Implications of re-oligotrophication in a large pre-alpine lake: studies on fish-zooplankton interactions and the ecology of mixotrophic chrysophytes

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Summary

Lake ecosystems are an essential part of our environment, inhabiting a large variety of living things. Lakes provide several advantages for human society, and their strategic importance in providing freshwater, food, and resources increased with human development. Especially in the last centuries, anthropogenic influences increased, and several problems emerged that affected the complex biological processes driving our aquatic ecosystems. Increased external nutrient loading caused eutrophication in many aquatic ecosystems all over the world. Eutrophication can cause profound changes in the food-webs and might favor the establishment of invasive species. The large pre-alpine Lake Constance located in central Europe experienced eutrophication in the second half of the 20th century due to an increased phosphorous intake generated by improved industrialization, housing, and land use. Yet, provisions to reduce the phosphorous load in the wastewater resulted in re-oligotrophication, displaying the lake's current state. Changing nutrient levels in Lake Constance were associated with shifts in the pelagic community structures that became a central part of researching the lake. Two prominent observations were highlighted in this thesis to better understand the consequences linked to re-oligotrophication in Lake Constance.

First, the increased invasive fish occurrence in the pelagic during the last years might have influenced the lakes' trophic structure. We investigated the impact of native whitefish and invasive sticklebacks on the zoo- and phytoplankton communities in a mesocosm experiment. The results revealed intense predation on the cladoceran *Daphnia longispina*. Since this large daphniid had a significant impact on the primary producers, we determined *D. longispina* as keystone species linking fish and phytoplankton in a trophic cascade. Furthermore, the impact of both fish species on the lower trophic levels was similar, indicating that the establishment of sticklebacks in the pelagic did not result in a changing zoo- and phytoplankton structure.

Second, the concomitant numeral and relative increase of mixotrophic chrysophytes while the total phytoplankton biomass decreased with declining phosphorous concentrations. Many chrysophytes are mixotrophic and combine photosynthesis with phagotrophy to fulfill their demands for energy and nutrients. In laboratory experiments, the growth of three mixotrophic chrysophytes was compared under the influence of different bacterial concentrations and different light intensities. The results show that the investigated species can be distinguished by their nutritional mode focused either on phototrophy or phagotrophy. With re-oligotrophication, especially the mixotrophic chrysophyte genus *Dinobryon* became dominant during summer. The evaluation of long-term data from 1952 to 2015 displayed the occurrences of three abundant *Dinobryon* species. While *Dinobryon* was common in the time

before eutrophication, the increased phosphorous level resulted in reduced abundances. With re-oligotrophication, *Dinobryon* recovered and became a dominant chrysophyte again.

The results illustrated in this thesis show that environmental changes like oligotrophication can influence the species found in the pelagic of Lake Constance. However, the zoo- and phytoplankton communities can show resilience towards a changing fish regime that primarily influences lower trophic levels through a single keystone species. Recovery from eutrophication can be found among mixotrophic chrysophytes that showed a positive response to decreased phosphorous levels and took advantage of the re-established conditions in the lake.

Zusammenfassung

Seeökosysteme sind ein essenzieller Teil unserer Umwelt, in dem eine Vielzahl von Lebewesen leben. Seen bieten unserer Gesellschaft verschiedene Vorteile und ihre strategische Bedeutung für die Versorgung mit Trinkwasser, Nahrung und Ressourcen stieg mit dem Fortschritt der Menschheit an. Insbesondere in den letzten Jahrhunderten nahmen die antrophogenen Einflüsse zu und es traten mehrere Probleme auf, die die komplexen biologischen Prozesse beeinflussten, die unsere aquatischen Ökosysteme antreiben. Ein erhöhter externe Nährstoffeintrag führte in vielen aquatischen Ökosystemen auf der ganzen Welt zu einer Eutropierung. Die Eutrophierung kann tiefgreifende Veränderungen in den Nahrungsnetzen verursachen und die Etablierung invasiver Arten begünstigen. Der große voralpine Bodensee in Mitteleuropa erlebte in der zweiten Hälfte des 20. Jahrhunderts eine Eutrophierung aufgrund erhöhtem Phosphoreintrags, die durch eine intensivierte Industrialisierung, Urbanisierung und Landnutzung verursacht wurde. Maßnahmen zur Verringerung der Phosphorbelastung in Abwässern führten jedoch zu einer erneuten Oligotrophierung, die den aktuellen Zustand des Sees widerspiegeln. Der Veränderte Nährstoffgehalt im Bodensee war mit Veränderungen in der pelagischen Tier- und Pflanzengemeinschaft verbunden, die zu einem zentralen Bestandteil der Seenforschung wurden. Zum besseren Verständnis der Folgen der Re-Oligotrophierung im Bodensee wurden in dieser Arbeit zwei herausragende Beobachtungen hervorgehoben.

Erstens, das vermehrte Auftreten invasiver Stichlinge im Pelagial der letzten Jahre, welches möglicherweise die trophischen Ebenen des Sees beeinflusst hat. In einem Mesokosmos-Experiment untersuchten wir die Auswirkungen von einheimischen Weißfischen und invasiven Stichlingen auf die Zoo- und Phytoplanktongemeinschaft. Die Ergebnisse weisen auf einen intensiven Prädationsdruck beider Fischarten auf *Daphnia longispina* hin. Da dieses große Daphnienart einen signifikanten Einfluss auf das Phytoplankton hatte, vermuten wir, dass *D. longispina* eine Schlüsselspezies darstellt, die Fische und Phytoplankton in einer trophischen Kaskade verbindet. Darüber hinaus war der Einfluss beider Fischarten auf die unteren trophischen Ebenen ähnlich, was darauf hinweist, dass die Etablierung der Stichlinge im pelagischen Bereich nicht zu einer Veränderung innerhalb der Zoo- und Phytoplanktonstrukturen führte.

Zweitens, nahmen die relativen und absoluten Abundanzen der Chrysophyten mit der Re-Oligotrophierung zu, während gleichzeitig die gesamte Phytoplanktonbiomasse mit sinkenden Phosphorkonzentrationen abnahm. Viele Chrysophyten sind mixotroph und kombinieren Photysythese und Heterotrophie, um ihren Energie- und Nährstoffbedarf zu decken. In

Laborexperimenten wurde das Wachstum von drei mixotrophen Chrysophyten unter dem Einfluss unterschiedlicher Bakterienkonzentrationen und unterschiedlicher Lichtintensitäten verglichen. Die Ergebnisse zeigen, dass die untersuchten Arten durch ihren Ernährungsmodus unterschieden werden können, der entweder auf Phototrophie oder Phagotrophie ausgerichtet ist. Mit der Re-Oligotrophierung dominierte während des Sommers insbesondere die mixotrophe Chrysophytengattung *Dinobryon*. Mithilfe der Auswertung von Langzeitdaten zwischen 1952 und 2015 konnte das Vorkommen von drei *Dinobryon*-Arten untersucht werden. Während *Dinobryon* schon in der Zeit vor der Eutrophierung häufig war, führte der erhöhte Phosphorgehalt zu einer verringerten Auftreten. Mit der erneuten Oligotrophierung erholte sich *Dinobryon* wieder und konnte erneut zu einem dominanten Gattung der Phytoplanktongemeinschaft werden. Die Ergebnisse dieser Arbeit zeigen auf, dass Umweltveränderungen wie Oligotrophierungen, die im pelagischen Bodensee vorkommenden Arten beeinflussen können. Die Zoo- und Phytoplanktongemeinschaft kann jedoch widerstandsfähig gegenüber einem sich ändernden Fischregimes präsentieren, das in erster Linie niedrigere trophische Ebenen durch eine einzelne Schlüsselart beeinflusst. Ein erneut starkes Auftreten nach der Eutrophierung kann bei mixotrophen Chrysophyten gefunden werden, die eine positive Reaktion auf verringerte Phosphorgehalte zeigten und die wiederhergestellten Bedingungen im See erfogreich ausnutzten.

Chapter I: General Introduction

Lakes are inland water bodies that can be linked to river systems and, compared to oceans, can be described as almost closed ecosystems. However, it is not easy to find an overarching description since our planet originated a broad variation of lakes. Parameters like the area or depth of a lake are easy to capture; some fluctuating ones like temperature, salinity, or the nutrient load need more effort. Every single lake certainly has its own characteristics heavily depending on biotic and abiotic factors. Among the stressors that might negatively influence the stability of lake ecosystems, human activities have become a factor of increased importance during the last decades. Evidence for anthropogenic influence along lakeshores can be found until the early time of our history. Lakes provide many benefits, primarily in most cases freshwater, but also recreational value, transport opportunities, fishery products, and raw materials. The increasing land use, along with the fast-developing industrialization of the last decades, caused some profound changes in many of our lakes. Overfishing, pollution, and an increased nutrient intake from nearby agriculture and housing lead to serious modifications of ecosystems in many cases. Especially the increased input of phosphorous promotes eutrophication in former oligotrophic lake systems, which interferes with existing food webs (Smith et al., 2006), negatively affects water quality (Lee et al., 1978), and reduces biodiversity (Glibert, 2017). However, techno-economic progress, together with public awareness for our natural environment, initiated a change. Provisions were concluded to reduce the phosphorous input that resulted in the restoration of the lakes' native trophic states (Sas, 1990; Jeppesen et al., 2005). This re-oligotrophication proceeded in many lakes across whole Europe during the last decades (Jeppesen et al., 2005). While eutrophication is connected to increased plant and algal growth due to the availability of former rare nutrients promoting photosynthesis (Schindler, 2006), a consequent oligotrophication makes those nutrients scarce again and might lead to an overall reduction of biomass (Jochimsen et al., 2013). Investigating the consequences of changing phosphorous concentrations on native lake communities can indicate whether a system might show resilience towards changes or is even able to recover when re-oligotrophication follows on human-made eutrophication. If a species is resilient to changes in the trophic state depends not only on the capacity to tolerate increased nutrient loads but also on changes in the whole community, some species might take advantage of the changed conditions and outcompete others who do not profit. Another disturbance comes with invasive species, which were not able to succeed in the original state but can establish themselves in an alternated environment. This might be crucial for species that have the potential capacity to recover after re-oligotrophication but experience a novel competition caused by the invaders. The consequences of re-oligotrophication need more investigation to better understand the dynamics in lakes strongly correlated with human activities. Fortunately, many lakes are well studied and have a long history of long-term recordings covering periods of eu- and oligotrophication.

Lake Constance as a case study

Lake Constance is one of the most noteworthy lakes which underwent re-oligotrophication. Located on the northern edge of the European Alps, in the tri-state-area of Austria, Germany, and Switzerland, the lake is one of the largest lakes in Central Europe. It covers an area of 536 km^2 and is portioned into two main basins, the larger Upper Lake Constance (473 km^2) and smaller Lower Lake Constance (63 km^2). Both basins are connected via the river Rhine which likewise builds the major in- and outflow of the lake system. Lake Constance has an average depth of 90 m, with its deepest point of 251 m in the upper part and 45 m in the shallower lower part. Early hydrological data records show that the lake's original state was oligotrophic with total phosphorous concentrations in the mixed layer (TP) below 10 µg l^{-1}. After 1950 the external nutrient intake initiated a strong increase of TP, resulting in concentrations above 80 µg l^{-1} in the late 1970s (IGKB 2020). Subsequently, the wastewater treatment measures reduced TP continuously in the following years until it reached concentrations again below 10 µg l^{-1} in the early 2000s.

Migration of the invasive three-spined stickleback into the pelagic

Lake Constance inhabits a rich and diverse fish community with at least thirty species recorded so far, and the native fauna is generally still existent (Alexander et al., 2016). However, before eutrophication, the pelagic zone was primarily dominated by salmonids. Besides minor species like lake char (*Salvelinus umbla*) and lake trout (*Salmo trutta lacustris*), the genus *Coregonus,* hereafter called whitefish, represented the most abundant group. Especially whitefish are of major importance to Lake Constance's fishery. The proportion of whitefish decreased with progressing eutrophication, whereas other fish like perch (*Perca fluviatilis*) and cyprinids increased in numbers (Eckmann and Rösch, 1998). With the upcoming re-oligotrophication situation changed, and whitefish became dominant again.

Recently, whitefish have faced a rising invasive competitor in the pelagic, the three-spined stickleback (*Gasterosteus aculeatus*). This small-sized fish was first reported in the first half of the 20th century and inhabited the littoral zone of Lake Constance (Nümann, 1972). Yet, in the last decade, *G. aculeatus* started to spread into the pelagic zone and increased intensively in numbers (Alexander et al., 2016). For the year 2014, Alexander et al. revealed in a large-scale survey that sticklebacks numerically represent 96% of the pelagic fish and 28% in terms of the total fish biomass. The emergence of sticklebacks in the pelagic zone directly affected whitefish by interspecific competition for food (Rösch et al., 2018). Whitefish are specialized in zooplankton feeding and rely on their narrow food spectrum existing in the pelagic (Eckmann et al., 2002). On the other hand, sticklebacks are generalist feeders that can handle a broader food spectrum if extended, which also includes eggs of the own species and other fish' larvae (Hynes, 1950; Delbeek and Williams, 1988, Mehlis et al., 2010). With the background that invaders might cause altered top-down effects, a mesocosm experiment was performed to compare the impact on the zoo- and phytoplankton community of whitefish and sticklebacks. We hypothesize that smaller and more evasive zooplankton species and combined groups of multiple zooplankton species are less susceptible to fish predation than large daphniid species and that whitefish as specialized planktivores consume more zooplankton than sticklebacks, which are generalist feeders. Furthermore, we tested the assumption if large daphniids cause stronger reductions in edible and total phytoplankton biovolumes than other zooplankton taxa, and consequently, fish stimulate increases in phytoplankton biovolumes by decreasing large daphniid densities.

Re-oligotrophication favors mixotrophy

A regime shift in the phytoplankton took place from a high biomass period (HBP) during eutrophic years to a low biomass period (LBP), showing that trajectories of eutrophication can be reversed but went along with changes in the community structure (Jochimsen et al., 2013). A concomitant increase of relative and absolute chrysophyte biomasses occurred with the shift from an HBP to an LBP during re-oligotrophication. Since many chrysophytes are mixotrophs, re-oligotrophication in Lake Constance might have favored this nutritional mode. Mixotrophic organisms can combine chemoheterotrophy with photoautotrophy to fulfill their energy and nutrients demands (Caron et al., 1993; Raven, 1997). Many mixotrophic algae are known to be important grazers on nutrient-rich bacteria and thus taking advance when dissolved nutrients in the environment are scarce (Bird and Kalff, 1986; Rothhaupt, 1996a). Furthermore, they can maintain positive growth through photosynthesis when enough light is available, even if bacterial prey concentration is low, which gives the ability to outcompete exclusive heterotrophs (Rothhaupt, 1996b). Inversely, the ability to use nutrients bound in bacterial prey for photosynthesis gives an advantage against pure photoautotrophic organisms when dissolved nutrients are low when photosynthetic growth is generally low. Due to the usage of the bacterial biomass for photosynthesis, mixotrophs might not necessarily remineralize nutrients taken up by bacterivory and, in addition to that, can indirectly suppress the growth of photoautotrophic competitors (Rothhaupt, 1996a). Mixotrophy in chrysophytes is usually not composed of phototrophy and heterotrophy in equal shares but is mostly shifted to one of the two nutritional modes (Caron et al., 1993; Zhang and Watanabe, 2001; Rottberger et al., 2013). They also can show considerable variability in using both modes when certain environmental conditions are given (Rottberger et al., 2013). In laboratory experiments, we tested how the growth of several mixotrophic chrysophytes is influenced by different bacterial densities and different light intensities. The cultures were grown in the absence of phosphorous or on low phosphorous levels to simulate an oligotrophic environment. The aim was to reveal how the chrysophytes differ in their nutritional strategy to use photosynthesis and phagotrophy. We assumed a species-specific reliance on either phototrophy or phagotrophy, which determines the primary nutritional mode of the respective chrysophyte. Furthermore, long-term data of the occurrence of *Dinobryon,* the most dominant chrysophyte in Lake Constance, was evaluated with the assumption that its appearance follows the characteristic progression of the phosphorous concentrations. We also assumed seasonal and spatial shifts in the occurrence of *Dinobryon* since the declined availability of free phosphorous in water might have shifted the seasonal succession of phytoplankton.

Chapter II: First Manuscript

Large daphniids are keystone species that link fish predation and phytoplankton in trophic cascades

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Abstract

Daphniids act as keystone species in lake ecosystems by controlling phytoplankton biomass and experiencing intense fish predation. However, the importance of single daphniid species as trophic links between phytoplankton and fish remains unclear, especially compared with other zooplankton taxa. To disentangle the role of individual zooplankton taxa in the food web of a large lake, we performed an in-situ mesocosm experiment with natural phytoplankton and zooplankton communities in three treatments with native, invasive or no fish predators, respectively. A large daphniid, *Daphnia longispina*, was the zooplankter most strongly predated by both fish species, and also had the highest top-down effects on phytoplankton. All other zooplankton taxa, including a small daphniid species, had minor roles in terms of both predation by fish and grazing on phytoplankton. We suggest that daphniid species with large body sizes can strongly link higher and lower trophic levels.

Keywords: Cladocera; *Daphnia*; food chain; ecosystem dynamics; oligotrophic lake; pelagic; trophic cascading

Introduction

Understanding the ecological roles of individual species and their interactions within food webs is essential for the characterisation of ecosystem dynamics. This is particularly true for keystone species, which have a strong influence on ecosystem functioning (Cottee-Jones & Whittaker 2012). For example, predator-mediated reductions in the abundance of keystone prey species can have indirect effects on lower trophic levels, resulting in trophic cascades (Carpenter et al. 1985; Moyle & Light 1996; Pagnucco et al. 2016). However, the influence of keystone prey species on food webs may change if the predation pressure they experience shifts, for example, due to the invasion of a new predator.

The development of strong trophic cascading effects depends on multiple factors, and disentangling top-down and bottom-up regulation within food webs may therefore be difficult. In general, bottom-up control is more pronounced at the base of food webs, whereas top-down control is stronger at higher trophic levels (Brett & Goldman 1997). Therefore, fish have a greater role in regulating zooplankton biomass and abundance than nutrients, but have less influence on phytoplankton (Post & McQueen 1987; Hansson et al. 2004; Vakkilainen et al. 2004). Multi-level cascades often occur in aquatic ecosystems (Jürgens 1994; Östman et al. 2016), especially when fish predation affects species with key roles in linking different trophic levels and transferring energy through food chains (Hansson et al. 2004).

Although zooplankton represent a link between primary producers and fish in freshwater ecosystems (Brooks & Dodson 1965; Hansson et al. 2007), not all zooplankters are keystone species that link trophic levels. Substantial differences in functional traits between species lead to their different effects on phytoplankton (Cyr & Curtis 1999; Sommer et al. 2003; Barnett et al. 2007), and conceptualising zooplankton as a homogeneous functional group thus masks species-specific roles in within food webs. Hence, studies considering the effects of total zooplankton biomass on phytoplankton have typically found no or only weak top-down effects (McQueen et al. 1989; Mehner et al. 2008). More pronounced cascading effects have been observed for zooplankton of the genus *Daphnia* (McQueen et al. 1986; McQueen et al. 1989), which are considered as keystone taxa that link primary and tertiary production due to their effective grazing on a broad size spectrum of edible phytoplankton (Lampert 1987). As well as influencing phytoplankton in green food webs, *Daphnia* can cause complex cascading effects in detritivore-based brown food webs by consuming microorganisms through non-selective grazing (Zöllner et al. 2003).

Body size is among the most important traits influencing both zooplankton filtration rates and therefore top-down control of lower trophic levels (Gianuca et al. 2016). Many *Daphnia* species are large zooplankters and are therefore keystone grazers with a high capacity to reduce phytoplankton biomass (Vakkilainen et al. 2004). Larger-bodied daphniids can outcompete smaller species due to consuming wider range of phytoplankton, and the growth and reproduction of largerbodied daphniids is greater when phytoplankton densities are low (Gliwicz 1990a; Gliwicz 1990b). Body size also influences fish predation, because most fish are size-selective predators that prefer larger and less evasive prey (Brooks & Dodson 1965). Daphnia densities can be strongly reduced by specialised planktivorous fish, which typically leads to an increase in phytoplankton biomass (Williams & Moss 2003).

Although many studies have considered fish effects on zooplankton and consequently on phytoplankton (Helminen & Sarvala 1997; Bertolo et al. 2000; Williams & Moss 2003; Vakkilainen et al. 2004), the importance of individual zooplankton species within trophic cascades is still poorly understood, even for potential keystone species such as daphniids. The role of individual zooplankton species in trophic cascades is challenging to investigate within natural ecosystems because species-specific effects are difficult to isolate. Equally, small-scale ex-situ top-down experiments designed to compare the feeding impacts of individual species on phytoplankton (Adrian & Schneider-Olt 1999; Gianuca et al. 2016) lack a natural zooplankton community structure as well as predation by fish. In-situ mesocosm experiments, which allow examination of natural zooplankton communities exposed to natural predation, rarely focus on the effects of single zooplankton species on phytoplankton (Lynch 1979; Bertolo et al. 2000; Hansson et al. 2004). The role of individual zooplankton species in lake food webs thus remains unclear, especially in deeper pelagic and oligotrophic environments.

To investigate the trophic roles of individual zooplankton species, we performed a mesocosm experiment in an offshore area of Upper Lake Constance, central Europe. This area allowed us to represent the pelagic environment using natural phytoplankton and zooplankton communities and the two most abundant pelagic fish species, the native whitefish (*Coregonus wartmanni*; hereafter, whitefish), and the invasive three-spined stickleback (*Gasterosteus aculeatus*; hereafter, stickleback). Our aim was to determine which zooplankters function as keystone species that represent strong links between fish predation and phytoplankton biomass, and which zooplankters are susceptible to changes in top-down predation in natural environments. We tested four hypotheses: 1) smaller and more evasive zooplankton species and combined groups of multiple zooplankton species are less susceptible to fish predation than large daphniid species; 2) as specialised planktivores, native whitefish consume more zooplankton than sticklebacks, which are generalist feeders; 3) large daphniids cause greater reductions in edible and total phytoplankton biovolumes than other zooplankton taxa; and 4) fish stimulate increases in phytoplankton biovolumes by decreasing large daphniid densities.

Methods

Study sites

Lake Constance is a large (536 km2), deep (maximum 251m) pre-Alpine lake situated at the border of Germany, Switzerland and Austria. It consists of two basins, the Lower Lake Constance and the larger Upper Lake Constance, in which our study was performed. Upper Lake Constance experienced several decades of anthropogenic eutrophication during the 20th century with phosphorus concentrations peaking in 1979, but has since returned to an oligotrophic state due to restoration efforts (IGKB 2013; Schotzko 2018). The most abundant zooplankton taxa in Upper Lake Constance are cyclopoid copepods, the calanoid copepod *Eudiaptomus gracilis*, and the cladocerans *Diaphanosoma brachyurum*, *Bosmina* spp., *Daphnia longispina* (formerly known as *D. hyalina*), *Daphnia galeata*, *Leptodora kindtii* and *Bythotrephes longimanus* (Straile & Geller 1998). Recently, a small daphniid species, *Daphnia cucullata*, has been recorded at high densities (IGKB 2018, Isanta Navarro et al. 2019). The fish fauna comprises approx. 30 species, of which three are common in the pelagic zone: the native lake char (*Salvelinus umbla*), the whitefish and the stickleback. The latter is an invasive, non-native species that has been occurring at high densities in the pelagic zone of Upper Lake Constance since 2012−2013 (Rösch et al. 2018; Eckmann & Engesser 2019), and in 2014 represented 96 % of the total abundance and 28 % of the biomass of pelagic fish (Alexander & Vonlanthen 2016).

Experimental design

The experiment was conducted in the Upper Guell bay of Upper Lake Constance between 27 June and 25 July 2019. It comprised 12 plastic cylindrical mesocosms made of transparent polyethylene, each with a 15-m depth, a 96-cm diameter and a 10 m3 volume. Mesocosms were heat-sealed at the base and open to the atmosphere above the water surface. Mesocosms were attached to pontoons, freely hanging in open water, with their bases approx. 2 m above the sediment. Each pontoon accommodated four mesocosms in a row at 25-cm intervals and in an east-west direction to prevent shading by adjacent mesocosms. Each mesocosm was covered with a transparent acrylic glass plate, to protect it from bird excrement and other external disturbances.

On June 19, the mesocosms were filled with filtered (< 300 µm) lake water and then inoculated with lake zooplankton. Zooplankton were sampled using vertical net tows (300 µm) from a depth of 15 m to the surface, filtering the same volume of water as in the 12 mesocosms, to ensure inoculation of zooplankton at natural densities at these depths. Samples were filtered through a 630-µm sieve to remove predatory zooplankton such as *L. kindtii* and *B. longimanus* as well as fish larvae; this process also removed the largest adult daphniids and copepods. The sampled zooplankton volume was then divided into 12 equal parts and one part added to each mesocosm. Zooplankton were then left to acclimatise within the mesocosms for eight days, allowing conditions to stabilise and zooplankton to develop to adult sizes (Schwartz 1984; Peterson 2001). The experiment lasted four weeks, to provide enough time for phytoplankton and zooplankton reproduction, community development and response to experimental conditions (Riebesell et al. 2011). To enable characterisation of well-pronounced top-down effects, the experimental period coincided with the lake's phytoplankton spring peak, which occurred unusually late in the study year i.e. around 1 July (unpublished data).

Treatments were randomly assigned to mesocosms, ensuring that each pontoon had at least one replicate of each treatment and that the treatments were placed in different orders within the pontoons. After the acclimatisation period ended (i.e. day 0, 27 June), the first samples were collected from all mesocosms (see below), and predatory zooplankton and fish were then added to treatments. Due to the patchy distribution of larger predatory zooplankton species and their potentially important roles in pelagic food webs, 200 *L. kindtii* and 40 *B. longimanus* were counted and added separately to each mesocosm, representing 1/4 and 1/5 of their typical maximum Lake Constance densities, respectively. These densities were limited by unusually low numbers of these two species in the lake during the study period. A treatment containing only zooplankton (including predatory taxa) acted as the control.

Two fish treatments received either four sticklebacks (mean ± SD, 0.31± 0.048 g) or four whitefish (0.32 ± 0.093 g). The body length of whitefish was generally greater than that of sticklebacks with the same biomass, although exact lengths were not measured. Total fish densities in mesocosms were 1.71± 0.096 g m−2 SD for sticklebacks and 1.77± 0.094 g m−2 SD for whitefish, representing natural densities in Lake Constance. Because fish densities in the lake are difficult to estimate precisely, annual fishery yields of adult whitefish were used as a reference, with 2007–2016 yields corresponding to 0.58 g m−2 (Kugler & Friedl 2018). Because adult whitefish dominate pelagic fish biomass (Alexander & Vonlanthen 2016) and because they are usually harvested soon after reaching their adult size (Eckmann & Rösch 1998), densities 2–3 times higher than annual whitefish yields might adequately represent actual densities in the lake.

Sampling and sample processing

Starting on day 0, abiotic parameters were measured and both phytoplankton and zooplankton samples were taken from mesocosms. Sampling frequency was different for each parameter. Twice a week, dissolved oxygen concentrations, pH and temperature were measured in each mesocosm and in the lake with a multiparameter probe (CTD-90 M; Sea&Sun Technology), and chlorophyll concentrations were measured with a fluorescence probe (bbe Moldaenke, TS 17-09). These abiotic measurements were taken by lowering probes from the surface to a depth of 12 m, prior to disturbance of the water column during biotic sample collection. Zooplankton were sampled using vertical net hauls (16.5-cm opening, 100-cm length and 100-µm mesh) from a depth of 12 m to the mesocosm surface, on days 0, 14, 21 and 28. Phytoplankton were sampled on days 0, 14 and 28 by performing a vertical tow with an integrated water sampler across the same depth range, which collected 2 L of water, from which 100-mL subsamples were taken for analysis.

Collected zooplankton samples were preserved at −20 °C until processing. After thawing, samples were divided into aliquots of ≥ 300 zooplankters, identified to the lowest taxonomic resolution possible (order to species), counted, and values used to back-calculate densities of each taxon. Although hybrids of the three *Daphnia* species probably occurred in our samples (Isanta Navarro et al. 2019), we designated each as only one of three species based on its morphological features. All copepod life stages were included except nauplii, due to their inconsistent sampling by a 100-µm mesh and identification difficulties. The five most abundant taxa were used for further analyses: *Bosmina* spp. (3.6 %), *Cyclopoida* (20.5 %), *E. gracilis* (10.1%), *D. cucullata* (44.9 %) and *D. longispina* (20.8 %). In addition, *D. longispina* and *D. cucullata* were combined as a total daphniid fraction. All other zooplankton taxa (including *D. galeata*) represented < 0.2 % of total zooplankton abundance and were therefore excluded from further analysis.

Phytoplankton samples were fixed in Lugol's acid solution + sodium acetate, identified to genus level using an inverted light microscope and counted following Utermöhl (1958). Biovolumes were calculated using taxon-specific body volumes established for Lake Constance (Pauli 1989; Kümmerlin & Bürgi 1989; Müller et al. 1991). Phytoplankton species were divided into two groups according to their edibility to large daphniids. Unicellular taxa < 40 µm in cell length were classified as edible, whereas those > 40 µm in cell length, colony-forming and mucilaginous species were classified as inedible (Lampert 1987); cell lengths were based on Rimet & Druart (2018).

Data analysis

Shapiro-Wilk tests confirmed that all zooplankton and phytoplankton data had normal distributions. Initial (day 0) total zooplankton densities were compared with a one-way ANOVA, to ensure comparability among treatments. To compare densities of different zooplankton species among treatments (hypothesis 1) including identification of differences between the whitefish and stickleback treatments (hypothesis 2), we performed a two-way repeated-measures (RM) ANOVA with treatment (control, whitefish, stickleback) as the between-subjects factor and day (0, 14, 21, 28) as the within-subjects factor. Mauchly's tests were used to test the RM ANOVA assumption of sphericity, with deviations from sphericity addressed using Greenhouse-Geisser corrections. Pairwise t-tests with Bonferroni corrections were used to identify differences between treatments on each day. Linear regression models (LM) were used to identify relationships between the densities of zooplankton taxa (as predictor variables) and total, edible and inedible fractions of phytoplankton biovolumes (as dependent variables; hypothesis 3). To test hypothesis 4, a second RM ANOVA and pairwise tests (as described above) were done to compare phytoplankton biovolumes in each treatment. All analyses were done in the statistical software R (R Core Development Team 2018), using the package afex (Singmann et al. 2020) to perform RM ANOVAs and Mauchly's tests, the basic R function lm() to run LM, and the package ggplot2 (Wickham 2016) to plot graphics.

Results

Abiotic conditions were stable during the experiment: dissolved oxygen ranged from 10–12 mg L–1 at the surface to 12–15 mg L–1 at a depth of 12 m, pH varied between 8.5–9.5 at all depths, and temperatures ranged from 22–25 °C at the surface to 10–12 °C at 12 m.
Zooplankton densities were comparable in all treatments at the start of the experiment (i.e. on day 0; one-way ANOVA $F_{2,6} = 0.03$, $p = 0.969$). Total zooplankton and total daphniid densities differed among treatments but not days, and no interactions were identified between day and treatment (RM ANOVA, Table 1). Both total zooplankton densities (pairwise t-test, $p = 0.012$) and total daphniid densities ($p = 0.004$) were higher in the control than in the whitefish treatment but not the stickleback treatment ($p > 0.05$).

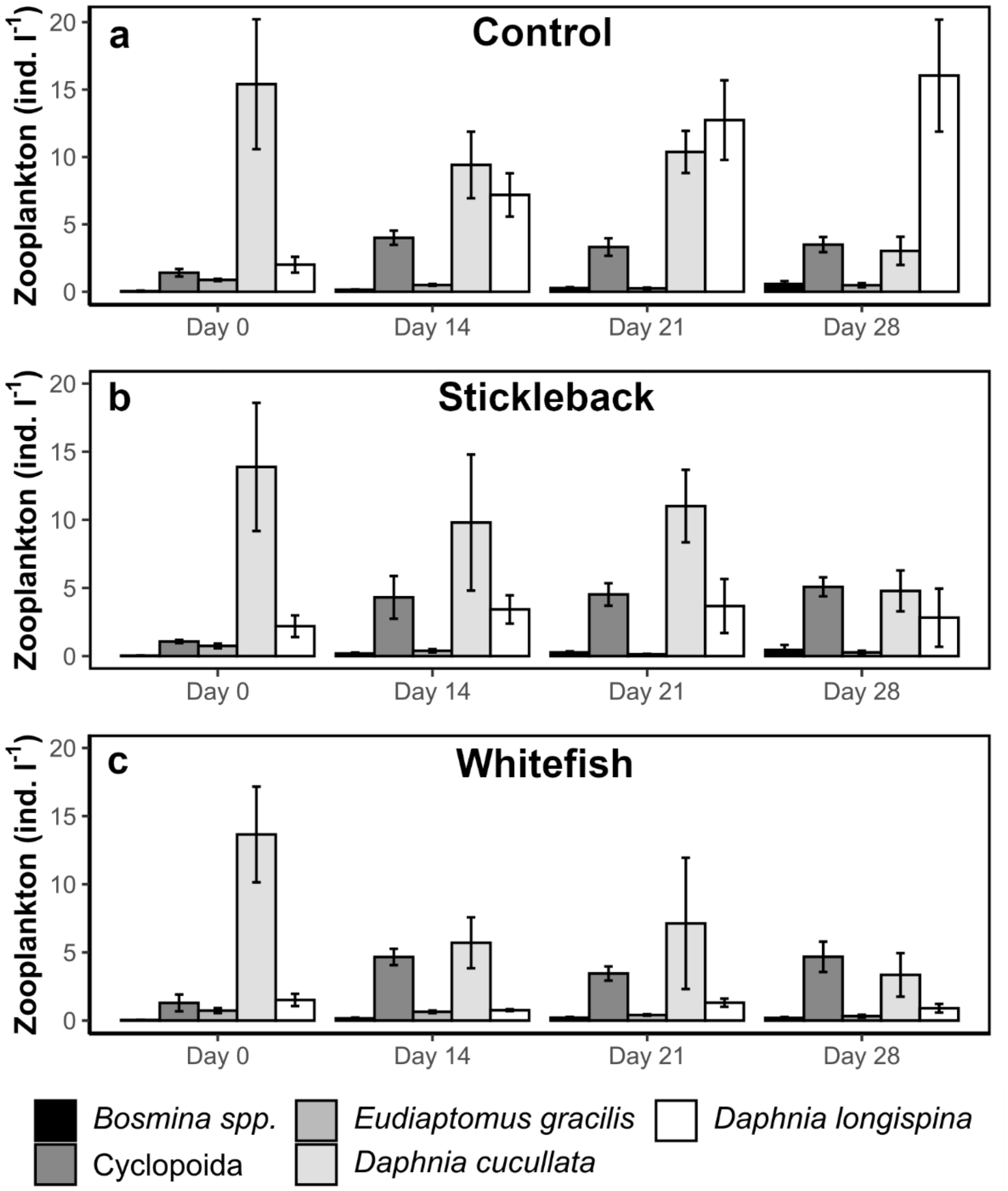

Fig.1. Mean (± 1 SD) zooplankton taxa densities (individuals [ind.] l–1) in control (a), stickleback (b) and whitefish (c) treatments on each sampling day.

Table 1. Results of two-way repeated-measures ANOVAs describing differences in zooplankton densities and total phytoplankton biovolumes among treatments, days and their interaction. Significant results (p < 0.05) are indicated in bold.

Species	Effect	df	F	p
Bosmina spp.	Treatment	2	1.190	0.367
	Date	**3**	**5.360**	**0.025**
	Treatment x Date	6	0.960	0.462
Cyclopoida	Treatment	2	0.82	0.484
	Date	**3**	**11.78**	**<0.001**
	Treatment x Date	6	1.25	0.336
Daphnia cucullata	Treatment	2	1.640	0.26
	Date	**3**	**5.170**	**0.036**
	Treatment x Date	6	1.210	0.353
Daphnia longispina	Treatment	**2**	**28.6**	**<0.001**
	Date	3	2.460	0.133
	Treatment x Date	**6**	**3.840**	**0.037**
Eudiaptomus gracilis	Treatment	2	2,48	0,164
	Date	**3**	**9.70**	**0.03**
	Treatment x Date	6	2.060	0.152
Daphnia	**Treatment**	**2**	**11.39**	**0.006**
	Date	3	1.28	0.306
	Treatment x Date	6	1.96	0.184
Total Zooplankton	Treatment	**2**	**6.69**	**0.024**
	Date	3	0.22	0.751
	Treatment x Date	6	1.77	0.212
Total Phytoplankton	**Treatment**	**2**	**11.28**	**0.005**
	Date	**2**	**147.728**	**<0.001**
	Treatment x Date	**4**	**8.546**	**0.006**

The large daphniid *D. longispina* was the only zooplankton taxon whose densities responded to fish, including a significant treatment effect and a significant treatment–day interaction (Table 1, Fig.1): while *D. longispina* densities were comparable on all days in both fish treatments, densities increased over time in the control treatment (Table 1, Fig. 2). *Daphnia longispina* densities were lower in the whitefish treatment compared to the control treatment on days 14 (pairwise t-test, $p = 0.005$), 21 ($p = 0.012$) and 28 ($p = 0.009$). Densities of this taxon were also higher in the stickleback treatment compared to the control on days 21 ($p = 0.038$) and 28 ($p = 0.013$). Densities of all other zooplankton taxa changed over time but did not differ among treatments, and no interactions were identified between treatment and time (Table 1, Fig.1). *Bosmina* spp. (pairwise t-test, $p = 0.013$) and *Cyclopoida* ($p < 0.001$) densities increased whereas *E. gracilis* and *D. cucullata* densities decreased ($p < 0.001$) between day 0 and day 28. Total phytoplankton biovolumes were negatively related to *D. longispina* densities on both day 14 (LM; $p = 0.049$) and day 28 ($p = 0.01$), and also to total daphniid densities on day 28 ($p = 0.03$; Table 2). Increasing *D. longispina* densities were related to a decline in edible phytoplankton biovolumes on day 14 (LM, R2 = 0.41, $p = 0.025$; Fig. 3a) and day 28 (R2 = 0.56, $p = 0.005$; Fig. 3b), whereas biovolumes of the inedible fraction did not respond to *D. longispina* densities (Fig. 3c–d).

Phytoplankton biovolumes were not related to densities of total zooplankton ($p = 0.07$) or of any other individual taxon ($p > 0.1$; Table 2). Phytoplankton biovolumes differed between days and treatments and a significant interaction was observed between treatment and date (Table 1). In all treatments, total phytoplankton biovolumes were higher on day 14 compared to day 0 (pairwise t-test, $p < 0.001$) then declined between days 14 and 28 ($p < 0.001$). Compared to the control, biovolumes were higher in the whitefish treatment on days 14 (pairwise t-test, $p = 0.001$) and 28 ($p < 0.001$) and in the stickleback treatment on day 28 ($p < 0.001$; Fig. 4). Biovolumes were higher in the whitefish treatment compared to the stickleback treatment on day 14 ($p = 0.002$; Fig. 4).

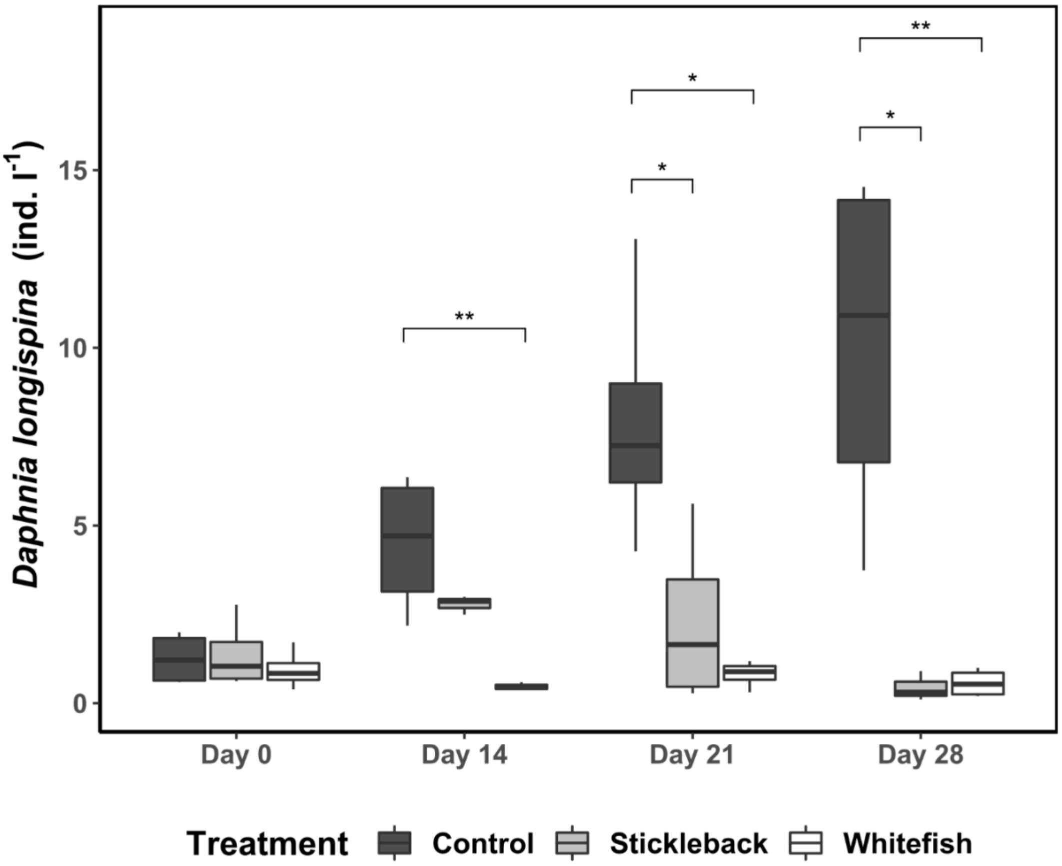

Fig. 2. Daphnia longispina densities (individuals [ind.] l–1) in each treatment on each sampling day. The results of the pairwise t-tests comparing treatments are indicated by brackets and asterisks (* $p < 0.05$, ** $p < 0.01$).

Table 2. Linear model results describing relationships between total phytoplankton biovolumes and densities of different zooplankton taxa and groups. Significant results ($p < 0.05$) are indicated in bold.

Zooplankton taxon/group	Day 14		Day 28	
	R^2	p	R^2	p
Bosmina spp.	0.04	0.54	0.06	0.45
Cyclopoida	0.00	0.89	0.24	0.11
Daphnia cucullata	0.12	0.27	0.00	0.98
Daphnia longispina	**0.33**	**0.049**	**0.51**	**0.01**
Eudiaptomus gracilis	0.20	0.14	0.18	0.17
Total daphniids	0.14	0.23	**0.39**	**0.03**
Total zooplankton	0.12	0.28	0.28	0.07

Discussion

Zooplankton of the genus *Daphnia* are typically described as keystone grazers that link primary production and higher trophic levels in lake ecosystems, but the roles of single daphniid species remain poorly known. Supporting our first hypothesis, densities of the largest-sized daphniid studied, *D. longispina*, decreased in response to fish predation. In contrast, densities of other zooplankters, including the small daphniid *D. cucullata*, did not decline. These findings demonstrate that considering the genus *Daphnia* as a keystone taxon is a generalisation that overlooks size-mediated differences between species. Whitefish and sticklebacks had comparable effects on zooplankton, which refutes our second hypothesis, that whitefish – as specialised planktivores – have a greater impact on zooplankton communities. Supporting our third hypothesis, phytoplankton biovolumes decreased primarily in response to *D. longispina* densities, likely their large body size enables filtration of a wider spectrum of phytoplankton taxa and also growth at lower phytoplankton densities (Gliwicz 1990b). Therefore, evidence of cascading effects was seen as higher phytoplankton biovolumes in fish treatments, caused by fish-mediated decreases in the densities of

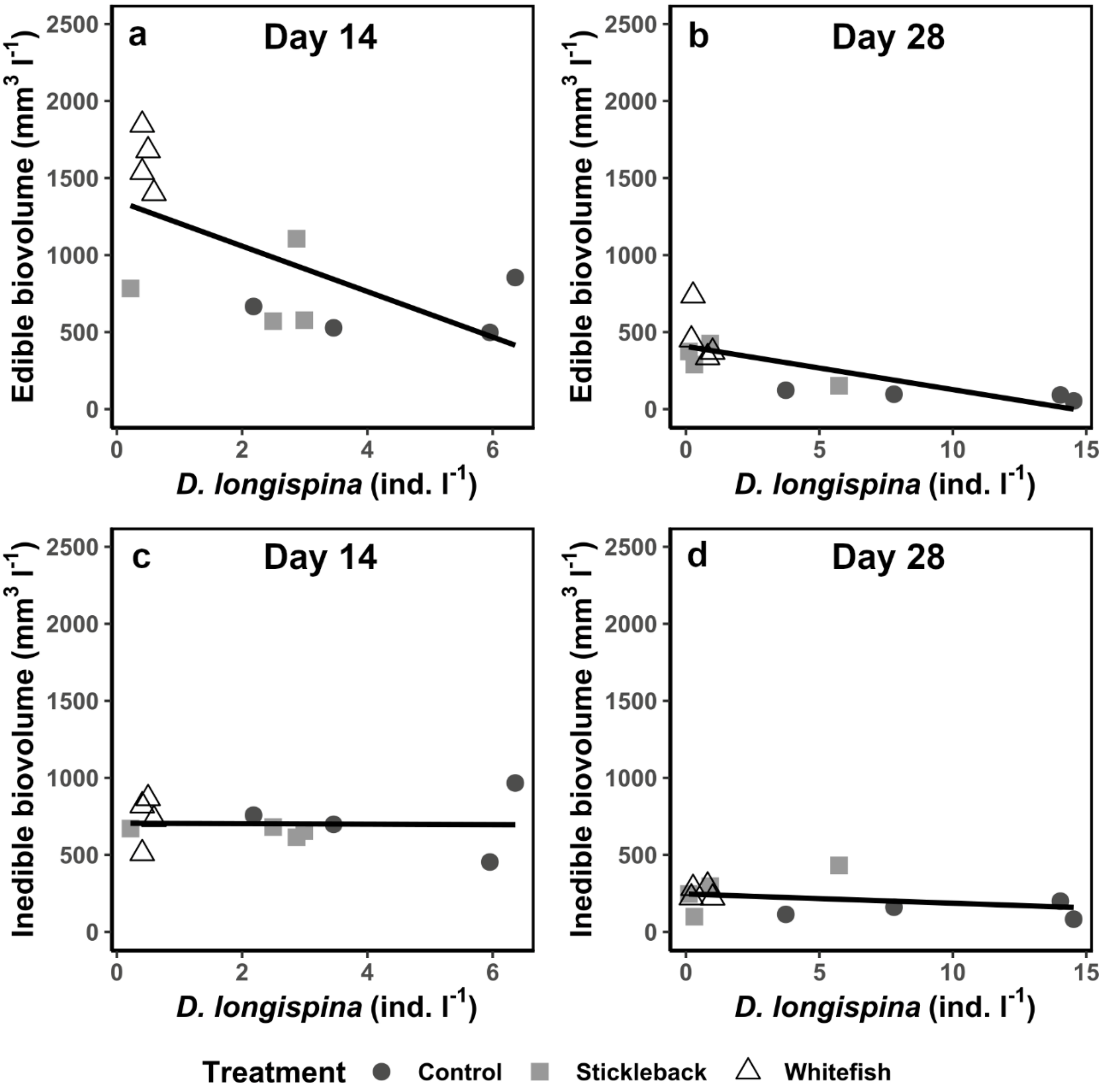

Fig. 3. Linear model (y = b*x) showing the relationship between Daphnia longispina densities (individuals [ind.] l–1) and biovolumes (mm3 l–1) of edible (< 40 µm) and inedible (> 40 µm) phytoplankton on day 14 (a, c) and day 28 (b, d).

large daphniids (Fig. 5), supporting our fourth hypothesis. Both fish treatments were linked to comparable increases in phytoplankton densities, which our results suggest were mediated by fish predation on zooplankton, in particular *D. longispina*. We thus suggest that *D. longispina* regulates trophic cascades by mediating top down effects in pelagic food webs including fish with both specialist and generalist feeding modes.

Fish predation on zooplankton

Compared to the initial lake conditions, *D. longispina* densities increased only in the fishless control treatments, reflecting release from vertebrate predation. Densities of other zooplankton taxa changed over time but not between treatments, suggesting that changes were not predation mediated. The increases in *Bosmina* spp. and cyclopoid copepods might be seasonal, because comparable increases were observed in the lake during the study.

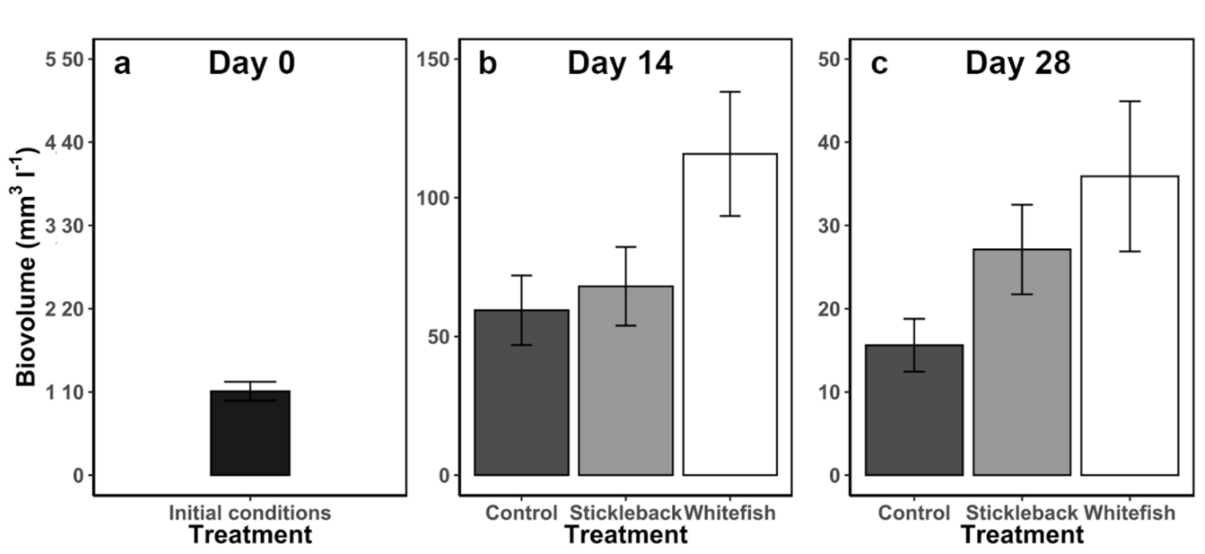

Fig. 4. Mean ±1 SD phytoplankton biovolumes (mm3 l–1) in control and fish treatments on sampling days 0 (a), 14 (b) and 28 (c). Identical phytoplankton biovolumes at day 0 are joined in one bar as initial conditions (a).

In addition, cyclopoid copepod densities might still have been increasing following inoculation, since their densities were lower on day 0 than on any other date. The drivers of temporal decreases in *D. cucullata* and *E. gracilis* are unclear but might reflect specific conditions within the closed mesocosm environment. Although densities of microcrustaceans other than large daphniids can be reduced when fish predation is high (Post & McQueen 1987; Bertolo et al. 2000), we did not observe such reductions, probably because fish densities were low, as is natural in oligotrophic Lake Constance. Mehner et al. (2008) also found no negative relationship between the biomass of planktivorous coregonid fish and zooplankton species other than *Daphnia* in an oligotrophic lake. Various factors contribute to reduced fish predation on zooplankton other than large daphniids. The contrasting movement pattern of cyclopoid and calanoid copepods makes them harder to capture compared to cladocerans such as *Daphnia* (Visser 2007; Peterka & Matěna 2011). Furthermore, due to their small size, taxa such as the genus *Bosmina* are less conspicuous and therefore less predated than larger zooplankters (Brooks & Dodson 1965). Although fish predate small and evasive zooplankton, densities of some zooplankters can increase at low fish densities due to the removal of their competitors by selective fish predation on larger and less evasive species (Brooks & Dodson 1965; Christoffersen et al. 1993). Comparable densities of the small daphniid *D. cucullata* in control and fish treatments may reflect its small size and also its morphology: it is slimmer and therefore less conspicuous than *D. longispina* of the same body length (Gliwicz 2001). The reactive distance of roach for detecting *D. longispina* is double that for *D. cucullata* of the same body length, resulting in a near-tenfold larger reactive field volume and thus higher fish predation on *D. longispina* (Gliwicz 2001). The dominance of small or large-sized zooplankton communities can thus largely reflect predation pressure in lakes (Jeppesen et al. 1997; Iglesias et al. 2017; Ersoy et al. 2017), as indicated in our study by the dominance of *D. cucullata* and cyclopoid copepods in fish treatments and *D. longispina* in non-fish treatments. The natural zooplankton community represented in our experiments included two large invertebrate predators, *B. longimanus* and *L. kindtii*. Although fish probably influenced their densities, we suggest that invertebrate predators did not cause pronounced differences in prey zooplankton composition between treatments. Invertebrate predation on zooplankton is typically much lower compared to vertebrate (fish) predation (Gliwicz & Pijanowska 1989; Šorf et al. 2014). In addition, due to their low densities, larger invertebrate predators were rarely observed in our zooplankton samples, and densities of smaller invertebrate predators (i.e. cyclopoid copepods) did not differ between treatments.

Cascading effects of fish species

Whitefish did not cause greater reductions in any zooplankton species compared to sticklebacks. However, cascading effects on phytoplankton via consumption of zooplankton (especially *D. longispina*) were initially more pronounced for whitefish than sticklebacks, as indicated by faster increases in phytoplankton biovolumes (i.e. by day 14). Whitefish are specialised zooplankton feeders with adaptations to pelagic environments whereas sticklebacks are generalist feeders (Morrow 1980; Becker & Eckmann 1992; Kottelat & Freyhof 2007), which probably enabled higher whitefish consumption of phytoplankton grazers. Although differences in zooplankton densities between fish treatments were not significant, moderately lower densities in the whitefish treatment might have been enough to cause significant differences in phytoplankton biovolume. Over time, differences in zooplankton densities and phytoplankton biovolumes between whitefish and sticklebacks became less pronounced, and within 28 days, sticklebacks and whitefish triggered similar trophic cascades.

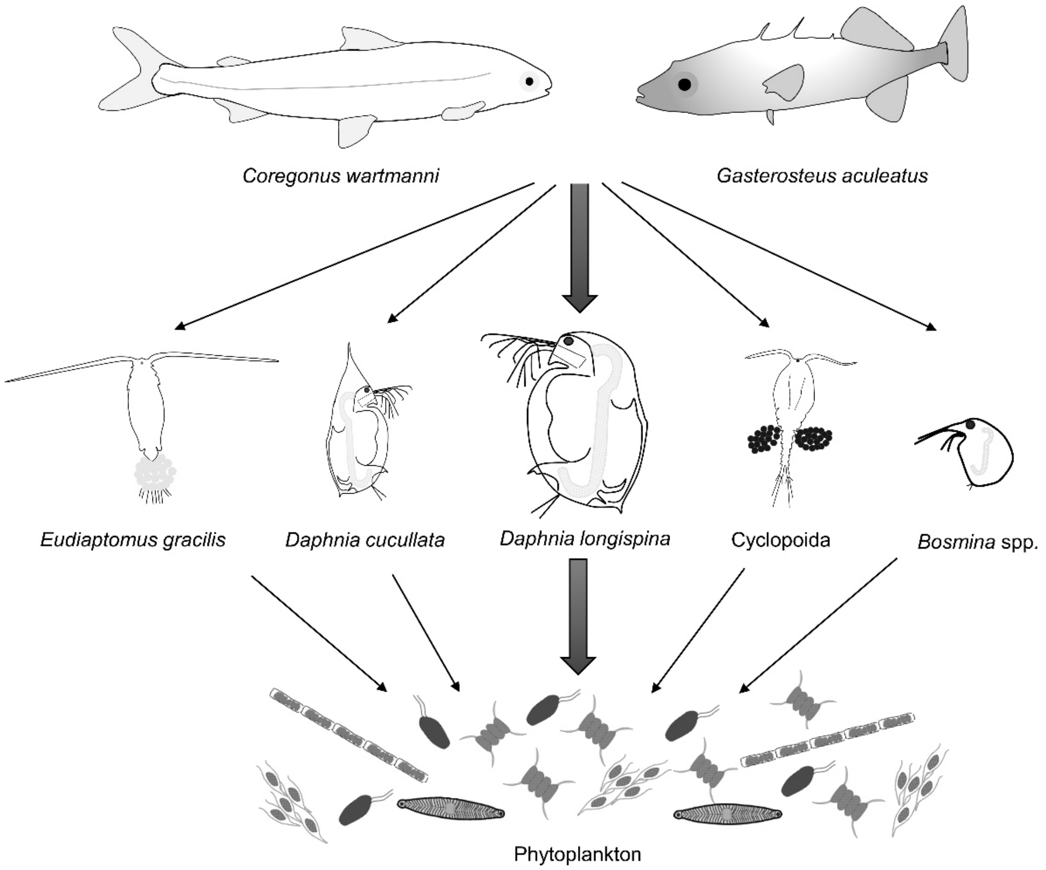

Fig. 5. A conceptual model of trophic cascading from fish (represented by a stickleback and a whitefish) to primary producers (phytoplankton) within a pelagic ecosystem. Thick arrows represent reductions in densities at lower trophic levels. Of all zooplankton taxa, only densities of the large daphniids were reduced by fish predation and thus caused declines in phytoplankton biovolumes, indicating their role as keystone species.

Other mesocosm experiments have indicated greater species-specific differences between fish, with specialised planktivores affecting zooplankton and consequently phytoplankton communities more strongly than facultative planktivorous fish, at least in shallow (<1 m) eutrophic littoral waters (Williams & Moss 2003; Des Roches et al. 2013). However, we are not aware of any previous studies with similarly low fish densities and/or sufficiently deep mesocosms to develop a thermocline and thus to represent cascading in pelagic and oligotrophic environments; these factors may explain the contrasting patterns observed in our study.

The effects of large daphniids on phytoplankton biomass

Many studies indicate that the genus *Daphnia* causes greater reductions in edible phytoplankton biovolumes than other zooplankton taxa (McQueen & Post 1988; Adrian & Schneider-Olt 1999; Mehner et al. 2008). In particular, McQueen et al. (1986; 1989) studied how body size influences the effects of *Daphnia* on phytoplankton biomass and linked large-sized daphniids to reduced biomass in mesoeutrophic and eutrophic environments. Our results build on such research by comparing the effects of different-sized daphniid species, and suggest that larger daphniids such as *D. longispina* are the main zooplankters that reduce phytoplankton biovolumes in natural lake communities. Although other zooplankton species, such as copepods, can have complementary effects to daphniids by grazing on different-sized phytoplankton species, thus contributing to greater overall reductions in biomass (Sommer et al. 2001; Sommer et al. 2003), they did not alter phytoplankton biovolumes at the natural densities represented in our communities. Total zooplankton densities had no significant effect on phytoplankton biovolume, due to the pronounced declines caused by *D. longispina* being offset by the limited effects of other, smaller taxa. Equally, the linear relationship between densities of the genus *Daphnia* and phytoplankton biovolumes on day 28 reflected only the pronounced effect of *D. longispina*, whereas *D. cucullata* had no effect on biovolumes. These results indicate that previous research may have overstated the importance of small *Daphnia* species, if genus-level responses were caused solely by larger, co-occurring congeners. Larger *D. longispina* also have higher potential than *D. cucullata* to increase their population densities due to higher birth and biomass production rates (Vijverberg & Richter 1982). This greater fecundity might have contributed to increasing *D. longispina* dominance in our fishless mesocosms, where increasing densities enabled greater control of phytoplankton.

Cascading links from fish to phytoplankton

Our results indicate that *D. longispina* was a keystone species that linked both planktivorous and generalist fish predators to phytoplankton communities (Fig. 5). No other zooplankton or daphniid taxa or groups were so greatly predated by fish or showed such strong phytoplankton control, and multi-taxon groups therefore formed much weaker cascading links between fish and primary producers. Although smaller zooplankton species can also contribute to trophic cascades from fish to phytoplankton if large cladocerans are absent (Christoffersen et al. 1993; Helminen & Sarvala 1997), most studies indicate that the genus *Daphnia* – if present – is the taxon most negatively affected by fish predation, and has also the strongest effects on phytoplankton densities (Post & McQueen 1987; Mehner et al. 2008). However, variability in the roles of different *Daphnia* species in trophic cascades remains poorly studied. Although fish can also have a positive effect on phytoplankton growth by remineralising nutrients, this effect is mostly limited to non-edible phytoplankton, whereas edible phytoplankton is more affected by zooplankton grazing (Vanni 2002). In our study, biovolumes of non-edible phytoplankton did not differ among treatments, whereas edible phytoplankton biovolumes decreased in response to *D. longispina* densities. These results suggest that phytoplankton biovolumes were strongly controlled by direct zooplankton (especially *D. longispina*) grazing, whereas fish effects such as remineralisation had a minor role.

Trophic cascades in a warming climate

Our results provide evidence that zooplankton taxa – including small *D. cucullata* – might have a more limited influence on phytoplankton biomass in large oligotrophic lakes than the larger *D. longispina*. Global warming is altering pelagic food webs by shrinking animal body sizes and shifting community compositions towards a higher proportion of small-sized species and individuals (Daufresne et al. 2009; Geerts et al. 2015). As such, increasing temperatures that cause intraspecific or interspecific decreases in zooplankton body sizes could alter the functioning of green and brown food webs in oligotrophic freshwaters. Indeed, *D. cucullata*, which was rarely observed in Upper Lake Constance before 2015, became a summer-dominant cladoceran in 2017 (IGKB 2018). How recent and ongoing changes in *Daphnia* composition are altering ecosystem dynamics is unknown. Further research is needed to determine how body size and other functional traits interact to determine the roles of different daphniid species in trophic cascading and energy transfer from phytoplankton to fish.

Conclusion

Our study advances our understanding of species-specific roles of zooplankton in food webs and trophic cascades in oligotrophic pelagic environments. We demonstrate that considering both zooplankton and daphniids as uniform taxonomic groups, as it is common praxis, might limit understanding of links between multiple trophic levels in aquatic ecosystems. We suggest that the genus *Daphnia* is not a keystone taxon, but rather that large *Daphnia* species such as *D. longispina* can control food web structure at trophic levels from phytoplankton to fish. Our observation of comparable top-down predation on *D. longispina* by fish with specialist and generalist feeding modes suggests that the keystone role of large daphniids in oligotrophic lake food webs could extend across ecosystems with different fish predation regimes.

Acknowledgements

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Supplementary material

The following graphs display the remaining zooplankton taxa according to Fig. 2 in the results paragraph.

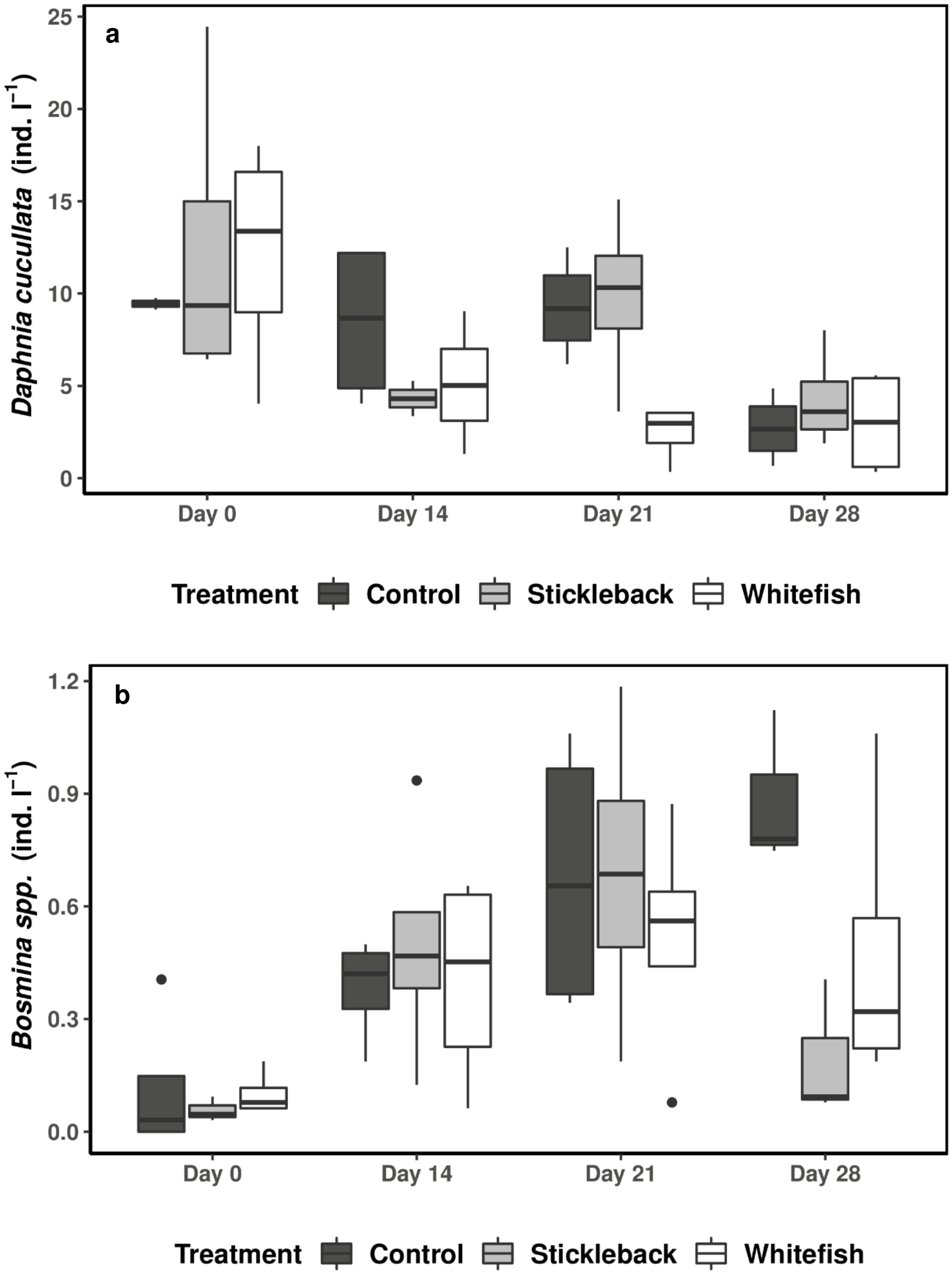

Fig. SM1. *Daphnia cucullata* (a) and *Bosmina spp.* (b) densities (individuals [ind.] l–1) in each treatment on each sampling day.

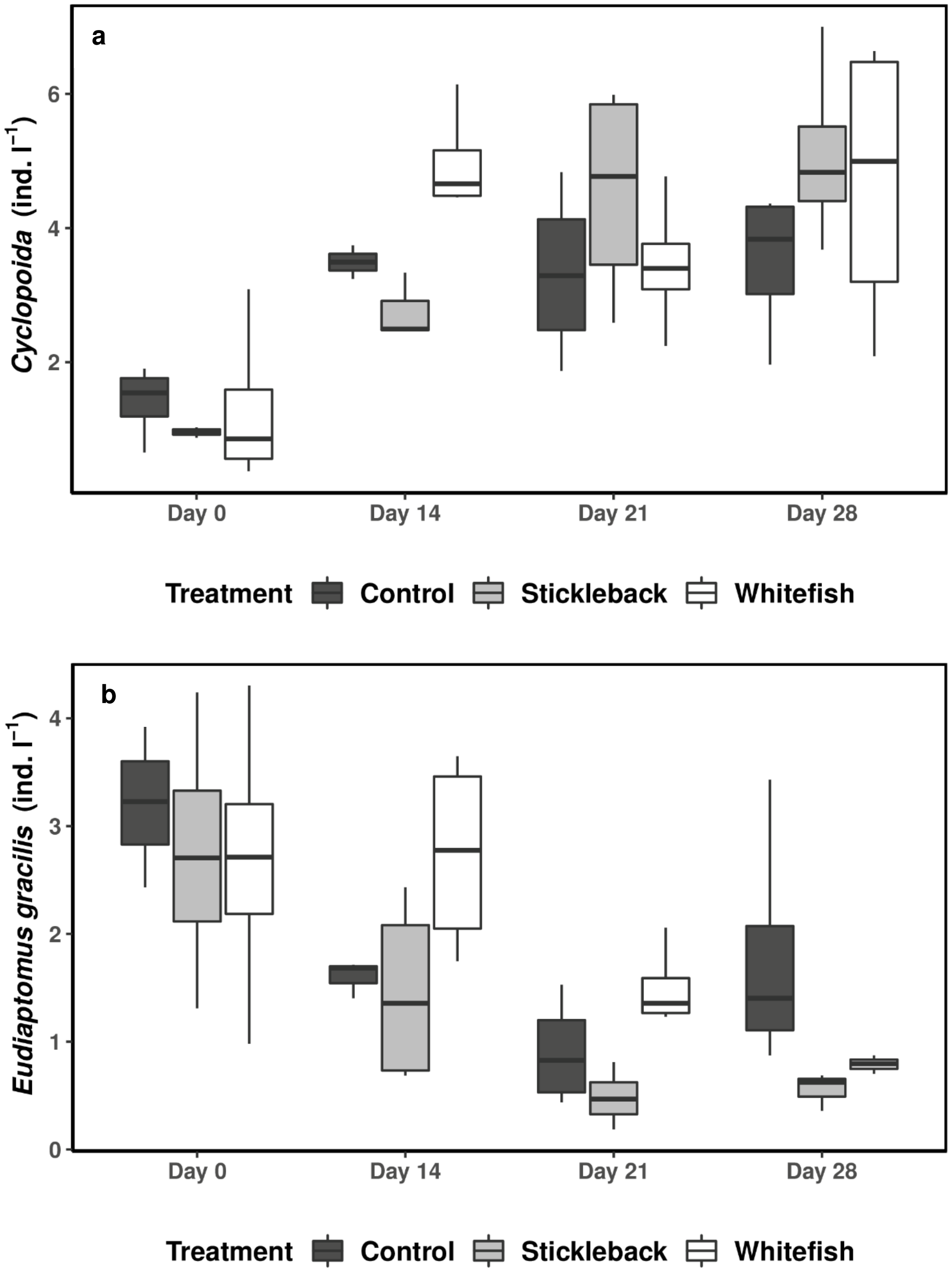

Fig. SM2. *Cyclopoida* (a) and *Eudiaptomus gracilis* (b) densities (individuals [ind.] l–1) in each treatment on each sampling day.

Chapter III: Second Manuscript

Growth experiments on chrysophytes native to Lake Constance considering re-oligotrophication

Carsten Wunsch, Samuel Kühn, Joshua Strittmatter and Karl-Otto Rothhaupt

Abstract

Mixotrophic chrysophytes combine the metabolic lifestyles of photoautorophy and heterotrophy to fulfill their demands for energy and nutrients. Especially in oligotrophic environments, mixotrophic organisms can take advantage when nutrients are mostly bound in bacterial biomass. When light is available, the combination of both nutritional modes might give the ability to outcompete exclusive heterotrophic or exclusive photoautotrophic phytoplankton. Mixotrophic chrysophytes can show strong variability in the expression of their mixotrophic trait, which can be directed to one of both strategies. Recent re-oligotrophication in many European lakes could explain the increased abundance of mixotrophic chrysophytes in the last decades. In laboratory experiments, we compared the response of different chrysophytes native to oligotrophic pre-alpine Lake Constance on different bacterial densities and different light intensities. We found that the investigated chrysophytes differed in their nutritional strategy, while *Poterioochromonas malhamensis* is more related to heterotrophy, species of the genus *Dinobryon* are primarily phototrophs.

Keywords: Mixotrophy; phytoplankton; chrysophytes; re-oligotrophication; lake ecosystems

Introduction

Mixotrophy in algae is characterized by the ability to satisfy an organism's demand for energy and nutrients through auto- and heterotrophy (Caron et al., 1993; Raven, 1997). With the combination of photosynthesis and the possibility to feed on bacterial biomass (Bird and Kalff, 1986; Rothhaupt, 1996b), several phytoplankton species from different taxa show a mixotrophic lifestyle (Boraas et al., 1988). Among these, chrysophytes are well-studied and geographically widespread representatives and can be found in many marine and freshwater ecosystems (Kristiansen, 2000; Unrein et al., 2010; Hartmann et al., 2013; Järvinen et al., 2013). However, chrysophytes' nutritional lifestyles are not straightforwardly mixotrophic; instead, a broad range of metabolic traits was found extending from obligate chemoheterotrophy to obligate photoautotrophy (Pålsson and Daniel, 2004; Lie et al., 2018). Especially in oligotrophic pelagic systems, mixotrophic chrysophytes are abundant (Berninger et al., 1992; Schlüter et al., 2016). Due to their availability to ingest bacteria, some mixotrophs might have an advantage over phototrophic phytoplankters in the competition for scarce mineral nutrients (Katechakis and Stibor, 2006). Furthermore, the availability of light in such nutrient-poor systems could increase with low biomasses, and mixotrophs may cover their energy demand through photosynthesis and, in addition to that, outcompete exclusive heterotrophic bacterivores (Tittel et al., 2003). Additionally, during past experiments, multiple factors influencing the growth of mixotrophic chrysophytes were found. Apart from the availability of carbon and light (Caron et al., 1993), the presence of phosphorous (Rothhaupt, 1996a), temperature (Wilken et al., 2013; Princiotta et al., 2016), and pH (Moser and Weisse, 2011) are essential factors which could favor the competitiveness in nature. Nevertheless, there is no comprehensive formula describing mixotrophic chrysophytes' success since every species relies on different parameters and its nutritional trait depending more or less on heterotrophy and phototrophy (Rottberger et al., 2013). During the last decades, mixotrophic chrysophytes showed increased abundances in lakes that passed through an event of re-oligotrophication (Jeppesen et al., 2005; Jacquet et al., 2014). Lake Constance, located in Central Europe, is a well-studied ecosystem and an exceptional example for re-oligotrophication, which is still proceeding. With the reduced phosphorous levels, a regime shift took place, and overall phytoplankton biomasses decreased (Jochimsen et al., 2013). It stands to reason that the lack of phosphorous and the greater availability of light, due to less biomass in the water column, might provide favorable growth conditions for mixotrophs and the possibility to outcompete local rivals.

In two laboratory experiments, we investigated the growth of several chrysophytes depending on [1] the presence and concentration of bacteria and [2] depending on light intensity. We used the three mixotrophic chrysophytes*, Poterioochromonas malhamensis, Dinobryon divergens, and Dinobryon sociale*, common in many European lakes as well as in Lake Constance. Our objectives were to determine how the three chrysophytes differ in their effectivity to grow on different bacterial concentration levels when no inorganic phosphorous source is present. Secondly, we wanted to show how the examined chrysophytes differ in their growth effectivity by implementing various light intensities and the dependence on bacteria under oligotrophic conditions with few inorganic phosphorous present. The colorless and exclusively heterotrophic chrysophyte *Spumella rivalis* was cultivated alongside to compare how growth on bacteria behaves independently from light. Due to the known mixotrophy of all three species (Bird and Kalff, 1986; Caron et al., 1990; Jones, 2000), we expected to find species-dependent relationships towards bacteria concentration and light intensity. Accordingly, we expected to find unique growth patterns with different species involved, referring to different feeding strategies depending more on heterotrophy or autotrophy.

Methods

Strains and culturing conditions

With *Poterioochromonas malhamensis*, *Dinobryon divergens*, and *Dinobryon sociale*, we included three mixotrophic strains and *Spumella rivalis* as a reference strain, a colorless obligate heterotrophic chrysophyte. *P. malhamensis* and *D. sociale* were isolated from Lake Constance. In contrast, *D. divergens* (strain FU 18K-A, Rottberger et al., 2013) origins from Lake Fuschelsee in Austria. *S.rivalis* (strain A_R4_A6) was isolated from River Fuschler Ache (A) and provided by the Experimental Phycology and Culture Collection of Algae at Goettingen University (SAG).

Standard cultures of chrysophytes were cultivated in liquid WC medium (Guillard and Lorenzen, 1972) and a carbon source for growth. To reduce the amount of phosphate and to avoid potential potassium toxicity for *Dinobryon* (Lehmann, 1976; Van Donk et al. 1988) before experiments, K_2HPO_4 (trihydrate) was substituted by 1 mg l^{-1} NaH_2PO_4 (dihydrate); as a potassium replacement for *P.malhamensis* and *S. rivalis*, 3 mg l^{-1} KCl was added. Three wheat grains were added to the flasks containing *S. rivalis*, and α-D-glucose (1.8 g l^{-1}) was dissolved in the medium of *P. malhamensis*. All strains were cultured in 250 ml borosilicate screw-top bottles under sterile conditions and aerated through a 0.2 µm PTFE air filter to ensure the sterile gas exchange and agitation of containing medium. The cultures grew in a temperated room at 18 °C and were illuminated by LED arrays imitating the natural spectrum of daylight with a light intensity of 50 µmol*m-^{2}*s in a daily light:dark cycle of 16:8 hours.

Bacteria for feeding experiments were isolated from Lake Constance by filtering collected water first through a set of mesh filters ranging from 100 µm to 25 µm and second through a polycarbonate membrane filter with a pore size of 5 µm. The filtrate was then checked for the bacteria via epifluorescence microscopy by staining them with 4′,6-Diamidin-2-phenylindol (DAPI). The isolated bacteria were cultivated in darkness at 18 °C using standard WC medium under the addition of α-D-glucose and yeast extract (both 1 g l^{-1}). After five days, the culture showed visible turbidity, and the bacteria were heat-inactivated in a water bath for 90 min at 75°C and then washed three times in a centrifuge-resuspend cycle (7500 rpm). The washed and condensed bacteria solution was then frozen and stored at -20 °C for later use. If not stated differently following methods are related to culturing conditions above.

Growth experiments

Experiments were performed in triplicates, which were deployed in closed boxes with adjustable LED arrays attached to the ceiling. In the first experiment with different bacteria concentrations and control without a constant light intensity of 100 µmol*m^{-2}*s^{-1} was adjusted. The phosphorous source NaH_2PO_4 was excluded from the WC medium to guarantee a phosphorus-free medium. The washed and cryopreserved lake bacteria were added in two different initial densities, low (LB treatment, 4.5*10^{6} cells ml^{-1}) and high (HB treatment, 2.7*10^{7} cells ml^{-1}). The control treatment was left without bacteria additions. The cultivation started with the inoculation of the respective chrysophyte. Inoculation doses for protists and bacteria were determined beforehand by performing counts of standard cultures under the epifluorescence microscope by staining cells with DAPI. Initial cell concentrations were around 10^{3} cells ml^{-1} for *P. malhamensis* and between 10^{1} and 10^{2} cells ml^{-1} for *D. divergens* and *D. sociale.* The same set-up was used for the second experiment but with various light intensities and a low phosphorous level. Five light treatments were prepared ranging from complete darkness (0 µmol*m^{-2}*s^{-1}), followed by 25 µmol*m^{-2}*s^{-1}, 50 µmol*m^{-2}*s^{-1}, 100 µmol*m^{-2}*s^{-1} and the highest light intensity at 200 µmol*m^{-2}*s^{-1}. To start the cultivation, flagellates from standard cultures were transferred to each replicate at an initial cell concentration of 1.2*10^{4} cells ml^{-1} for *P. malhamensis* and *S. rivals* and 1.5*10^{4} cells ml^{-1} for both *Dinobryon* strains. NaH_2PO_4 (dihydrate) was reduced to 0.01 mg l^{-1} to receive a phosphate concentration of about 0.006 mg l^{-1}, which corresponds to the common concentration in oligotrophic lakes.

Sampling and flow cytometric determination of cell densities

Samples were taken daily inside a clean bench with laminar airflow to prevent contamination of cultures and samples. To determine protists' densities, 200 µl of each replicate were transferred to a 96-well microtiter plate, and cells were then analyzed alive by using a FACSVerse^{TM} (BD Biosciences) flow cytometer. The cytometer was equipped with three lasers: violet (405 nm), blue (488 nm), and red (633 nm), and the device was calibrated with CS&T Research Beads (BD Biosciences) before experimental set-up. To count the chlorophyll-containing flagellates *P. malhamensis, D. divergens, and D. sociale,* the autofluorescence of chlorophyll a was detected using the red laser and a 700/54 nm bandpass filter. Due to the absence of chlorophyll a in heterotrophic *S. rivalis,* the cells were stained with DAPI beforehand. The stained samples were afterward analyzed using the violet laser and a 448/54 nm bandpass filter. The cell sizes of all species were detected by the forward scatter (FSC), which describes the intensity of light that is scattered at a small angle when hitting a cell and can be detected proportional to cell size. With both the fluorescence and the FSC, the cell density could be determined after visualization in a biplot by setting gates to target populations using the flow cytometry software FlowJo (v. 10.04.1, FlowJo LLC). We used the mean chlorophyll a autofluorescence intensity to ascertain the photosynthetic activity of chlorophyll a containing species. Flow cytometric count was performed with a flow-rate of 120 µl min^{-1} and started directly after sampling to ensure the viability of cells and avoid changes in the chlorophyll a content due to biasing light fluctuations.

Data analysis

Growth rates (µ) were determined using the slope of linear models applied on log10 transformed cell numbers against time during the period of exponential growth. The exponential phase was determined by the best fitting model consisting of three linear regressions describing the lag- and exponential phase and the steady-state. Comparison of growth rates between bacterial density treatments was performed using one-way ANOVA (cell number ~ treatment). For the second experiment introducing light intensities, the method was extended to two-way ANOVA (cell number ~ light intensity + treatment). Comparison of chlorophyll autofluorescence intensities between treatments and light intensity + treatment was also performed using one-way ANOVA or two-way ANOVA, respectively. Autofluorescence was compared during the steady-state of each flagellates' culture. Normal distribution of data was confirmed using Shapiro-Wilk tests, and homogeneity of variances was checked using Levene's test. Growth rates and fluorescence intensities were pairwise compared with the respective light intensity using Tukey's HSD test (honestly significant difference). All analyses were done in the statistical software R (R Core Team 2018); Linear models and ANOVAs were performed using the basic "lm" and "aov" function, the package "ggplot2" (Wickham 2016) was used to plot graphics.

Results

The influence of bacterial density

The growth curves of *P. malhamensis* showed a net increase in cell densities under HB and LB conditions while the densities of the control treatment remained low (Fig. 1). Growth in the HB treatment was faster than in the LB treatment, and steady-state was achieved earlier than the LB treatment, where growth was less and steady-state was achieved later.

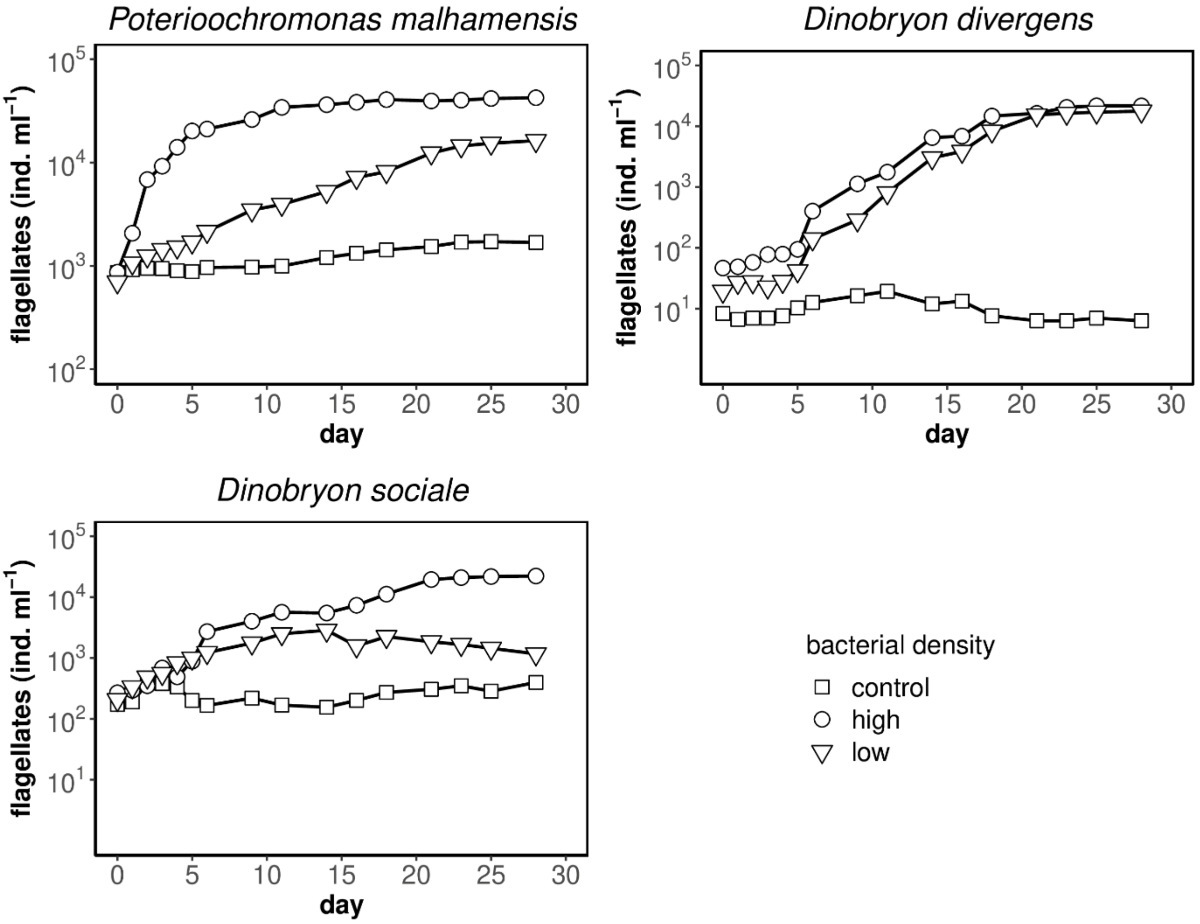

Fig. 1. Time course displaying growth curves of examined chrysophytes responding to different bacterial densities. Datapoints represent the mean values of replicates.

The maximal cell yield was highest in the HB treatment with final cell concentrations around $4x10^4$ cells ml^{-1}. Cell densities in the LB treatment reached final cell concentrations of around $2x10^4$ cells ml^{-1}. Compared to the bacteria treatments, growth in the control treatment was minor, and cell densities stayed close to the starting densities. Growth rates deviated significantly between treatments for *P. malhamensis* (ANOVA, F (2, 6) = 138.1, $p < 0.001$). The highest growth rate was determined HB treatment ($\mu = 0.74$) compared to the LB treatment's maximal growth rate ($\mu = 0.38$). The growth rate in the control treatment without bacteria was the lowest ($\mu = 0.04$). Pairwise comparisons revealed higher growth rates with increasing bacterial densities for *P. malhamensis* (Tukey's HSD: $p < 0.01$, Fig. 2).

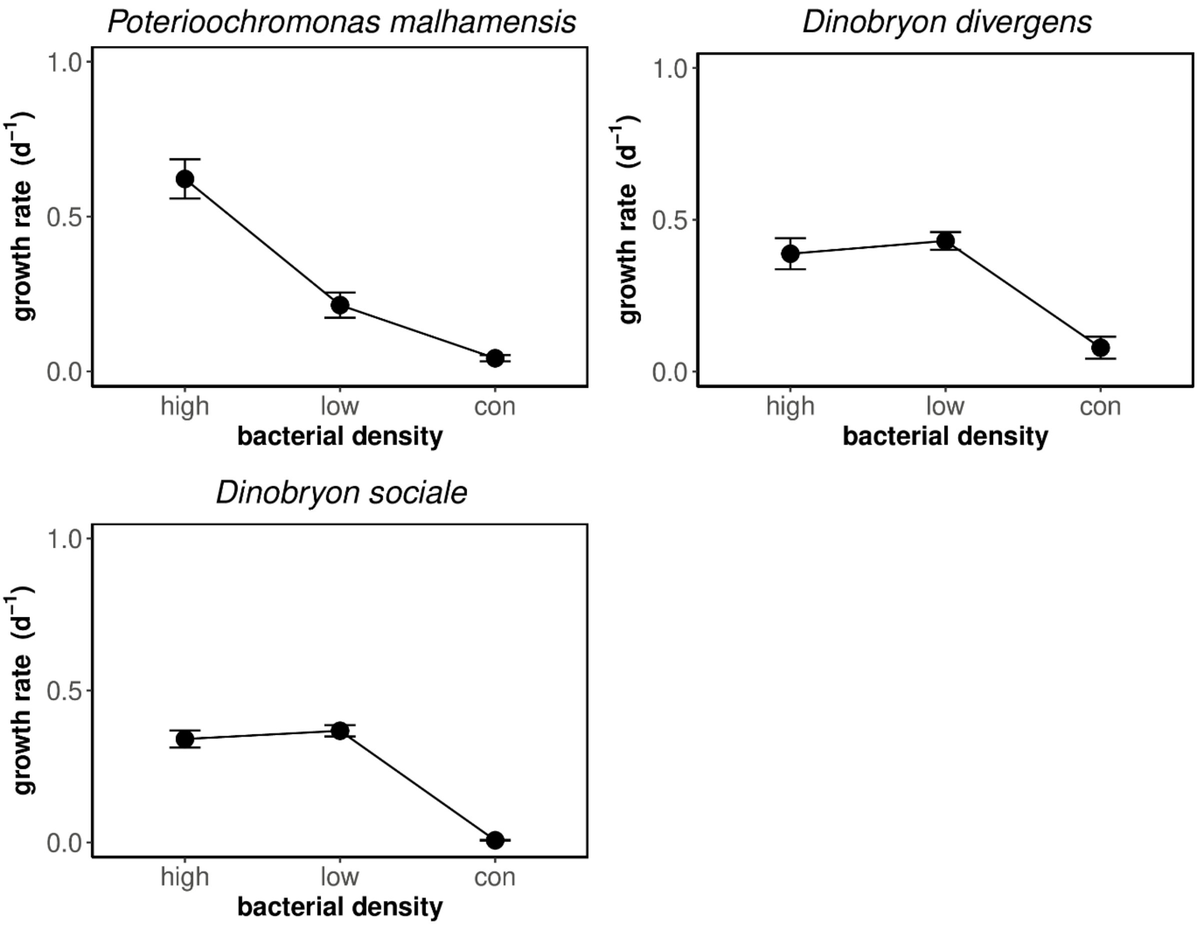

Fig. 2. Average growth rates of examined chrysophytes during the phase of exponential growth responding to different bacterial densities. Datapoints represent the mean values of the respective three replicates. Error bars display standard deviations.

Growth curves of *D. divergens* showed a similar growth progression at both bacterial densities. In both cases, the maximal cell yield was around $2x10^4$ cells ml^{-1}. In contrast, growth in the control treatment was minor compared to bacteria treatments, and cell densities were fluctuating around the starting densities. Growth curves of *D. sociale* showed a similar course at both bacterial densities at the beginning (Fig. 1). Nevertheless, the maximal cell yield was tenfold higher in the HB treatment ($2x10^4$ cells ml^{-1}) than in the LB treatment ($2x10^3$ cells ml^{-1}). Growth rates differed significantly between treatments for *D. divergens* (ANOVA, F (2, 6) = 69.5, $p < 0.001$) and *D. sociale* (F (2, 6) = 320.5, $p < 0.001$). The growth rate for *D. diverges* was highest in the LB treatment ($\mu = 0.59$) and thus compared to the HB treatments' growth rate ($\mu = 0.55$). For the control treatment, a growth rate of $\mu = 0.08$ was found. Although *D. sociale* showed growth activity in the LB treatment, cell densities decreased slightly after 14 days. Growth in the control treatment was minor, and cell densities stayed close to the starting densities. Growth rates were highest in the HB treatment ($\mu = 0.46$), and flagellates in the LB treatment showed a lower growth rate with $\mu = 0.33$. Growth in the control treatment was low with $\mu = 0.006$. Both *Dinobryon* species showed higher growth rates in bacteria treatments than in the control treatment ($p < 0.001$) but no differences between high and low bacteria treatments (Fig. 2).

Chlorophyll autofluorescences differed between treatements for each chrysophyte: *P malhamensis* (ANOVA, F (2, 42) = 4.5, p = 0.018), *D. divergens* (F (2, 42) = 53.6, $p < 0.001$) and *D. sociale* (F (2, 42) = 71.4, $p < 0.001$). Pairwise comparison of *P. malhamensis'* fluorescence between treatments revealed significantly higher values in the control treatment compared to both bacteria treatments (Tukey's HSD, $p < 0.05$, Fig. 3). Fluorescence of both *Dinobryon* species was different between all treatments ($p < 0.001$), showing lowest values in the control treatment and increasing with bacterial density (Fig. 3).

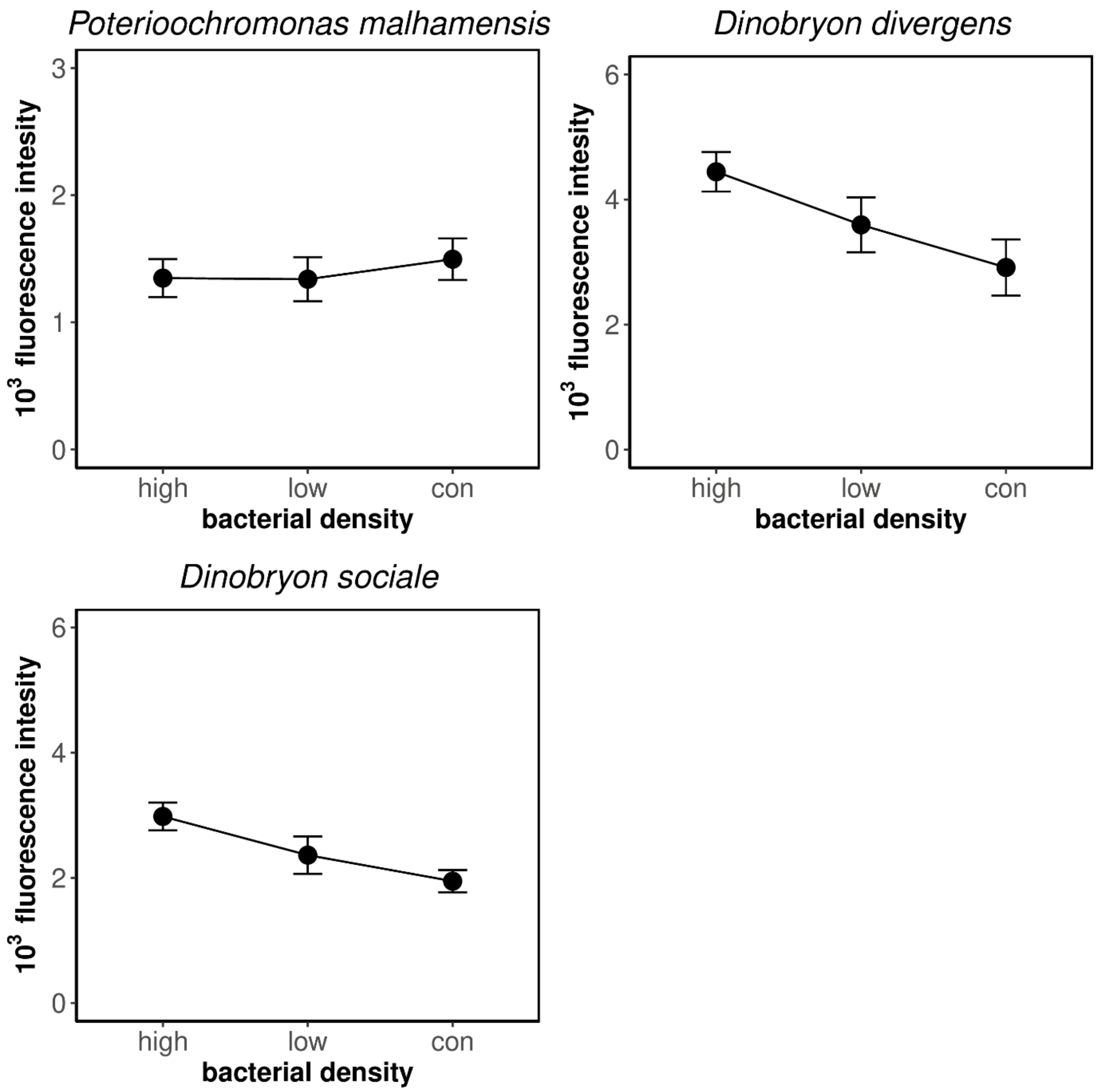

Fig. 3. Average chlorophyll a autofluorescence intensities of examined chrysophytes during the phase of exponential growth responding to different bacterial densities. Datapoints represent the mean values of the respective three replicates. Error bars display standard

The influence of light

Growth activity of *P. malhamensis* was observed at both bacterial densities for all light intensities, with a maximal cell yield around 10^5 cells ml^{-1} (HB) and 6×10^4 cells ml^{-1} (LB). Without light, cell numbers decreased after four days at both bacterial densities (Fig. 4). Growth rates for *P. malhamensis* were highest at 0 µmol m^{-2}s^{-1} followed by 25 µmol m^{-2}s^{-1}, 50 µmol m^{-2}s^{-1}, 100 µmol m^{-2}s^{-1} and 200 µmol m^{-2}s^{-1} (Tab. 1). In the LB treatment, *P. malhamensis* showed the highest growth activity at 200 µmol m^{-2}s^{-1}, while the least growth was observed at 50 µmol m^{-2}s^{-1}. Comparison of *P. malhamensis'* growth rates between light intensities and treatments revealed significant differences between light intensities (ANOVA, $F(4, 24) = 5.1$, $p = 0.004$) and a higher growth in the HB treatment (ANOVA, $F(1, 24) = 26.8$, $p < 0.001$). Pairwise comparisons between light intensities yielded a higher growth of *P. malhamensis* when the light intensity was above 25 µmol m^{-2}s^{-1} (Tukey's HSD, $p < 0.05$). No differences were found between darkness and 25 µmol m^{-2}s^{-1} and between the higher light intensities (Fig. 6). The colorless *S. rivalis* grew at both bacterial density treatments and with each light intensity. Growth curves showed similar courses at both bacterial densities with a maximal cell yield of around 10^5 and 10^6 cells ml^{-1} (Fig. 4). Growth rates revealed no difference between light intensities at both bacterial densities but between treatments (ANOVA, $F(1, 24) = 45.7$, $p < 0.001$, Tab. 1), resulting in an increased growth rate when bacterial densities were high (Fig. 5). *D. divergens* grew with all light intensities but cultures starved in darkness. In the treatment with 200 µmol m^{-2}s-1, *D. divergens* commenced dying directly after the start of the experiment; therefore, the treatment was not considered in the comparison between light intensities. Maximal cell yields for both bacterial densities were between 1×10^4 and 5×10^4 cells ml^{-1} (Fig. 5). Comparison *D. divergens'* growth rates revealed significant differences between light intensities (ANOVA, $F(4,24) = 13.4$, $p < 0.001$, Tab. 1) but no differences between bacterial densities. Pairwise comparison between light intensities featured increased growth of *D. divergens* when the light intensity exceeded 25 µmol m^{-2}s^{-1} (Tukey's HSD, $p < 0.05$, Fig. 6). *D. sociale* could grow in light at both bacterial densities but starved after few days in darkness and at 25 µmol m^{-2}s^{-1}. The maximal cell yields with light intensities from 50-200 µmol m^{-2}s^{-1} were around $6 \cdot 10^4$ cells ml^{-1} at both bacterial densities (Fig. 5). Growth rates significantly depended on light intensity (ANOVA, $F(4,24) = 250.5$, $p < 0.001$, Tab. 1) bot not on bacteria concentration. Comparing light intensities displayed increasing growth rates with increased light intensities compared to the dark treatment (Tukey's HSD, $p < 0.001$, Fig. 6).

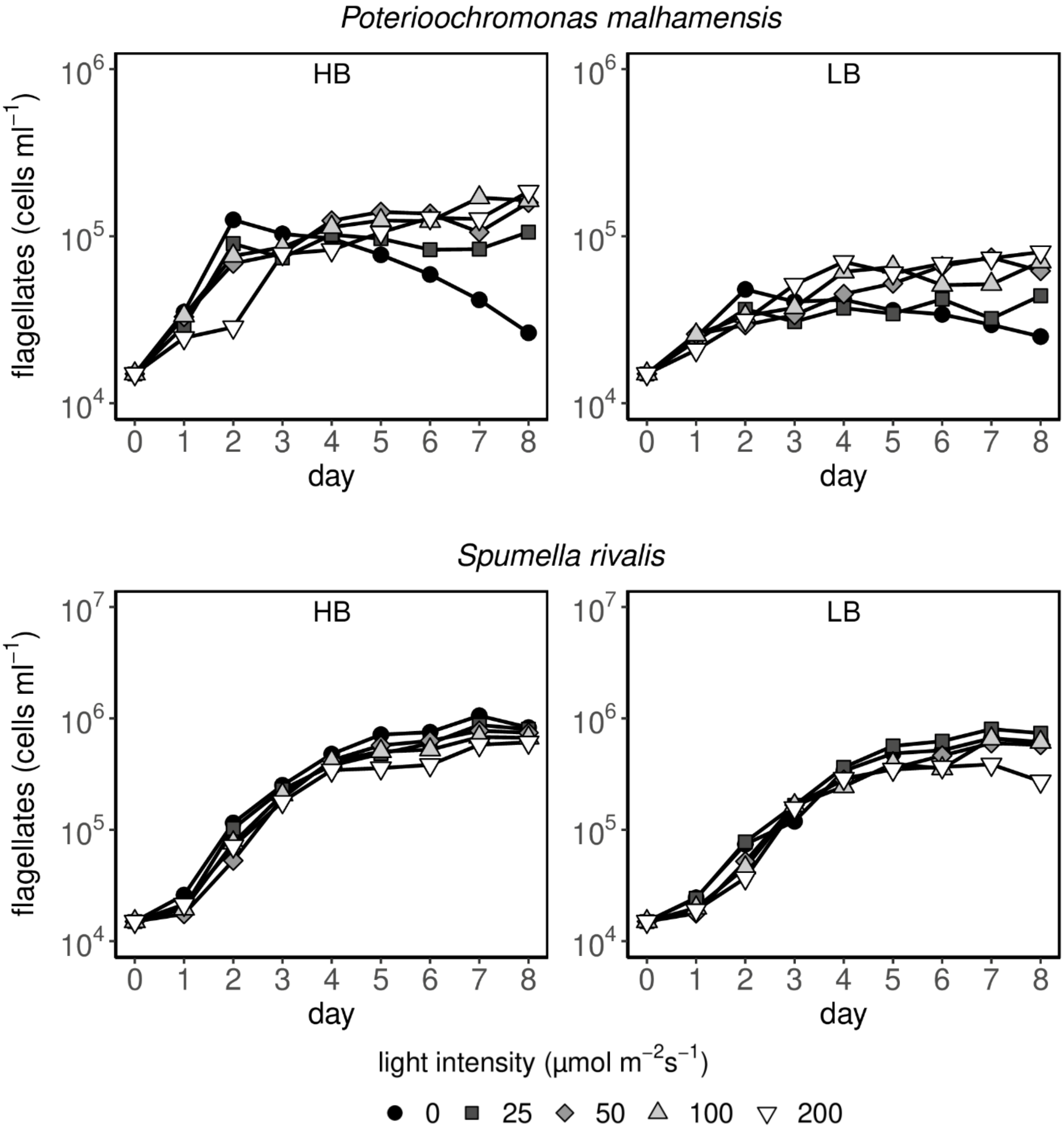

Fig. 4. Time course displaying growth curves of *P. malhamensis* and *S. rivalis*, grown with high bacterial (HB) and low bacterial (LB) densities, responding to different light intensities.

Chlorophyll autofluorescence measured for *P. malhamensis* was depended on the light intensity (ANOVA, F (4,84) = 110.8, $p < 0.001$) but not on bacterial density. The pairwise comparison revealed the highest values at 25 µmol m^{-2}s^{-1} and a gradual decrease with increasing light intensities (Tukey's HSD, $p < 0.05$, Fig. 7). However, the lowest fluorescence was measured in the dark treatment ($p < 0.001$). Similar dependency on the light intensity but not on bacterial density was found for *D. divergens* (ANOVA, F (4,84) = 167.1, $p < 0.001$) and

D. sociale (F (4,84) = 570.9, $p < 0.001$). Corresponding to that, for both *Dinobryon* species, the highest value was measured at 25 µmol $m^{-2}s^{-1}$ with a subsequent gradual decrease of the fluorescence (Tukey's HSD, $p < 0.05$, Fig. 7). Fluorescence was lowest in the darkness compared to other light treatments ($p < 0.001$).

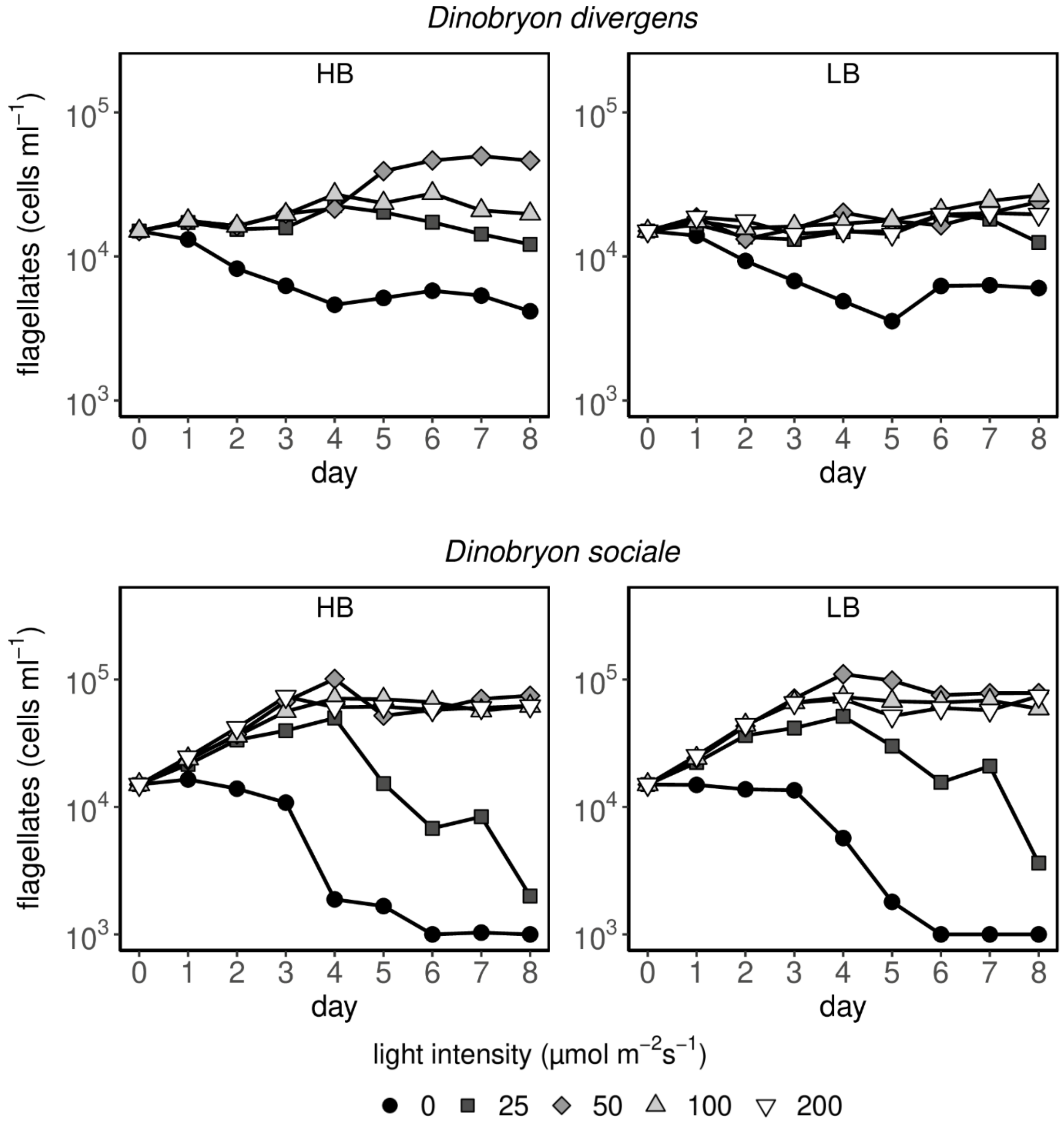

Fig. 5. Time course displaying growth curves of *D. divergens* and *D. sociale*, grown with high bacterial (HB) and low bacterial (LB) densities, responding to different light intensities.

Table 1. Growth rates of examined chrysophytes, grown with high bacterial density or low bacterial density, in dependence on the respective light intensity. Growth rates represent mean values ± SD determined during the exponential growth phase.

	high bacterial density (HB)		low bacterial density (LB)	
species	light intensity (μmol m^{-2} s^{-1})	growth rate (μ)	light intensity (μmol m^{-2} s^{-1})	growth rate (μ)
Poterioochromonas malhamensis	200	0.51 ± 0.04	200	0.41 ± 0.10
	100	0.57 ± 0.14	100	0.39 ± 0.05
	50	0.62 ± 0.07	50	0.34 ± 0.01
	25	0.89 ± 0.09	25	0.42 ± 0.16
	0	1.01 ± 0.14	0	0.57 ± 0.12
Spumella rivalis	200	0.84 ± 0.03	200	0.80 ± 0.03
	100	0.90 ± 0.02	100	0.77 ± 0.03
	50	0.91 ± 0.02	50	0.81 ± 0.01
	25	0.89 ± 0.01	25	0.82 ± 0.03
	0	0.91 ± 0.04	0	0.78 ± 0.03
Dinobryon divergens	200	-	200	0.12 ± 0.01
	100	0.16 ± 0.06	100	0.11 ± 0.07
	50	0.16 ± 0.03	50	0.11 ± 0.01
	25	0.08 ± 0.01	25	0.03 ± 0.04
	0	0.00 ± 0.00	0	0.00 ± 0.00
Dinobryon sociale	200	0.53 ± 0.03	200	0.49 ± 0.02
	100	0.44 ± 0.02	100	0.41 ± 0.02
	50	0.50 ± 0.01	50	0.49 ± 0.01
	25	0.38 ± 0.05	25	0.36 ± 0.03
	0	0.07 ± 0.04	0	0.01 ± 0.01

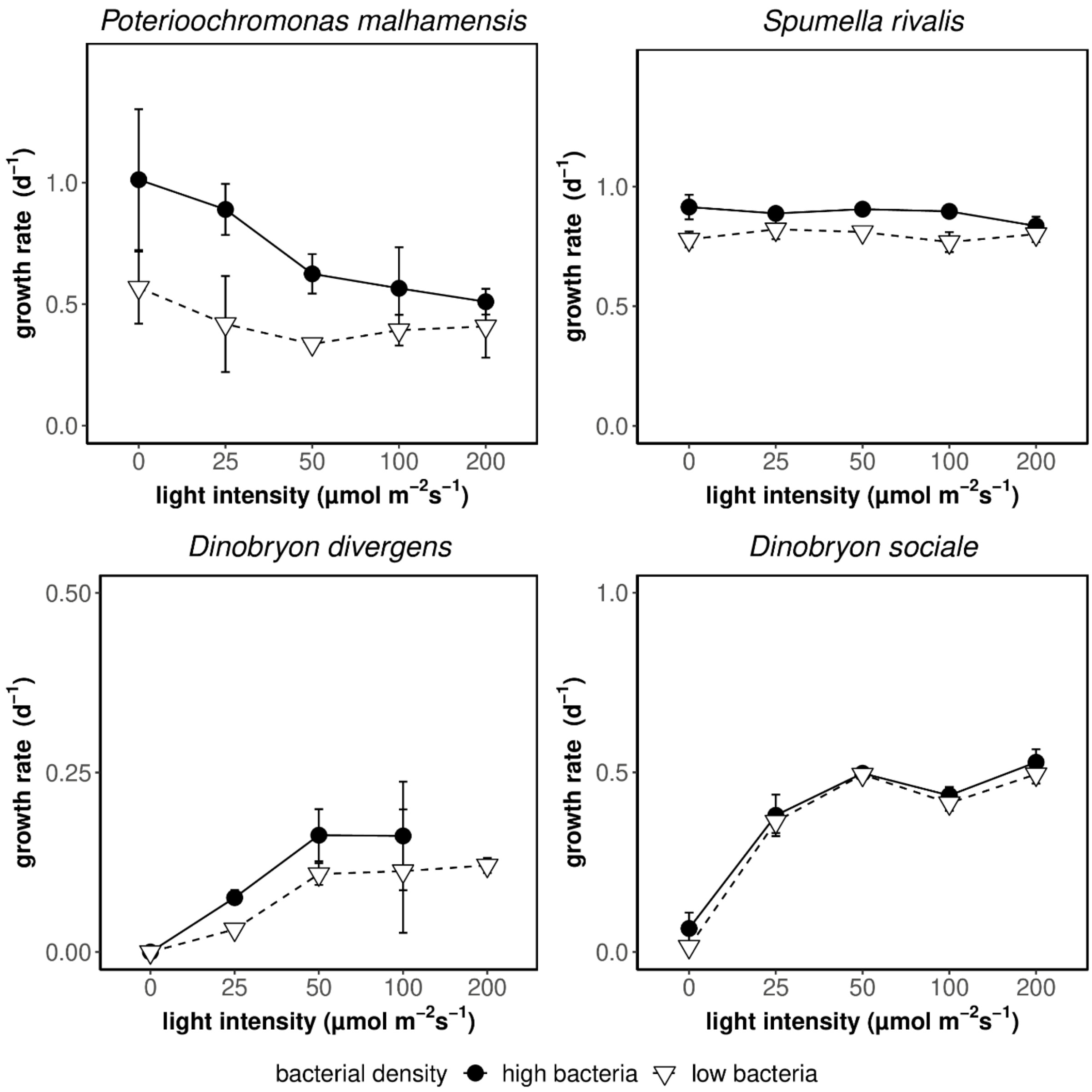

Fig. 6. Average growth rates of examined chrysophytes during the phase of exponential growth responding to different light intensities and different bacterial densities (HB & LB). Datapoints represent the mean values of the respective three replicates. Error bars display standard deviations.

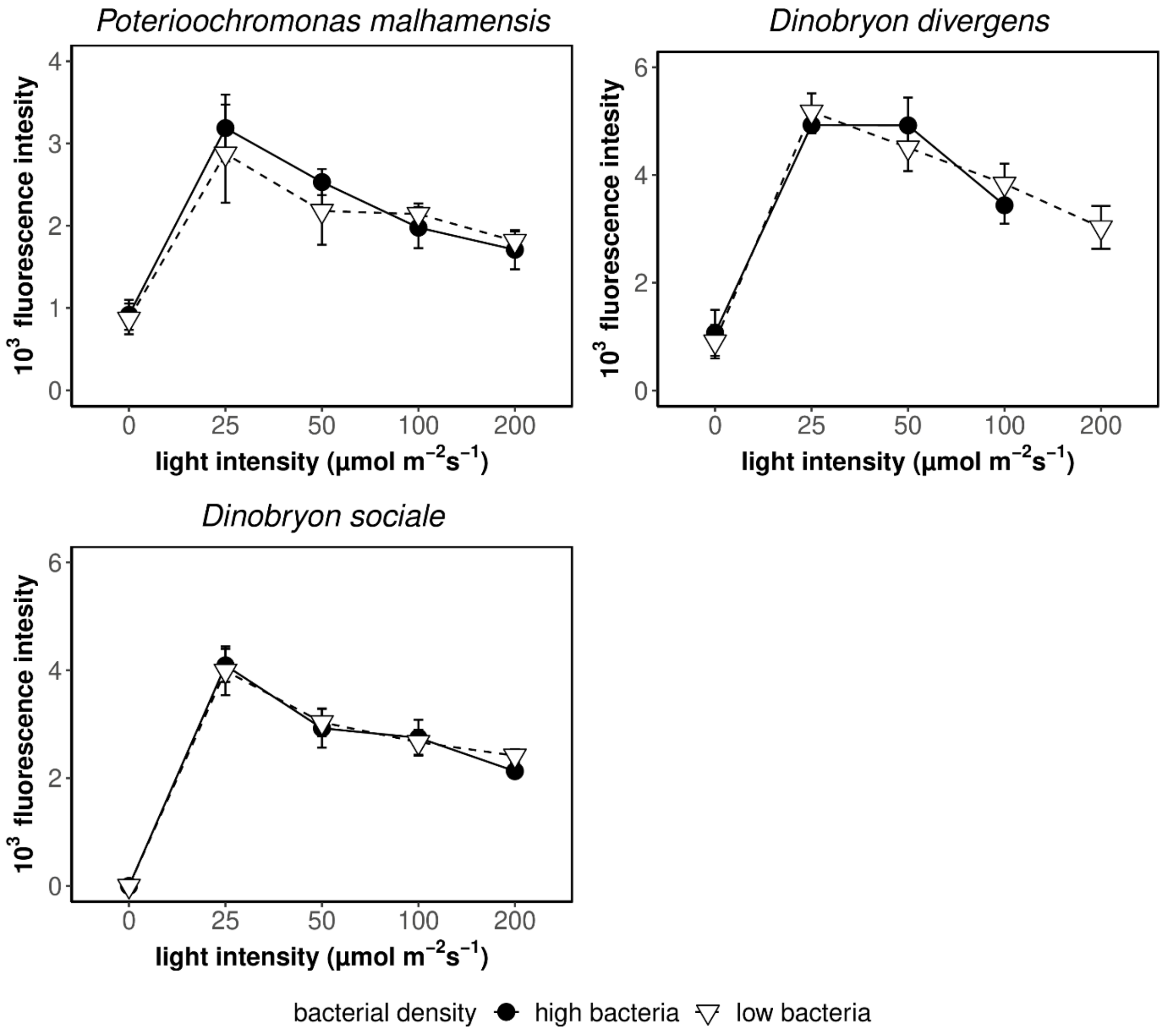

Fig. 7. Average chlorophyll a autofluorescence intensities of examined chrysophytes during the phase of exponential growth responding to different light intensities and different bacterial densities (HB & LB). Datapoints represent the mean values of the respective three replicates. Error bars display standard deviations.

Discussion

Nutritional traits of chrysophytes can be manifold, ranging from obligate heterotrophic to obligate phototrophic species (e.g., Andersson et al., 1989; Caron et al., 1993; Rottberger et al., 2013). A wide range of different strategies can be found between these extremes, giving advantages to mixotrophs when specific environmental parameters are met (Rottberger et al., 2013). The fight for nutrients like phosphorous, essential for algal growth, is highly competitive in aquatic ecosystems. While primary production relies on scarce dissolved phosphorous, heterotrophic organisms can satisfy their P demand by ingesting phosphorous-rich particles such as bacteria. In contrast, the availability of sunlight provides photoautotrophs a stable source of energy while heterotrophs strictly rely on carbon-rich food, which is often limited. Mixotrophy combines both strategies, which can be advantageous when either P or energy supply is limited. With a background that re-oligotrophication in Lake Constance might favor mixotrophy, we conducted experiments under phosphorous limitation to investigate the influence of bacterial densities and different light intensities on the growth of native mixotrophic chrysophytes. The chrysophytes examined in our study represent this range and fulfilled our expectations that bacterial densities and various light intensities are critical factors for their growth.

Heterotrophy as a major strategy of *P. malhamensis*

When no dissolved inorganic phosphorous but bacterial biomass was present, *P. malhamensis* intensive growth activity was found, indicating that P. malhamensis can drive its whole growth with nutrients bound in bacteria. An increase in cell numbers was most intensive at high bacterial densities, while lower bacterial density yielded less growth, and the steady-state was achieved later. The increased growth rates by growing on high bacterial densities suggest that *P. malhamensis* heavily relies on phagotrophy when dissolved inorganic phosphorous is not present. Increased growth of *P. malhamensis* when bacteria were present was yet observed by Caron et al. (1990). A related study reported a gradient growth with increasing bacterial densities (Sanders et al., 1990). Both studies described *P. malhamensis* as predominantly heterotrophic since growth rates and ingestion rates were independent of light present, and the flagellate covers its demand for C, P, and N mostly through bacteria. The related chrysophyte *Ochromonas sp.* is another example of a primarily heterotrophic chrysophyte showing increased growth with higher bacterial densities (Andersson et al., 1989; Rothhaupt, 1996a). Light, however, did not influence heterotrophy of *Ochromonas sp.* but was a crucial factor for the viability of the flagellate. Mixotrophic chrysophytes like *P. malhamensis* and *Ochromonas sp.* appear to follow similar mixotrophic strategies, primarily based on heterotrophy. *P. malhamensis* showed the highest growth rates without light, indicating that heterotrophic growth in the absence of light is highly effective, and *P. malhamensis* can increase its own densities fast under these conditions. However, cell numbers decreased after four days of intensive growth. Even if *P. malhamensis* grows primarily heterotrophic, photoautotrophy must contribute some essential amount of energy since viability was given when illuminated with all intensities (Sanders et al., 1990; Zhang and Watanabe, 2001). However, increasing light intensities lead to a reversed decrease in growth rates and autofluorescence. *P. malhamensis* might have a minimal demand for photosynthetic carbon acquisition to survive, which can be induced according to the prevailing light conditions. Different light intensities do not enhance growth rates suggesting that growth is primarily based on heterotrophy and the photosynthetic performance is kept on a consistent level. The growth of *P. malhamensis* in the different light conditions was comparable to the growth of *S. rivalis*, with the highest performances in darkness or under low-light conditions. Nevertheless, the total yield in cell numbers of *S. rivalis* was higher, indicating that *P. malhamensis* does not reach an exclusive heterotrophic chrysophyte's full growth performance. However, even if heterotrophic growth is the favorite feeding strategy, light might indirectly influence growth. Holen (1999) showed that the chlorophyll a concentration in *P. malhamensis* decreased with the introduction of bacteria and increased again when bacteria became limiting. Thus, light could become an essential factor when prey densities are low and photosynthetic growth represents an alternative that can be enabled (Sanders et al., 1990, Holen, 1999). In the present study, no differences in autofluorescence were observed for *P. malhamensis*, confirming these results. Our results were similar to other studies where *P. malhamensis* showed strong tolerance towards different light intensities (Zhang and Watanabe, 2001), and its energy budget was not necessarily bound to phototrophy (Sanders et al., 1990). Sanders et al. (1990) determined phagocytosis as a relevant factor for growth and not photosynthesis, which represents a long-term strategy and can be induced during times of starvation when prey densities are low. However, bacterial densities in our experiments were considerably high in both treatments and were not a limiting factor, and changing nutrition might not be necessary.

Photoautotrophy as a major strategy of *D. divegrens* and *D. sociale*

In contrast to *P. malhamensis, Dinobryon* relies more on phototrophy and might use nutrients obtained by phagotrophy to promote its photosynthesis. Caron et al. (1993) found out that *Dinobryon cylindricum* is an obligate phototroph that is not able to survive in darkness and may build up the majority (≥ 75 %) of its carbon through photosynthesis and the uptake of bacterial C, P, and N contributes with up to 25 %. However, in darkness and under low light conditions, *D. cylindicum* survived, but no growth was observed. Caron et al. (1993) suggested that *D. cylindricum* cannot utilize the full amount of incorporated bacterial biomass for its biomass under these conditions and releases it to the environment re-mineralized or as organic compounds. In the present study, both *Dinobryon* species showed similar behavior and grew with bacteria when phosphorous was absent in the medium. However, a higher bacterial density did not enhance growth rates for *D. divergens* and *D. sociale*, which suggests primarily phototrophic growth. Both species showed slight initial growth in darkness but could not survive, showing that the accessibility to light, not the presence of bacteria, is crucial for viability. Nevertheless, the genus *Dinobryon* is found to be an important grazer on bacteria in multiple systems with a different trophic state from eutrophic (Sanders et al., 1989) over mesotrophic (Bird and Kalff, 1987) to oligotrophic (Domaizon et al., 2003).

The comparison between light intensities revealed a higher growth performance of *D. divergens* at light intensities above the low-light condition of 25 µmol $m^{-2}s^{-1}$. Additionally, growth rates between bacterial densities at each light intensity were not different, and the similarities between bacteria treatments at different light intensities are consistent with the first experiment. Accordingly, growth rates of *D. sociale* also increased with more intensive light, and dependence on bacteria was not found. This shows that also *D. sociales'* growth is obligately bound to photosynthesis but not necessarily to the concentration of bacteria. Caron et al. (1993) found strong dependencies on light intensities the availability of light for *D. cylindricum.* While light intensities of 150 µmol $m^{-2}s^{-1}$ and 400 µmol $m^{-2}s^{-1}$ lead to strong growth, low light conditions and complete darkness yielded no growth. When grown in light, both *Dinobryon* species showed no differences among the chlorophyll autofluorescence, indicating that increased light intensities do not promote a more substantial photosynthetic production. Data of *D. divergens* at 200 µmol $m^{-2}s^{-1}$ was excluded from data since all replicates died suddenly after the start of the experiment. The reason remains unclear, but potential contamination cannot be excluded. One can assume that *D. divergens* can grow at 200 µmol $m^{-2}s^{-1}$ with the given bacterial densities since growth at high bacterial densities was positive in the first experiment, and Rottberger et al. (2013) reported photosynthetic activity at even higher light intensities.

Both *Dinobryon* species showed only poor growth performance compared to the heterotrophic chrysophyte *S. rivalis*. Phosphorous was mostly bound in heat-killed bacteria and not present or very limited in the medium. Assuming that *Dinobryon* is primarily phototrophic and only partly capable of using bacterial phosphorous, we can conclude that the ability of feeding bacteria does not necessarily compensate P limitations in the water. However, our experimental conditions definitively favor mixotrophs like *P. malhamensis* that have a strong preference for heterotrophy and approximately reach the growth performance of an exclusive heterotrophic protist like *S. rivalis*.

Re-oligotrophication might enhance mixotrophy in lakes

Especially in oligotrophic environments, mixotrophy might result in a great advantage over exclusive phototrophic competitors and heterotrophic competitors (Katechakis and Stibor, 2006). When bacteria are present, growth can be enhanced to fulfill mixotrophic organisms' demand for carbon and nutrients through heterotrophy (Rothhaupt, 1996a), where phototrophs are bound to dissolved nutrients to promote their photosynthesis. On the other hand, the availability to grow through phototrophy (*Dinobryon*) or at least to maintain viability (*P. malhamensis*) gives mixotrophs an advantage over heterotrophs when prey densities are low. In a large lake like Lake Constance, which passed through a process of re-oligotrophication in the last decades, reduced phosphorous concentrations lead to a shift from a high biomass period to a low biomass period (Jochimsen et al., 2013). In contrast, the proportion of chrysophytes increased in the last years, indicating that mixotrophy is in advance. The genus *Dinobryon* might play an important role among the chrysophytes (Sommer et al., 1993) since it is assumed that *Dinobryon* is responsible for a large proportion of bacterial grazing and occurs seasonally occurs in high densities when environmental conditions are convenient (Kamjunke et al., 2007).

We can conclude that investigating three different mixotrophic chrysophytes revealed fundamental differences in feeding strategies. While *P. malhamensis* is more on the heterotrophic end of the nutritional range*, Dinobryon* seems to be more dependent on phototrophy. However, even between both *Dinobryon* species, differences were found. While the light was a growth factor for both species, a higher light intensity only promoted the growth of *D. sociale*. Nevertheless, under optimal conditions regarding light availability, dissolved nutrients, and prey density, exclusive photo- and heterotrophs might show the best performance. In environments where nutrients are scarce and only found in bacterial biomass coupled with increased light intensity, the possibility of using heterotrophy and phototrophy represents a powerful strategy to outcompete competitors bound to a unilateral feeding strategy.

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Chapter IV: Third Manuscript

Long term development and spatial distribution of the chrysophyte genus *Dinobryon* in a large pre-alpine lake along a process of re-oligotrophication

Carsten Wunsch

Abstract

A declined total phytoplankton biomass accompanied re-oligotrophication in lake Constance, and coincidently the amount of chrysophytes increased. Many chrysophyte species are mixotrophic, combining phototrophy and heterotrophy. A lower phosphorous level might have favored mixotrophic organisms in Lake Constance during the last decades. The genus *Dinobryon* is one of the most dominant chrysophytes in the lake and can reach remarkable quantities during the summer months. With the aid of a long-term data set, the development of three *Dinobryon* species was evaluated between 1952 and 2015. During this period, the initially oligotrophic lake experienced eutrophication in the 1970s and 1980s, followed by re-oligotrophication in the following decades. The results show that the abundances of *Dinobryon* declined during eutrophication and shifted towards later time points in the year. With the reduction of phosphorous, *Dinobryon* was able to recover in terms of its abundancies and seasonal occurrence.

Keywords: *Dinobryon*; chrysophytes; mixotrophy; long-term data; re-oligotrophication; oligotrophic lake

Introduction

Anthropogenic influences such as intensive agriculture, urbanization, and industrialization caused a process of eutrophication in many lakes worldwide due to increased nutrient loads (Smil, 2000; Dokulil *et al.*, 2008; Jeppesen *et al.*, 2010). Primarily responsible for changing trophic states is phosphorus, congruently causing a strong increase in total phytoplankton biovolume (Kalff and Knoechel, 1978). How quickly the manipulation of total phosphorous turns the lake ecosystem into a eutrophic state was shown by reference to several small lakes in northwestern Ontario, Kanada (Schindler, 1974). However, while the addition of phosphorous to these lakes directly induced eutrophication, a subsequent deduction caused a recovery of the trophic state. Since the trophic conditions of lakes can be reversed (Jeppesen et al., 2005), the question arises if eutrophication with subsequent re-oligotrophication also shows reversibility inside the phytoplankton communities through phytoplankton biovolumes and species composition. Changing phosphorous concentrations can affect the total phytoplankton biomass (Carpenter et al., 1998) and species compositions of phytoplankton communities (Anneville et al., 2005; Dokulil & Teubner, 2005). Lake Constance, a large pre-alpine lake in Central Europe, was no exception in the late 20th century. The total phosphorous in the lake increased from concentrations lower than 10µg/L in the early 1950s to a maximum of 87 µg/L in the late 1970s (IGKB, 2018). Yet, provisions to reduce the phosphorous load in wastewater resulting in recent concentrations similar to the state in 1950. During the first decade of oligotrophication, Sommer et al. (1993) described a decline of mean summer phytoplankton biovolume, roughly at the same rate as total phosphorous. Two decades later, the decrease of phytoplankton biomass was described as regime shift, from a high biomass period, during eutrophic times towards a low biomass period during oligotrophication initiated by a biomass drop within a few years in the late 1980s, when the phosphorous decline was steepest (Jochimsen *et al.*, 2013). However, both studies noted an opposed increase in chrysophyte biomass. Furthermore, Sommer (Sommer et al.,1993) reported that the analyzed *Chrysophycaea* were practically only represented by the genus *Dinobryon.* Mostly found in oligotrophic waters (Hutchinson, 1944), this genus attracted some attention in the past due to its widespread distribution and specific seasonal periodicity (Lehman, 1976; Hutchinson, 1944). Several pioneer studies described its ecophysiological traits (Ahlstrom, 1937; Bird & Kalff, 1986, 1987; Lehman, 1976; Caron et al., 1993;) and highlighted *Dinobryon* as important planktonic algae considering environmental changes (Lehman, 1976; Wirth *et al.*, 2019). Like many chrysophytes, *Dinobryon* is mixotrophic and can be important grazers on bacteria (Bird & Kalff, 1986, 1987; Unrein et al., 2010), which is advantageous in oligotrophic waters where dissolved nutrients are scarce (Unrein *et al.*, 2014;

Hartmann *et al.*, 2012; Nygaard and Tobiesen, 1993). To date, the importance of the genus *Dinobryon* within the phytoplankton community could be reported for several temperate lakes (Dokulil and Skolaut, 1991; Domaizon *et al.*, 2003; Reynolds *et al.*, 1993; Taş *et al.*, 2010; Kamjunke *et al.*, 2007). As one of those lakes, Lake Constance is a prominent example, and with its alternating trophic states in the past, an exciting system to study *Dinobryon*.

This study aims to display long-term changes in biovolumes of the abundant *Dinobryon* species *Dinobryon divergens*, *Dinobryon sociale*, and *Dinobryon sertularia* during the decades of eutrophication and re-oligotrophication on the base of a dataset compiled from routine sampling in Lake Constance between 1952 and 2015. One can assume that the fluctuating phosphorous concentrations markedly influenced the annual occurrence of *Dinobryon* in the past. Due to the possibility to classify the historic trophic state of Lake Constance in three different periods, oligotrophic followed by eutrophication followed by re-oligotrophication, it is hypothesized that the course of *Dinobryon* appearances mirror the phosphorous curve coupled to the lake (H1). Additional to the long-term reflection, the seasonal variability of *Dinobryon* species is compared among the different trophic periods. Here the second hypothesis (H2) supposes *Dinobryon* to appear early in the year or even twice during the oligotrophic periods, due to the overall low phosphorous load, and late in the year during the eutrophic period when phosphorous is depleted with the phytoplankton bloom in summer. Furthermore, phytoplankton was collected to compare the recent spatial distribution of *D. divergens* and *D. sociale* with data from 1955. The third hypothesis (H3) assumes that *Dinobryon* shows similar spatial distributions in both oligotrophic periods.

Methods

Lake Constance is a large pre-alpine and temperate-monomictic lake located in the three-state-area between Austria, Germany, and Switzerland. Divided into two main basins, Upper and Lower Lake Constance, it claims an area of 536 km^2 and has a maximum depth of 251. The year-round, long-term dataset of *Dinobryon* occurrence covered the period from 1952 to 2015 and was provided by the "Internationale Gewässerschutzkommission für den Bodensee" (IGKB). Through the decades, sampling took place in two places in Upper Lake Constance, the Überlingen Basin (1952-1963) and the middle of the main basin (1964 – 2015) between Fischbach (D) and Uttwil (CH). Sampling for analysis of the spatial distribution was performed bi-weekly for nine months between March and November 2018 in the Überlingen Basin of Upper lake Constance. A water sampler was used to collect water samples incrementally from the surface to a maximal depth of 40 meters. The hauls were performed more frequently at the first 10 meters (0, 1, 2, 4, 8, 10m) and less frequently in deeper layers (15, 20, 25, 30, 40m). For the conservation of containing phytoplankton, sampled water of each increment was mixed with 6ml Lugol's acid solution + sodium acetate in brown 200ml screw-top jars and stored in an insulation box to prevent insolation and overheating. The water was not additionally filtered for large particles and zooplankton to prevent loss of large *Dinobryon* colonies. Afterward, the samples were directly brought to the lab, and containing *Dinobryon* was counted following Utermöhl (1958). To ensure a reliable count of recently living *Dinobryon* cells on-site, only unspoiled cells with intact surrounding loricae were included, cysts were excluded.

To analyze long-term changes on *Dinobryon*, a generalized additive model (GAM) was applied on the annual mean biovolumes (mm^3 m^{-3}) with time (year) and total phosphorous (µg L^{-1}). The model was computed in R (R Team, 2018) using the package “mgcv” (Wood, 2020). The dataset was log10 transformed previously, and the number of base functions for every smooth term was set to k = 5.

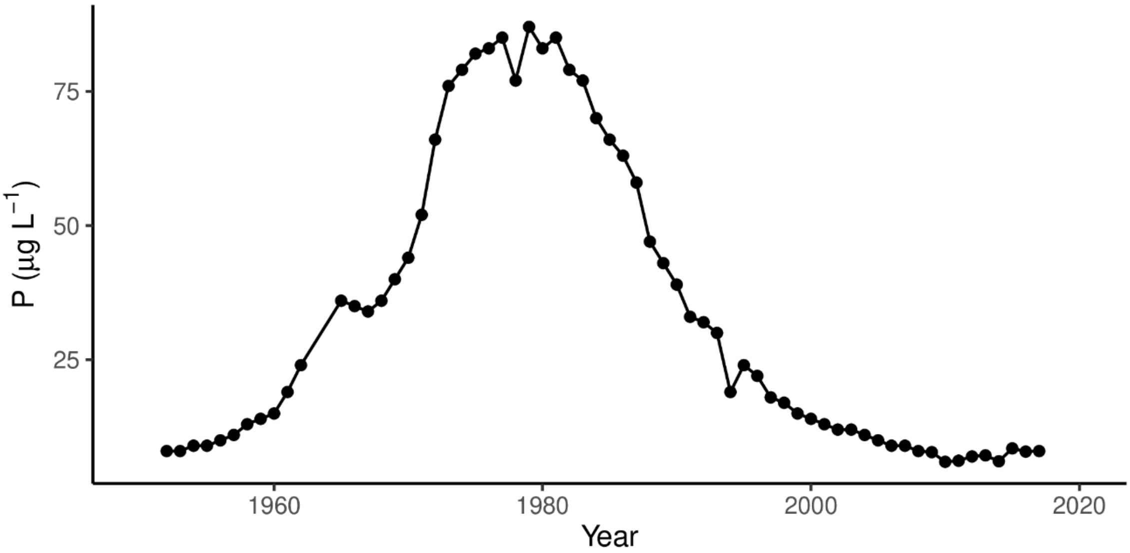

Fig. 1. Annual development of total phosphorous concentrations in upper Lake Constance. Data: © BOWIS - Daten aus dem Bodensee Wasserinformationssystem der Internationalen Gewässerschutzkommission für den Bodensee (IGKB)

Results

At the beginning of the time series, upper Lake Constance was in an oligotrophic state with phosphorous concentrations below 10 µg L^{-1}. Eutrophication took place after 1960 and reached a maximal P concentration during the late 1970s and early 1980s with concentrations above 80 µg L^{-1}. Subsequently, re-oligotrophication started and stable P concentrations similar to those of the early 1950s measured after 2005 (Fig. 1). The applied GAM suggested significant long-term variability for *D. divergens* ($p < 0.001$), *D. sociale* ($p < 0.001$) and *D. sertularia* ($p = 0.022$, Tab.1). The applied GAM suggested a decrease in mean annual biovolumes for *D. divergens* between the early 1950s and the mid of 1970s (Fig. 2A). Subsequently, biovolumes increased again until the mid of 2000s and remained stable until the end of the time-series. A similar decline in mean annual biovolumes was observed for *D. sociale* between the early 1950s and the early 1970s.

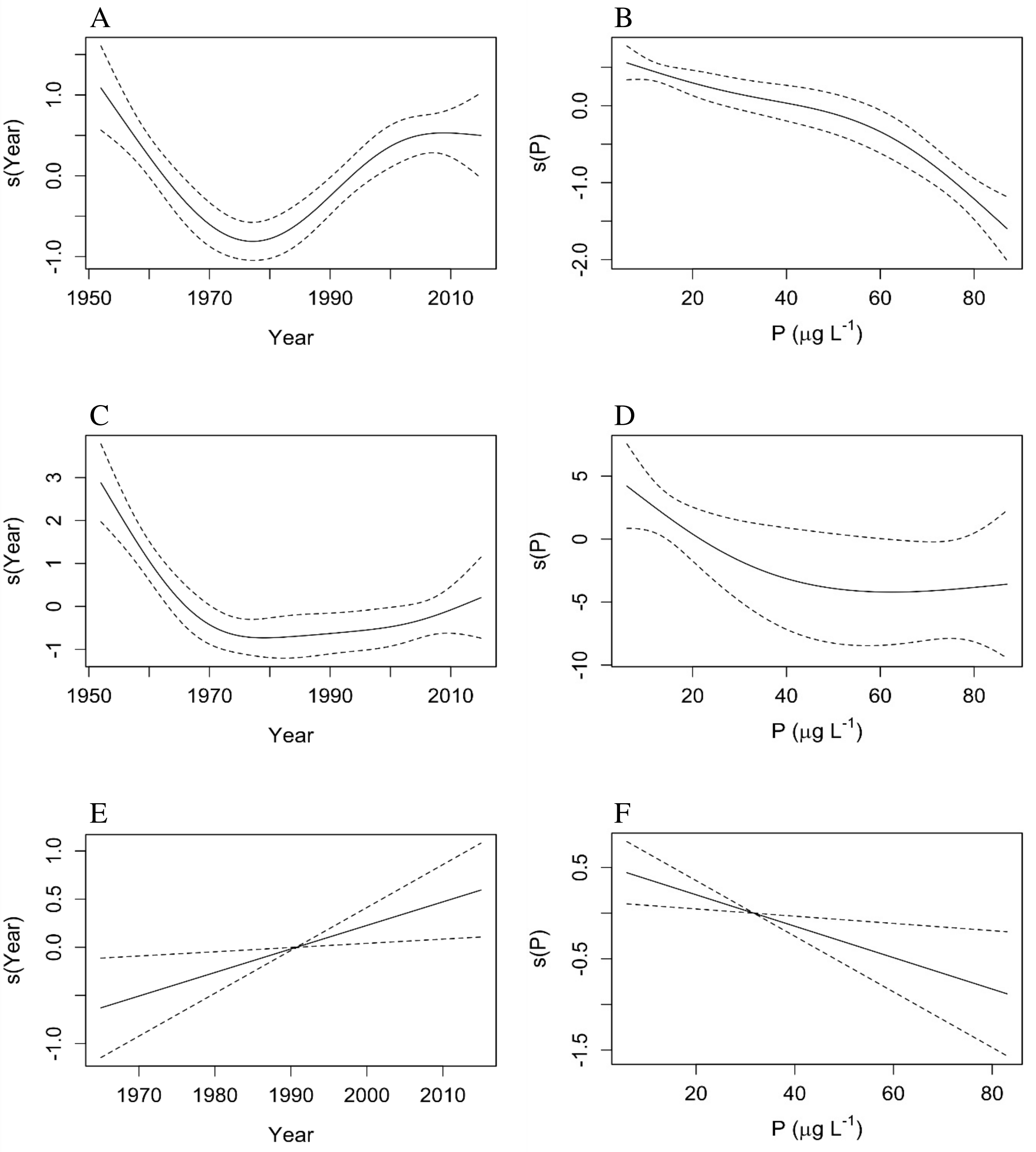

Fig. 2. Long-term changes in biovolumes of investigated Dinobryon species in Upper Lake Constance (A, C & E) and the relationship between their biovolumes and total phosphorous concentrations (μg L^{-1}) (B, D & F). Single frames A and B show *Dinobryon divergens*, C and D *Dinobryon sociale*, E and F *Dinobryon sertularia*. The fit of the generalize additive model

In the following decades, the biovolumes of *D. sociale* increased slightly until the end of the time series. First observations of D. sertularia were available in 1965, and in the following decades, the GAM suggests a linear increase of mean annual biovolumes. A negative relationship of mean annual biovolumes with increased phosphorous concentrations was suggested by the GAM for *D. divergens* ($p < 0.001$), *D. sociale* ($p = 0.02$) and *D. sertularia* ($p = 0.015$, Tab.1). While non-linear relationships were displayed for *D. divergens* and *D. sociale*, a linear relationship was suggested for *D. sertularia* (Tab.1, Fig.2).

Evaluation of monthly mean biovolumes displays a peak occurrence of *D. divergens* in July between the years 1952 and 1956. In the following two decades the peak shifted from July to August between 1963 and 1967 and from August to October between 1976 and 1980. Afterward, an opposed shift occurred back to a biovolume peak in July between 2011 and 2015. *D. sociale* showed two peaks between 1952 and 1956 in May and September. Between the years 1963 and 1967, only the peak in September remained and shifted afterward to August between 1976 and 1980. No records were found for *D. sertularia* between 1952 and 1956. In the period between 1963 and 1967, two peaks were found for *D. sertularia* in August and October. Later, the autumn peak was substituted, but D. sertularia was found to have larger biovolumes in spring between April and May. However, in the 1990s, the later peak found in the decades before shifted to August. In total, biovolumes of D. sertularia were sparse in the 1960s but became more and more frequent to date.

Table 1. Generalized mixed model (GAM) between the species biovolumes and the smooth functions of time (Year) and phosphorous concentration (Tpmix).

Species	Variable	EDF	Ref.df	F-value	p-value
Dinobryon divergens	Time (Year)	3.491	3.851	13.93	< 0.001***
	P (Tpmix)	2.546	3.042	29.86	< 0.001***
Dinobryon sociale	Time (Year)	3.263	3.702	11.73	< 0.001***
	P (Tpmix)	1.913	2.323	3.891	0.02*
Dinobryon sertularia	Time (Year)	1	1	5.935	0.022*
	P (Tpmix)	1	1	6.751	0.015*

Discussion

Mixotrophy favors growth when inorganic nutrients are limited (Nygaard and Tobiesen, 1993; Unrein *et al.*, 2014) since mixotrophs can compete with obligate phototrophic phytoplankton for dissolved nutrients due to their ability to obtain nutrients through the ingestion of bacteria (Rothhaupt, 1996; Katechakis and Stibor, 2006). The anthropogenic increase of phosphorous in Lake Constance went along with an increase in total phytoplankton biomass and, moreover, affected the structure of the phytoplankton community (Kümmerlin, 1991; Wessels *et al.*, 1999; Jochimsen *et al.*, 2013). With declining phosphorous concentrations during re-oligotrophication, a shift from a high biomass phase to a low biomass phase occurred (Jochimsen et al., 2013).

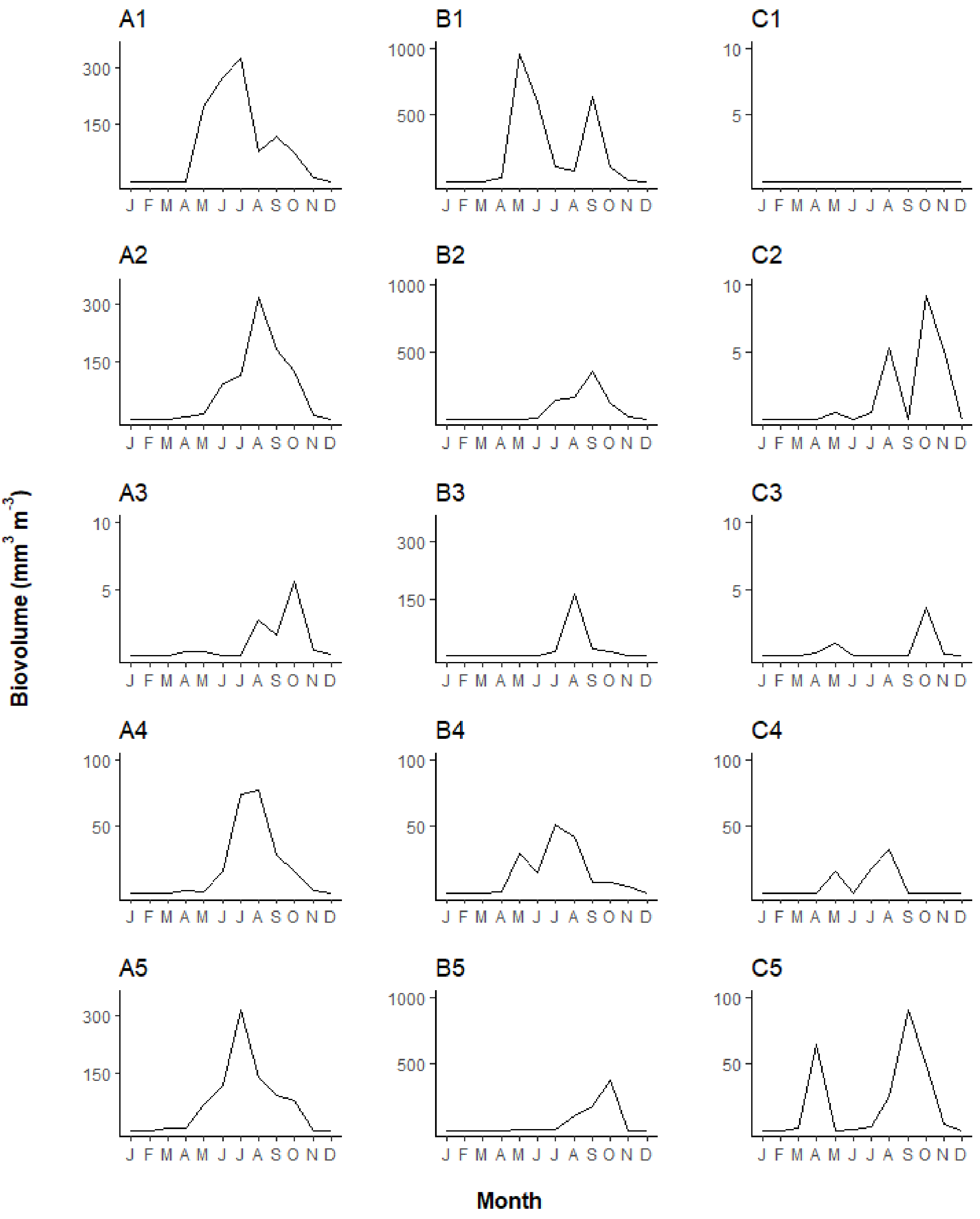

Fig. 3. Seasonal distribution of the five-year means abundances of investigated *Dinobryon* species troughout a whole year. Panels A1 to A5 display D. divergens, B1 to B5 D. sociale and C1 to C5 D. sertularia. The numbers 1 to 5 constantly represent the five-year means of the different trophic periods, which show the years 1952-1956 (1), 1963-1967 (2), 1976-1980 (3), 1995-1999 (4) and 2011-2015 (5). Please note the different scales of the y-axis.

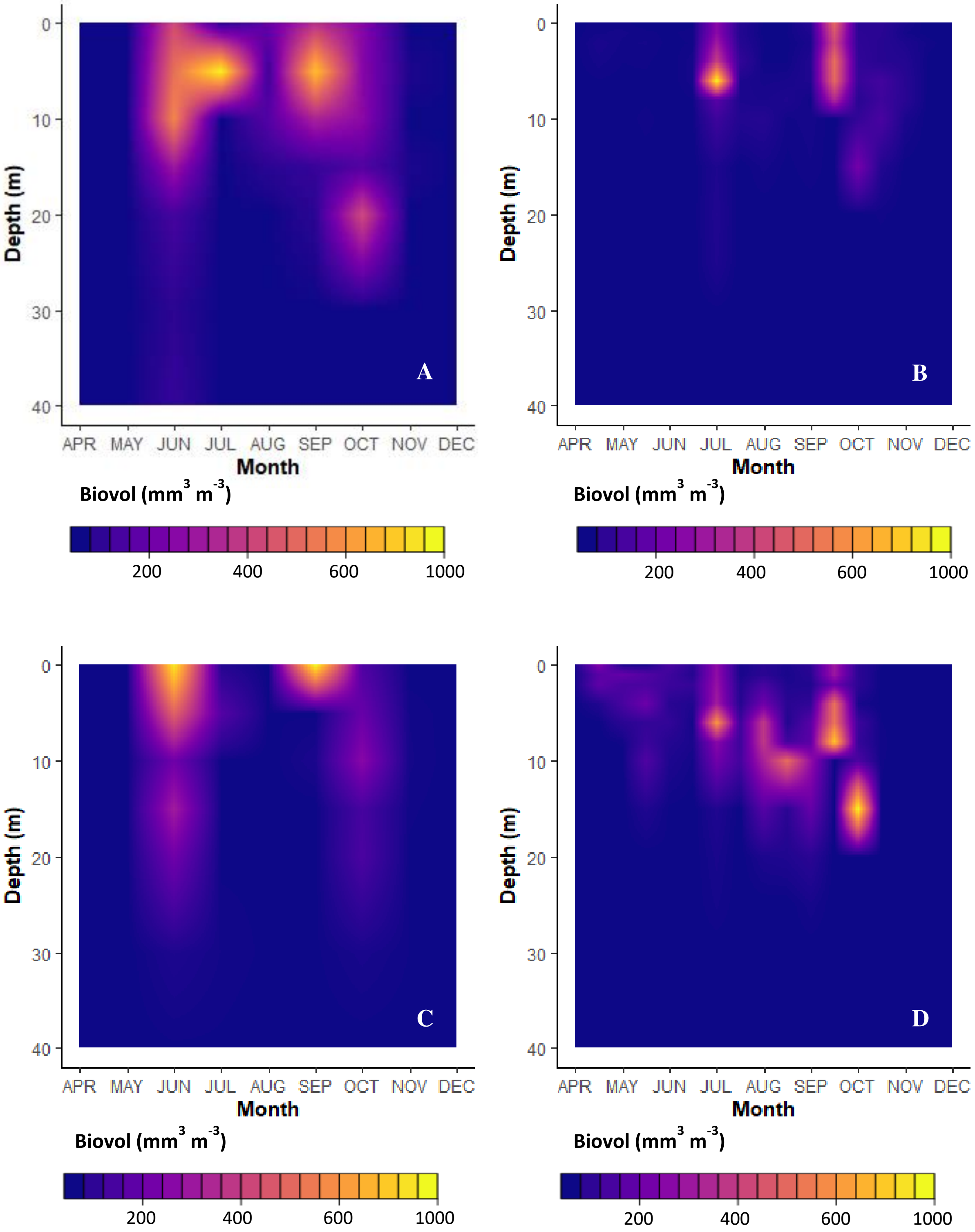

Fig. 4. Spatio-temporal distribution of *D. divergens* and *D. sociale* in upper Lake Constance during the summer and autumn months from April to December. Panels A and B display D. divergens in 1955 (A) and in 2018 (B). Panels C and D show D. sociale in 1955 (A) and in 2018 (B).

As a result, with this shift, the relative and absolute abundance of mixotrophic algae increased, and dominance of Dinobryon was observed (Sommer *et al.*, 1993). Especially in oligotrophic systems, the ability to combine phagotrophy and photosynthesis gives mixotrophs an advantage against phototrophic competitors for dissolved nutrients and can make them important grazers on bacteria.

The present study revealed large variations in the long-term development and the seasonal abundance of the investigated *Dinobryon* species. The changing environment of Lake Constance, coupled with the dynamic history of *Dinobryon,* shows how eutrophication with subsequent re-oligotrophication may affect the phytoplankton community. With *Dinobryon divergens* and *Dinobryon sociale*, the most abundant species of this genus in the past decades were included (Sommer, 1986; Kamjunke *et al.*, 2007), which also occurred in high numbers seasonally. With D. sertularia, I included a species that was recorded infrequently in the past but emerged more recently with re-oligotrophication. In my study, I found a strong relationship between the abundance of all three *Dinobryon* species and the predominant phosphorous concentration. Especially the progression of *D. divergens* mirrors the phosphorous curve in a contradictory way. Since phosphorous is an important limiting factor for algal growth in Lake Constance (Tilzer and Beese, 1988) and the ability of mixotrophic *Dinobryon* species to compensate for lacking inorganic nutrients by the ingestion of bacteria, these circumstances outline the base of a robust explanation for their prosperity during re-oligotrophication. This indicates that the occurrence of *D. divergens* is highly dependent on the predominant trophic state.

A similar relationship could be found for *D. sociale*. However, recovery from eutrophication seems not to be as consistent as it was observed for *D. divergens*. The renewed increase occurred much later, so that low abundances were observed until the early 2000s. The less significant relationship of *D. sociale* with the phosphorous concentrations indicates that lower P concentrations may not favor the growth of *D. sociale* as it does in the case of *D. divergens*. Furthermore, it can be assumed that *D. sociale* necessarily needs oligotrophic conditions to profit in contrast *to D. divergens*, which is also directly linked to the phosphorous load but also may increase numbers even in mesotrophic waters. Despite data from the oligotrophic years prior to 1960 for *D. sertularia* were not available, a positive trend from eutrophication to re-oligotrophication could be observed, which, together with a negative correlation with the P concentrations, indicates that *D. sertularia* profits from oligotrophic conditions as well. In situ experiments with different *Dinobryon* species, including *D. sociale* and *D.sertularia*, revealed that phosphorous is not toxic even at higher concentrations (Lehmann, 1976).

Due to their capability of phagocytosis, *Dinobryon* can be vital grazers on bacteria to obtain mineral nutrients (Bird and Kalff, 1987; Kamjunke *et al.*, 2007) which gives them the ability to compete with obligate phototrophs when dissolved nutrients are limiting (Caron *et al.*, 1993). Assuming that the light irradiance in the water column increased with decreasing phytoplankton biomass with re-oligotrophication (Kamjunke *et al.*, 2007), stronger yielding photosynthesis could additionally promote the growth of *Dinobryon*.

Considering the whole timeframe, with upcoming eutrophication in the late 1950s, *D. divergens* and *D. sociale* abundances went along with the increasing phosphorous concentrations resulting in much lower abundances. *D. divergens* is outstanding by showing a congruent response to changing trophic conditions. The corresponding negative correlation with increasing P concentrations indicates that *D. divergens* showed a strong response towards the changing phosphorous concentrations in Lake Constance. Thus, the long-term progression of *D. divergens* shows a reversed coincidence with the characteristic curve explaining the phosphorous concentrations over the years and, in addition to that, full reversibility, with today's abundances analog to those in the early 1950s. Nevertheless, since *Dinobryon* is a known indicator for oligotrophy, it may also be abundant in meso- and eutrophic waters (Dokulil and Skolaut, 1991; Sanders *et al.*, 1990; Celewicz-Goldyn, 2005).

The shown long-term development of *Dinobryon* in my study revealed an increasing trend during the past years of re-oligotrophication which reflects former results surveying this period (Sommer *et al.*, 1993; Kamjunke *et al.*, 2007). *D. divergens* thus could be seen as a capable indicator for eutrophication or oligotrophication events. Furthermore, with re-oligotrophication, *D. divergens* reached abundances recorded in pre-eutrophic times and, in addition to that, showed a strong capacity to recover from eutrophic periods. A similar response to increasing phosphorous concentration was found for *D. sociale* that also was numerous before eutrophication and then firmly declined. However, the recovery of *D. sociale* was not that rigorously bound to the phosphorous curve and took place later in the early 2000s. The available dataset ended in 2015, and further data analysis needs to be done for recent years. The abundancies found for *D. sociale* in the spatio-temporal survey in 2018 might indicate that in the years after 2016, *D. sociale* was able to increase in numbers during summer months massively.

Additional to the advantage of phagotrophy, increased light availability could have favored the growth of *Dinobryon* in lake Constance. Since Lake Constance shifted from a high biomass phase during eutrophy to a low biomass phase with re-oligotrophication, light attenuation ceased with the decreasing assemblages of phytoplankton (Jochimsen *et al.*, 2013). A disappearing shading effect may give Dinobryon the advantage to optimize its capacity in photosynthesis.

The trophic state of Lake Constance influences the long-term development of *Dinobryon,* but other factors might also play an important role. Several studies reported dependence of physiological processes like photosynthesis and ingestion of bacteria on temperature for *Dinobryon* (Bird and Kalff, 1987; Hitchman and Jones, 2000; Kamjunke et al., 2007). In oligotrophic Lake Lacawac, high abundances of *Dinobryon* were associated with a narrow temperature range between 9-18°C (Heinze et al., 2013). The rates of photosynthesis and phagocytosis are temperature-dependent for *D. sociale*, with maximal ingestion rates and photosynthetic performance at around 16°C (Princiotta et al., 2016). Higher and lower temperatures resulted in lower performance of both nutritional modes. During summer, increasing surface water temperatures could explain why the abundance of *D. sociale* drifts towards deeper layers between 5 m to 20 m in summer, where the temperature is below 20°C (IGKB, 2020). Parallel to re-oligotrophication and the related shift of the phytoplankton biomass, the average annual water temperature increased (Jochimsen et al., 2013; IGKB, 2020). No statistical relations between the changes of phytoplankton biomass and water temperature were found, but the involvement of temperature cannot be excluded, e.g., in triggering regime shift timing (Jochimsen et al., 2013). Lower surface temperatures in the 1950s might indicate why *D. sociale* was found with high numbers in the upper layers of the water column and a spatially deeper occurrence in the late summer of 2018.

During the eutrophic period, the periodicity of phytoplankton was characterized by a spring bloom with the onset of thermal stratification and was followed by the succession of zooplankton in mid-summer (Sommer, 1986). This clear-water phase (Lampert and Schober, 1978) was followed again by increasing phytoplankton due to the lack of food for the zooplankton. In some years, it took place that this second bloom was followed by a cyanophyte and *Dinobryon* assemblage (Sommer, 1986). The seasonal occurrences evaluated in this study show that the investigated *Dinobryon* species were mostly found in late summer and autumn, reflecting the findings of Sommer (1986). Under oligotrophic conditions, before eutrophication, *D. divergens* and *D. sociale* were both recorded earlier in the year and showed a broader distribution during summer months. *D. sociale* even showed two distinct peaks, one in spring and one in late summer. While the clear-water phase during eutrophication was mostly induced by increased grazing of zooplankton, in oligotrophic times when thermal stratification took place in spring, phosphorous was depleted directly after the first bloom. Mixotrophs like *Dinobryon* had better conditions to grow earlier in the year and were able to appear in high numbers during the whole summer. With the re-oligotrophication occurrence of *D. divergens* retroverted again to earlier time points in the year and was again broader across the summer months. This shows that overall competition with other phytoplankton was low again, and phosphorous was the limiting factor again early in the summer. The abundance of *D. sociale* also went back to the earlier summer months with re-oligotrophication in the 1990s. Between 2011 and 2015, *D.sociale* was again found late in the year, which cannot be explained with a later succession caused by eutrophication. This corresponds with the data of the 1950s when *D. sociale* also showed this autumn peak. Apart from this, the strong spring peak of pre-eutrophic times could not be observed with progressed re-oligotrophication. *D. sociale* could again recover from eutrophication in numbers, but seasonal periodicity could not be reconstituted.

D. divergens and *D. sociale* showed similar seasonal patterns in the spatio-temporal survey. *D. divergens* were found between June and July and later between September and October in both years, which underlines the recovery from eutrophication and shows that *D. divergens can show* the same behavior after re-oligotrophication. However, the occurrence of *D. sociale* shifted slightly backward in the year and was also found in deeper layers during late summer. This might be a further indicator that *D. sociale* could not fully recover its structure of pre-eutrophic years, and its occurrence patterns might have changed permanently. Unfortunately, *Dinobryon* sampling in different depths was only performed in the 1950s. A comparison between both oligotrophic periods with the eutrophic period in the 1970s and 1980s could give insights into how the spatial distribution of *Dinobryon* potentially had changed with increased phosphorous concentrations.

Acknowledgements

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Chapter V: General Discussion

Aquatic ecosystems constantly underlie multiple stressors, which might cause significant shifts in the community structures of the present organisms (Albaret and Laë, 2003; Søndergaard and Jeppesen, 2007; Jeppesen et al., 2010; Smol, 2010). Changes in the nutrient level initiated prominent environmental changes in European lakes during the past decades (Straile and Geller, 1998; Jeppesen et al., 2005). The decline in phosphorous proceeded in Lake Constance resulted in straightforward alterations in the food web that were well studied in the past years (Sommer et al., 1993; Gaedke and Wickham, 2004; Jochimsen et al., 2013; Murphy et al., 2018; Sabel et al., 2020). The manuscripts in this thesis highlight the two following prominent examples of how re-oligotrophication may affect a large lake ecosystem.

As a consequence of the reduced phosphorous concentrations, invasive species were able to take advantage of the novel conditions and could establish themselves. Among these, the three-spined stickleback is of particular importance since it might have severe top-down effects on lower trophic levels (Jakobsen et al., 2004; Candolin et al., 2013)). Our mesocosm experiment results give evidence of how this small invasive fish influences the zooplankton community compared to native whitefish. With the identification of *Daphnia longispina* as keystone species, the indirect impact of pelagic fish could be demonstrated.

Also, the prosperity of mixotrophic chrysophytes in the last decades can be referred to re-oligotrophication (Sommer et al., 1993; Kamjunke et al., 2007). The results of the laboratory experiments comparing the growth of mixotrophic flagellates depending on bacterial density and the light intensity revealed different nutrition modes. Agreeing with findings of the past (Caron et al., 1993; Zhang and Watanabe; 2001; Rottberger, 2013), we could show that some chrysophytes are more located to the photosynthetic end (*Dinobryon)*, and others are more relying on heterotrophy (*P. malhamensis*). Together with the results of the lab experiments, the evaluation of long-term data describing the progression of three *Dinobryon* species, it was possible to find indications why especially, the genus *Dinobryon* increased in numbers while total phytoplankton experienced a decline in biomass during re-oligotrophication.

The first part contained in this thesis displays the top-down effect of the two predominant pelagic fish species found in Lake Constance. While whitefish are native to the lake and dominated in pre eutrophic times (Eckmann and Rösch, 1998), the three-spined stickleback *Gasterosteus aculeatus* is invasive and could establish themselves in the pelagic recently (Alexander et al., 2016). Since whitefish are known as specialized zooplankton feeders, the emergent role of the generalistic sticklebacks is not holistically clear yet. Our results show that a strong predation pressure of both fish species is experienced by the large cladoceran *Daphnia longispina*. Within the zooplankton community of lake ecosystems, daphniids play a major role as grazers of primary producers (Straile and Geller, 1998; Stich et al., 2005). In this intermediate role, large daphniids can be seen as keystone species connecting trophic levels. With our results, we found strong evidence that *D. longispina* acts as a mediator between fish in a high trophic level and the phytoplankton as primary producers. Both fish species showed intense predation on *D. longispina*, while the impact on other zooplankters was low. It is also the presence of *D. longispina* which markedly influenced the structure of the contained phytoplankton community. We suggested that with the invasion of sticklebacks, fundamental changes in the food web were forced, and the former top-down control by whitefish was displaced. We did not find any differences between sticklebacks and whitefish concerning the predation on different zooplankton taxa. Moreover, large *D. longispina* was also intensively grazed by sticklebacks. With these findings, we cannot conclude that the invasion of sticklebacks altered the trophic cascade from fish to phytoplankton. With our experiment, we found evidence that the establishment of sticklebacks in the pelagic does not change the structure of the zooplankton community since large daphniids as keystone species were successfully controlled by both fish species, a change of dominant fish might also not lead to alterations in the structure of phytoplankton communities.

Considering the rest of the investigated zooplankters in our experiment, no significant relationships towards the presence or absence were found. Especially the small *Daphnia cucullata* did not profit from the high predation on the larger *D. longispina.* Nevertheless, the ratio between *D. longispina* and *D. cucullata* species was higher when fish were present. It is surprising that *D. cucullata,* which mostly occur in eutrophic waters with high fish predation, was rare during the eutrophic period but rose with re-oligotrophication. Our results show that small fish, sticklebacks, as well as juvenile whitefish mostly feed on large daphniids, and small *D. cucullate* successfully resists predation. Before the establishment of sticklebacks in the pelagic, whitefish were dominant, and the overall number of present small fish was lower than today (Eckmann and Engesser, 2019). Nowadays, sticklebacks constitute the numerical majority of fish (Alexander et al., 2016) and in addition to that might have increased the total predation of *D. longispina* lake. Even with a moderate amount of fish, the predation pressure is high due to the small size of sticklebacks which might favor the spread of *D. cucullata* due to their effective avoidance of fish predation (Gliwicz, 2001). Since 2016, a few years after the intrusion of sticklebacks into the pelagic, D. cucullate is one of the most dominant daphniids in Lake Constance (IGKB, 2020), which indicates that their occurrence is a consequence of the presence of the stickleback invasion. This shows that prominent environmental shifts like the re-oligotrophication in Lake Constance can also indirectly lead to lagged reactions towards alterations taking place beforehand.

Our mesocosm experiments were a temporary assessment of the trophic processes proceeding in the pelagic. With the free-floating set-up in the lake, we tried to adapt as many natural factors as possible. Besides abiotic factors like temperature and natural light conditions, we also inoculated our mesocosms with the seston of the surrounding lake. Nevertheless, trophic interactions in the pelagic are very complex and might follow seasonal fluctuations and changing weather conditions. Since our mesocosms were closed systems, they might have developed apart from the surrounding lake. Especially a divergent progression of the containing phytoplankton cannot be excluded since internal processes affecting the nutrient balance like sedimentation, a lack of water movement, and microbial and algal growth on the walls are difficult to be estimated.

Also, the involvement of other predating species could impact lower trophic levels. In the lake's pelagic, other fish species are present, which could take part in the feeding on zooplankton but may target other species like whitefish and sticklebacks. Nevertheless, it is assumable that they might not have a strong influence since the fishery survey of Alexander et al. (2016) revealed that stickleback and whitefish cover more than 97 % of the fish biomass in the pelagic. More suspicious to have a relevant grazing impact on the zooplankton community might be invertebrate predators like cyclopoid copepods, *Bythotrephes longimanus*, and *Leptodora kindtii.* In our experiments, we did not observe any increase of invertebrate predators in the control when fish was absent. High daphniid numbers in control suggest that fish are the controlling factor, and predation by invertebrates is, if present, just a minor factor not prohibiting the succession of daphniids. The same results were recently found explicit for predatory cyclopid copepods that showed only low predation compared to fish (Sorf et al., 2014). It is assumable that invertebrate predation, especially on large daphniids, is weak since, in the case of cyclopoids, *Bythotrephes longimanus* and *Leptodora kindtii*, small organisms like rotifers are the preferred prey (Stemberger and Evans, 1984; Plaßmann et al., 1997)

The occurrence of fish and zooplankton is traditionally well recorded in Lake Constance (e.g., Alexander et al., 2016; IGKB, 2020), and reliable data is available to receive insights into the long-term development of respective taxa. However, the interaction between species inside the food web remains unclear in many cases. Environmental changes do not always cause direct responses, like in the case of the delayed stickleback migration, and it might take some time until they are detectable. It is always a difficult task to disentangle potential relationships influenced by many factors. Mesocosm experiments that try to simulate the natural conditions thus can be an important tool to broad view on food webs going on in an ecosystem since it is possible that many biological processes can be monitored simultaneously under controlled conditions on a manageable scale. This leads to the big advantage of mesocosm experiments: under controlled conditions, biotic and abiotic parameters can be manipulated to simulate potential future scenarios but also to reconstruct events in the past.

In the second manuscript, we investigated the growth of different chrysophytes exposed to different bacterial concentrations and light intensities. Many chrysophytes are mixotrophic and display a dominant algae group in freshwater systems and marine environments all over the world. For our experiments, we chose representatives that are commonly found in Lake Constance. Two of them, *Dinobryon divergens* and *Dinobryon sociale,* occur in large numbers during the summer months and have become more dominant during re-oligotrophication. Due to their availability to use photosynthesis and phagocytosis, they might have taken advantage of the lowered phosphorous concentrations in the water.

Our results show that *Poterioochromonas malhamensis* has a mixotrophic trait that heavily relies on phagocytosis. *P. malhamensis* was highly effective in growing on bacteria and showed higher growth rates and a higher maximal cell yield with increased bacterial concentrations. Nevertheless, phototrophy must at least contribute some proportion of the energy budget since *P. malhamensis* could not survive permanently in darkness. Despite its strong dependence on bacterial prey density, *P. malhamensis* is not as effective using phagocytosis as the exclusively heterotrophic comparison flagellate *Spumella rivalis.* In contrast to the high heterotrophic proportion, both *Dinobryon* species were more dependent on photosynthesis. Growth on different bacterial densities did not result in increased growth rates, but with higher light intensities, growth was influenced positively. Growth was almost similar at light intensities at 50 µmol $m^{-2}s^{-1}$ and above, indicating that photosynthetic growth shows its highest performance under these conditions. At a low light intensity (25 µmol $m^{-2}s^{-1}$) grow was negligible, and the cultures started to die after few days; the same was observed in darkness, where *Dinobryon* could not survive from the start. Although *Dinobryon* could not survive in darkness and under low light, both species were showed tolerance towards higher light intensities indicating that *Dinobryon* is able to grow successfully in a broad range of light regimes. In the lake, under natural conditions, this could be important regarding the spatial occurrence in different depths where light is progressively decreasing. Also, in environments where light is seasonally limited due to variable weather conditions or shading effects in the water column caused by phytoplankton blooms (Shigesada and Okubo, 1981), this could be an advantageous adaption that might ensure survival.

Considering their strategies in fulfilling the demands for energy and nutrients, chrysophytes are a very diverse taxon inside the phytoplankton community. Their characteristics range from exclusively heterotrophic (e.g., *Poteriospumella lacustris* and *S. rivalis*) towards exclusively photoautotrophic species (e.g., *Mallomonas annulata*). Inbetween exists many manifestations of mixotrophy shifted to one or the other side (Rottberger et al., 2013). On the one hand, chrysophytes like *p. malhamensis* depend on the availability of prey organisms to prosper, and their photosynthetic trait can ensure the energy supply when prey concentrations are low and thus represents a long-term surviving strategy. On the other hand, *Dinobryon* grows mostly photosynthetically and might use food organisms as an additional source for nutrients and energy. In an oligotrophic environment like Lake Constance where both, light and bacteria are available, the conditions for growth are convenient. We could show that *Dinobryon* can effectively utilize phosphorous bound in bacteria, but the density of bacteria does not influence growth.

Together with the total phytoplankton in Lake Constance, the bacterial biomass leads to a depletion of dissolved phosphorous in spring followed by the succession of zooplankton. The following clear-water phase is characterized by a low phosphorous level, low phytoplankton biovolumes, and a suffering zooplankton community (Sommer, 1986). Nutrient-rich bacteria are now an exclusive source of phosphorous, which mixotrophs can effectively utilize. Besides, light availability increases in the water column due to the reduced zoo- and phytoplankton biovolumes (Jochimsen et al., 2013) which might have created convenient for mixotrophic growth during summer. The long-term data show that *Dinobryon* is very successful during summer, indicating that *Dinobryon* effectively uses the conditions during the clear-water phase. Its nutritional mode is focused on gaining energy through photosynthesis, and the necessary nutrients are obtained from bacteria. This could rapidly boost growth and cause a rapid and unrivaled increase of biovolume (Kamjunke et al., 2007). Mixotrophs like *P. malhamensis* gain energy mostly through phagotrophy, which generally also might be advantageous in the clear-water phase, but since they are not that effective in phototrophy, they might be limited to the available bacterial biomass. Especially in oligotrophic systems, bacteria might be a limited resource that is exhausted after some time. Assuming that photosynthesis is the promoting factor for strong growth during summer in Lake Constance, this could explain the recent and past success of *Dinobryon*. Another factor favoring *Dinobryon* blooms could be a low grazing pressure by zooplankters. The cells of *Dinobryon* are covered by a lorica mostly consisting of cellulose, and many species, including *D. divergens* and *D. sociale,* show colony formation. A selection against *Dinobryon* cells was found for *Daphniids* with the assumption that the cells (20-30 µm) were too large for ingestion (Knisely and Geller, 1986). *D. divergens* showed a strong resistance towards grazing of *Daphnia longispina* and copepods. Evidence was found for a poor food-quality of *D. divergens* due to its low lipid content and a high C:P ratio (Vad et al., 2020). In a stable isotope approach, the study also highlights that the carbon transfer from *Dinobryon* to zooplankton is not efficient.

During eutrophication in Lake Constance, *Dinobryon* was mostly found at the end of the clear-water phase taking advantage of the low nutrient level and the lack of competitors. With re-oligotrophication, its occurrence shifted towards an earlier point of time in the year. The lower amount of phosphorous in the water was already depleted earlier, which led to an earlier succession of *Dinobryon* (Kamjunke et al., 2007). Low phosphorous concentrations in the upper layers were found in July and afterward during the eutrophic period but already occurred in May under oligotrophic conditions (Gaedke, 1998). The results of my study can confirm these dynamics for the investigated species. *D. divergens* shows the most distinct shift to a later time point in summer with eutrophication and subsequent recovery with re-oligotrophication. Similar sequence was found for *D.sociale* and *D. sertularia*. However, with re-oligotrophication, *D. sociale* occurred earlier until the 1990s, but in the beginning 21st century, it shifted back again towards late summer. Furthermore, prior to eutrophication, two peaks were found for *D. sociale* during summer, while only one peak remained during re-oligotrophication. For *D. Sertularia* comparison of the occurrences during re-oligotrophication with the pre-eutrophic times cannot be made since respective data is not available. Nevertheless, *D. Sertularia* abundances increased during the last decades and compared with the occurrence during eutrophication, and it also became abundant in late spring during re-oligotrophication.

The long-term analysis results revealed an immediate response of *D. divergens* towards increasing phosphorous levels and a strong recovery with re-oligotrophication. The course of *D. divergens'* abundance in addition to that perfectly mirrors the characteristic phosphorous curve of Lake Constance. A similar trend was also found for *D. sociale,* but its succession is not that clear, especially internal variability during re-oligotrophication remains unexplained. Presumably, *D. sociale* might take longer to recover, and the fluctuations in its occurrence are the consequences of changes taking place independently from the re-oligotrophication, which already proceeds for several decades. The spatio-temporal recordings of *D. sociale* in 2018 could hint that abundances might have increased in the last years and suggest an occurrence earlier in the summer.

Conclusion

The provisions to reduce phosphorous in the wastewater were concluded in the second half of the 20th century, and the associated re-oligotrophication progresses for about thirty years. Lake Constance is an outstanding example of a well-studied lake, and much knowledge about eu- and oligotrophication could be collected. However, considering events like the recent establishment of sticklebacks in the pelagic or still alternating zoo- and phytoplankton communities, many mechanisms remain unclear. The question if lakes are resilient towards changing nutrient levels or can recover themselves from such changes is difficult to answer. The demonstrated examples in this thesis might give some indications. The sticklebacks invaded Lake Constance during the first half of the 20th century but firstly rose in the pelagic in recent years, which implemented a new member into the fish community. The cascading effect on lower trophic levels showed similar characteristics for sticklebacks and whitefish and was mostly based on one keystone species. Here we can conclude that the presence of a new fish species might not influence the food web differently, like native species and the system show a certain amount of resilience. Nevertheless, it is indispensable to make further investigations and to observe the future development of the lakes' fish. A high degree of reversibility was shown by reference to *Dinobryon divergens*. This species closely mirrored the progression of the phosphorous curve and can be looked upon as a good indicator for staggering phosphorous concentrations. The abundance of the other *Dinobryon* species also showed an increasing trend with re-oligotrophication, but their succession might still continue. Mixotrophic chrysophytes may undoubtedly be one of the dominant members of the phytoplankton community. They show an enormous capacity to exploit declining phosphorous levels and adapt to changing environments due to their adjustable nutritional modes. Studying mixotrophy will be challenging in the future since our knowledge of the metabolism of algae is no longer based on primary production. Mixotrophic chrysophytes like *P. malhamensis* and *Dinobryon* can be likewise both primary producers and consumers.

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Record of achievements

Chapter II

Žiga Ogorelec, Carsten Wwunsch, Alessandra Janina Kunzmann and Jana Isanta Navarro designed the study. All authors conducted the experiment. Carsten Wunsch analysed the data, drawings by Jana Isanta Navarro. Žiga Ogorelec and Carsten Wunsch prepared the manuscript with contributions from all authors. Žiga Ogorelec and Carsten Wunsch contributed equally to this work and share the first authorship.

Chapter III

The study was designed by Karl-Otto Rothhaupt and Carsten Wunsch. Joshua Strittmatter, Samuel Kühn and Carsten Wunsch performed the laboratory work and accomplished the growth experiments including flow cytometry. Carsten Wunsch analysed the data and created the graphics. Carsten Wunsch wrote the manuscript with contributions of Joshua Strittmatter and Samuel Kühn.

Chapter IV

The study design, sampling, data analysis and writing of the manuscript was done by Carsten Wunsch.

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