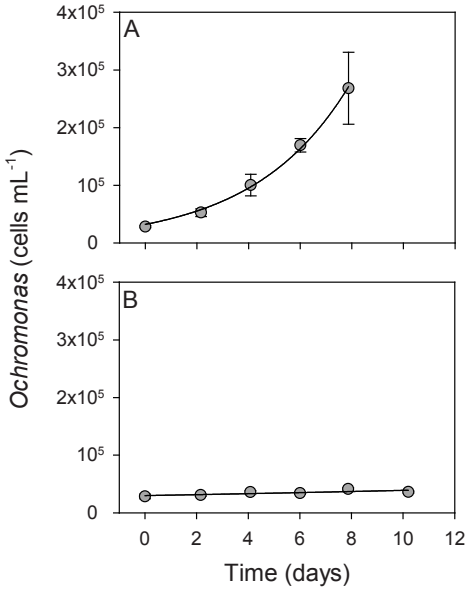

Figure 6.2: Growth of *Ochromonas* sp. in batch culture provided with either (A) ammonium or (B) nitrate as nitrogen source. Error bars indicate the standard deviation of triplicate measurements. The solid lines are based on linear regression of the ln-transformed population abundances versus time.

In monoculture chemostats, both *Microcystis* and *Ochromonas* grew well with ammonium as nitrogen source (Fig. 6.3). The cellular nitrogen contents of both species showed transient nitrogen storage during the first few days, when the ambient ammonium concentration was not yet depleted (Fig. 6.3B,D). At steady state, *Microcystis* reduced the ammonium concentration to 0.001 ± 0.011 (SE) µM, whereas *Ochromonas* reduced the ammonium concentration to only 0.091 ± 0.008 (SE) µM (Fig. 6.3E). Therefore, *Microcystis* has the lower critical nutrient requirement (i.e., a lower R^* sensu Tilman 1982), and, hence, resource competition theory predicts that *Microcystis* will be a better competitor for ammonium than *Ochromonas*. We were unable to grow *Ochromonas* in monoculture chemostats with nitrate as nitrogen source. In contrast, *Microcystis* grew well on nitrate, and reached almost identical monoculture abundances of ~560,000 cells mL^{-1} on both nitrogen sources (Fig. 6.4A,B, before inoculation of *Ochromonas*). The model captured the monoculture population dynamics of both species quite well (Figs. 6.3 and 6.4). The model parameters estimated from the monoculture experiments are provided in Table 6.1.

Inoculation of *Ochromonas* into the steady-state monocultures of *Microcystis* led to a steep decline in *Microcystis* abundances through grazing, irrespective of whether nitrate or ammonium was used as nitrogen source (Fig. 6.4). However, the subsequent population dynamics differed between the two treatments. With ammonium as nitrogen source, *Microcystis* suffered from intraguild predation and was suppressed to near-extinction, while *Ochromonas* became the dominant species (Fig. 6.4A). We note that *Microcystis* did not

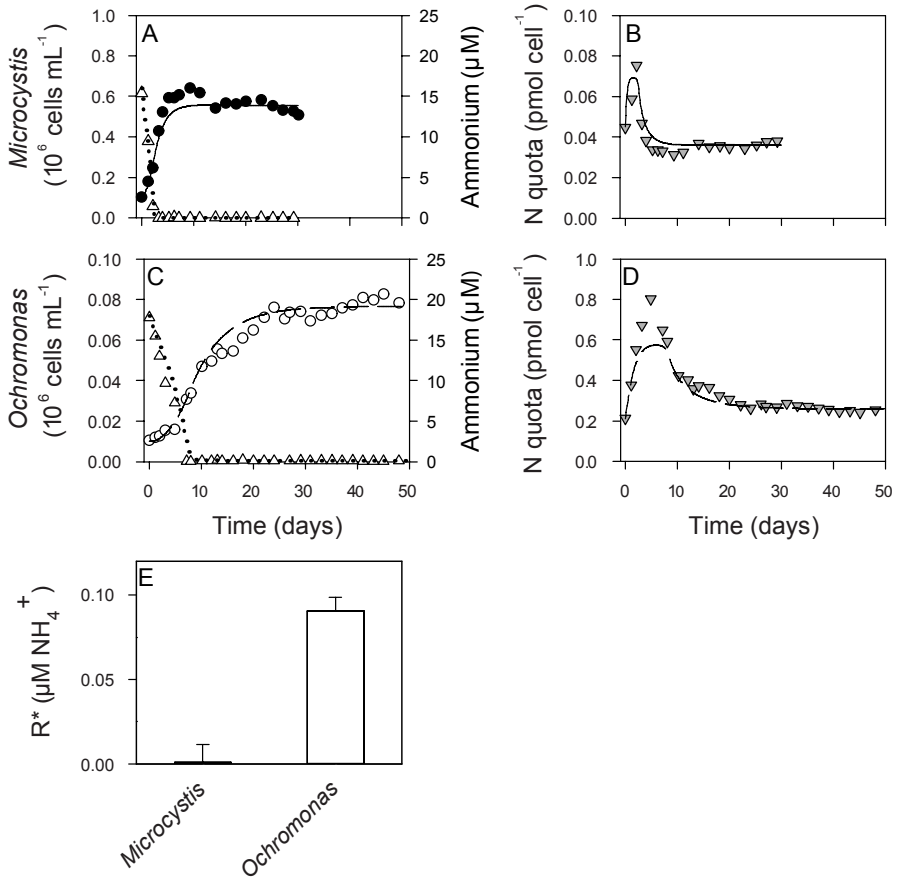

Figure 6.3: Monoculture chemostats with ammonium as nitrogen source. (A) Dynamic changes in population density (black circles), ammonium concentration (open triangles) and (B) cellular nitrogen content (grey triangles) of the autotroph *Microcystis*. (C) Dynamic changes in population density (open circles), ammonium concentration (open triangles) and (D) cellular nitrogen content (grey triangles) of the mixotroph *Ochromonas* sp. Symbols represent chemostat data, lines show the model predictions based on the parameter values in Table 1. (E) Steady-state ammonium concentrations (*R**) in the monoculture chemostats of *Microcystis* and *Ochromonas*. Error bars indicate the standard error of repeated measurements during steady state (n = 12).

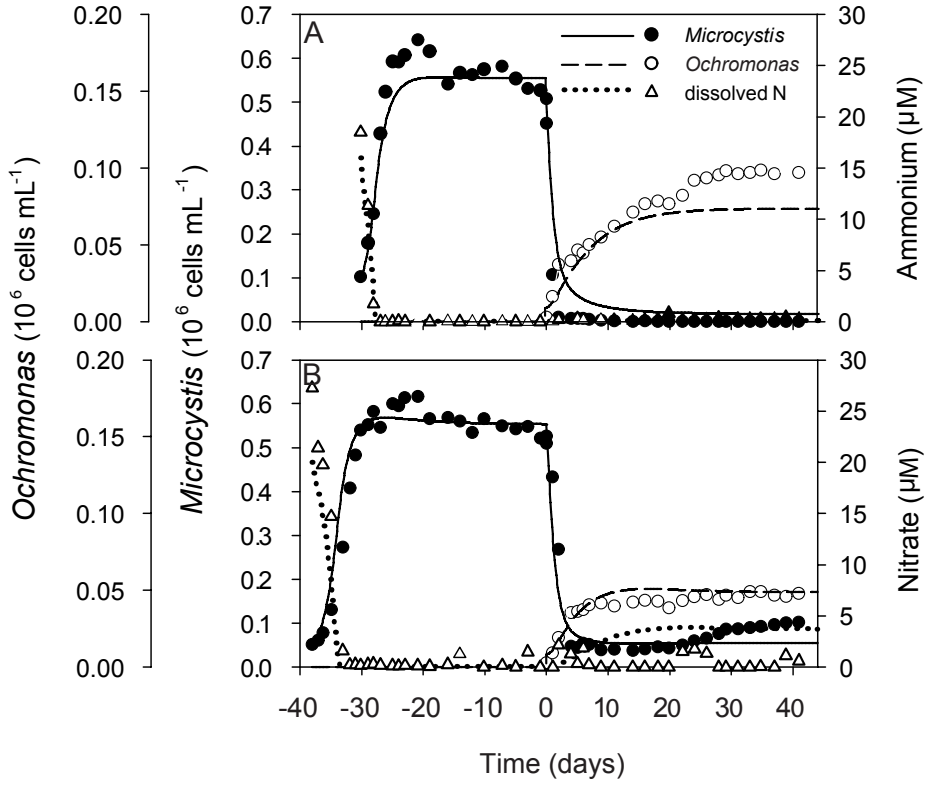

Figure 6.4: Population dynamics in the species mixtures. The autotroph *Microcystis* is grown until it has established a steady-state monoculture. Subsequently, the mixotroph *Ochromonas* is inoculated at t=0. (A) With ammonium as nitrogen source, *Ochromonas* acts as intraguild predator and suppresses *Microcystis* to near extinction. (B) With nitrate as nitrogen source, *Ochromonas* acts as top-down predator and *Microcystis* is suppressed much less. Symbols represent chemostat data, lines show the model predictions based on the parameter values in Table 6.1.

go completely extinct but persisted throughout the entire experiment at trace abundances of 100 to 500 cells mL^{-1}. In contrast, with nitrate as nitrogen source, *Microcystis* suffered from top-down predation only. In this case, *Ochromonas* reached lower population abundances, while *Microcystis* was suppressed but remained numerically the dominant species at a population abundance fluctuating between 45,000 and 100,000 cells mL^{-1} (Fig. 6.4B). Although the model slightly overestimated the nitrate concentration during top-down predation (Fig. 6.4B), these striking differences in the population dynamics with and without intraguild predation were qualitatively in good agreement with the model predictions (Fig. 6.4).

Table 6.1: Definition and values of model parameters

Symbol	Definition	Value		Unit	Source
State variables					
N	Dissolved nitrogen concentration			mol L^{-1}	
P_A	Population density of the autotroph			cells L^{-1}	
P_M	Population density of the mixotroph			cells L^{-1}	
Q_A	Cellular nitrogen content of the autotroph			mol $cell^{-1}$	
Q_M	Cellular nitrogen content of the mixotroph			mol $cell^{-1}$	
System parameters					
D	Dilution rate	0.12		day^{-1}	a
N_{in}	Nitrogen supply concentration	2.0×10^{-5}		mol L^{-1}	a
Species parameters		***Microcystis***	***Ochromonas***		
$u_{max,i}$	Maximum N-uptake rate				
	-Ammonium	12.0×10^{-14}	24.0×10^{-14}	mol $cell^{-1}$ day^{-1}	b
	-Nitrate	12.0×10^{-14}	0	mol $cell^{-1}$ day^{-1}	b
K_i	Half-saturation constant				
	-Ammonium	6.0×10^{-7}	6.0×10^{-7}	mol L^{-1}	b
	-Nitrate	2.0×10^{-6}	n.a.	mol L^{-1}	b
$\mu_{max,i}$	Maximum specific growth rate	1	2	day^{-1}	b
$Q_{min,i}$	Minimum cellular nitrogen content	2.8×10^{-14}	7.2×10^{-14}	mol $cell^{-1}$	b
$Q_{max,i}$	Maximum cellular nitrogen content	9.5×10^{-14}	32×10^{-13}	mol $cell^{-1}$	b
f_{max}	Maximum prey ingestion rate	n.a.	53.0	cells $cell^{-1}$ day^{-1}	c
k_M	Half-saturation constant for prey ingestion	n.a.	4.0×10^{8}	cells L^{-1}	d
b	Curvature of type III functional response	n.a.	2.37	-	c

a: measured system parameter; b: estimated from our monoculture chemostats; c: based on the grazing experiment of Wilken et al. (2010); d: tuning parameter estimated from the model fit to the species mixtures.

Discussion

A simple manipulation of the nitrogen source offered a unique opportunity to compare specialist predation to intraguild predation without confounding effects of differences in species composition. Our results confirm a key prediction of intraguild predation theory that prey abundances are suppressed much more strongly by intraguild predation than by specialist predation. Intraguild predation by *Ochromonas* reduced the population density of the toxic cyanobacterium *Microcystis* by more than two orders of magnitude in comparison to suppression by specialist predation.

Our results also allow comparison with other predictions of intraguild predation theory. In particular, there is considerable debate on the mechanisms that enable coexistence of the intraguild predator and its prey (e.g., Holt and Polis 1997, Křivan and Diehl 2005, Amarasekare 2008, Abrams 2011, Hin et al. 2011). Most models predict that coexistence of both species is possible in habitats of intermediate productivity, while intraguild prey will be excluded from productive environments due to intense predation by the intraguild predator (Holt and Polis 1997, Diehl and Feissel 2000, Mylius et al. 2001, Van de Wolfshaar et al. 2006). These predictions are partially supported by microcosm experiments with aquatic protists (Morin 1999, Diehl and Feissel 2000, 2001) and by field experiments with an insect and its parasitoids (Borer et al. 2003). However, they are contradicted by the common occurrence of intraguild predation in natural communities over a wide range of productivity levels (Polis and Strong 1996, Arim and Marquet 2004).

Interestingly, in our experiments, *Microcystis* was not excluded by intraguild predation, but persisted at very low densities throughout the entire duration of the experiment. According to intraguild predation theory, a necessary (but not sufficient) condition for coexistence of the intraguild predator and its prey is that the prey should be the better competitor for the limiting resource (Polis and Holt 1992, Holt and Polis 1997, Diehl and Feissel 2000). This is in good agreement with our experimental results, where the autotroph *Microcystis* was indeed a better competitor for ammonium (had a lower R^* for ammonium) than the mixotroph *Ochromonas* (Fig. 6.3E). Since mixotrophy requires investments into the physiological and biochemical machinery for both phototrophic and heterotrophic nutrition, the low competitive strength of *Ochromonas* might reflect a more general trait of mixotrophic organisms (Raven 1997).

The persistence of *Microcystis* at very low abundances in the intraguild predation experiment is further supported by the type III functional response of the intraguild predator, a mechanism for coexistence that has also been mentioned by other studies (Gismervik and Andersen 1997, Abrams and Fung 2010a). This can best be seen by a closer look at the population dynamics of *Microcystis*, described by equation (6.1). The per capita mortality

rate of *Microcystis* due to predation is $f_M(P_A)P_M/P_A$. At very low population densities of *Microcystis* (i.e., $P_A << k_M$), the functional response of equation (6.8) can be approximated as $f_M(P_A)=c(P_A)^b$, where $c=f_{max}/(k_M)^b$. Hence, the per capita mortality rate is $c(P_A)^{b-1}P_M$. This shows that, for a type III functional response (i.e., for $b>1$), the per capita mortality rate vanishes to zero when P_A approaches zero. This implies that *Microcystis* experiences negligible predation when present at very low abundances. Thus, because *Microcystis* is the better competitor for ammonium, it can always invade a monoculture of the intraguild predator and persist at low population densities.

The type III functional response is often referred to as a switching response used by predators that feed preferentially on the more abundant prey. In mixotrophs, autotrophic and heterotrophic nutrition require different cellular structures and biochemical pathways that can largely operate independently from each other. A strong decline in intraguild prey abundance may therefore reduce investment of mixotrophs into their cellular machinery for heterotrophic nutrition. This is reflected in reduced clearance rates at low prey abundances, and hence a shift towards predominantly autotrophic nutrition, as captured by the type III functional response (Wilken et al. 2010). The equilibrium population density of *Ochromonas* in the intraguild predation experiment was only slightly higher than that in monoculture (compare Fig. 6.4A vs Fig. 6.3B). This indicates that, after the strong decline of *Microcystis*, *Ochromonas* was indeed growing largely autotrophically in the species mixture.

Our findings are based on the observation that *Ochromonas* can utilize ammonium, but not nitrate as nitrogen source. To what extent this is a more common property among mixotrophic organisms is not yet known. However, phytoplankton species differ in their ability to utilize different forms of nitrogen, and a general preference for ammonium and small organic nitrogen molecules is commonly observed (Eppley et al. 1969, McCarthy et al. 1977, Lomas and Glibert 1999, Taylor et al. 2006, L'Helguen et al. 2008). Several phytoplankton species cannot utilize nitrate, because they lack enzymes for nitrate uptake and assimilation (DeYoe and Suttle 1994, Moore et al. 2002). Given the costs of nitrate reduction, the acquisition of diverse forms of reduced nitrogen with the ingestion of their prey might even favour a reduced capability for nitrate assimilation in mixotrophic organisms. As our results demonstrate, changes in the composition of the dissolved nitrogen pool can therefore have major effects on the trophic position of mixotrophs, and hence on the structure of microbial food webs.

Intraguild predation has received considerable attention in the context of invasion ecology and pest control (Rosenheim et al. 1995, Straub et al. 2008, Crowder and Snyder 2010, Hall 2011, van Maanen et al. 2012). In several of these studies, the target for

biological control is not the intraguild prey but the basal prey species. In this case, intraguild predation among its natural enemies can diminish the effectiveness of biological control (Rosenheim et al. 1995, Straub et al. 2008). However, engagement in intraguild predation can increase the efficacy of a biological control agent, if the target species is the intraguild prey (Bampfylde and Lewis 2007). The latter is the case for *Microcystis*, a widespread freshwater cyanobacterium favoured by eutrophic conditions, which can produce a variety of cyanotoxins posing a major threat to water quality and human health (Chorus and Bartram, 1999, Codd et al. 2005, Huisman et al. 2005, Paerl and Huisman 2008). Intraguild predators that attack *Microcystis* through both competition and predation, like *Ochromonas*, might be effective biological control agents of this harmful cyanobacterium. However, our results show that this depends on the nitrogen source. *Ochromonas* can act as intraguild predator on ammonium but not on nitrate. As a consequence, *Ochromonas* is expected to be a much more effective control agent of *Microcystis* in ammonium-rich waters than in nitrate-rich waters.

In conclusion, we have used a simple aquatic microbial community to test several of the key predictions of intraguild predation theory. In particular, our experiment demonstrates (i) that prey species can be suppressed much more strongly by intraguild predation than by the classic predator-prey interaction of a linear food chain, (ii) that a type III functional response may enable the persistence of the intraguild prey at low population abundances, and (iii) that intraguild predation can be context-dependent; a change in nitrogen source turned an intraguild predator into a classic 'top-down' predator. These experimental results may open up the opportunity to apply intraguild predation for the biological control of microbial pest species, including toxic cyanobacteria.

Chapter 7

Biological control of harmful cyanobacteria by mixotrophic predators: an experimental test of intraguild predation theory

Abstract

Intraguild predators both feed on and compete with their intraguild prey. In theory, intraguild predators can therefore be very effective as biological control agents of intraguild prey species, especially in productive environments. We investigated this hypothesis using the mixotrophic chrysophyte *Ochromonas* as intraguild predator and the harmful cyanobacterium *Microcystis aeruginosa* as its prey. *Ochromonas* can grow photoautotrophically, but can also graze efficiently on *Microcystis*. Hence, it competes with its prey for inorganic resources. We developed a mathematical model and parameterized it for our experimental food web. The model predicts dominance of *Microcystis* at low nutrient loads, coexistence of both species at intermediate nutrient loads, and an increase of *Ochromonas* but decrease of *Microcystis* with further nutrient enrichment. We tested these theoretical predictions in chemostat experiments supplied with three different nitrogen concentrations. *Ochromonas* initially suppressed the *Microcystis* abundance by > 97% compared to the *Microcystis* monocultures. Thereafter, however, *Microcystis* gradually recovered to ~20% of its monoculture abundance at low nitrogen loads, but to 50-60% at high nitrogen loads. Hence, *Ochromonas* largely lost control over the *Microcystis* population at high nitrogen loads. We explored several mechanisms that might explain this deviation from theoretical predictions, and found that intraspecific interference at high *Ochromonas* densities reduced their grazing rates on *Microcystis*. These results illustrate the potential of intraguild predation to control pest species, but also show that the effectiveness of their biological control can be reduced in productive environments.

This chapter is based on the manuscript: Wilken S, Verspagen JMH, Naus-Wiezer S, Van Donk E, and Huisman J. Biological control of harmful cyanobacteria by mixotrophic predators: an experimental test of intraguild predation theory. submitted

Introduction

Studies of competition and predation, the two major structuring forces of ecological food webs, have played a key role in the development of community ecology. Intraguild predation encompasses both types of species interaction (Polis et al. 1989, Polis and Holt 1992). Intraguild predators feed upon their prey but also compete against their prey for shared resources. In other words, intraguild predators eat their competitors. Attacking prey from two sides, through both competition and predation, can be a very efficient strategy to suppress the population abundances of intraguild prey (Polis and Holt 1992, Thingstad et al. 1996). Therefore, intraguild predators that utilize pest species as their intraguild prey might be very effective as biological control agents (Bampfylde and Lewis 2007).

Most models predict coexistence of intraguild predators and their prey in habitats of intermediate productivity, provided that the intraguild prey is a better competitor for the common resource (Polis and Holt 1992, Holt and Polis 1997, Diehl and Feissel 2000). However, intraguild prey populations are predicted to decrease with increasing productivity due to an enhanced predation pressure, and intraguild predators will tend to exclude their intraguild prey from highly productive environments (Holt and Polis 1997, Diehl and Feissel 2000, Mylius et al. 2001, Van de Wolfshaar et al. 2006). These theoretical predictions are supported to various degrees by microcosm experiments with aquatic protists (Morin 1999, Diehl and Feissel 2000, 2001) and by field experiments with an insect and its parasitoids (Borer et al. 2003). Yet, other experiments observed persistence of intraguild prey in productive environments (Liess and Diehl 2006, Montserrat et al. 2008). Moreover, coexistence of intraguild predators and their prey appears widespread in natural communities, including productive environments (Polis and Strong 1996, Arim and Marquet 2004). During recent years, theoretical studies have therefore proposed a plethora of possible mechanisms that promote the persistence of intraguild prey in productive environments (e.g., Křivan and Diehl 2005, Finke and Denno 2006, Janssen et al. 2007, Amarasekare 2008, Rudolf and Armstrong 2008, Abrams and Fung 2010b, Hin et al. 2011).

Intraguild predation has received considerable interest in agricultural studies (Rosenheim et al. 1995, Straub et al. 2008, Van Maanen et al. 2012), but may also find application in other fields including water management. In particular, cyanobacterial blooms are favoured by high nutrient loads, and have increasingly become a major nuisance in many lakes and reservoirs (Chorus and Bartram 1999, Huisman et al. 2005, Paerl and Huisman 2008). Dense cyanobacterial blooms may cause nighttime oxygen depletion, sometimes leading to large fish kills. In addition, the high turbidity of cyanobacterial blooms reduces the growth of aquatic macrophytes, suppressing important underwater habitat for other aquatic organisms (Scheffer 1998). Furthermore, several cyanobacterial species can produce

toxins causing serious and sometimes fatal liver, digestive and neurological diseases in birds, mammals (e.g., pets, cattle) and humans (Codd 1995, Carmichael et al. 2001). Cyanobacterial blooms are therefore a major concern in water quality management (Codd et al. 2005, Verspagen et al. 2006, Qin et al. 2010).

Mixotrophic organisms combine autotrophic and heterotrophic nutrition. For instance, many photosynthetic microalgae can also ingest bacteria and phytoplankton species by phagocytosis (Stoecker 1998, Jones 2000). Hence, mixotrophs often act as intraguild predators that compete with phytoplankton species for nutrients and light, but also graze upon them (Thingstad et al. 1996). Mixotrophs are widespread in plankton communities of both freshwater and marine ecosystems (Isaksson 1998, Zubkov and Tarran 2008, Flynn et al. 2013, Hartmann et al. 2012), and several species are capable to form dense blooms in eutrophic environments (Burkholder et al. 2008). In particular, some mixotrophic chrysophytes of the *Ochromonas* genus can graze efficiently on toxic cyanobacteria, and are even capable of degrading cyanobacterial toxins (Van Donk et al. 2009, Wilken et al. 2010). Their function as intraguild predators might make these mixotrophs ideal candidates as biological control agents against harmful cyanobacteria in eutrophic lakes.

In this paper, we test the predictions of intraguild predation theory with the toxic cyanobacterium *Microcystis aeruginosa* as intraguild prey and the mixotroph *Ochromonas* sp. as intraguild predator. We first develop a simple model to describe the population dynamics generated by intraguild predation along a productivity gradient. The model predictions are tested in a series of chemostat experiments at three different nitrogen levels to create an experimental productivity gradient. The experimental results are used to evaluate the potential for mixotrophic predators to control the development of harmful cyanobacterial blooms in productive environments.

Theory

Model structure

Our model is a variant of the earlier intraguild predation models developed by Holt and Polis (1997) and Diehl and Feissel (2000). These authors assumed that the basal resource was a living organism (e.g., a bacterium) obeying logistic growth, the intraguild prey was a heterotroph feeding on the basal prey, and the intraguild predator consumed both the basal and intraguild prey. In our application, the basal resource is an inorganic nutrient, the intraguild prey is an autotrophic organism, and the intraguild predator is a mixotroph combining autotrophic growth with grazing upon the intraguild prey.

Let N denote the concentration of dissolved inorganic nutrient, P_A the population density of the autotroph, and P_M the population density of the mixotroph. We consider a nutrient-limited system, where the autotroph takes up dissolved nutrients from the environment, while the mixotroph obtains its nutrients through both uptake of dissolved nutrients and ingestion of its autotrophic prey. Our intraguild predation model then reads:

$$\frac{dN}{dt} = D(N_{in} - N) - u_A(N)P_A - u_M(N)P_M \tag{7.1}$$

$$\frac{dP_A}{dt} = \left(1/Q_A\right)u_A(N)P_A - m_A P_A - f_M(P_A)P_M \tag{7.2}$$

$$\frac{dP_M}{dt} = \left(1/Q_M\right)u_M(N)P_M + \left(Q_A/Q_M\right)f_M(P_A)P_M - m_M P_M \tag{7.3}$$

Here, D is the nutrient turnover rate, N_{in} is the nutrient load, $u_A(N)$ and $u_M(N)$ are the nutrient uptake rates of the autotroph and mixotroph, Q_A and Q_M are their cellular nutrient contents, $f_M(P_A)$ is the functional response of the mixotroph grazing upon its autotrophic prey, and m_A and m_M are the mortality rates of the autotroph and mixotroph, respectively.

We assume that the nutrient uptake rates of the autotroph and mixotroph increase with the ambient nutrient concentration according to Michaelis-Menten kinetics:

$$u_i(N) = \frac{u_{max,i}N}{K_i + N} \qquad i = A,M \tag{7.4}$$

where $u_{max,i}$ is the maximum nutrient uptake rate of species i and K_i is its half-saturation constant for nutrient uptake. Furthermore, we assume that the mixotroph feeds upon the autotroph with a Holling type III functional response, in accordance with the experimental data of Wilken et al. (2010):

$$f_M(P_A) = \frac{f_{max}(P_A)^b}{(k_M)^b + (P_A)^b} \tag{7.5}$$

where f_{max} is the maximum ingestion rate, k_M is the half-saturation constant for prey ingestion, and b describes the curvature of the type III functional response.

Parameter estimates

The model was parameterised based on our earlier chemostat experiments with monocultures of the autotroph *Microcystis aeruginosa* and the mixotroph *Ochromonas* sp., using ammonium as the limiting nutrient (Chapter 6). We assume that the specific loss rates of both species were governed by the dilution rate of the chemostat (i.e., $m_i = D$ for all i). The functional response of *Ochromonas* grazing on *Microcystis* was obtained from the grazing experiments of Wilken et al. (2010), adopting their values for the maximum ingestion rate (f_{max}) and the curvature of the functional response (b), but using the half-saturation constant (k_M) as a tuning parameter to account for differences in predator-prey encounter rates between the batch experiments of Wilken et al. (2010) and the much more turbulent conditions in our chemostat experiments. The parameter values are provided in Table 7.1.

Model predictions

The model predictions are illustrated in Fig. 7.1. From these results, which are in good agreement with other intraguild predation models (Holt and Polis 1997, Diehl and Feissel 2000), we deduce the following theoretical predictions: (i) at low nutrient loads, the autotroph dominates; (ii) at intermediate nutrient loads, the autotroph and mixotroph coexist; (iii) with a further increase in nutrient loads, the mixotroph becomes dominant, while the autotroph decreases in abundance; (iv) the dissolved nutrient concentration shows a slight increase after the mixotroph has entered the system, but remains rather low; (v) the community does not show alternative stable states or non-equilibrium dynamics such as predator-prey oscillations (Fig. 7.1).

With respect to prediction (iii), we note that the type III functional response of the mixotroph implies that its grazing pressure on the autotroph becomes negligible when the autotroph becomes rare. Hence, the model predicts that the mixotroph will suppress the autotroph at high nutrient loads, but will not drive it to extinction (Chapter 6). Furthermore, we note that prediction (v) is specific for our parameter estimates, while intraguild predation models can display alternative stable states and predator-prey oscillations for other parameter combinations (cf. Holt and Polis 1997; Verdy and Amarasekare 2010; Hiltunen et al. in press).

Table 7.1: Definition and values of model parameters

Symbol	Definition	Value		Unit	Source
State variables					
N	Dissolved nitrogen concentration	-		mol N L^{-1}	
P_A	Autotroph abundance	-		cells L^{-1}	
P_M	Mixotroph abundance	-		cells L^{-1}	
System parameters					
D	Dilution rate	0.12		day^{-1}	a
N_{in}	Nitrogen load	10^{-8} – 10^{-2}		mol N L^{-1}	a
Species parameters		***Microcystis***	***Ochromonas***		
$u_{max,\,i}$	Maximum N-uptake rate	2.0×10^{-14}	8.00×10^{-14}	mol N $cell^{-1}$ day^{-1}	b
K_i	Half saturation constant for N-uptake	1.23×10^{-7}	1.60×10^{-7}	mol N L^{-1}	b
Q_i	Cellular nitrogen content	3.57×10^{-14}	2.94×10^{-13}	mol N $cell^{-1}$	b
f_{max}	Maximum ingestion rate	n.a.	53.0	cells $cell^{-1}$ day^{-1}	c
k_M	Half saturation constant for prey ingestion	n.a.	4.0×10^{8}	cells L^{-1}	d
b	Curvature of type-III functional response	n.a.	2.37	-	c

a: measured system parameter; b: estimated from monoculture chemostats; c: based on the grazing experiment of Wilken et al. (2010); d: tuning parameter estimated from the model fit to the species mixtures

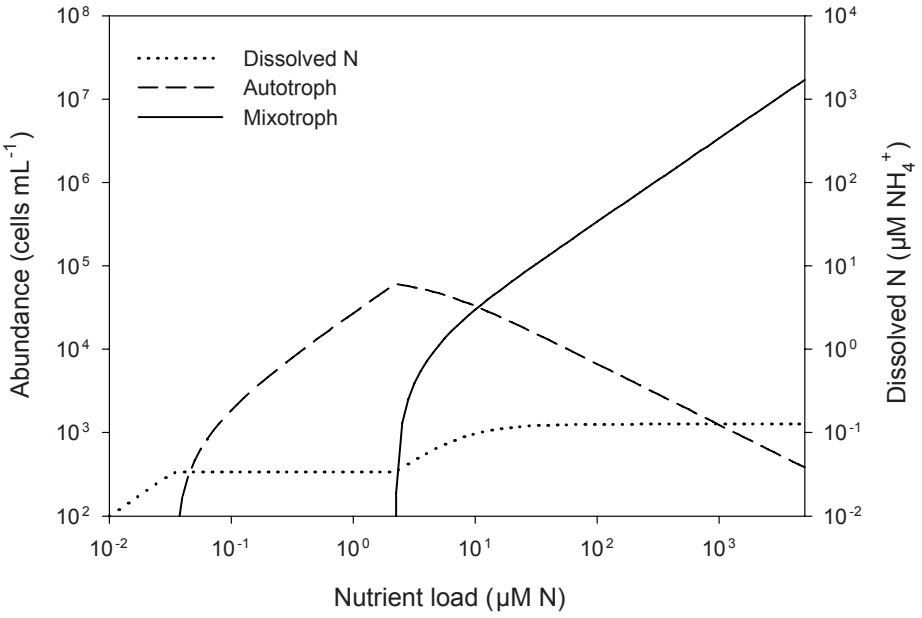

Figure 7.1: Model predictions of the steady-state abundances of the autotroph and mixotroph and the dissolved nitrogen concentration as function of the nutrient load. Parameter values are provided in Table 7.1.

Materials and methods

Species

Our experiments were performed with the mixotrophic chrysophyte *Ochromonas* sp. as intraguild predator, and the toxic cyanobacterium *Microcystis aeruginosa* PCC 7806 as intraguild prey. Our *Ochromonas* strain is probably *Ochromonas globosa* Skuja (determined by Dr R. Bijkerk, Koeman & Bijkerk B.V., Ecological Research and Advice, Haren, The Netherlands). It was originally detected as infection in a large-scale mesocosm experiment with *Microcystis*, from which it was isolated with micro-needle techniques (Van Donk et al. 2009). *Microcystis* PCC 7806 is a single-celled strain, and did not show any colony formation during the experiments.

Chemostat experiments

The theoretical predictions were tested in chemostat experiments, using three different nitrogen concentrations in the mineral medium (N_{in} = 20, 100 and 500 µM of NH_4Cl) to generate an experimental productivity gradient. All other nutrients were provided in excess

using a nutrient-rich mineral medium (Wilken et al. 2013). First, monoculture chemostats of *Microcystis* and *Ochromonas* were grown at each nitrogen level. After the monocultures had been maintained at steady state for at least two weeks, mixed cultures were produced by cross-inoculating each monoculture with 5% from the monoculture of the other species at the same nitrogen level. This resulted in two mixed cultures at each nitrogen level, one in which a small amount of *Ochromonas* was added to a steady-state monoculture of *Microcystis* and the other in which a small amount of *Microcystis* was added to a steady-state monoculture of *Ochromonas*. Hence, this procedure provided a straightforward method to test for alternative stable states.

The chemostat experiments were performed in 1.6 L flat culture vessels as described in Huisman et al. (1999), with a dilution rate of $D = 0.12$ d^{-1}. The temperature was kept constant at 23 ± 1 °C by a metal cooling finger connected to a water bath. Light was supplied by white fluorescent tubes (Philips PL-L 24W/840/4P, Eindhoven, The Netherlands) at a constant incoming irradiance (I_{in}) of 50 ± 2 µmol photons m^{-2} s^{-1}. The chemostats were aerated with filter-sterilized air at a flow rate of 20 L h^{-1}. To prevent introduction of gaseous ammonium, the air flow was cleaned by leading it through a 0.5 N phosphoric acid solution before entering the chemostat cultures. Carbon limitation was avoided by enriching the air flow with low concentrations of CO_2 using Brooks Mass Flow Controllers (Brooks Instruments, Hatfield, PA, USA), targeted to stabilize the pH at 7.8. Wall growth was removed daily by scraping the walls with a magnetic stirrer.

The chemostats were sampled approximately every other day. Samples for cell counts were fixed with a mixture of glutaraldehyde and formaldehyde (final concentration of 0.025 and 0.0037 percent by mass, respectively) and stored at 4 °C until counting at a flow cytometer (MoFlo XDP cell sorter, Beckman Coulter, Miami, FL, USA). The flow cytometer distinguished between *Ochromonas* and *Microcystis* based on their cell size and pigmentation. Samples for determination of the dissolved ammonium concentration were centrifuged for 10 min at 4,000 rpm and the supernatant was stored at -20 °C until further analysis. Ammonium concentrations were measured using the fluorometric method described in Holmes et al. (1999).

Results

Chemostat experiments

The autotroph *Microcystis* and the mixotroph *Ochromonas* both grew well in monoculture, and increased in abundance with increasing nitrogen load (Fig. 7.2). *Ochromonas* had a lower growth rate than *Microcystis*, as can be seen from the longer time

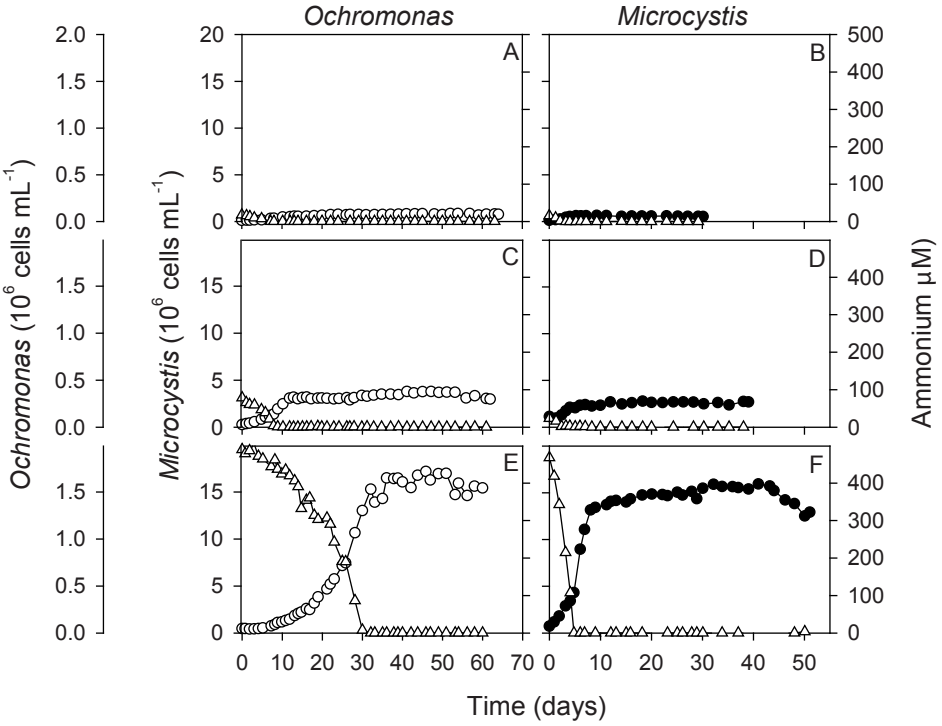

Figure 7.2: Monoculture experiments of *Ochromonas* (left panels) and *Microcystis* (right panels). The experiments were performed at (A,B) low nitrogen load of 20 µM ammonium, (C,D) intermediate nitrogen load of 100 µM ammonium, and (E,F) high nitrogen load of 500 µM ammonium. Open circles = *Ochromonas*, closed circles = *Microcystis*, open triangles = ammonium concentration.

span needed until steady state was reached (compare Fig. 7.2E and F). The linear increase in steady-state abundance of both species with increasing nitrogen load confirmed that nitrogen was indeed the limiting factor, even at the highest nitrogen level (Fig. 7.3A). Due to its smaller cell size, *Microcystis* reached about 10 times higher cellular abundances than *Ochromonas* (Fig. 7.3A). At steady state, *Microcystis* reduced the dissolved ammonium concentration to lower levels than *Ochromonas* (paired t-test: $t_2 = 5.05$, $p < 0.05$; Fig. 7.3B). Hence, *Microcystis* has a lower critical ammonium concentration (i.e., a lower *R** sensu Tilman 1982), and resource competition theory therefore predicts that *Microcystis* will be the better competitor for ammonium.

After inoculation of *Microcystis* into the monocultures of *Ochromonas*, the *Microcystis* abundance remained low for several weeks and then slowly increased (Fig. 7.4A,C,E). At low nitrogen load, the *Ochromonas* population was not much affected by the invasion

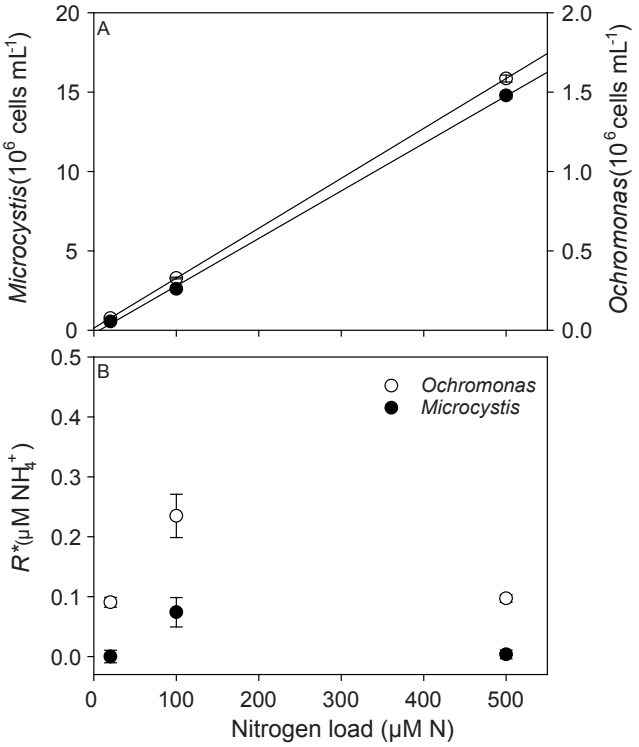

Figure 7.3: (A) Steady-state abundances of *Ochromonas* (open circles) and *Microcystis* (closed circles) in the monoculture experiments plotted as function of the nitrogen load. (B) Steady-state concentration of dissolved ammonium (*R**) in the monocultures of *Ochromonas* and *Microcystis* plotted as function of the nitrogen load. Error bars indicate standard errors of repeated measurements during steady state.

of *Microcystis* (Fig. 7.4A). However, at intermediate and high nitrogen load, the increase of *Microcystis* came along with a clear decline in *Ochromonas* abundance (Fig. 7.4C,E). Conversely, after inoculation of *Ochromonas* into the *Microcystis* monocultures, *Ochromonas* showed high initial grazing rates on the *Microcystis* populations, resulting in a rapid increase of the *Ochromonas* population. *Microcystis* quickly declined within the first 1-2 weeks, from 450,000 to less than 1,000 cells mL^{-1} at the low nitrogen load (Fig. 7.4B), from 2.6 million to 50,000 cells mL^{-1} at the intermediate nitrogen load (Fig. 7.4D), and from 12 million to 300,000 cells mL^{-1} at the high nitrogen load (Fig. 7.4F). Hence, within the first few weeks *Microcystis* was reduced by > 97% at all three nutrient treatments. After some time, however, the growth rates of *Ochromonas* dwindled, and concomitantly *Microcystis* was able to slowly grow up again (Fig. 7.4B,D,F). Steady-state abundances of *Ochromonas* and *Microcystis* converged to similar levels, irrespective of whether the

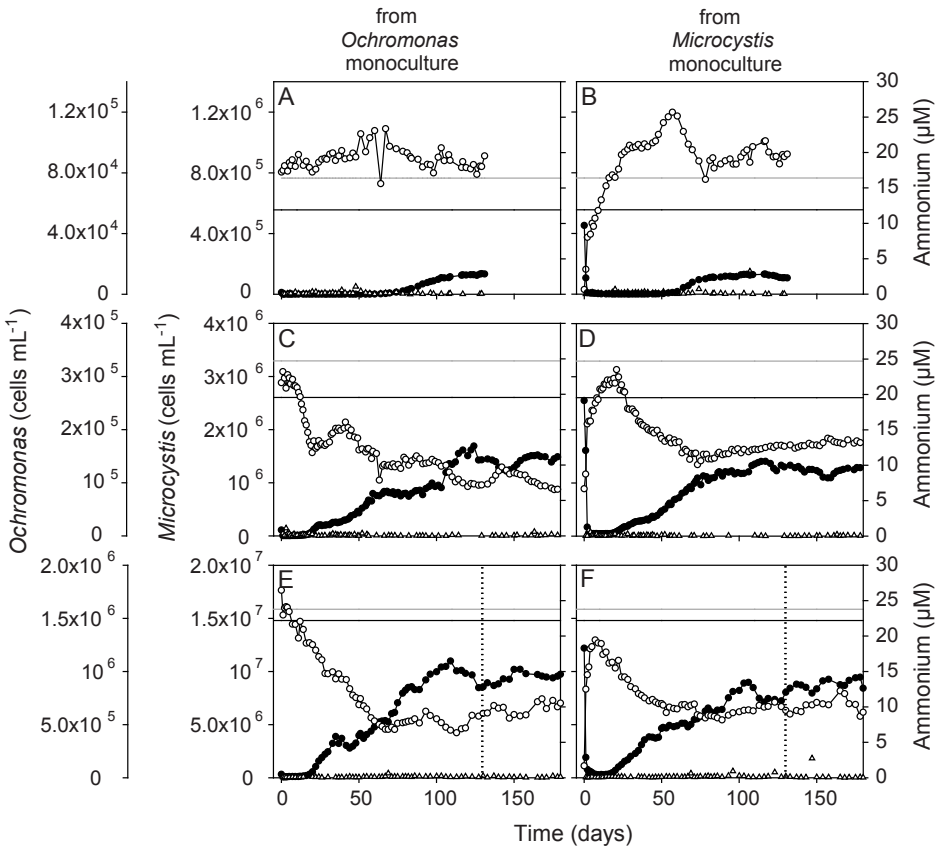

Figure 7.4: Intraguild predation experiments. In the left panels, a small amount of *Microcystis* was inoculated into monocultures of *Ochromonas*. In the right panels, a small amount of *Ochromonas* was inoculated into monocultures of *Microcystis*. The experiments were performed at (A,B) low nitrogen load of 20 µM ammonium, (C,D) intermediate nitrogen load of 100 µM ammonium, and (E,F) high nitrogen load of 500 µM ammonium. Open circles = *Ochromonas*, closed circles = *Microcystis*, open triangles = ammonium concentration. For comparison, horizontal lines give the steady-state abundances of *Ochromonas* (grey lines) and *Microcystis* (black lines) in monoculture. The vertical dotted lines in panels E and F indicate the time at which the incident light intensity was increased.

species mixtures were started from the *Ochromonas* or *Microcystis* monocultures (compare left column versus right column in Fig. 7.4; Fig. 7.5A). This indicates that the outcome of the species interactions was independent of the initial conditions. The species abundances at high nitrogen load seemed to fluctuate mildly, suggestive of small-amplitude predator-prey oscillations (Fig. 7.4E,F). The dissolved ammonium concentration remained low in all treatments.

In the species mixtures, both *Ochromonas* and *Microcystis* increased in steady-state abundance with increasing nitrogen load (Fig. 7.5A). At the lowest nitrogen load, *Ochromonas* reached slightly higher steady-state abundances than in monoculture and suppressed *Microcystis* by 80% relative to its monoculture abundance (Fig. 7.5B). Hence, the mixotroph was facilitated by the presence of its prey at low nitrogen loads. In contrast, at the intermediate and high nitrogen loads *Ochromonas* reached lower steady-state abundances than in monoculture, while *Microcystis* was suppressed by only 40-50%. Thus, although both species increased with the nitrogen load, the intraguild prey *Microcystis* benefitted relatively more from the increasing nitrogen load than its intraguild predator *Ochromonas*. The dissolved ammonium concentration in the species mixture was slightly higher than in the *Microcystis* monocultures, while it was slightly lower than in the *Ochromonas* monocultures (Fig. 7.5C; Friedmann ANOVA $X^2 = 2.67$, df = 2, p = 0.26).

Evaluation of theoretical predictions

We can now evaluate to what extent the theoretical predictions are supported by the experiments:

Prediction (i): at low nutrient load, the autotroph dominates. The autotroph *Microcystis* was not the dominant species at the lowest nutrient load of 20 µM. In fact, the mixotroph *Ochromonas* strongly suppressed its autotrophic prey (Fig. 7.4A,B). However, the parameterised model predicts dominance of the autotroph only at very low nitrogen loads of $N_{in} < 2$ µM, but coexistence of the autotroph and mixotroph at higher nitrogen loads (Fig. 7.1). Hence, the applied nutrient load of 20 µM was apparently not low enough to enable dominance of the autotroph.

Prediction (ii): at intermediate nutrient load, the autotroph and mixotroph coexist. This prediction is supported by the experiments.

Prediction (iii): with a further increase in nutrient load, the mixotroph becomes dominant while the autotroph decreases in abundance. This prediction is not supported. The autotroph and mixotroph both increased in abundance with the nutrient load (Fig. 7.5A). In relative terms, the dominance of the mixotroph *Ochromonas* even decreased and the autotroph *Microcystis* was suppressed less strongly at high nutrient loads (Fig. 7.5B).

Prediction (iv): the dissolved nutrient concentration slightly increases after the mixotroph has entered the system, but remains rather low. As predicted, there was a slight increase in the dissolved ammonium concentration in the species mixtures compared to the *Microcystis* monocultures, but the ammonium concentrations clearly remained low in all experiments.

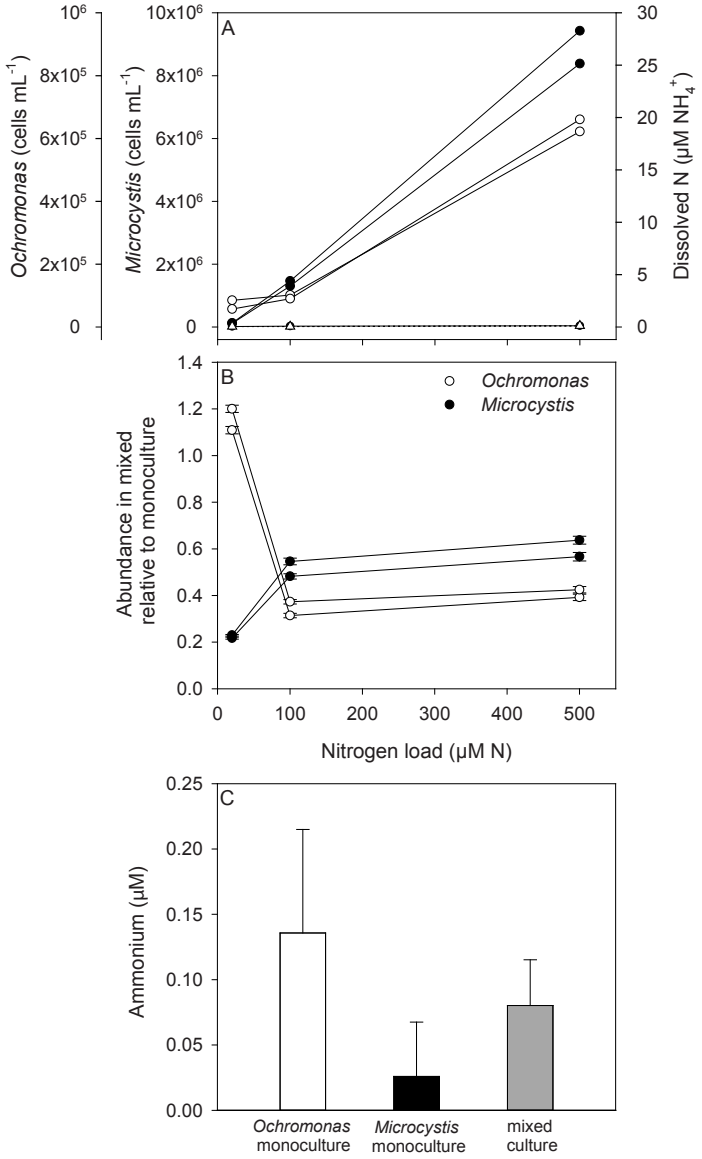

Figure 7.5: (A) Steady-state abundances of *Ochromonas* (open circles) and *Microcystis* (closed circles) in the intraguild predation experiments plotted as function of the nitrogen load. The open triangles show the steady-state concentration of dissolved ammonium. (B) The abundances of *Ochromonas* and *Microcystis* in the intraguild predation experiments relative to their abundances in monoculture. (C) Steady-state dissolved ammonium concentrations in *Ochromonas* and *Microcystis* monocultures compared to the intraguild predation experiment. Shown are the means and standard deviations calculated over the three nitrogen loads.

Prediction (v): the community does not show alternative stable states or predator-prey oscillations. Despite completely different initial conditions, the two experiments at each nutrient level rapidly converged to the same population dynamics. Hence, we did not find evidence for alternative stable states. The population dynamics suggest convergence to equilibrium at low nutrient load, but mild predator-prey oscillations at high nutrient load.

Summarizing, several of the model predictions are supported by the experiments. However, the experimental results clearly contradict the expectation based upon prediction (iii) that mixotrophs (intraguild predators) will dominate at high nutrient loads, while autotrophs (intraguild prey) will decrease with increasing nutrient loads.

Explanations for the discrepancy between theory and experiments

Similar to our findings, earlier studies have been puzzled by the persistence of intraguild prey species in productive environments (Holt and Polis 1997, Amarasekare 2007). This has recently inspired a series of theoretical papers analysing possible mechanisms that allow coexistence of intraguild predators and their prey (Křivan and Diehl 2005, Finke and Denno 2006, Janssen et al. 2007, Amarasekare 2008, Abrams and Fung 2010b, Urbani and Ramos-Jiliberto 2010, Hin et al. 2011). However, only very few studies have tested these mechanisms experimentally (e.g., Amarasekare 2007). Here, we experimentally explore several potential mechanisms to find a suitable explanation for the observed increase of both the intraguild predator and its prey with increasing nitrogen loads. Detailed descriptions of the experimental methods can be found in Appendix B.

(i) Genetic diversity within the prey: Genetic variation within the prey population can result in rapid prey evolution (Yoshida et al. 2003), and may select for predator-resistant genotypes that allow persistence of the prey at high predator densities (Meyer et al. 2006). We therefore tested for genetic variation in the Microcystis population using denaturing gradient gel electrophoresis (DGGE) of three different parts of the internal transcribed spacer (ITS) between the 16S and 23S rRNA coding regions. This approach allows detection of different *Microcystis* strains at high resolution (Janse et al. 2003; Kardinaal et al. 2007). Samples from our chemostat resulted in only one band in the DGGE gel, confirming the presence of only the strain PCC 7806 in the chemostats (Fig. 7.6). Hence, we did not detect genetic variation within the *Microcystis* population, at least not at the ITS region. We note that some degree of mutation-derived genetic variation might also occur within a clonal culture and would escape detection by DGGE of the ITS region. Yet, it seems unlikely that de novo mutations during the experiments can explain our results, because the similarity of the trajectories in the different chemostats indicates that this

would require similar mutations at almost the same time points in several independent chemostats. Hence, although we cannot exclude the possibility of prey evolution in our chemostats, it does not seem to be a very plausible explanation of our results.

(ii) Inducible defences within the prey: Predator resistance can also be induced as a phenotypic response of the prey to enhanced predation pressure. Such inducible defences can prevent oscillations in predator-prey systems (Verschoor et al. 2004) and may enable intraguild prey to coexist with their intraguild predators at high levels of productivity (Abrams and Fung 2010b, Urbani and Ramos-Jiliberto 2010). We used a standard protocol to test for inducible defences (Hessen and Van Donk 1993), in which we exposed the intraguild prey *Microcystis* to the filtrates of other *Microcystis* cultures grown either with or without *Ochromonas* as grazer. In case of an inducible defence, one would expect a lower grazing rate on the 'induced' *Microcystis*. However, grazing rates were about 20 % higher on the 'induced' *Microcystis* than on the control (Student's t-test: $t_4 = -7.53$, $p < 0.005$; data not shown). Thus, there was no evidence for an inducible defence against grazing in *Microcystis*.

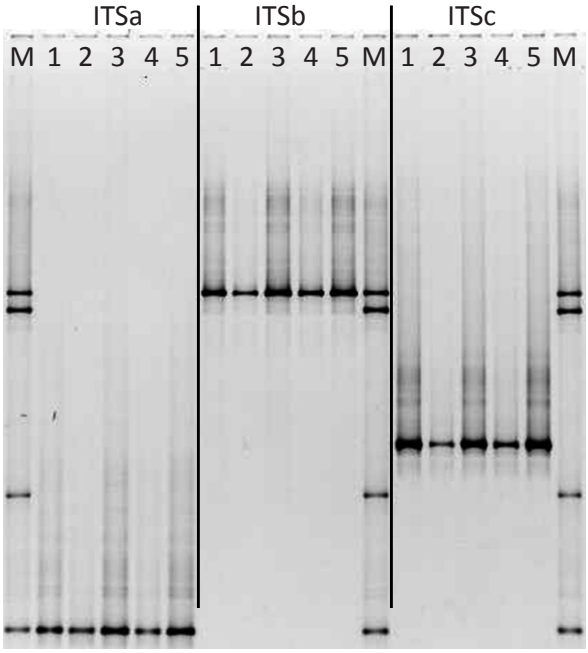

Figure 7.6: DGGE-gels for the ITSa, ITSb, and ITSc regions of *Microcystis* sampled from the experiments performed at high nitrogen load of 500 µM ammonium. The lanes represent the following samples: M = marker; 1 = day 48 of monoculture; 2 = day 9 of mixed culture; 3 = day 24 of mixed culture; 4 = day 78 of mixed culture; 5 = day 165 of mixed culture.

(iii) Changes in nutritional quality of the prey: The nutritional quality of prey species may vary along nutrient gradients (Sterner and Elser 2002, Andersen et al. 2004). Because predatory chrysophytes can alter their grazing rates in response to changes in the nitrogen content of their prey (John and Davidson 2001), we tested whether nitrogen deprivation of the intraguild prey *Microcystis* affected the performance of its predator *Ochromonas*. The results show that nitrogen deprivation caused a clear decrease of the cellular nitrogen content of *Microcystis* (Fig. 7.7A). Although this did not lead to a decrease in grazing rate (Fig. 7.7B), it strongly suppressed the growth rate of *Ochromonas* on N-deprived *Microcystis* cells (Fig. 7.7C). This suggests, however, that *Ochromonas* will be a weaker predator at low nitrogen loads, whereas our chemostat experiments indicated a reduced predation pressure at high nitrogen loads. Hence, it seems unlikely that such a change in nutritional quality of *Microcystis* can explain the observed patterns in population abundance.

(iv) A shift towards competition for light: An increase in nutrient load allows accumulation of higher biomass, which reduces light availability due to self-shading, and may shift the species interactions from competition for nutrients to competition for light (Passarge et al. 2006, Brauer et al. 2012). Assuming that the autotroph *Microcystis* is the better competitor for light, this may tend to favour *Microcystis* at high nitrogen loads. Light limitation in the mixotroph might furthermore result in lower grazing rates (Li et al. 1999), and thus reduce the grazing pressure on *Microcystis*. We indeed observed that the depth-averaged light intensity was lowest in the chemostats receiving the highest nitrogen load (Appendix B, Fig. B1). Therefore, we increased the light supply (I_{in}) for the chemostats with the highest nitrogen load to reach the same depth-averaged light intensity as in the chemostats with lower nitrogen

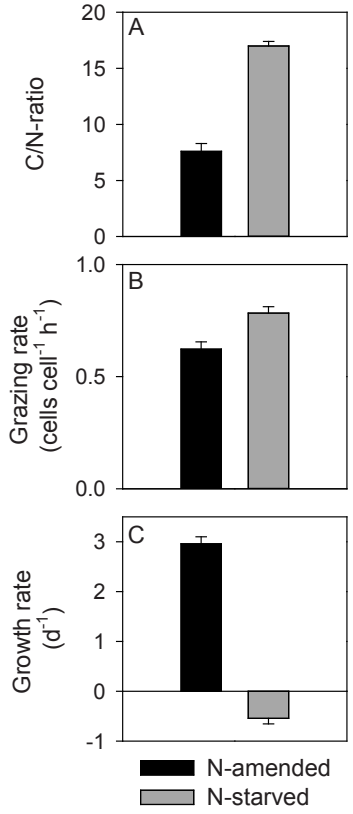

Figure 7.7: Differences in nutritional quality of N-amended versus N-starved cells of *Microcystis*. (A) C:N ratios of *Microcystis*. (B) Grazing rates of *Ochromonas* on *Microcystis*. (C) Growth rates of *Ochromonas* on *Microcystis*. Error bars give standard deviations of triplicate measurements.

loads. The time point at which the light supply was increased is indicated by the vertical dotted line in Fig. 7.4E and F. The results show that an increase in the light supply did not affect the steady-state abundances of the species. This provides further confirmation that even the chemostat experiments at the highest nitrogen load were nitrogen-limited rather than light-limited, in agreement with the linear increase of the monoculture population abundances with increasing nitrogen load (Fig. 7.3A). Changes in light availability along the nutrient gradient are therefore unlikely to explain the observed patterns.

(v) Intraspecific interference within the predator population: Grazing rates of the predators may depend not only on prey abundance, but may also change with the abundance of the predator. For instance, grazing rates may decrease with increasing predator abundances due to intraspecific interference within the predator population, which may allow coexistence of the intraguild predator and prey at high levels of productivity (Amarasekare 2008). In mixotrophic populations, physical interactions among the individuals might interfere with the ingestion of prey, while nutrient uptake by mixotrophs is less likely to be affected by interference. Because we observed physical interactions among *Ochromonas* individuals under the microscope, we tested for intraspecific predation interference within *Ochromonas* by investigating the dependence of its grazing rates on its own population density. This confirmed that grazing rates decreased at high abundances of *Ochromonas* (Fig. 7.8). These results suggest that intraspecific predation interference might explain the reduced predation pressure of *Ochromonas* on *Microcystis* at high nitrogen loads.

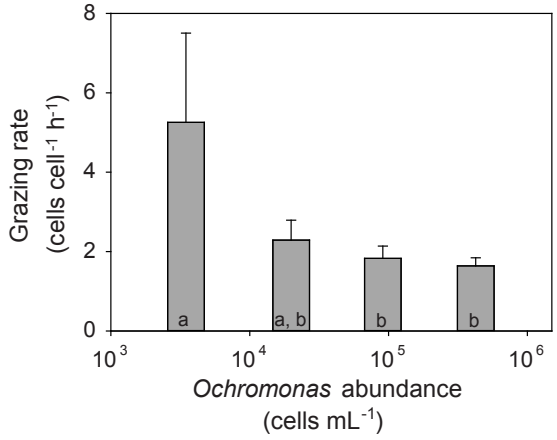

Figure 7.8: Grazing rates of *Ochromonas* on *Microcystis as* function of *Ochromonas* abundance. Error bars give standard deviations of triplicate measurements. Bars with different letters are significantly different (ANOVA after log-transformation of data to improve homogeneity of variance: $F_{3,8} = 8.03$; $p < 0.01$).

To explore this possible explanation in further detail, we rewrote the type III functional response to account for intraspecific interference among the predators (Beddington 1975, DeAngelis et al. 1975):

$$f_M(P_A, P_M) = \frac{f_{max}(P_A)^b}{(k_M)^b + (P_A)^b + cP_M} \tag{7.6}$$

The functional response thus becomes a function $f_M(P_A,P_M)$ of the abundances of both the autotroph and mixotroph. The parameter c determines the strength of the interference. All other parameters remained the same as in Table 7.1. This new type III functional response alters the model predictions considerably (Fig. 7.9). In particular, the model predicts that predation interference will cause an increase of both the autotroph and mixotroph with increasing nutrient loads. Therefore, interference within the intraguild predator population offers a possible explanation for the discrepancy between the experimental results and the theoretical expectations.

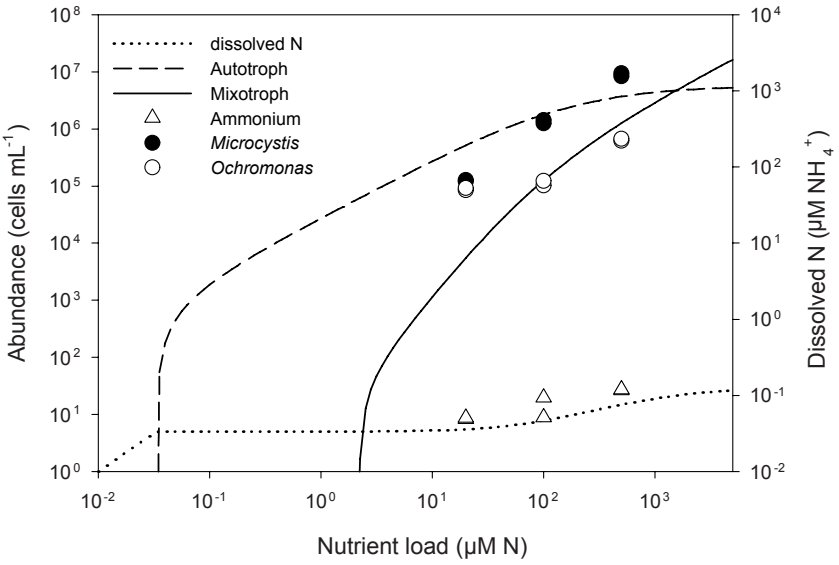

Figure 7.9: Model predictions when intraspecific interference among the predators is included (eqn 7.6). The graph shows steady-state abundances of the autotroph and mixotroph and the dissolved nitrogen concentration as function of the nutrient load. Symbols represent experimental measurements, while lines give model predictions. Parameter values are the same as in Fig. 7.1 (see Table 7.1). The interference parameter c takes a value of 3×10^{-5} L cell^{-1}. In some cases, the measurements were too similar to be visibly distinguishable in the graph.

Discussion

Our experimental results show that the toxic cyanobacterium *Microcystis* and its mixotrophic predator coexisted across a wide range of nutrient loads. Based on intraguild predation theory, a necessary (but not sufficient) condition for coexistence is that the autotrophic prey *Microcystis* is the better competitor for the shared resource (Holt and Polis 1997). Indeed, *Microcystis* had a lower critical ammonium concentration (i.e., a lower R^*) than *Ochromonas* during autotrophic growth (Fig. 7.3A), and hence is the better competitor for ammonium (Tilman 1982). The lower competitive strength of *Ochromonas* might reflect the high costs of mixotrophic growth, which requires investments into the biochemical machinery for both autotrophic and heterotrophic nutrition (Raven 1997).

Our results contradicts the model prediction that the intraguild prey would be more strongly suppressed with increasing nutrient load, which is widely regarded as one of the key predictions of intraguild predation theory (Holt and Polis 1997, Diehl and Feissel 2000, Mylius et al. 2001). Its function as intraguild predator enabled *Ochromonas* to strongly suppress *Microcystis* at the lowest nitrogen load. However, although *Ochromonas* increased in abundance with a further increase in nitrogen load, this did not result in a stronger suppression of its prey. Instead, the autotrophic prey *Microcystis* increased in abundance with increasing nutrient load, both in absolute terms and relative to its abundance in monoculture (Fig. 7.5). The ability of *Ochromonas* to suppress *Microcystis* therefore declined at high nitrogen loads.

Motivated by earlier studies aiming to explain the frequently observed coexistence of intraguild prey and predator species (e.g., Liess and Diehl 2006, Amarasekare 2008, Abrams and Fung 2010b, Urbani and Ramos-Jiliberto 2010), we explored a number of potential explanations for our experimental results. One interesting aspect of our experiments is that the intraguild predator *Ochromonas* uses a type III functional response (Wilken et al. 2010), whereas most previous theory developed for intraguild predation assumed a type I or type II functional response (e.g., Holt and Polis 1997, Diehl and Feissel 2000; but see Gismervik and Andersen 1997). The type III functional response indicates that *Ochromonas* switches to autotrophic growth when prey becomes rare, which reduces the per capita mortality rate of the prey species. As a consequence, a type III functional response enables persistence of the intraguild prey at high nutrient loads. Yet, despite its persistence, Fig. 7.1 shows that incorporation of the type III response in our model still leads to a reduction in prey abundance with increasing nutrient load. Hence, the type III response is not sufficient to explain the experimental results. Furthermore, we did not find experimental evidence for several other possible explanations for the observed pattern, such as genetic diversity within the prey, inducible defences, a reduced nutritional quality,

or a shift from competition for nutrient to competition for light. However, we did find experimental support for intraspecific interference within the *Ochromonas* population. Moreover, model simulations show that predation interference results in an increase of both the autotroph and mixotroph with increasing nutrient loads. Therefore, interference within the intraguild predator population offers a plausible mechanism explaining the steady state abundances observed in our experiments.

However, the list of mechanisms we tested experimentally is not exhaustive and we can therefore not exclude other factors that were not investigated. For instance, the temporal dynamics suggest a slow decrease in grazing pressure before *Microcystis* abundances started to increase again in the mixed cultures (Fig. 7.4). It could well be that, due to genotypic or phenotypic changes, *Ochromonas* might have inactivated part of its cellular machinery for heterotrophic metabolism once *Microcystis* was suppressed to sufficiently low abundances. That is, *Ochromonas* might have shifted its trophic position from an intraguild predator to a predominantly autotrophic competitor during the experiment. Since *Ochromonas* is a weaker competitor for ammonium than *Microcystis*, this might explain the initial decline and subsequent partial recovery of the *Microcystis* populations observed in several of the experiments (Fig. 7.4B,D,F). While evolution of the nutritional strategy of mixotrophs has been shown experimentally (Reboud and Bell 1997, Bell 2013), we have not developed new theory or ran additional experiments to investigate such alternative explanations in further detail.

Natural communities offer a much more complex setting than our experimental system. The abundance of *Ochromonas* is typically lower in natural communities than in our chemostat experiments (Van Donk et al. 2009), which will most likely reduce the importance of predation interference due to lower encounter rates among *Ochromonas* individuals. However, other factors that may favour coexistence of *Ochromonas* with its intraguild prey might become more important in natural waters. For instance, *Ochromonas* is known to induce colony formation in some strains of *Microcystis* (Burkert et al. 2001), and the large *Microcystis* colonies often observed in eutrophic lakes offer efficient protection against flagellate grazing (Yang and Kong 2012). In addition, grazing by protists can act as selection factor on *Microcystis* populations, and evolution towards less edible strains is likely to occur in natural populations (Van Wichelen et al. 2010). Furthermore, in their natural habitat *Ochromonas* and *Microcystis* will not interact in isolation. *Ochromonas* can utilize alternative prey organisms, such as heterotrophic bacteria (Wilken et al. 2013), while both species are subject to predation by and competition with other species. All these aspects may affect the abundance patterns of *Microcystis* and *Ochromonas* along natural productivity gradients.

Nutrient enrichment of aquatic ecosystems, through agriculture, urbanization, and other human activities, has increased the risk of toxic cyanobacterial blooms (Chorus and Bartram 1999, Huisman et al. 2005, Paerl and Huisman 2008). To what extent can mixotrophic species like *Ochromonas* suppress or even prevent cyanobacterial bloom development? Our experiments showed that *Ochromonas* initially reduced the abundance of the toxic cyanobacterium *Microcystis* by more than 97%. After several weeks, however, *Microcystis* partially recovered to about 20% of its original monoculture abundance at low nitrogen loads, and to about 50-60% of its monoculture abundance at high nitrogen loads. Furthermore, in previous experiments we showed that *Ochromonas* cannot grow on nitrate, and therefore *Ochromonas* is capable to suppress *Microcystis* populations much more strongly with ammonium than with nitrate as nitrogen source (Chapter 6). Taken together, these findings suggest that mixotrophic chrysophytes like *Ochromonas* are most effective as biological control agents of toxic cyanobacteria in relatively oligotrophic waters with ammonium as the dominant nitrogen source. In eutrophic waters, where the problem of cyanobacterial blooms is generally most severe, a newly invading *Ochromonas* population may have a transient effect but will ultimately have less control over cyanobacterial populations.

In conclusion, while intraguild predators may act as effective biological control agents against pest species, our study highlights the potential of several mechanisms to reduce this control. Understanding the relative importance of these mechanisms is therefore crucial to make predictions about the possibilities for biological control in specific communities. The mixotroph *Ochromonas* is capable of strongly suppressing toxic cyanobacteria at low nutrient loads, but intraspecific predation interference is one of the mechanisms that may prevent its effective control over toxic cyanobacteria at high nutrient loads.

Chapter 8

Synthesis

The aim of this thesis is to better understand the functional role of mixotrophs in food webs. Because mixotrophs can act both as primary producers and as consumers, a key determinant of their functional role in the environment is their nutritional balance. Hence, an understanding of the factors influencing the nutritional balance of mixotrophs is a prerequisite for understanding their ecological impact, including a potential application as biological control agents against harmful cyanobacteria. For this reason, I started off with physiological investigations of the interaction and balance between the two nutritional pathways in mixotrophs (Chapter 2 and 3). I then set out to more specifically study the interaction of the mixotrophic chrysophyte *Ochromonas* sp. with toxic cyanobacteria, in particular *Microcystis aeruginosa* (Chapter 4 and 5). Finally, I investigated the population dynamics of a mixotroph and its autotrophic prey, using *Ochromonas* and *Microcystis* as a model system (Chapter 6 and 7).

Mixotrophs combine 'plant-like' photoautotrophic and 'animal-like' organoheterotrophic nutrition. But, does this allow us to understand mixotrophs based on our knowledge on autotrophic and heterotrophic eukaryotes? In this chapter, I will discuss in how far mixotrophs can be understood as the sum of their autotrophic and heterotrophic components, both with respect to their physiology and their ecological impact. The key findings presented in the previous chapters will be linked to the potential role of mixotrophs under different environmental conditions. Furthermore, the remaining question, whether mixotrophs might serve as biological control agents against toxic cyanobacteria in natural ecosystems, will be addressed. I will close this chapter with a brief outline of some of the many open questions that will need to be tackled in future research.

Summing up the parts - Photoautotrophy and organoheterotrophy

The two nutritional pathways of mixotrophs, photoautotrophy and organoheterotrophy, are often assumed to be additive. In that case the metabolic rates for each of the two nutritional pathways would depend only on the availability of the resources required for the respective pathway, e.g. light, inorganic carbon, and nutrients for photoautotrophic and prey for organoheterotrophic growth (Chapter 7, Thingstad et al. 1996, Jost et al. 2004, Hammer and Pitchford 2005). This approach has been criticized for its lack of physiological realism (Flynn and Mitra 2009, Mitra and Flynn 2010), and indeed the interaction between photoautotrophy and organoheterotrophy is often more intricate. An increasing availability of resources for one metabolic pathway can cause metabolic rates of the other pathway to increase (Caron et al. 1993, Li et al. 1999, Kim et al. 2008) or decrease (Sanders et al. 1990, Skovgaard 1996), as the two pathways can either act substitutable with respect to a specific resource, or be complementary in the sense that they supply different resources required for growth. Hence, the two nutritional pathways do not operate in isolation, but might show a close interaction and concerted regulation on the subcellular level.

A fundamental prediction of the Metabolic Theory of Ecology is that heterotrophic processes increase more strongly with temperature than autotrophic processes (Allen et al. 2005, Lopez-Urrutia et al. 2006, Rose and Caron 2007, Lopez-Urrutia 2008). Our comparison of the effect of temperature on autotrophic and heterotrophic growth of the mixotroph *Ochromonas* confirmed this prediction (Chapter 2). As a consequence, mixotrophs become more heterotrophic with increasing temperature. This general result suggests a good predictability of mixotrophic growth based on knowledge of the autotrophic and heterotrophic components. Nevertheless, there remains a less predictable component, because mixotrophs can change their relative investments in autotrophic versus heterotrophic metabolism. The activation energy of 0.50 eV for electron transport rates implies an increase by a factor of 3.8 with a temperature increase from 13 to 33 ºC, while the activation energy for ingestion rates describes an increase by a factor of 9.5. Without alterations of the biochemical machineries, this would result in a decrease of the relative contribution of autotrophy to growth by a factor of 2.5. However, in addition, the mixotroph also reduced its pigment content. Overall, this resulted in a 10-fold reduction of the autotrophic contribution to mixotrophic growth with rising temperature. The ability of mixotrophs to adjust their cellular investments into the two metabolic pathways can therefore modify their temperature response. This modification is difficult to predict from the two separate components, and might differ between mixotrophs with different nutritional strategies (Sanders et al. 1990, Li et al. 1999).

Mixotrophs do not only utilize two different sources of carbon, but also two different sources of energy. But do they just add up the carbon and energy supplied via the different nutritional pathways? Or do the two pathways specialize into the utilization of either carbon or energy? The altered composition of the photosynthetic machinery during mixotrophic growth compared to photoautotrophic growth suggests a division of labour between the biochemical machineries for both nutritional pathways (Chapter 3). Mixotrophs might primarily utilize light as source of energy and organic carbon from their prey as source of carbon and therefore grow as photoheterotrophs (Fig. 8.1). In this case, mixotrophy is not the pure sum of photoautotrophy and organoheterotrophy, but a mixed strategy that combines the best of both worlds. A tendency towards photoheterotrophy could allow mixotrophs to reduce a significant part of their enzymatic machinery for both autotrophic and organotrophic processes. In this case, the costs for investment into both nutritional pathways would be less than the sum of the parts, alleviating the strength of the trade-off commonly assumed to be present in mixotrophs (Raven 1997, Litchman et al. 2007). Furthermore, in the case of photoheterotrophic growth the impact of mixotrophs on biogeochemical cycles is distinctly different from the sum of photoautotrophy and organoheterotrophy. Photosynthesis is not used for carbon fixation, but instead substitutes respiration by light-driven ATP-synthesis. This allows higher carbon assimilation efficiencies from the prey and prevents carbon losses by photorespiration. Such a contribution of photosynthesis to the carbon cycle would escape an assessment with standard techniques based on, e.g., ^{14}C carbon fixation. While this problem is known for photoheterotrophic prokaryotes (Kolber et al. 2001, Zubkov 2009), it might also be relevant for some eukaryotes.

Interestingly, a tendency towards photoheterotrophic growth might have implications for the temperature dependence of mixotrophs. The temperature-dependent rate-limiting steps are thought to be carbon fixation for light-saturated photosynthesis and aerobic

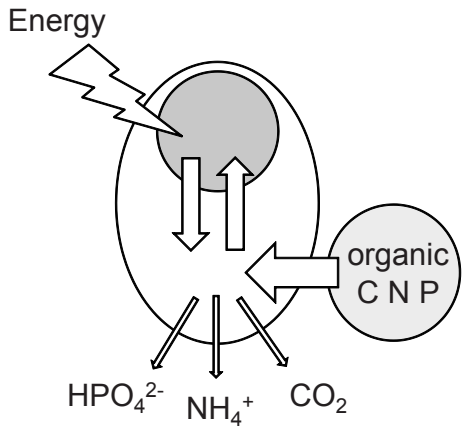

Figure 8.1: Resource utilization during photoheterotrophic growth. Energy is derived from sunlight, and carbon and nutrients from the ingestion of prey, resulting in only little remineralization.

respiration for organoheterotrophic growth (Brown et al. 2004, Allen et al. 2005). Hence, it is the process of carbon acquisition limiting the rate of photosynthesis and the process of energy generation limiting organoheterotrophic growth rates. During photoheterotrophy, however, the photosynthetic apparatus serves the acquisition of energy and ingestion of prey serves as carbon source. Especially the weaker temperature dependence of carbon fixation would thus be removed and photoheterotrophic growth is expected to respond more strongly to temperature than photoautotrophic growth.

Summing up the parts - Competition and predation

The ability of mixotrophs to utilize alternative sources of carbon, energy and nutrients (Nygaard and Tobiesen 1993, Rothhaupt 1996b, Flöder et al. 2006) has received considerable attention in studies of the competitive interaction of mixotrophs with specialist autotrophs or heterotrophs (Rothhaupt 1996a, Tittel et al. 2003, Ptacnik et al. 2004). Acquiring nutrients from ingested prey enables mixotrophs to coexist with superior competitors for dissolved nutrients (Rothhaupt 1996a). However, mixotrophs often feed directly on the species they compete with (Thingstad et al. 1996), whether these are heterotrophic bacteria or other phytoplankton species. Due to this simultaneous interaction by both competition and predation, mixotrophs act as intraguild predators. This results in a more complex interaction between two species than either competition or predation alone (Polis and Holt 1992).

Intraguild predation theory provides a number of predictions about the interactions and coexistence of the intraguild prey and predator. For instance, the intraguild prey needs to be the stronger competitors for the shared resource to not be excluded by its intraguild predator (Polis and Holt 1992). We found *Ochromonas* to be a weaker competitor for ammonium than the autotroph *Microcystis* (Chapter 6 and 7), and this might be a more general trait of mixotrophs for two reasons. First, prey species are often smaller than their mixotrophic predators (although there are some impressive exceptions) and competitive strength for dissolved nutrients is inversely related with size (Chisholm 1992, Edwards et al. 2012), and second, the competitive strength for nutrients might be impaired by a trade-off between the two nutritional pathways in mixotrophs (Raven 1997, Litchman et al. 2007). Another consequence of this trade-off might be the type III functional response of *Ochromonas*, as the prey is dropped from the diet and the mixotroph switches to photosynthesis when the prey abundance becomes too low (Chapter 5, 6, and 7). Taken together, the weaker competitive strength and type III functional response of the mixotroph could enable coexistence with their intraguild prey over a broad range of environmental conditions.

Intraguild predation theory does not only provide a relevant framework for the ecology of mixotrophs, but conversely, mixotrophs provide a suitable model system to test predictions made by intraguild predation theory. In particular, the inability of *Ochromonas* sp. to utilize nitrate provided a unique opportunity to manipulate the food web structure and directly compare an intraguild predation system with a linear food chain (Chapter 6). This allowed us to confirm one of the key predictions of intraguild predation theory, that it is indeed the combination of competition and predation that enables intraguild predators to strongly suppress their prey (Chapter 6). This implies that factors altering the relative importance of competition and predation will affect the population dynamics of a mixotroph and its intraguild prey.

Another key prediction of intraguild predation theory is the stronger suppression of intraguild prey by intraguild predators at higher levels of productivity (Holt and Polis 1997, Diehl and Feissel 2000, Mylius et al. 2001). This prediction could not be confirmed by our chemostat experiments (Chapter 7). Instead, the intraguild prey *Microcystis* increased with nutrient enrichment. We showed that intraspecific predation interference among *Ochromonas* individuals is a likely explanation for the deviation from this theoretical prediction in our experimental system. Although intraspecific interference is less likely to play a role at the lower abundances of *Ochromonas* in most natural waters, the pattern found in our chemostat experiments is in agreement with the occurrence of chrysophytes over a wide range of trophic states and with their higher contribution to the total phytoplankton biomass in oligotrophic lakes (Chapter 4, Sandgren 1988, Siver 1995, Duff et al. 1997). In addition to intraspecific interference, there is a plethora of other mechanisms that could potentially cause similar deviations from intraguild predation theory in natural waters and prevent intraguild prey from being strongly suppressed in productive environments (Chapter 7, Liess and Diehl 2006, Amarasekare 2008, Abrams and Fung 2010b). Mechanisms preventing mixotrophs from dominating over their intraguild prey in eutrophic habitats are therefore expected to be quite species and habitat specific.

In contrast to the ingestion of prey, autotrophic growth requires the independent uptake of several resources from the environment. While our chemostat experiments were performed with nitrogen as the only growth-limiting factor, a mixotroph can compete with its autotrophic prey for several resources. The relative availability of these resources changes along environmental gradients and the uptake capacity for different resources differs among taxonomic groups. In the following, I will discuss the relative importance of competition and predation in the interaction of mixotrophs with their prey under different environmental conditions.

Linking the physiology of mixotrophs to their role in the food web

While the ability to grow photoautotrophically generally enables mixotrophs to utilize inorganic resources, not all of these resources will be equally available to different mixotrophic species. The uptake of complex organic compounds might come along with a reduced ability to utilize some inorganic resources. Examples are the inability to utilize nitrate (Chapter 6) or the absence of carbon concentrating mechanisms in chrysophytes (Maberly et al. 2009). Mixotrophic chrysophytes could therefore dominate as intraguild predators in habitats, where reduced nitrogen compounds represent the major source of bioavailable nitrogen, and CO_2 is available in high concentrations. Such conditions can be found in acidic waters, where low pH can impair nitrification and thereby preserve high concentrations of ammonium (Rudd et al. 1988, Strauss et al. 2002) or in nitrogen limited and strongly stratified waters such as the oligotrophic ocean, where the transport of nitrate through the thermocline is restricted and regenerated nitrogen represents the major nitrogen source for primary producers (Dugdale and Goering 1967, Eppley and Peterson 1979, Glibert et al. 1982, Berman and Bronk 2003). However, in these areas nitrogen fixation constitutes an important source of 'new' nitrogen and in particular the unicellular nitrogen fixers (Montoya et al. 2004) would be susceptible to grazing by mixotrophs, but could escape competition for nitrogen. Irrespective of the nitrogen source, an increase in nutrient availability will ultimately cause other resources like light or inorganic carbon to become limiting for autotrophic growth (Ibelings and Maberly 1998, Huisman et al. 2004, Brauer et al. 2012). As a consequence, the functional role of mixotrophs, in particular chrysophytes, in the food web might shift from intraguild predators of other phytoplankton species in oligotrophic environments to specialist predators under carbon limitation in eutrophic environments.

Not only the relative availability of different resources, but also the physiological interaction between the two nutritional pathways will affect the functional role of mixotrophs in food webs. Interestingly, a primarily photoheterotrophic mixotroph would not compete for inorganic carbon (Chapter 3; Figure 8.1), but might still compete with other phytoplankton for light. Increasing atmospheric concentrations of CO_2 might not only alleviate carbon limitation in phytoplankton, but could also shift the functional role of a photoheterotroph from a predator that utilizes light as a substitutable source of energy towards an intraguild predator competing with its prey for light.

Because increasing temperatures cause the nutritional balance of mixotrophs to shift towards heterotrophy (Chapter 2), they might cause the interaction of mixotrophs with their intraguild prey to be more and more dominated by grazing and less by competition. While the much higher grazing rates reached at high temperature might allow a faster

reduction in prey abundances, the relatively weaker autotrophic component might result in a lower competitive strength, ultimately decreasing the ability of mixotrophs to suppress their intraguild prey. However, in how far the nutritional balance at saturating resource concentrations (as provided in the experiments described in Chapter 2) can be translated to the competitive strength under resource limitation, still remains open.

Can mixotrophs control harmful cyanobacteria in natural ecosystems?

Several species of the chrysophyte genera *Ochromonas* and *Poterioochromonas* can graze efficiently on toxic cyanobacteria and algae (Chapter 4 and 5, Cole and Wynne 1974, Zhang and Watanabe 2001, Zhang et al. 2008). They can reach high growth rates on *Microcystis* as prey without being affected by the toxic microcystins (Chapter 4 and 5). For this reason these mixotrophic flagellates had been suggested as biological control agents against blooms of *Microcystis*. Compared to several purely heterotrophic protists that can also graze efficiently on *Microcystis* (Dryden and Wright 1987, Kim et al. 2006, Van Wichelen et al. 2010), the mixotrophic nutrition might give some advantages. For instance, mixotrophs do not depend on the uptake of polyunsaturated fatty acids with their food, as their autotrophic metabolism enables them to synthesize these fatty acids themselves (Boëchat et al. 2007). Furthermore, mixotrophs can compete with their cyanobacterial prey for dissolved nutrients and light, and their function as intraguild predators enables them to suppress *Microcystis* populations more strongly than possible by predation alone (Chapter 6). Based on intraguild predation theory (Holt and Polis 1997, Diehl and Feissel 2000), high nutrient loads are expected to result in an even stronger suppression of *Microcystis* by its mixotrophic predator *Ochromonas*. All these factors are supporting the idea to use mixotrophs as biological control agents against cyanobacteria.

However, there are also numerous arguments questioning the success of such an application. In contrast to the theoretical predictions, *Microcystis* increased more strongly with increasing nutrient load than *Ochromonas* in our chemostat experiments (Chapter 7). Thus, *Microcystis* is favoured by eutrophication even in the presence of its intraguild predator. In the chemostats this pattern was probably caused by intraspecific predation interference in *Ochromonas*. However, there are more mechanisms that could counteract the suppression of *Microcystis* in natural waters. For instance, *Microcystis* can form colonies in response to grazing by *Ochromonas* (Burkert et al. 2001), and the large colonies that *Microcystis* usually forms in nature are efficient defiances against flagellate grazing (Yang and Kong 2012). In addition to phenotypic responses, grazing by protists can be an important selection factor, resulting in evolution towards less edible strains in the *Microcystis* population (Van Wichelen et al. 2010). Furthermore, dense *Microcystis* blooms

often cause carbon depletion and a concomitant increase in pH (Ibelings and Maberly 1998, Van de Waal et al. 2011), which might be less favourable for *Ochromonas* as the lack of a carbon concentrating mechanism prevents it from competing effectively with *Microcystis* for inorganic carbon. All these factors cause a natural population of *Microcystis* to be well defended against grazing.

To assess a potential effect of *Ochromonas* grazing on a natural phytoplankton community dominated by *Microcystis* I performed a preliminary experiment with a plankton community collected during a *Microcystis* bloom from Lake Westeinderplassen, The Netherlands. Water was collected from the lake in mid August 2010 and incubated in the lab at an ambient temperature of 19 ºC. Treatments consisted of two factors (i) the addition of pre-cultured *Ochromonas* in a relatively high abundance (1.8×10^4 cells mL^{-1}) and (ii) removal of large zooplankton by filtration through a 150 µm net. *Microcystis* colonies had a mean equivalent spherical diameter of 134 µm. While the addition of *Ochromonas* resulted in only a small increase in the particulate organic carbon concentration (Fig. 8.2A; repeated measures ANOVA: $F_{1,8}$=19.4, p<0.01), it caused a slightly higher growth of *Microcystis* during the first 6 days of incubation, and a much slower decline of its biomass thereafter (Fig. 8.2B, repeated measures ANOVA: $F_{1,8}$=19.4, p<0.0001). These preliminary results indicate that a natural community of *Microcystis* cannot only be well defended against

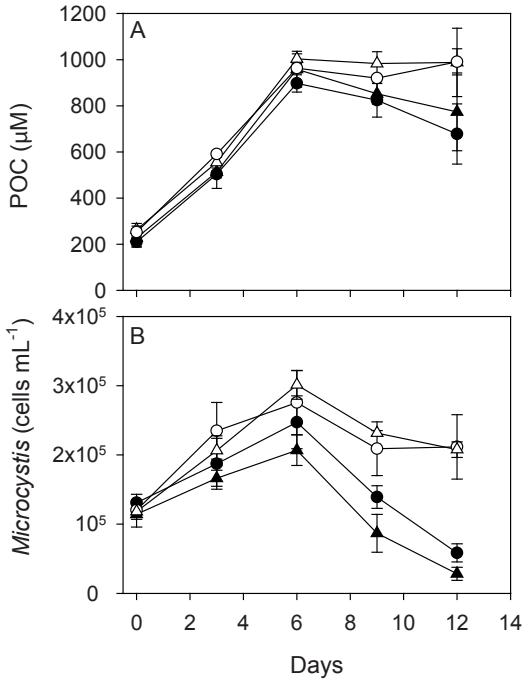

Figure 8.2: (A) Concentration of particulate organic carbon and (B) *Microcystis* abundance in a natural phytoplankton community taken from Westeinderplassen, The Netherlands, during a *Microcystis* bloom. Treatments represent the addition of *Ochromonas* sp. (white symbols), or the addition of an equivalent amount of cell-free *Ochromonas* sp. culture filtrate (black symbols) and removal of large zooplankton by filtration through a 150 µm net (triangles) or incubation of the whole plankton community (circles). Error bars give one standard deviation (n=3).

grazing, but might even be favoured by the addition of *Ochromonas* to the community. This can either be due to nutrient recycling by *Ochromonas* or, as the abundance of *Ochromonas* decreased by 50% over the course of the first 6 days, by the additional nutrients contained in the biomass of *Ochromonas* ultimately ending up in the biomass of *Microcystis*. While these preliminary data do not allow any conclusion about the specific pathways of nutrient and carbon flows, they indicate that the attempt to use *Ochromonas* as biological control agent might not only be unsuccessful, but can even intensify and prolong the problem of cyanobacterial blooms.

Outlook - Mixotrophs in a changing world

Human impact causes dramatic changes in environmental conditions with severe consequences for life in both lakes and oceans. Burning of fossil fuels leads to increasing atmospheric concentrations of CO_2 and higher dissolved CO_2 concentrations in aquatic ecosystems. The rise in atmospheric CO_2 concentration is accompanied by global warming. In addition to the direct effects of increasing temperature on biological processes, there are many indirect effects on lakes and oceans. For instance, rising temperatures intensify thermal stratification, resulting in an extension of permanently stratified areas of the ocean and a longer duration of seasonal stratification in temperate lakes and oceans (Peeters et al. 2007, Polovina et al. 2008). A stronger stratification in turn reduces nutrient availability in the upper mixed layer and thereby reduces the primary productivity in oligotrophic waters (Behrenfeld et al. 2006), while it increases the risk of harmful algal bloom events in eutrophic waters (Smayda 1997, Huisman et al. 2004, Jöhnk et al. 2008, Paerl and Huisman 2008). Furthermore, altered precipitation patterns will change the runoff from terrestrial ecosystems and hence the input of nutrients and organic matter into freshwater ecosystems and the ocean. This is predicted to result in an increase of organic carbon concentrations in boreal lakes (Larsen et al. 2011). The combined consequences of all these changes will certainly be complex and predicting their effect on aquatic ecosystems is one of the major challenges of future research. But where do mixotrophs enter the picture? And will they be an important part of the puzzle? While these questions remain to be answered in the future, I will briefly discuss some potential implications of global change on the occurrence of mixotrophs here.

Increasing concentrations of CO_2 could directly stimulate some mixotrophic taxa, such as chrysophytes, that lack carbon concentrating mechanisms and therefore are unable to utilize bicarbonate (Maberly et al. 2009). The reduced pH accompanying higher CO_2 concentrations might furthermore cause a shift towards a higher contribution of ammonium to the total nitrogen pool (Rudd et al. 1988) and hence, indirectly favour mixotrophic

species like our strain of *Ochromonas* that cannot utilize nitrate. The combination of an increasing strength and longer duration of the stratification is another factor that might favour mixotrophs. Many mixotrophic species can occur in thin layers in strongly stratified water columns (Bird and Kalff 1989). But maybe more importantly, mixotrophs can efficiently compete with specialist autotrophs and heterotrophs in oligotrophic environments (Rothhaupt 1996a) and, moreover, can suppress other phytoplankton species through intraguild predation (Chapter 6). Indeed, algal taxa commonly represented by mixotrophic species have increased in abundance over the course of re-oligotrophication in several deep peri-alpine lakes (Sommer et al. 1993, Van Donk et al. 2008). A reduced nutrient availability due to increasing strength and duration of stratification might have a similar effect on the phytoplankton community composition. In eutrophic environments, on the other hand, an increasing strength of stratification can favour the formation of harmful algal blooms, many of which are dominated by mixotrophic dinoflagellates in marine waters (Smayda 1997, Burkholder et al. 2008). Also, increasing concentrations of dissolved organic carbon are expected to favour mixotrophs, as phytoplankton assemblages in humic lakes are often dominated by mixotrophic taxa (Jansson et al. 1996). The dissolved organic carbon concentrations can either be utilized by mixotrophs directly (Flöder et al. 2006), or promote the growth of bacteria that, through their efficient utilization of inorganic nutrients, serve as source of both carbon and nutrients to mixotrophic phytoplankton species (Jansson et al. 1996). In combination all these factors suggest an increasing importance of mixotrophs with global change.

Mixotrophy is increasingly recognized as an important nutritional strategy, especially among the small (< 3µm) eukaryotic phytoplankton species inhabiting the permanently stratified oligotrophic areas of the ocean (Zubkov and Tarran 2008, Hartmann et al. 2012). The ingestion of bacterial prey by eukaryotic picophytoplankton represents both an important phosphorus source for primary production in these areas and a major loss process for the bacterial community (Hartmann et al. 2011, 2012). The eukaryotic picophytoplankton has been found to be tremendously diverse, with ecologically dominant groups not represented in culture (Moon-van der Staay et al. 2001, Shi et al. 2009). As a consequence, very little is known about their physiological traits. Given their impact as both primary producers and consumers of other microorganisms (Grob et al. 2007, Jardillier et al. 2010, Hartmann et al. 2012), and the presumed expansion of permanently stratified ocean waters with global warming (Polovina et al. 2008), there is a strong need for a better understanding of the picoeukaryotic community in these areas including their mixotrophic representatives.

In this thesis I have presented a series of physiological and ecological studies showing that mixotrophic organisms become more heterotrophic with increasing temperature, suggesting the potential for photoheterotrophic growth, and investigating the capability

of mixotrophs to suppress their autotrophic prey. In how far these findings based on the primarily heterotrophic chrysophyte *Ochromonas* can be generalized towards mixotrophs with a stronger autotrophic component or a more diverse mixotrophic community still remains open. Assessing the relative contribution of autotrophic and heterotrophic nutrition to the growth of mixotrophs in a natural community is challenging. However, given that the nutritional balance of mixotrophs is a major determinant of their population dynamics and ecological impact, such studies are a necessary step towards a mechanistic understanding of food web dynamics and carbon cycling in the wide variety of habitats dominated by mixotrophs.

Appendix A

Parameter estimates of the light-response curves during autotrophic growth

Table A1: Parameter estimates of the light-response curves during autotrophic growth. Parameters were estimated by fitting eqn (2.4) to the electron transport rate (A) and specific growth rate (B) data for each temperature (*T*) shown in Fig. 2.1. Bracketed values give 95% confidence intervals. For the electron transport rates, α and β are given in 10^{-3} fmol electrons cell^{-1} (μmol quanta m^{-2})$^{-1}$, and P_{sat} and P_0 are given in fmol electrons cell^{-1} s^{-1}. For the specific growth rates, α and β are given in d^{-1} (μmol quanta m^{-2} s^{-1})$^{-1}$, and P_{sat} and P_0 are given in d^{-1}. In all cases, n=16.

A) Electron transport rates

T (°C)	α	β	P_{sat}	P_0	R^2
13	2.75	0.48	0.07	0.017	0.81
	(0.29 – 5.79)	(-0.83 – 1.78)	(0.01– 0.14)	(-0.003 – 0.039)	
17	2.10	2.04	0.16	0.006	0.74
	(-0.44 – 4.65)	(-26.45 – 30.54)	(-1.46 – 1.78)	(-0.011 – 0.023)	
21	1.69	1.49	0.17	0.007	0.92
	(0.62 – 2.76)	(-12.67 – 15.65)	(-0.98 – 1.33)	(-0.003 – 0.017)	
25	2.97	1.52	0.20[1)]	0.019	0.92
	(1.12 – 4.82)	(-5.33 – 8.37)	(-0.33 – 0.73)	(0.001 – 0.037)	
29	3.63	0.12	0.11	0.024	0.92
	(0.79 – 6.47)	(-0.35 – 0.59)	(0.07 – 0.16)	(-0.002 – 0.050)	
33	1.61	0.41	0.20[1)]	0.017	0.82
	(-1.26 – 4.48)	(-21.60 – 22.41)	(-4.08 – 4.49)	(-0.019 – 0.053)	

B) Specific growth rates

T (°C)	α	β	P_{sat}	P_0	R^2
13	0.007	0.012	0.54	0.000	0.46
	(-0.005 – 0.020)	(-0.333 – 0.356)	(-12.06 – 13.13)	(-0.084 – 0.084)	
17	0.010	0.000	0.23	0.000	0.90
	(0.000 – 0.020)	(-0.001 – 0.001)	(0.14 – 0.31)	(-0.063 – 0.063)	
21	0.008	0.000	0.29	0.000	0.95
	(0.003 – 0.014)	(-0.001 – 0.001)	(0.12 – 0.45)	(-0.045 – 0.045)	
25	0.007	0.001	0.47	0.000	0.97
	(0.002 – 0.011)	(-0.007 – 0.008)	(-0.66 – 1.60)	(-0.045 – 0.045)	
29	0.004	0.004	1.00[1)]	0.014	0.96
	(0.001 – 0.007)	(-0.181 – 0.185)	(-31.13 – 33.13)	(-0.026 – 0.055)	
33	0.004	0.003	0.60	0.081	0.90
	(0.000 – 0.008)	(-0.094 – 0.099)	(-13.43 – 14.64)	(0.029 – 0.133)	

[1)] Note that in some model fits the estimates of β and P_{sat} were coupled and ran away to extremely high values. In those cases, we constrained the estimates by setting P_{sat} to a more realistic maximum value of P_{sat} = 0.20 fmol electrons $cell^{-1}$ s^{-1} for electron transport rates and P_{sat} = 1.00 d^{-1} for specific growth rates.

Appendix B

Detailed methods of experimental tests of coexistence mechanisms

We experimentally explored several potential coexistence mechanisms that would explain why both the intraguild predator *Ochromonas* and its prey *Microcystis* increased with nutrient enrichment. In particular, we tested for the presence of (i) genetic diversity within the prey, (ii) inducible defences within the prey, (iii) changes in nutritional quality of the prey, (iv) a shift towards competition for light, and (v) intraspecific interference within the predator population. The methodology applied for these tests is described below.

(i) Genetic diversity within the prey

To test for the potential presence of different strains of *Microcystis*, we performed a denaturing gradient gel electrophoresis (DGGE) of three different parts of the internal transcribed spacer (ITS) between the 16S and 23S rRNA coding regions. The highly variable ITS-region has been demonstrated to give a good resolution between different *Microcystis* strains (Janse et al. 2003; Kardinaal et al. 2007), and is therefore suitable to test for the presence of intraspecific diversity.

We chose 5 samples from the chemostat experiment with the highest nitrogen load that had been started from a monoculture of *Microcystis* and was later inoculated with *Ochromonas*. The first sample was taken from the *Microcystis* monoculture (day 48 in Fig. 2F of the main text), while the remaining samples were taken after inoculation of *Ochromonas* (days 9, 24, 78 and 165 in Fig. 6.4F of the main text). DNA was extracted from material collected on glass fibre filters by a phenol extraction procedure. For PCR

amplification, we used primer sets for the ITSa, ITSb and ITSc regions described in Janse et al. (2003). The amplification was performed in a 50 µL reaction mixture containing 1.25 U GoTaq DNA Polymerase and GoTaq buffer (Promega), 0.25 mM of each primer, 0.2 mM of each nucleotide, and 400 ng μL^{-1} bovine serum albumin. A touch down procedure was used with pre-incubation at 95 °C for 2 min, followed by a total of 30 cycles, where the reaction mixture was exposed to 95 °C for 30 s, annealing temperature (T_a) for 40 s, and 72 °C for 60 s. In the first 20 cycles, T_a was reduced by 1 °C in every second cycle, from 62 °C in the first cycle to 52 °C in the twentieth cycle. For the remaining 10 cycles a T_a of 52 °C was used. The PCR was performed in a Peltier thermal cycler (PTC-200, MJ-Research).

The DGGE was performed as described in Janse et al. (2003). PCR products were separated on a 1.5 mm thick vertical gel containing 8% (wt/vol) polyacrylamide (37.5:1 acrylamide:bisacrylamide) and a linear gradient of the denaturants urea and formamide, increasing from 30% at the top of the gel to 40% at the bottom. Here, 100% denaturant is defined as 7 M urea and 40% (vol/vol) formamide. Electrophoresis was performed in a buffer containing 0.04 M Tris-acetate and 0.001 M EDTA (pH 7.6) (0.5× TAE buffer) for 16 h at 75 V. After staining of the gel in water containing 0.5 µg of ethidium bromide mL^{-1}, an image of the gel was recorded with a CCD camera system (Imago, B & L Systems).

(ii) Inducible defences within the prey

To test whether *Microcystis* showed an inducible defence against grazing, we used the approach described by Hessen and Van Donk (1993). *Microcystis* was grown in 100 mL Erlenmeyer flasks. The *Microcystis* cultures were exposed to an 'induced' treatment receiving filtrate from a mixed culture of *Ochromonas* grazing on *Microcystis*, while a control treatment received filtrate from a monoculture of *Microcystis*. Both treatments were performed in triplicate. For the production of the filtrates both mono- and mixed cultures were diluted with fresh medium to a *Microcystis* abundance of 3×10^6 cells mL^{-1}, prior to filtration through 0.2 µm polycarbonate filters (Nucleopore, Whatman). The fresh medium ensured a sufficiently high nutrient concentration in the filtrate for further growth of *Microcystis*. The daily addition of 30 mL fresh filtrate resulted in a replacement of half of the culture volume every day over a period of one week.

After one week a grazing experiment was performed in 100 mL Erlenmeyer flasks to compare grazing rates of *Ochromonas* grazing on the 'induced' and 'control' *Microcystis* cultures. *Ochromonas* was first precultured on saturating abundances of *Microcystis* from the respective treatments for 4 days prior to the experiments. *Microcystis* cultures from the 'induced' and 'control' treatments were diluted to a saturating prey abundance of 2.5×10^5 cells mL^{-1}. For each of these *Microcystis* cultures, a grazing treatment was inoculated with 2×10^4 cells mL^{-1} of *Ochromonas*, while a grazing control was performed

without *Ochromonas*. Each treatment was run in triplicate. Experiments were incubated under constant illumination of 90 µmol quanta m^{-2} s^{-1} for 10 h. Samples were taken at 2 h intervals, fixed with a mixture of formaldehyde and glutaraldehyde (final concentration of 0.025 and 0.0037 percent by mass, respectively), and stored at 4 °C until counting at a flow cytometer (MoFlo XDP cell sorter, Beckman Coulter).

Growth and grazing rates were calculated over periods of exponential growth. Specific growth rates were calculated as:

$$\mu = \frac{\ln(N_2) - \ln(N_1)}{t_2 - t_1} \tag{B1}$$

where N_1 and N_2 represent the cellular abundances at time steps t_1 and t_2. Grazing rates were calculated as described by Heinbokel (1978):

$$G = \frac{(\mu_C - \mu_T)N_M}{N_O} \tag{B2}$$

where μ_C and μ_T represent the specific growth rates of *Microcystis* in the grazing control and grazing treatment, respectively, and N_M and N_O represent the mean abundances of *Microcystis* and *Ochromonas* during the grazing experiment.

(iii) Changes in nutritional quality of the prey

In this test we investigated whether nitrogen deprivation of *Microcystis* affected the grazing and growth rates of *Ochromonas*. We first grew *Microcystis* in batch culture with 500 µM ammonium provided in the medium until the cells became nitrogen-starved at the stationary phase. *Ochromonas* was then pregrown for a period of 2 weeks receiving saturating abundances of the nitrogen-starved *Microcystis* as food. All cultures were incubated under constant illumination of 90 µmol quanta m^{-2} s^{-1}. One day prior to the grazing experiment both *Ochromonas* and *Microcystis* precultures were diluted with N-free medium and split into cultures either remaining N-limited or receiving a pulse of ammonium (250 µM). Grazing rates on N-deficient and N-amended *Microcystis* cells were then compared in a grazing experiment, as described above. For measurement of the cellular nitrogen and carbon content of *Microcystis*, monocultures were filtered onto pre-combusted glassfibre filters (Whatman GF/F), dried at 60 °C overnight, and analysed in an organic elemental analyser (Flash 2000; Thermo Fisher Scientific).

(iv) A shift towards competition for light

Both the incident light intensity (I_{in}) and the light intensity penetrating through the chemostat cultures (I_{out}) were measured at 10 points distributed over the front surface and back surface of the chemostat vessel, respectively, using a quantum photometer (LI-250;

LI-COR Biosciences). Assuming that the light gradient in the chemostat can be described by Lambert-Beer's law, the depth-averaged light intensity (I_{avg}) was calculated as (Huisman et al. 2002):

$$I_{avg} = \frac{I_{in} - I_{out}}{\ln I_{in} - \ln I_{out}} \quad \text{(B3)}$$

The depth-averaged light intensity decreases with increasing population abundances of *Microcystis* and *Ochromonas*, because their photosynthetic pigments absorb light. To test whether growth in the densest cultures might have become light-limited, we therefore raised the incident light intensity from 50 to 70 µmol quanta m^{-2} s^{-1} for the chemostat vessels with the highest nitrogen load to obtain a depth-averaged light intensity equal to the chemostat vessels with lower nitrogen loads (Fig. B1). This adjustment was made on day 130 of the mixed cultures and the time point is indicated as dotted line in Fig. 6.4E and F. Sampling was continued thereafter to detect potential changes in the steady-state abundances of the species.

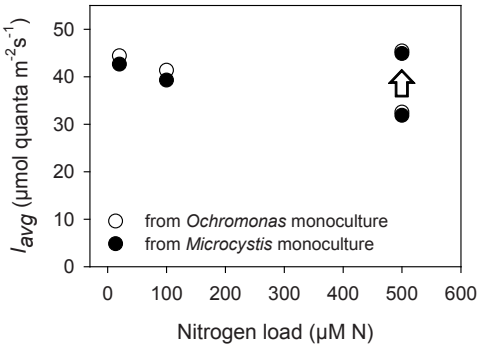

Figure B1: Depth-averaged light intensity (I_{avg}) during steady state in the mixed culture chemostats. The arrow indicates the adjustment of the light intensity of the cultures with high nitrogen load during steady state.

(v) Intraspecific interference within the predator population

To test for intraspecific interference within the *Ochromonas* population, we investigated whether the grazing rate of *Ochromonas* depended on its own abundance. *Ochromonas* was precultured on saturating abundances of *Microcystis* for 4 days prior to the experiment. In the grazing experiments *Microcystis* was inoculated at a saturating abundance of 1×10^6 cells mL^{-1} in all treatments. We used 5 different levels of *Ochromonas* abundance, including a control treatment without *Ochromonas* and four grazing treatments with *Ochromonas* abundances of 3.5×10^3, 2.0×10^4, 8.8×10^4, and 4×10^5 cells mL^{-1}, respectively. Each of the treatments was performed in triplicate. The experiments were incubated for 3 h and samples were taken after 20, 40, 60, 80, 120, 150 and 180 min. Samples were analysed and grazing rates were calculated as described above.

References

Abrams PA (2011) Simple life-history omnivory: responses to enrichment and harvesting in systems with intraguild predation. American Naturalist 178: 305-319.

Abrams PA, and Fung SR (2010a). Prey persistence and abundance in systems with intraguild predation and type-2 functional responses. Journal of Theoretical Biology 264: 1033-1042.

Abrams PA, and Fung SR (2010b) The impact of adaptive defence on top-down and bottom-up effects in systems with intraguild predation. Evolutionary Ecology Research 12: 307-325.

Adolf JE, Stoecker DK, and Harding LW (2006) The balance of autotrophy and heterotrophy during mixotrophic growth of *Karlodinium micrum* (Dinophyceae). Journal of Plankton Research 28: 737-751.

Allen AE, LaRoche J, Maheswari U, Lommer M, Schauer N, Lopez PJ, Finazzi G, Fernie AR, and Bowler C (2008) Whole-cell response of the pennate diatom *Phaeodactylum tricornutum* to iron starvation. Proceedings of the National Academy of Sciences USA 105: 10438-10443.

Allen AP, Gillooly JF, and Brown JH (2005) Linking the global carbon cycle to individual metabolism. Functional Ecology 19: 202–213.

Amarasekare P (2007) Trade-offs, temporal variation, and species coexistence in communities with intraguild predation. Ecology 88: 2720-2728.

Amarasekare P (2008) Coexistence of intraguild predators and prey in resource-rich environments. Ecology 89: 2786-2797.

Andersen T, Elser JJ, and Hessen DO (2004) Stoichiometry and population dynamics. Ecology Letters 7: 884-900.

Anderson DM, Glibert PM, and Burkholder JM (2002) Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. Estuaries 25: 704-726.

Andersson A, Falk S, Samuelsson G, and Hagström Å (1989) Nutritional characteristics of a mixotrophic nanoflagellate, *Ochromonas* sp. Microbial Ecology 17: 251-262.

Archibald JM, and Keeling PJ (2002) Recycled plastids: a 'green movement' in eukaryotic evolution. Trends in Genetics 18: 577-584.

Arim M, and Marquet PA (2004) Intraguild predation: a widespread interaction related to species biology. Ecology Letters 7: 557-564.

Arvola L, and Salonen K (2001) Plankton community of a polyhumic lake with and without *Daphnia longispina* (Cladocera). Hydrobiologia 445: 141-150.

Bampfylde CJ, and Lewis MA (2007) Biological control through intraguild predation: case studies in pest control, invasive species and range expansion. Bulletin of Mathematical Biology 69: 1031-1066.

Beddington JR (1975) Mutual interference between parasites or predators and its effect on searching efficiency. Journal of Animal Ecology 44: 331-340.

Behrenfeld MJ, O'Malley RT, Siegel DA, McClain CR, Sarmiento JL, Feldman GC, Milligan AJ, Falkowski PG, Letelier RM, and Boss ES (2006) Climate-driven trends in contemporary ocean productivity. Nature 444: 752-755.

Béjà O, Spudich EN, Spudich JL, Leclerc M, and DeLong EF (2001) Proteorhodopsin phototrophy in the ocean. Nature 411: 786-789.

Bell G (2013) Experimental evolution of heterotrophy in a green alga. Evolution 67:468–476.

Bergström AK, Jansson M, Drakare S, and Blomqvist P (2003) Occurrence of mixotrophic flagellates in relation to bacterioplankton production, light regime and availability of inorganic nutrients in unproductive lakes with differing humic contents. Freshwater Biology 48: 868-877.

Bennett SJ, Sanders RW, and Porter KG (1990) Heterotrophic, autotrophic, and mixotrophic nanoflagellates: seasonal abundances and bacterivory in a eutrophic lake. Limnology and Oceanography 35: 1821-1832.

Berman T, and Bronk DA (2003) Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. Aquatic Microbial Ecology 31: 279-305.

Bernacchi CJ, Singsaas EL, Pimentel C, Portis AR, and Long SP (2001) Improved temperature response functions for models of Rubisco-limited photosynthesis. Plant Cell and Environment 24: 253-259.

Bernát G, Waschewski N, and Rögner M (2009) Towards efficient hydrogen production: the impact of antenna size and external factors on electron transport dynamics in *Synechocystis* PCC6803. Photosynthesis Research 99: 205-216.

Berninger UG, Caron DA, and Sanders RW (1992) Mixotrophic algae in three ice-covered lakes of the Pocano Mountains, USA. Freshwater Biology 28: 263-272.

Bird DF, and Kalff J (1987) Algal phagotrophy: regulating factors and importance relative to photosynthesis in *Dinobryon* (Chrysophyceae). Limnology and Oceanography 32: 277-284.

Bird DF, and Kalff J (1989) Phagotrophic sustenance of a metalimnetic phytoplankton peak. Limnology and Oceanography 34: 155-162.

Blackman FF (1905) Optima and limiting factors. Annals of Botany 19: 281-296.

Boëchat IG, Weithoff G, Krüger A, Gücker B, and Adrian R (2007) A biochemical explanation for the success of mixotrophy in the flagellate *Ochromonas* sp. Limnology and Oceanography 52: 1624-1632

Boenigk J, and Arndt H (2000) Particle handling during interception feeding by four species of heterotrophic nanoflagellates. Journal of Eukaryotic Microbiology 47: 350-358.

Boenigk J, Matz C, Jürgens K, and Arndt H (2001) The influence of preculture conditions and food quality on the ingestion and digestion process of three species of heterotrophic nanoflagellates. Microbial Ecology 42: 168-176.

Boenigk J, and Stadler P (2004) Potential toxicity of chrysophytes affiliated with *Poterioochromonas* and related '*Spumella*-like' flagellates. Journal of Plankton Research 26: 1507-1514.

Borer ET, Briggs CJ, Murdoch WW, and Swarbrick SL (2003) Testing intraguild predation theory in a field system: does numerical dominance shift along a gradient of productivity? Ecology Letters 6: 929-935.

Boxhorn JE, Holen DA, and Boraas ME (1998) Toxicity of the chrysophyte flagellate *Poterioochromonas malhamensis* to the rotifer *Brachionus angularis*. Hydrobiologia 388: 283-287.

Brauer VS, Stomp M, and Huisman J (2012) The nutrient-load hypothesis: patterns of resource limitation and community structure driven by competition for nutrients and light. American Naturalist 179: 721-740.

Brown JH, Gillooly JF, Allen AP, Savage VM, and West GB (2004) Toward a metabolic theory of ecology. Ecology 85: 1771-1789.

Burkert U, Hyenstrand P, Drakare S, and Blomqvist P (2001) Effects of the mixotrophic flagellate *Ochromonas* sp. on colony formation in *Microcystis aeruginosa*. Aquatic Ecology 35: 9-17.

Burkholder JM, Glibert PM, and Skelton HM (2008) Mixotrophy, a major mode of nutrition for harmful algal species in eutrophic waters. Harmful Algae 8: 77-93.

Callieri C, Caravati E, Morabito G, and Oggioni A (2006) The unicellular freshwater cyanobacterium *Synechococcus* and mixotrophic flagellates: evidence for a functional association in an oligotrophic, subalpine lake. Freshwater Biology 51: 263-273.

Carmichael WW (2001) Health effects of toxin-producing cyanobacteria: 'the cyanoHABs'. Human and Ecological Risk Assessment 7: 1393-1407.

Carmichael WW, Azevedo S, An JS, Molica RJR, Jochimsen EM, Lau S, Rinehart KL, Shaw GR, and Eaglesham GK (2001) Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. Environmental Health Perspectives 109: 663-668.

Caron DA, Goldman JC, and Dennett MR (1986) Effect of temperature on growth, respiration, and nutrient regeneration by an omnivorous microflagellate. Applied and Environmental Microbiology 52: 1340-1347.

Caron DA, Porter KG, and Sanders RW (1990) Carbon, nitrogen, and phosphorus budgets for the mixotrophic phytoflagellate *Poterioochromonas malhamensis* (Chrysophyceae) during bacterial ingestion. Limnology and Oceanography 35: 433-443.

Caron DA, Sanders RW, Lim EL, Marrase C, Amaral LA, Whitney S, Aoki RB, and Porter KG (1993) Light-dependent phagotrophy in the freshwater mixotrophic chrysophyte *Dinobryon cylindricum*. Microbial Ecology 25: 93-111.

Carpenter SR, Kitchell JF, and Hodgson JR (1985) Cascading trophic interactions and lake productivity. Bioscience 35: 634-639.

Chisholm SW (1992) Phytoplankton size. In: Falkowski PG, and Woodhead AD (eds.) Primary productivity and biogeochemical cycles in the sea. Plenum, New York, USA.

Choi JW, and Peters F (1992) Effects of temperature on two psychrophilic ecotypes of a heterotrophic nanoflagellate, *Paraphysomonas imperforata*. Applied and Environmental Microbiology 58: 593-599.

Chorus I, and Bartram J (1999) Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. E and FN Spon, London, UK.

Christoffersen K (1996) Effect of microcystin on growth of single species and on mixed natural populations of heterotrophic nanoflagellates. Natural Toxins 4: 215-220.

Codd GA (1995) Cyanobacterial toxins: occurrence, properties and biological significance. Water Science and Technology 32: 149-156.

Codd GA (2000) Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. Ecological Engineering 16: 51-60.

Codd GA, Bell SG, Kaya K, Ward CJ, Beattie KA, and Metcalf JS (1999) Cyanobacterial toxins, exposure routes and human health. European Journal of Phycology 34: 405-415.

Codd GA, Morrison LF, and Metcalf JS (2005) Cyanobacterial toxins: risk management for health protection. Toxicology and Applied Pharmacology 203: 264-272.

Cole GT, and Wynne MJ (1974) Endocytosis of *Microcystis aeruginosa* by *Ochromonas danica*. Journal of Phycology 10: 397-410.

Coles JF, and Jones RC (2000) Effect of temperature on photosynthesis-light response and growth of four phytoplankton species isolated from a tidal freshwater river. Journal of Phycology 36: 7-16.

Compeau G, Alachi BJ, Platsouka E, and Levy SB (1988) Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. Applied and Environmental Microbiology 54: 2432-2438.

Crowder DW, and Snyder WE (2010) Eating their way to the top? Mechanisms underlying the success of invasive generalist predators. Biological Invasions 12: 2857-2876.

Dawson RM (1998) The toxicology of microcystins. Toxicon 36: 953-962.

DeAngelis DL, Goldstein RA, and O'Neill RV (1975) A model for trophic interaction. Ecology 56: 881-892.

DeMott WR, Zhang QX, and Carmichael WW (1991) Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and 3 species of *Daphnia*. Limnology Oceanography 36: 1346-1357.

DeYoe HR, and Suttle CA (1994) The inability of the Texas "brown tide" alga to use nitrate and the role of nitrogen in the initiation of a persistent bloom of this organism. Journal of Phycology 30: 800-806.

Diehl S, and Feissel M (2000) Effects of enrichment on three-level food chains with omnivory. American Naturalist 155: 200-218.

Diehl S, and Feissel M (2001) Intraguild prey suffer from enrichment of their resources: a microcosm experiment with ciliates. Ecology 82: 2977-2983.

Dittmann E, Neilan BA, Erhard M, von Döhren H, and Börner T (1997) Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. Molecular Microbiology 26: 779-787.

Dittmann E, and Wiegand C (2006) Cyanobacterial toxins: occurrence, biosynthesis and impact on human affairs. Molecular Nutrition and Food Research 50: 7-17.

Droop MR (1973) Some thoughts on nutrient limitation in algae. Journal of Phycology 9: 264-272.

Dryden RC, and Wright SJL (1987) Predation of cyanobacteria by protozoa. Canadian Journal of Microbiology 33: 471-482.

Ducobu H, Huisman J, Jonker RR, and Mur LR (1998) Competition between a prochlorophyte and a cyanobacterium under various phosphorus regimes: comparison with the Droop model. Journal of Phycology 34: 467-476.

Duff KE, Zeeb BA, and Smol JP (1997) Chrysophyte cyst biogeographical and ecological distributions: a synthesis. Journal of Biogeography 24: 791-812.

Dugdale RC, and Goering JJ (1967) Uptake of new and regenerated forms of nitrogen in primary productivity. Limnology and Oceanography 12: 196-206.

Edwards KF, Thomas MK, Klausmeier CA, and Litchman E (2012) Allometric scaling and taxonomic variation in nutrient utilization traits and maximum growth rate of phytoplankton. Limnology and Oceanography 57: 554-566.

Englund G, Öhlund G, Hein CL, and Diehl S (2011) Temperature dependence of the functional response. Ecology Letters 14: 914-921.

Eppley RW (1972) Temperature and phytoplankton growth in the sea. Fishery Bulletin 70: 1063-1085.

Eppley RW, Coatsworth JL, and Solórzano L (1969) Studies of nitrate reductase in marine phytoplankton. Limnology and Oceanography 14: 194-205.

Eppley RW, and Peterson BJ (1979) Particulate organic matter flux and planktonic new production in the deep ocean. Nature 282: 677-680.

Estep KW, Davis PG, Keller MD, and Sieburth J McN (1986) How important are oceanic algal nanoflagellates in bacterivory? Limnology and Oceanography 31: 646-650.

Falconer IR (1999) An overview of problems caused by toxic blue-green algae (cyanobacteria) in drinking and recreational water. Environmental Toxicology 14: 5-12.

Falkowski PG, and Raven JA (2007) Aquatic Photosynthesis. 2nd Edition, Princeton University Press, Princeton, NJ, USA.

Fastner J, Flieger I, and Neumann U (1998) Optimised extraction of microcystins from field samples: a comparison of different solvents and procedures. Water Research 32: 3177-3181.

Finke DL, and Denno R (2006) Predator diversity and the functioning of ecosystems: the role of intraguild predation in dampening trophic cascades. Ecology Letters 8: 1299-1306.

Flöder S, Hansen T, and Ptacnik R (2006) Energy-dependent bacterivory in *Ochromonas minima*: a strategy promoting the use of substitutable resources and survival at insufficient light supply. Protist 157: 291-302.

Flynn KJ, and Mitra A (2009) Building the "perfect beast": modelling mixotrophic plankton. Journal of Plankton Research 31: 965-992.

Flynn KJ, Stoecker DK, Mitra A, Raven JA, Glibert PM, Hansen PJ, Graneli E, and Burkholder JM (2013) Misuse of the phytoplankton-zooplankton dichotomy: the need to assign organisms as mixotrophs within plankton functional types. Journal of Plankton Research 35: 3-11.

Fulton RS, and Paerl HW (1987a) Effect of colonial morphology on zooplankton utilization of algal resources during blue-green algal (*Microcystis aeruginosa*) blooms. Limnology and Oceanography 32: 634-644.

Fulton RS, and Paerl HW (1987b) Toxic and inhibitory effects of the blue-green alga *Microcystis aeruginosa* on herbivorous zooplankton. Journal of Plankton Research 9: 837-855.

Gebauer G, and Meyer M (2003) 15N and 13C natural abundance of autotrophic and mycoheterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. New Phytologist 160: 209-223.

Ghadouani A, Pinel-Alloul B, Plath K, Codd GA, and Lampert W (2004) Effects of M*icrocystis aeruginosa* and purified microcystin-LR on the feeding behavior of *Daphnia pulicaria*. Limnology and Oceanography 49: 666-679.

Gibbs PB, and Biggins J (1989) Regulation of the distribution of excitation energy in *Ochromonas danica*, an organism containing a chlorophyll-A/C/carotenoid light harvesting antenna. Photosynthesis Research 21: 81-91.

Gibbs SP (1978) The chloroplasts of *Euglena* may have evolved from symbiotic green algae. Canadian Journal of Botany 56: 2883-2889.

Gibbs SP (1981) The chloroplasts of some algal groups may have evolved from endosymbiotic eukaryotic algae. Annals of the New York Academy of Sciences 361: 193-208.

Gillooly JF, Brown JH, West GB, Savage VM, and Charnov EL (2001) Effects of size and temperature on metabolic rate. Science 293: 2248-2251.

Gismervik I, and Andersen T (1997) Prey switching by *Acartia clausi*: experimental evidence and implications of intraguild predation assessed by a model. Marine Ecology Progress Series 157: 247-259.

Glibert PM, Lipschultz F, McCarthy JJ, and Altabet MA (1982) Isotope dilution models of uptake and remineralization of ammonium by marine plankton. Limnology and Oceanography 27: 639-650.

Gómez-Consarnau L, Gonzáles JM, Coll-Lladó M, Gourdon P, Pascher T, Neutze R, Pedrós-Alió C, and Pinhassi J (2007) Light stimulates growth of proteorhodopsin-containing marine Flavobacteria. Nature 445: 210-213.

Grob C, Ulloa O, Claustre H, Huot Y, Alarcon G, and Marie D (2007) Contribution of picoplankton to the total particulate organic carbon concentration in the eastern South Pacific. Biogeosciences 4: 837-852.

Gulati RD, and Van Donk E (2002) Lakes in the Netherlands, their origin, eutrophication and restoration: state-of-the-art review. Hydrobiologia 478: 73-106.

Gustafsson S, Rengefors K, and Hansson LA (2005) Increased consumer fitness following transfer of toxin tolerance to offspring via maternal effects. Ecology 86: 2561-2567.

Hairston NG, Smith FE, and Slobodkin LB (1960) Community structure, population control, and competition. American Naturalist 44: 421-425.

Hall RJ (2011) Eating the competition speeds up invasions. Biology Letters 7: 307-311.

Hammer AC, and Pitchford JW (2005) The role of mixotrophy in plankton bloom dynamics, and the consequences for productivity. ICES Journal of Marine Science 62: 833-840.

Hansen PJ (2011) The role of photosynthesis and food uptake for the growth of marine mixotrophic dinoflagellates. Journal of Eukaryotic Microbiology 58: 203-214.

Hansson LA, Gustafsson S, Rengefors K, and Bomark L (2007) Cyanobacterial chemical warfare affects zooplankton community composition. Freshwater Biology 52: 1290-1301.

Hartmann M, Grob C, Scanlan DJ, Martin AP, Burkill PH, and Zubkov MV (2011) Comparison of phosphate uptake rates by the smallest plastidic and aplastidic protists in the North Atlantic subtropical gyre. FEMS Microbiology Ecology 78: 327-335.

Hartmann M, Grob C, Tarran GA, Martin AP, Burkill PH, Scanlan DJ, and Zubkov MV (2012) Mixotrophic basis of Atlantic oligotrophic ecosystems. Proceedings of the National Academy of Sciences USA 109: 5756-5760.

Havskum H, and Hansen AS (1997) Importance of pigmented and colourless nano-sized protists as grazers on nanoplankton in a phosphate-depleted Norwegian fjord and in enclosures. Aquatic Microbial Ecology 12: 139-151.

Heinbokel JF (1978) Studies on the functional role of tintinnids in the Southern California Bight. I. Grazing and growth rates in laboratory cultures. Marine Biology 47: 177-189.

Hessen DO, and Van Donk E (1993) Morphological changes in *Scenedesmus* induced by substances released from *Daphnia*. Archiv für Hydrobiologie 127: 129-140.

Hiltunen, T, Jones LE, Ellner SP, and Hairston Jr NG. (in press) Temporal dynamics of a simple community with intraguild predation: an experimental test. Ecology. doi: org/10.1890/12-0786.1.

Hin V, Schellekens T, Persson L, and de Roos AM (2011) Coexistence of predator and prey in intraguild predation systems with ontogenetic niche shifts. American Naturalist 178: 701-714.

Holen DA (1999) Effects of prey abundance and light intensity on the mixotrophic chrysophyte *Poterioochromonas malhamensis* from a mesotrophic lake. Freshwater Biology 42: 445-455.

Holling CS (1959) The components of predation as revealed by a study of small mammal predation of the European pine sawfly. Canadian Entomologist 91: 293-320.

Holmes RM, Aminot A, Kerouel R, Hooker BA, and Peterson BJ (1999) A simple and precise method for measuring ammonium in marine and freshwater ecosystems. Canadian Journal of Fisheries and Aquatic Sciences 56: 1801-1808.

Holt RD, and Polis GA (1997) A theoretical framework for intraguild predation. American Naturalist 149: 745-764.

Holyoak M, and Sachdev S (1998) Omnivory and the stability of simple food webs. Oecologia 117: 413-419.

Hoppe HG, Gocke K, Koppe R, and Begler C (2002) Bacterial growth and primary production along a north-south transect of the Atlantic Ocean. Nature 416: 168-171.

Huisman J, Jonker RR, Zonneveld C, and Weissing FJ (1999) Competition for light between phytoplankton species: experimental tests of mechanistic theory. Ecology 80: 211-222.

Huisman J, Matthijs HCP, Visser PM, Balke H, Sigon CAM, Passarge J, Weissing FJ, and Mur LR (2002) Principles of the light-limited chemostat: theory and ecological applications. Antonie van Leeuwenhoek 81: 117-133.

Huisman J, Sharples J, Stroom J, Visser PM, Kardinaal WEA, Verspagen JMH, and Sommeijer B (2004) Changes in turbulent mixing shift competition for light between phytoplankton species. Ecology 85: 2960-2970.

Huisman J, Matthijs HCP, and Visser PM (2005) Harmful cyanobacteria. Springer, Dordrecht, The Netherlands.

Ibelings BW, and Maberly SC (1998) Photoinhibition and the availability of inorganic carbon restrict photosynthesis by surface blooms of cyanobacteria. Limnology and Oceanography 43: 408-419.

Isaksson A (1998) Phagotrophic phytoflagellates in lakes: a literature review. Archiv für Hydrobiologie, Special Issues, Advances in Limnology 51: 63-90.

Isaksson A, Bergstrom AK, Blomqvist P, and Jansson M (1999) Bacterial grazing by phagotrophic phytoflagellates in a deep humic lake in northern Sweden. Journal of Plankton Research 21: 247-268.

Jang MH, Ha K, Joo GJ, and Takamura N (2003) Toxin production of cyanobacteria is increased by exposure to zooplankton. Freshwater Biology 48: 1540-1550.

Janse I, Meima M, Kardinaal WEA, and Zwart G (2003) High-resolution differentiation of cyanobacteria by using rRNA-internal transcribed spacer denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 69: 6634-6643.

Janssen A, Sabelis MW, Magalhães S, Montserrat M, and van der Hammen T (2007) Habitat structure affects intraguild predation. Ecology 88: 2713-2719.

Jansson M, Blomqvist P, Jonsson A, and Bergström AK (1996) Nutrient limitation of bacterioplankton, autotrophic and mixotrophic phytoplankton, and heterotrophic nanoflagellates in Lake Örträsket. Limnology and Oceanography 41: 1552-1559.

Jardillier L, Zubkov MV, Pearman J, and Scanlan DJ (2010) Significant CO2 fixation by small prymnesiophytes in the subtropical and tropical northeast Atlantic Ocean. ISME Journal 4: 1180-1192.

John EH, and Davidson K (2001) Prey selectivity and the influence of prey carbon:nitrogen ratio on microflagellate grazing. Journal of Experimental Marine Biology and Ecology 260: 93-111.

Jöhnk KD, Huisman J, Sharples J, Sommeijer B, Visser PM, and Stroom JM (2008) Summer heatwaves promote blooms of harmful cyanobacteria. Global Change Biology 14: 495-512.

Johnson MD (2011) The acquisition of phototrophy: adaptive strategies of hosting endosymbionts and organelles. Photosynthesis Research 107: 117-132.

Johnson MD, and Stoecker DK (2005) Role of feeding in growth and photophysiology of *Myrionecta rubra*. Aquatic Microbial Ecology 39: 303-312.

Jones H (1997) A classification of mixotrophic protists based on their behaviour. Freshwater Biology 37: 35-43.

Jones RI (2000) Mixotrophy in planktonic protists: an overview. Freshwater Biology 45: 219-226.

Jordan DB, and Ogren WL (1984) The CO_2/O_2 specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase: dependence on ribulosebisphosphate concentration, pH and temperature. Planta 161: 308-313.

Jost C, Lawrence CA, Campolongo F, van de Bund W, Hill S, and DeAngelis DL (2004) The effects of mixotrophy on the stability and dynamics of a simple planktonic food web model. Theoretical Population Biology 66: 37-51.

Jungmann D (1992) Toxic compounds isolated from *Microcystis* PCC7806 that are more active against *Daphnia* than two microcystins. Limnology and Oceanography 37: 1777-1783.

Kardinaal WEA, Janse I, Kamst-van Agterveld M, Meima M, Snoek J, Mur LR, Huisman J, Zwart G, and Visser PM (2007) Microcystis genotype succession in relation to microcystin concentrations in freshwater lakes. Aquatic Microbial Ecology 48: 1-12.

Katechakis A, and Stibor H (2006) The mixotroph *Ochromonas tuberculata* may invade and suppress specialist phago- and phototroph plankton communities depending on nutrient conditions. Oecologia 148: 692-701.

Keller MD, Shapiro LP, Haugen EM, Cucci TL, Sherr EB, and Sherr BF (1994) Phagotrophy of fluorescently labeled bacteria by an oceanic phytoplankter. Microbial Ecology 28: 39-52.

Kilham SS, Kreeger DA, Lynn SG, Goulden CE, and Herrera L (1998) COMBO: a defined freshwater culture medium for algae and zooplankton. Hydrobiologia 377: 147-159.

Kim BR, Nakan S, Kim BH, and Han MS (2006) Grazing and growth of the heterotrophic flagellate *Diphylleia rotans* on the cyanobacterium *Microcystis aeruginosa*. Aquatic Microbial Ecology 45: 163-170.

Kim S, Kang YG, Kim HS, Yih W, Coats DW, and Park MG (2008) Growth and grazing responses of the mixotrophic dinoflagellate *Dinophysis acuminata* as functions of light intensity and prey concentration. Aquatic Microbial Ecology 51: 301-310.

Kiørboe T (2008) A mechanistic approach to plankton ecology. Princeton University Press, Princeton, USA.

Kolber ZS, Plumley FG, Lang AS, Beatty JT, Blankenship RE, VanDover CL, Vetriani C, Koblizek M, Rathgeber C, and Falkowski PG (2001) Contribution of aerobic photoheterotrophic bacteria to the carbon cycle in the ocean. Science 292: 2492-2495.

Kovacs L, Wiessner W, Kis M, Nagy F, Mende D, and Demeter S (2000) Short- and long-term redox regulation of photosynthetic light energy distribution and photosystem stoichiometry by acetate metabolism in the green alga *Chlamydobotrys stellata*. Photosynthesis Research 65: 231-247.

Kratina P, Greig HS, Thompson PL, Carvalho-Pereira TSA, and Shurin JB (2012) Warming modifies trophic cascades and eutrophication in experimental freshwater communities. Ecology 93: 1421-1430.

Krienitz L, Ballot A, Kotut K, Wiegand C, Putz S, Metcalf JS, Codd GA, and Pflugmacher S (2003) Contribution of hot spring cyanobacteria to the mysterious deaths of Lesser Flamingos at Lake Bogoria, Kenya. FEMS Microbiology Ecology 43: 141-148.

Křivan V, and Diehl S (2005) Adaptive omnivory and species coexistence in tri-trophic food webs. Theoretical Population Biology 67: 85-99.

Laemmli UK (1970) Cleavage of structural proteins during assembly of head of bacteriophage-T4. Nature 227: 680-685.

Lampert W (1987) Laboratory studies on zooplankton-cyanobacteria interactions. New Zealand Journal of Marine and Freshwater Research 21: 483-490.

Larsen S, Andersen T, and Hessen DO (2011) Climate change predicted to cause severe increase of organic carbon in lakes. Global Change Biology 17: 1186-1192.

Lawler SP, and Morin PJ (1993) Food web architecture and population dynamics in laboratory microcosms of protists. American Naturalist 141: 675-686.

L'Helguen S, Maguer JF, and Caradec J (2008) Inhibition kinetics of nitrate uptake by ammonium in size-fractionated oceanic phytoplankton communities: implications for new production and f-ratio estimates. Journal of Plankton Research 30: 1179-1188.

Li AS, Stoecker DK, and Adolf JE (1999) Feeding, pigmentation, photosynthesis and growth of the mixotrophic dinoflagellate *Gyrodinium galatheanum*. Aquatic Microbial Ecology 19: 163-176.

Li AS, Stoecker DK, and Coats DW (2000) Mixotrophy in *Gyrodinium galatheanum* (Dinophyceae): grazing responses to light intensity and inorganic nutrients. Journal of Phycology 36: 33-45.

Liess A, and Diehl S (2006) Effects of enrichment on protist abundances and bacterial composition in simple microbial communities. Oikos 114: 15-26.

Litchman E, Klausmeier CA, Schofield OM, and Falkowski PG (2007) The role of functional traits and trade-offs in structuring phytoplankton communities: scaling from cellular to ecosystem level. Ecology Letters 10: 1170-1181.

Lomas MW, and Glibert PM (1999) Interactions between NH_4^+ and NO_3^- uptake and assimilation: comparison of diatoms and dinoflagellates at several growth temperatures. Marine Biology 133: 541-551.

Long BM, Jones GJ, and Orr PT (2001) Cellular microcystin content in N-limited *Microcystis aeruginosa* can be predicted from growth rate. Applied and Environmental Microbiology 67: 278-283.

Lopez-Urrutia A, San Martin E, Harris RP, and Irigoien X (2006) Scaling the metabolic balance of the oceans. Proceedings of the National Academy of Sciences USA 103: 8739-8744.

Lopez-Urrutia A (2008) The metabolic theory of ecology and algal bloom formation. Limnology and Oceanography 53: 2046-2047.

Lürling M (2003) *Daphnia* growth on microcystin-producing and microcystin-free *Microcystis aeruginosa* in different mixtures with the green alga *Scenedesmus obliquus*. Limnology and Oceanography 48: 2214-2220.

Lürling M, and Van Donk E (1997) Morphological changes in *Scenedesmus* induced by infochemicals released in situ from zooplankton grazers. Limnology and Oceanography 42: 783-788.

Maberly SC, Ball LA, Raven JA, and Sueltemeyer D (2009). Inorganic carbon acquisition by chrysophytes. Journal of Phycology 45: 1052-1061.

MacKintosh C, Beattie KA, Klumpp S, Cohen P, and Codd GA (1990) Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Letters 264: 187-192.

Maxwell K, and Johnson GN (2000) Chlorophyll fluorescence: a practical guide. Journal of Experimental Botany 51: 659-668.

McCarthy JJ, Taylor WR, and Taft JL (1977) Nitrogenous nutrition of the plankton in the Chesapeake Bay. I. Nutrient availability and phytoplankton preferences. Limnology and Oceanography 22: 996-1011.

McFadden GI (2001) Primary and secondary endosymbiosis and the origin of plastids. Journal of Phycology 37: 951-959.

Mereschkowski C (1905) Über Natur und Ursprung der Chromatophoren im Pflanzenreiche. Biologisches Centralblatt 25: 593-604.

Meyer JR, Ellner SP, Hairston NG, Jones LE, and Yoshida T (2006) Prey evolution on the time scale of predator-prey dynamics revealed by allele-specific quantitative PCR. Proceedings of the National Academy of Sciences USA 103: 10690-10695.

Mitchell TD, and Jones PD (2005) An improved method of constructing a database of monthly climate observations and associated high-resolution grids. International Journal of Climatology 25: 693-712.

Mitra A, and Flynn KJ (2010) Modelling mixotrophy in harmful algal blooms: more or less the sum of the parts? Journal of Marine Systems 83: 158-169.

Moe SJ, Dudley B, and Ptacnik R (2008) REBECCA databases: experiences from compilation and analyses of monitoring data from 5,000 lakes in 20 European countries. Aquatic Ecology 42: 183-201.

Montoya JP, Holl CM, Zehr JP, Hansen A, Villareal TA, and Capone DG (2004) High rates of N2 fixation by unicellular diazotrophs in the oligotrophic Pacific Ocean. Nature 430: 1027-1031.

Montserrat M, Magalhães S, Sabelis MW, de Roos AM, and Janssen A (2008) Patterns of exclusion in an intraguild predator-prey system depend on initial conditions. Journal of Animal Ecology 77: 624-630.

Moon-van der Staay SY, De Wachter R, and Vaulot D (2001) Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nature 409: 607-610.

Moore LR, Post AF, Rocap G, and Chisholm SW (2002) Utilization of different nitrogen sources by the marine cyanobacteria *Prochlorococcus* and *Synechococcus*. Limnology and Oceanography 47: 989-996.

Morel FMM (1987) Kinetics of nutrient uptake and growth in phytoplankton. Journal of Phycology 23: 137-150.

Morin P (1999) Productivity, intraguild predation, and population dynamics in experimental food webs. Ecology 80: 752-760.

Moustaka-Gouni M, Vardaka E, Michaloudi E, Kormas KA, Tryfon E, Mihalatou H, Gkelis S, and Lanaras T (2006) Plankton food web structure in a eutrophic lake with a history of toxic cyanobacterial blooms. Limnology and Oceanography 51: 715-727.

Müller-Navarra DC, Brett MT, Liston AM, and Goldman CR (2000) A highly unsaturated fatty acid predicts carbon transfer between primary producers and consumers. Nature 403: 74-77.

Mylius SD, Klumpers K, De Roos AM, and Persson L (2001) Impact of intraguild predation and stage structure on simple communities along a productivity gradient. American Naturalist 158: 259-276.

Nishibe Y, Kawabata Z, and Nakano S (2002) Grazing on *Microcystis aeruginosa* by the heterotrophic flagellate *Collodictyon triciliatum* in a hypertrophic pond. Aquatic Microbial Ecology 29: 173-179.

Nygaard K, and Hessen DO (1990) Use of C-14 protein-labelled bacteria for estimating clearance rates by heterotrophic and mixotrophic flagellates. Marine Ecology Progress Series 68: 7-14.

Nygaard K, and Tobiesen A (1993) Bacterivory in algae: a survival strategy during nutrient limitation. Limnology and Oceanography 38: 273-279.

O'Connor MI, Piehler MF, Leech DM, Anton A, and Bruno JF (2009) Warming and resource availability shift food web structure and metabolism. PLoS Biology 7: e1000178.

Oksanen L, Fretwell SD, Arruda J, and Niemelä P (1981) Exploitation ecosystems in gradients of primary productivity. American Naturalist 118: 240-261.

Olrik K, and Nauwerck A (1993) Stress and disturbance in the phytoplankton community of a shallow, hypertrophic lake. Hydrobiologia 249: 15-24.

Olrik K (1998) Ecology of mixotrophic flagellates with special reference to Chrysophyceae in Danish lakes. Hydrobiologia 370: 329-338.

O'Neil JM, Davis TW, Burford MA, and Gobler CJ (2012) The rise of harmful cyanobacteria blooms: the potential roles of eutrophication and climate change. Harmful Algae 14: 313-334.

Ou DY, Song LR, Gan NQ, and Chen W (2005) Effects of microcystins on and toxin degradation by *Poterioochromonas* sp. Environmental Toxicology 20: 373-380.

Paerl HW, and Huisman J (2008) Blooms like it hot. Science 320: 57-58.

Pålsson C, and Daniel C (2004) Effects of prey abundance and light intensity on nutrition of a mixotrophic flagellate and its competitive relationship with an obligate heterotroph. Aquatic Microbial Ecology 36: 247-256.

Park S, Brett MT, Müller-Navarra DC, Shin SC, Liston AM, and Goldman CR (2003) Heterotrophic nanoflagellates and increased essential fatty acids during *Microcystis* decay. Aquatic Microbial Ecology 33: 201-205.

Passarge J, Hol S, Escher M, and Huisman J (2006) Competition for nutrients and light: stable coexistence, alternative stable states, or competitive exclusion? Ecological Monographs 76: 57-72.

Peeters F, Straile D, Lorke A, and Livingstone DM (2007) Earlier onset of the spring phytoplankton bloom in lakes of the temperate zone in a warmer climate. Global Change Biology 13: 1898-1909.

Pfandl K, Posch T, and Boenigk J (2004) Unexpected effects of prey dimensions and morphologies on the size selective feeding by two bacterivorous flagellates (*Ochromonas* sp. and *Spumella* sp.). Journal of Eukaryotic Microbiology 51: 626-633.

Platt T, Gallegos CL, and Harrison WG (1980) Photoinhibition of photosynthesis in natural assemblages of marine phytoplankton. Journal of Marine Research 38: 687-701.

Polis GA, Myers CA, and Holt RD (1989) The ecology and evolution of intraguild predation: potential competitors that eat each other. Annual Review of Ecology and Systematics 20: 297-330.

Polis GA, and Holt RD (1992) Intraguild predation: the dynamics of complex trophic interactions. Trends in Ecology and Evolution 7: 151-154.

Polis GA, and Strong DR (1996) Food web complexity and community dynamics. American Naturalist 147: 813-846.

Polovina JJ, Howell EA, and Abecassis M (2008) Ocean's least productive waters are expanding. Geophysical Research Letters 35: L03618.

Porter KG (1988) Phagotrophic phytoflagellates in microbial food webs. Hydrobiologia 159: 89-97.

Portmann C, Blom JF, Kaiser M, Brun R, Jüttner F, and Gademann K (2008) Isolation of aerucyclamides C and D and structure revision of microcyclamide 7806A: heterocyclic ribosomal peptides from *Microcystis aeruginosa* PCC 7806 and their antiparasite evaluation. Journal of Natural Products 71: 1891-1896.

Power ME (1990) Effects of fish in river food webs. Science 250: 811-814.

Pringsheim EG (1958) Über Mixotrophie bei Flagellaten. Planta 52: 405-430.

Ptacnik R, Sommer U, Hansen T, and Martens V (2004) Effects of microzooplankton and mixotrophy in an experimental planktonic food web. Limnology and Oceanography 49: 1435-1445.

Qin B, Zhu G, Gao G, Zhang Y, Li W, Paerl HW, and Carmichael WW (2010) A drinking water crisis in Lake Taihu, China: linkage to climatic variability and lake management. Environmental Management 45: 105-112.

Rantala A, Fewer DP, Hisbergues M, Rouhiainen L, Vaitomaa J, Börner T, and Sivonen K (2004) Phylogenetic evidence for the early evolution of microcystin synthesis. Proceedings of the National Academy of Sciences USA 101: 568-573.

Raven JA (1997) Phagotrophy in phototrophs. Limnology and Oceanography 42: 198-205.

Real LA (1977) Kinetics of functional response. American Naturalist 111: 289-300.

Reboud X, and Bell G (1997) Experimental evolution in *Chlamydomonas*. III. Evolution of specialist and generalist types in environments that vary in space and time. Heredity 78: 507-514.

Reynolds CS, and Walsby AE (1975) Water blooms. Biological Reviews 50: 437-481.

Rohrlack T, Dittmann E, Henning M, Börner T, and Kohl JG (1999a) Role of microcystins in poisoning and food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 65: 737-739.

Rohrlack T, Henning M, and Kohl JG (1999b) Mechanisms of the inhibitory effect of the cyanobacterium *Microcystis aeruginosa* on *Daphnia galeata*'s ingestion rate. Journal of Plankton Research 21: 1489-1500.

Rohrlack T, Christoffersen K, Hansen PE, Zhang W, Czarnecki O, Henning M, Fastner J, Erhard M, Neilan BA, and Kaebernick M (2003) Isolation, characterization, and quantitative analysis of microviridin J, a new *Microcystis* metabolite toxic to *Daphnia*. Journal of Chemical Ecology 29: 1757-1770.

Rose JM, and Caron DA (2007) Does low temperature constrain the growth rates of heterotrophic protists? Evidence and implications for algal blooms in cold waters. Limnology and Oceanography 52: 886-895.

Rosenheim JA, Kaya HK, Ehler LE, Marois JJ, and Jaffee BA (1995) Intraguild predation among biological control agents: theory and evidence. Biological Control 5: 303-335.

Rothhaupt KO (1996a) Laboratory experiments with a mixotrophic chrysophyte and obligately phagotrophic and phototrophic competitors. Ecology 77: 716-724.

Rothhaupt KO (1996b) Utilization of substitutable carbon and phosphorus sources by the mixotrophic chrysophyte *Ochromonas* sp. Ecology 77: 706-715.

Rudd JWM, Kelly CA, Schindler DW, and Turner MA (1988) Disruption of the nitrogen cycle in acidified lakes. Science 240: 1515-1517.

Rudolf VHW, and Armstrong J (2008) Emergent impacts of cannibalism and size refuges in prey on intraguild predation systems. Oecologia 157: 675-686.

Safi KA, and Hall JA (1999) Mixotrophic and heterotrophic nanoflagellate grazing in the convergence zone east of New Zealand. Aquatic Microbial Ecology 20: 83-93.

Sagan L (1967) On the origin of mitosing cells. Journal of Theoretical Biology 14: 255-274.

Sanders RW, Porter KG, and Caron DA (1990) Relationship between phototrophy and phagotrophy in the mixotrophic chrysophyte *Poterioochromonas malhamensis*. Microbial Ecology 19: 97-109.

Sanders RW (1991) Mixotrophic protists in marine and freshwater ecosystems. Journal of Protozoology 38: 76-81.

Sanders RW, Berninger UG, Lim EL, Kemp PF, and Caron DA (2000) Heterotrophic and mixotrophic nanoplankton predation on picoplankton in the Sargasso Sea and on Georges Bank. Marine Ecology Progress Series 192: 103-118.

Sanders RW, Caron DA, Davidson JM, Dennett MR, and Moran DM (2001) Nutrient acquisition and population growth of a mixotrophic alga in axenic and bacterized cultures. Microbial Ecology 42: 513-523.

Sandgren CD (1988) The ecology of chrysophyte flagellates: their growth and perennation strategies as freshwater phytoplankton. In: Sandgren CD (ed.) Growth and reproductive strategies of freshwater phytoplankton. Cambridge University Press, Cambridge, UK.

Savage VM, Gillooly JF, Brown JH, West GB, and Charnov EL (2004) Effects of body size and temperature on population growth. American Naturalist 163: 429-441.

Scheffer M (1998) Ecology of shallow lakes. Chapman and Hall, London, UK.

Scheibe R (2004) Malate valves to balance cellular energy supply. Physiologia Plantarum 120: 21-26.

Seelert H, Poetsch A, Dencher NA, Engel A, Stahlberg H, and Müller DJ (2000) Structural biology: proton-powered turbine of a plant motor. Nature 405: 418-419.

Selosse MA, and Roy M (2009) Green plants that feed on fungi: facts and questions about mixotrophy. Trends in Plant Science 14: 64-70.

Shapiro J, and Wright DI (1984) Lake restoration by biomanipulation: Round Lake, Minnesota, the first two years. Freshwater Biology 14: 371-383.

Shi XL, Marie D, Jardillier L, Scanlan DJ, and Vaulot D (2009) Groups without cultured representatives dominate eukaryotic picophytoplankton in the oligotrophic south east Pacific Ocean. Plos One 4: e7657.

Shurin JB, Borer ET, Seabloom EW, Anderson K, Blanchette CA, Broitman B, Cooper SD, and Halpern BS (2002) A cross-ecosystem comparison of the strength of trophic cascades. Ecology Letters 5: 785-791.

Siver PA (1995) The distribution of chrysophytes along environmental gradients: their use as biological indicators. In: Sandgren CD, Smol JP, and Kristiansen J (eds.) Chrysophyte algae: ecology, phylogeny and development. Cambridge University Press, Cambridge, UK.

Skovgaard A (1996) Mixotrophy in *Fragilidium subglobosum* (Dinophyceae): growth and grazing responses as functions of light intensity. Marine Ecology Progress Series 143: 247-253.

Smayda TJ (1997) Harmful algal blooms: their ecophysiology and general relevance to phytoplankton blooms in the sea. Limnology and Oceanography 42: 1137-1153.

Sokal RR, and Rohlf FJ (1995) Biometry: the principles and practice of statistics in biological research. W.H. Freeman and Company, New York, USA.

Sommer U, Gaedke U, and Schweizer A (1993) The 1st decade of oligotrophication of Lake Constance. 2. The response of phytoplankton taxonomic composition. Oecologia 93: 276-284.

Sterner RW, and Elser JJ (2002) Ecological stoichiometry: the biology of elements from molecules to the biosphere. Princeton University Press, Princeton, NJ, USA.

Stibor H, and Sommer U (2003) Mixotrophy of a photosynthetic flagellate viewed from an optimal foraging perspective. Protist 154: 91-98.

Stoecker DK, and Michaels AE (1991) Respiration, photosynthesis and carbon metabolism in planktonic ciliates. Marine Biology 108: 441-447.

Stoecker DK (1998) Conceptual models of mixotrophy in planktonic protists and some ecological and evolutionary implications. European Journal of Protistology 34: 281-290.

Straub CS, Finke DL, and Snyder WE (2008) Are the conservation of natural enemy biodiversity and biological control compatible goals? Biological Control 45: 225-237.

Strauss EA, Mitchell NL, and Lamberti GA (2002) Factors regulating denitrification in aquatic sediments: effects of organic carbon, nitrogen availability and pH. Canadian Journal of Fisheries and Aquatic Sciences 59: 554-563.

Sukenik A, Eshkol R, Livne A, Hadas O, Rom M, Tchernov D, Vardi A, and Kaplan A (2002) Inhibition of growth and photosynthesis of the dinoflagellate *Peridinium gatunense* by *Microcystis* sp. (cyanobacteria): a novel allelopathic mechanism. Limnology and Oceanography 47: 1656-1663.

Taylor GT, Gobler CJ, and Sañudo-Wilhelmy SA (2006) Speciation and concentrations of dissolved nitrogen as determinants of brown tide *Aureococcus anophagefferens* bloom initiation. Marine Ecology Progress Series 312: 67-83.

Tcherkez GGB, Farquhar GD, and Andrews TJ (2006) Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. Proceedings of the National Academy of Sciences USA 103: 7246-7251.

Tcherkez G, Boex-Fontvieille E, Mahe A, and Hodges M (2012) Respiratory carbon fluxes in leaves. Current Opinion in Plant Biology 15: 308-314.

Thingstad TF, Havskum H, Garde K, and Riemann B (1996) On the strategy of "eating your competitor'': a mathematical analysis of algal mixotrophy. Ecology 77: 2108-2118.

Thompson RM, Hemberg M, Starzomski BM, and Shurin JB (2007) Trophic levels and trophic tangles: the prevalence of omnivory in real food webs. Ecology 88: 612-617.

Tilman D (1982) Resource competition and community structure. Princeton University Press, Princeton, NJ, USA.

Tittel J, Bissinger V, Zippel B, Gaedke U, Bell E, Lorke A, and Kamjunke N (2003) Mixotrophs combine resource use to outcompete specialists: implications for aquatic food webs. Proceedings of the National Academy of Sciences USA 100: 12776-12781.

Tonk L, Visser PM, Christiansen G, Dittmann E, Snelder E, Wiedner C, Mur LR, and Huisman J (2005) The microcystin composition of the cyanobacterium *Planktothrix agardhii* changes toward a more toxic variant with increasing light intensity. Applied and Environmental Microbiology 71: 5177-5181.

Tonk L, Welker M, Huisman J, and Visser PM (2009) Production of cyanopeptolins, anabaenopeptins, and microcystins by the harmful cyanobacteria *Anabaena* 90 and *Microcystis* PCC 7806. Harmful Algae 8: 219-224.

Tooming-Klunderud A, Rohrlack T, Shalchian-Tabrizi K, Kristensen T, and Jakobsen KS (2007) Structural analysis of a non-ribosomal halogenated cyclic peptide and its putative operon from *Microcystis*: implications for evolution of cyanopeptolins. Microbiology 153: 1382-1393.

Unrein F, Massana R, Alonso-Saez L, and Gasol JM (2007) Significant year-round effect of small mixotrophic flagellates on bacterioplankton in an oligotrophic coastal system. Limnology and Oceanography 52: 456-469.

Urbani P, and Ramos-Jiliberto R (2010) Adaptive prey behavior and the dynamics of intraguild predation systems. Ecological Modelling 221: 2628-2633.

Van de Waal DB, Verspagen JMH, Finke JF, Vournazou V, Immers AK, Kardinaal WEA, Tonk L, Becker S, Van Donk E, Visser PM, and Huisman J (2011) Reversal in competitive dominance of a toxic versus non-toxic cyanobacterium in response to rising CO_2. ISME Journal 5: 1438-1450.

Van de Wolfshaar KE, de Roos AM, and Persson L (2006) Size-dependent interactions inhibit coexistence in intraguild predation systems with life-history omnivory. American Naturalist 168: 62-75.

Van Donk E, Hessen DO, Verschoor AM, and Gulati RD (2008) Re-oligotrophication by phosphorus reduction and effects on seston quality in lakes. Limnologica 38: 189-202.

Van Donk E, Cerbin S, Wilken S, Helmsing NR, Ptacnik R, and Verschoor AM (2009) The effect of a mixotrophic chrysophyte on toxic and colony-forming cyanobacteria. Freshwater Biology 54: 1843-1855.

Van Maanen R, Broufas G, Oveja MF, Sabelis MW, and Janssen A (2012) Intraguild predation among plant pests: western flower thrips larvae feed on whitefly crawlers. BioControl 57: 533-539.

Van Wichelen J, van Gremberghe I, Vanormelingen P, Debeer AE, Leporcq B, Menzel D, Codd GA, Descy JP, and Vyverman W (2010) Strong effects of amoebae grazing on the biomass and genetic structure of a *Microcystis* bloom (Cyanobacteria). Environmental Microbiology 12: 2797-2813.

Vasseur DA, and McCann KS (2005) A mechanistic approach for modeling temperature-dependent consumer-resource dynamics. American Naturalist 166: 184-198.

Verdy A, and Amarasekare P (2010) Alternative stable states in communities with intraguild predation. Journal of Theoretical Biology 262: 116-128.

Verschoor AM, Vos M, and van der Stap I (2004) Inducible defences prevent strong population fluctuations in bi- and tritrophic food chains. Ecology Letters 7: 1143-1148.

Verspagen JMH, Passarge J, Jöhnk KD, Visser PM, Peperzak L, Boers P, Laanbroek HJ, and Huisman J (2006) Water management strategies against toxic *Microcystis* blooms in the Dutch delta. Ecological Applications 16: 313-327.

Visser PM, Ibelings BW, Mur LR, and Walsby AE (2005) The ecophysiology of the harmful cyanobacterium *Microcystis*: features explaining its success and measures for its control. In: Huisman J, Matthijs HCP, and Visser PM (eds.) Harmful cyanobacteria. Springer, Dordrecht, The Netherlands.

von Elert E, Martin-Creuzburg D, and Le Coz JR (2003) Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*). Proceedings of the Royal Society of London Series B 270: 1209-1214.

Wacker A, and von Elert E (2001) Polyunsaturated fatty acids: evidence for non-substitutable biochemical resources in *Daphnia galeata*. Ecology 82: 2507-2520.

Wan MX, Liu P, Xia JL, Rosenberg JN, Oyler GA., Betenbaugh MJ, Nie ZY, and Qiu GZ (2011) The effect of mixotrophy on microalgal growth, lipid content, and expression levels of three pathway genes in *Chlorella sorokiniana*. Applied Microbiology and Biotechnology 91: 835-844.

Weis D, and Brown AH (1959) Kinetic relationships between photosynthesis and respiration in the algal flagellate *Ochromonas malhamensis*. Plant Physiology 34: 235-239.

Welker M, and von Dohren H (2006) Cyanobacterial peptides: nature's own combinatorial biosynthesis. FEMS Microbiology Reviews 30: 530-563.

Wiedner C, and Nixdorf B (1998) Success of chrysophytes, cryptophytes and dinoflagellates over blue-greens (cyanobacteria) during an extreme winter (1995/96) in eutrophic shallow lakes. Hydrobiologia 370: 229-235.

Wiedner C, Visser PM, Fastner J, Metcalf JS, Codd GA, and Mur LR (2003) Effects of light on the microcystin content of *Microcystis* strain PCC 7806. Applied and Environmental Microbiology 69: 1475-1481.

Wilken S, Wiezer S, Huisman J, and Van Donk E (2010) Microcystins do not provide anti-herbivore defence against mixotrophic flagellates. Aquatic Microbial Ecology 59: 207-216.

Wilken S, Huisman J, Naus-Wiezer S, and Van Donk E (2013) Mixotrophic organisms become more heterotrophic with rising temperature. Ecology Letters 16: 225-233.

Wohlers J, Engel A, Zöllner E, Breithaupt P, Jürgens K, Hoppe HG, Sommer U, and Riebesell U (2009) Changes in biogenic carbon flow in response to sea surface warming. Proceedings of the National Academy of Sciences USA 106: 7067-7072.

Wu QL, Boenigk J, and Hahn MW (2004) Successful predation of filamentous bacteria by a nanoflagellate challenges current models of flagellate bacterivory. Applied and Environmental Microbiology 70: 332-339.

Y**ang Z, Kong FX, Shi XL, and Cao HS** (2006) Morphological response of *Microcystis aeruginosa* to grazing by different sorts of zooplankton. Hydrobiologia 563: 225-230

Yang Z, Kong FX, Yang Z, Zhang M, Yu Y, and Qian SQ (2009) Benefits and costs of the grazer induced colony formation in *Microcystis aeruginosa*. Annales de Limnologie – International Journal of Limnology 45: 203-208.

Yang Z, and Kong F (2012) Formation of large colonies: a defense mechanism of *Microcystis aeruginosa* under continuous grazing pressure by flagellate *Ochromonas* sp. Journal of Limnology 71: 61-66.

Yoshida T, Jones LE, Ellner SP, Fussmann GF, and Hairston NG (2003) Rapid evolution drives ecological dynamics in a predator-prey system. Nature 424: 303-306.

Yvon-Durocher G, Jones JI, Trimmer M, Woodward G, and Montoya JM (2010) Warming alters the metabolic balance of ecosystems. Philosophical Transactions of the Royal Society B 365: 2117-2126.

Zehr JP, Bench SR, Carter BJ, Hewson I, Niazi F, Shi T, Tripp HJ, and Affourtit JP (2008) Globally distributed uncultivated oceanic N(2)-fixing cyanobacteria lack oxygenic photosystem II. Science 322: 1110-1112.

Zhang XM, Watanabe MM, and Inouye I (1996) Light and electron microscopy of grazing by *Poterioochromonas malhamensis* (Chrysophyceae) on a range of phytoplankton taxa. Journal of Phycology 32: 37-46.

Zhang XM, and Watanabe MM (2001) Grazing and growth of the mixotrophic chrysomonad *Poterioochromonas malhamensis* (Chrysophyceae) feeding on algae. Journal of Phycology 37: 738-743.

Zhang X, Hu HY, Hong Y, and Yang J (2008) Isolation of a *Poterioochromonas* capable of feeding on *Microcystis aeruginosa* and degrading microcystin-LR. FEMS Microbiology Letters 288: 241-246.

Zubkov MV (2009) Photoheterotrophy in marine prokaryotes. Journal of Plankton Research 31: 933-938.

Zubkov MV, and Tarran GA (2008) High bacterivory by the smallest phytoplankton in the North Atlantic Ocean. Nature 455: 224-226.

Summary and conclusion

Mixotrophs combine traits from plants (autotrophs) and animals (heterotrophs). They can use sunlight for photosynthesis but also feed on other organisms. Understanding the intricate interaction and regulation of these autotrophic and heterotrophic processes within mixotrophs is essential for understanding their functional role in ecosystem processes, including their potential application as biological control agents against toxic cyanobacteria. This thesis combines lab experiments with mathematical models to study the ecophysiology and population dynamics of mixotrophs. The results shed new light on the applicability of several general ecological concepts to mixotrophs.

In **Chapter 2** we confirmed one of the key predictions of the Metabolic Theory of Ecology that rates of heterotrophic processes increase more strongly with temperature than those of autotrophic processes. Consequently, mixotrophs become more heterotrophic with rising temperature. While these results suggest that mixotrophic growth can be predicted from autotrophic and heterotrophic metabolism, **Chapter 3** illustrates that the interaction between the two metabolic pathways can also be more complex. Structural changes in the photosynthetic machinery of *Ochromonas danica* during mixotrophic growth indicate that its photosynthesis merely serves to provide energy rather than to fix inorganic carbon. Hence, mixotrophs might have a tendency towards photoheterotrophic growth.

Chapter 4 and **5** confirm the ability of the mixotrophic chrysophyte *Ochromonas* to efficiently feed on harmful cyanobacteria. Not only can *Ochromonas* degrade the toxic microcystins, but the comparison of its growth on a microcystin-containing strain of

Microcystis and its microcystin-deficient mutant revealed that growth remained unaffected by the presence of the toxins in its prey (**Chapter 5**). Conversely, also the intracellular microcystin content of *Microcystis* was not affected by grazing through *Ochromonas*. Furthermore, a dataset from Scandinavian lakes showed frequent co-occurrence of *Microcystis* and *Ochromonas*, with a relatively constant biomass of *Ochromonas* over a wide range of nutrient loads (**Chapter 4**).

In **Chapter 6** and **7** we used the mixotroph *Ochromonas* and the cyanobacterium *Microcystis* to test several of the key predictions of intraguild predation theory. The inability of *Ochromonas* to utilize nitrate allowed alteration of the food-web structure from an intraguild predation system to a linear food chain by providing either ammonium or nitrate as limiting nutrient (**Chapter 6**). In this way, we could demonstrate that the combination of competition and predation indeed enables intraguild predators to suppress their prey more strongly than specialist predators. However, contrary to theoretical predictions the intraguild prey *Microcystis* was favored relatively more by nutrient enrichment than its intraguild predator (**Chapter 7**). These results agree with the natural abundance of both species (**Chapter 4**) and can potentially be caused by several mechanisms, with intraspecific interference being a likely explanation during our chemostat experiments.

In conclusion, it is the balance between autotrophic and heterotrophic processes that defines the position of mixotrophs in food webs. The combination of competition and predation allows mixotrophs to strongly suppress their intraguild prey. This thesis demonstrates that the relative importance of alternative sources of carbon, energy and nutrients for mixotrophic growth depends not only on their respective availability, but also on temperature, the chemical form of the required elements and the regulation and interaction of the different nutritional pathways on the subcellular level. Hence, all these factors can affect the grazing efficiency of mixotrophs and their competitive strength for inorganic resources. In turn, this will affect the population dynamics of mixotrophs and their prey and alter the functional role of mixotrophs in food webs.

Despite the remarkable ability of *Ochromonas* to graze on toxic cyanobacteria and to strongly suppress their abundance in controlled lab experiments, *Ochromonas* is unlikely to successfully control cyanobacterial growth in natural waters. Many of the common bloom-forming cyanobacteria, such as *Microcystis*, seem to be well protected against grazing by mixotrophic flagellates. Especially nutrient enrichment benefits the growth of *Microcystis* even in the presence of its intraguild predator.

Samenvatting en conclusie

Mixotrofen bezitten eigenschappen van zowel planten (autotrofen) als dieren (heterotrofen). Ze kunnen gebruik maken van zonlicht voor hun fotosynthese maar kunnen zich ook voeden met andere organismen. Om de functionele rol van mixotrofen in ecosystemen te doorgronden is het van essentieel belang om de ingewikkelde interacties en regulatie van autotrofe en heterotrofe groeiprocessen in mixotrofe organismen te begrijpen. Een beter begrip van mixotrofe planktonsoorten zou nieuwe mogelijkheden kunnen bieden om deze in te zetten bij de biologische bestrijding van toxische cyanobacteriën (blauwalgen). In dit proefschrift worden laboratorium experimenten gecombineerd met wiskundige modellen om de ecofysiologie en populatie-dynamiek van mixotrofen te bestuderen. De resultaten geven meer inzicht in de wijze waarop verschillende algemene ecologische concepten toepasbaar zijn op mixotrofen.

In **Hoofdstuk 2** bevestigen we één van de belangrijkste voorspellingen van de Ecologische Metabolisme Theorie, namelijk dat snelheden van heterotrofe processen sterker toenemen met temperatuur dan die van autotrofe processen. Dit heeft tot gevolg dat mixotrofen meer heterotroof gaan groeien met een toename in temperatuur. Terwijl deze resultaten suggereren dat mixotrofe groei voorspeld kan worden vanuit het autotrofe en heterotrofe metabolisme laten de resultaten van **Hoofstuk 3** zien dat de interactie tussen de twee metabolische routes complex kan zijn. De structurele veranderingen in het fotosynthese-apparaat van de mixotrofe goudalg *Ochromonas danica*, waargenomen tijdens mixotrofe

groei, zijn een aanwijzing dat zijn fotosynthese meer dient als energie-voorziening dan voor de vastlegging van anorganisch koolstof. Gebaseerd op deze resultaten zou men kunnen stellen dat mixotrofen de neiging lijken te hebben tot fotoheterotrofe groei.

De resultaten uit **Hoofdstukken 4** en **5** bevestigen de mogelijkheid van de mixotrofe goudalg *Ochromonas* om efficiënt giftige cyanobacteriën weg te eten. *Ochromonas* kan de gifstof microcystine afbreken en zijn groei wordt niet negatief beïnvloed door deze gifstof. Dit is gebleken uit een vergelijkend onderzoek tussen de groei van *Ochromonas* op de microcystine bevattende cyanobacterie *Microcystis* en op een niet-giftige mutant van *Microcystis* (**Hoofdstuk 5**). Omgekeerd beïnvloedde begrazing door *Ochromonas* niet de hoeveelheid intracellulaire microcystine in *Microcystis*. Bovendien laten planktonmonsters uit Scandinavische meren zien dat *Microcystis* en *Ochromonas* vaak samen voorkomen, met een relatief constante biomassa van *Ochromonas* over een brede range van nutriëntenconcentraties (**Hoofdstuk 4**).

In **Hoofdstukken 6** en **7** hebben we gebruik gemaakt van de mixotroof *Ochromonas* en de cyanobacterie *Microcystis* om verschillende voorspellingen te testen van de zogenaamde "Intraguild Predatie Theorie". Intraguild predatoren zijn predatoren die hun concurrenten opeten. Met andere woorden, ze jagen niet alleen op prooisoorten maar concurreren ook met hun prooisoorten om dezelfde voedingsstoffen. Het onvermogen van *Ochromonas* om nitraat te gebruiken gaf ons de mogelijkheid om de voedselweb-structuur te laten schakelen tussen een intraguild predatie systeem en een lineaire voedselketen door ammonium of wel nitraat als limiterend nutriënt toe te dienen (**Hoofdstuk 6**). Op deze manier kon experimenteel worden aangetoond dat de combinatie van concurrentiee en predatie intraguild predatoren in staat stelt hun prooi veel sterker te onderdrukken dan gespecialiseerde predatoren, zoals voorspeld door de theorie. Echter in tegenstelling tot theoretische voorspellingen werd de intraguild prooi *Microcystis* relatief meer begunstigd door nutriëntverrijking dan zijn intraguild predator (**Hoofdstuk 7**). Deze afwijkende resultaten komen overeen met de natuurlijke abundanties van beide soorten (**Hoofdstuk 4**) en kunnen worden veroorzaakt door verschillende mogelijke mechanismen. Onderlinge interferentie tussen de intraguild predatoren is de meest waarschijnlijke verklaring voor hun minder effectieve onderdrukking van *Microcystis* bij een hoog nutriëntenaanbod.

Concluderend, kunnen we stellen dat het de balans is tussen autotrofe en heterotrofe processen die de positie bepaalt van mixotrofen in voedselwebben. De combinatie van concurrentie en predatie geeft mixotrofen in principe de mogelijkheid om hun intraguild-prooi sterk te onderdrukken. Dit proefschrift laat zien dat het relatieve belang van alternatieve bronnen van koolstof, energie en nutriënten voor mixotrofe groei niet alleen afhangt van hun beschikbaarheid, maar ook van de temperatuur, de chemische vorm van

de beschikbare voedingsstoffen, en de regulatie en interactie van de verschillende metabole paden op cellulair niveau. Al deze factoren kunnen daarom de graasefficiëntie van mixotrofen en hun concurrentiekracht voor anorganische voedselbronnen beïnvloeden. Dit zal ook weer de populatie-dynamiek van mixotrofen en hun prooi beïnvloeden en de functionele rol van mixotrofen in voedselwebben veranderen.

Ondanks het opmerkelijke vermogen van de mixotroof *Ochromonas* om in gecontroleerde laboratorium experimenten op giftige cyanobacteriën te grazen en hun abundantie krachtig te onderdrukken, is het onwaarschijnlijk dat *Ochromonas* met succes de groei van cyanobacteriën in natuurlijke wateren volledig kan controleren. Veel bloeivormende cyanobacteriën, zoals *Microcystis*, lijken relatief goed beschermd te zijn tegen begrazing door mixotrofe flagellaten. Vooral nutriëntenverrijking bevoordeelt de groei van *Microcystis*, zelfs in de aanwezigheid van zijn intraguild-predator die deze groei slechts ten dele kan intomen.

Acknowledgement

After 5 years full of reading, experimenting, discussing, thinking and rethinking, more experimenting, analysing, writing and rewriting, I finally arrived at the very last lines of this thesis. This is the time to look back and thank all those many people, who contributed in one or another way during the various stages of my PhD-experience and without whom this book could not have been put together.

First of all, I want to thank my advisors for their support of my work over the last 5 years. Ellen and Jef, I enjoyed feeling equally welcome and at home at both NIOO and UvA. While working on the combination of opposing nutritional strategies in mixotrophs, I also learned a lot about combining opposing supervision strategies. All your positive traits combined in one person would surely give the perfect supervisor! While such a combination seems impossible due to the trade-offs involved, I was very lucky to still experience it with the two of you as my advisors. Ellen, your doors were always open. I greatly appreciate your positive, supportive, and encouraging attitude. Thank you also, for giving me the freedom to follow my own ideas. Jef, you have been of great support especially during the last year. Thanks, for that! Your critical attitude, fast thinking and clear reasoning together with your enthusiasm and patience make working with you a very motivating experience. Discussing with you is always a pleasure and I hope there will still be plenty of opportunities to do so in the future.

Furthermore, I want to thank all my co-authors. Suzanne, thank you for risking a serious lack of sunlight with the countless hours you spent in the dark MoFlow room! Without your help this thesis would certainly be thinner. I will also remember our many discussions about all the non-scientific issues in the everyday scientific life. Jolanda, thanks for joining into my project at a late stage. I hope you didn't regret saying yes to what was originally meant to be about 2 weeks of work… Your contribution greatly improved the two chemostat chapters. Hans, collaborating with you was great fun. Your enthusiasm is infectious! I am happy that our work resulted in a chapter of this thesis. Merijn, thank you for the help with the Western blots.

As the work presented in this thesis has been performed to almost equal extent in two working groups and four different locations, I spent a significant amount of time searching for and setting up all kinds of equipment. Bas, Corrien, Nico, Michaela, Pieter, and Suzanne, thank you for minimizing this time by answering many questions, giving introductions to various instruments and advice on experimental set-ups, and by taking care that the labs keep running. Nico, thanks for feeding all my samples to the elemental analyzer. Maybe I should ask for an official tin-ball folding competition once? Pieter, thank you for the HPLC-measurements.

Next to the support in the lab all the mental support in the form of discussions, encouraging words or listening ears have been invaluable. Alena, we shared both the many happy moments, but also the frustrations involved in the life of a PhD-student. Thanks for your company on the many trips we did together. And Suzanne, what about planning some future SAS-events in Berlin and California? Anne, travelling through Japan with you and Alena was great. Good luck with finishing your project! Anthony and Dedmer, it was great to share the office with you in the beginning of my project. Thanks to both of you for all the discussions and advice during that time. Dedmer, I am looking forward to our future collaboration! Tania, thanks for supplying me with chocolade during the stressfull last weeks. Verena, thanks for hosting me in Amsterdam. I hope we will meet again sometime soon! Steven, I will miss your pizza and movie company. Katie and Jitske thanks for your commitment.

Working in two different places can be exhausting at times, but I was lucky to feel at home in two groups! All my colleagues from NIOO and UvA, Amanda, Annette, Anouk, Barbara, Bart, Bas, Bert, Casper, Daan, Dennis, Dina, Dirk, Elisa, Fleur, Giovanni, Harry, Jan, Joost, Judith, Koos, Koos, Kostas, Liesbeth, Lisette, Luuk, Maayke, Martijn, Michael, Michiel, Pascale, Pedro, Petra, Slawek, Spiros, Sven, Thijs, Tibor, and Wolf, thank you for the pleasant atmosphere, shared coffee breaks, social events and many scientific and non-scientific discussions.

I also want to thank Judith Blom for hosting me in Zurich for two weeks and Kevin Flynn for inviting me to the Leverhulme Mixotrophy workshop in Swansea without knowing whom he was inviting.

Andrea and Alena, thanks for supporting me during my defence. Frank, thank you for always believing in me, especially at times when I don't. Kristina and Fuji, thank you for making me feel at home in Wageningen.

Und zu guter Letzt vielen Dank an meine lieben Eltern! Ihr habt den Grundstein für mein Interesse an der Natur gelegt. Die Gewissheit Eurer stetigen Unterstützung war mir immer eine große Hilfe. Oft habe ich die viel gehörte Frage 'Und wann sehen wir Dich mal wieder?' mit einem Zeitpunkt in ferner Zukunft beantwortet. Und leider sieht es nicht so aus, als würde sich daran in den nächsten drei Jahren etwas ändern…

CV and publications

Curriculum Vitae

Susanne was born on the 4th of May 1981 in Pinneberg, Germany. After completing her secondary school in Pinneberg and Flensburg, she moved from the northernmost to the southernmost part of Germany to start her biology studies at Albert-Ludwigs University in Freiburg, where she obtained her Vordiplom in 2002. Her interest in marine biology brought her back to the north, first for one semester to the University of Bergen, Norway, and then to Christian-Albrechts University Kiel, Germany. In Kiel Susanne focused on biological oceanography with minors in marine chemistry and zoology. It was here where she got fascinated by the 'power of the small', the tremendous impact that microorganisms have on biogeochemical cycles and thereby on the conditions for life on earth. After graduation in 2007 she worked as a junior researcher at the Leibniz Institute for Marine Science in Kiel for 6 months. She started her PhD-project at the Netherlands Institute of Ecology (NIOO) in 2008. The research performed at the NIOO and the University of Amsterdam (UvA) resulted in this thesis. Susanne will continue to study mixotrophs as a postdoc in the group of Dr. Alexandra Worden at the Monterey Bay Aquarium Research Institute (MBARI) in Moss Landing, California.

Publications

Submitted:

Wilken S, Verspagen JMH, Naus-Wiezer S, Van Donk E, Huisman J: Biological control of harmful cyanobacteria by mixotrophic predators: an experimental test of intraguild predation theory.

Wilken S, Verspagen JMH, Naus-Wiezer S, Van Donk E, Huisman J: Experimental comparison of predator-prey interactions with and without intraguild predation.

Published:

Wilken S, Huisman J, Naus-Wiezer S, Van Donk E (2013) Mixotrophic organisms become more heterotrophic with rising temperature. Ecology Letters 16: 225-233.

Wilken S, Hoffmann B, Hersch N, Kirchgessner N, Dieluweit S, Rubner W, Hoffmann LJ, Merkel R., Peeken I (2011) Diatom frustules show increased mechanical strength and altered valve morphology under iron limitation. Limnology and Oceanography 56: 1399-1410.

Wilken S, Wiezer S, Huisman J, Van Donk E (2010) Microcystins do not provide anti-herbivore defence against mixotrophic flagellates. Aquatic Microbial Ecology 59: 207-216.

Van Donk E, Cerbin S, **Wilken S**, Helmsing NR, Ptacnik R, Verschoor AM (2009) The effect of a mixotrophic chrysophyte on toxic and colony forming cyanobacteria. Freshwater Biology 54: 1843-1855.